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Characterization, *in vitro* Trypsin Digestibility and Antioxidant Activity of Fermented Soybean Protein Meal with *Lactobacillus plantarum* Lp6

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Abstract: In this study, soybean protein meal was subjected to solid state fermentation with *Lactobacillus plantarum* Lp6 either in the presence or absence of a protease. The extracts were investigated for changes in mineral composition, amino acid composition, *in vitro* trypsin digestibility, DPPH radical scavenging activities and electrophoretic pattern. The amino acid and mineral element compositions showed significant ($p < 0.001$) variations among the samples. The Fermented Soybean Protein Meal (FSPM) with protease added (FSPMe) showed higher total free amino acid (4.8467 g/100 g sample) compared to 0.2523 g/100 g sample obtained for unfermented Soybean Protein Meal (SPM). The FSPMe had the highest *in vitro* trypsin digestibility and showed a single polypeptide with estimated molecular weight of 14.4 kDa in the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) assay.

Key words: *Lactobacillus plantarum* Lp6, fermented soybean protein meal, DPPH radical, *in vitro* trypsin digestibility

INTRODUCTION

Soybeans are abundant sources of food proteins that have been recognized for high nutritional value and excellent functional properties in food systems (Smith and Circle, 1976). Fermentation is one of the oldest technologies used for food preservation. Many benefits are attributed to fermentation. It preserves and enriches food, improves digestibility and enhances the taste and flavor of foods (Motarjemi, 2002). Fermentation processes have been used to prepare traditional soybean foods and these fermented soy foods are highly digestible and nutritious (Lee, 1998; Kim *et al.*, 1999; Matsuo, 2006).

In the past few years, there has been increasing interest in research about antioxidants, such as phenolic compounds and antioxidant peptides derived from protein hydrolysates since they can protect the human body from free radicals and retard the progress of many chronic diseases such as cancer and cardiovascular diseases (Göktürk *et al.*, 2007; Rajapakse *et al.*, 2005; Amadou *et al.*, 2009). The antioxidant properties of these hydrolysates have been ascribed to the cooperative effect of a number of properties, including their ability to scavenge free radicals, to act as metal-ion chelator, oxygen quencher or hydrogen donor and to the possibility of preventing the penetration of lipid oxidation initiators by forming a membrane around oil droplets (Moure *et al.*, 2006).

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Studies have confirmed the degradation of soybean allergens during fermentation by microbial proteolytic enzymes in soy sauce, miso, soybean ingredients and feed-grade soybean meals (Hong *et al.*, 2004; Kobayashi, 2005; Yamanihi *et al.*, 1995). Fermentation of legumes has been reported generally to improve nutritional and functional properties compared to original products (Granito *et al.*, 2005). Frias *et al.* (2008) showed that soybean flour fermented with *Lactobacillus* sp. (*L. plantarum*) was able to further break down and use available proteins as nutrient sources thereby enriching the fermented product.

The objective of this study was to characterize soybean protein meal proteins in terms of amino acid composition, molecular weight estimation of the polypeptides by electrophoresis and *in vitro* trypsin digestibility and to investigate the antioxidant potential of the protein meals before and after fermentation.

MATERIALS AND METHODS

Sodium Dodecyl Sulfate (SDS) and Coomassie Brilliant Blue R-250 were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). Trypsin, β -mercaptoethanol (β ME) and protein standard were obtained from Sigma Chemical Co. St. Louis, MO. The strain *Lactobacillus plantarum* Lp6 was obtained from the culture collection of Jiangnan University (Wuxi, China). Soybean protein meal was of food grade and was obtained from Sun-Green Biotech Co. Ltd (Nantong, China). All other chemicals were obtained from the Chemical Reagent Co., China and were of analytical grade quality. This research was conducted in the State Key Laboratory and School of Food Science and Technology Laboratory of Jiangnan University, Wuxi from July 2008 to August 2009.

Fermentation and Preparation of Fermented Soy Protein Meal Hydrolysate Extract

The microorganism *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 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 mm×200 mm) and vacuum sealed. Also, of 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.

The FSMP extract was prepared according to the method described by Ye *et al.* (2003). Five grams of fermented soy protein meal 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.

Minerals Composition

Samples were digested in 100 mL micro-Kjeldahl flask with HNO₃/HClO₄ until the solution became colorless. The sample was cooled and diluted to volume in a 25 mL volumetric flask with 0.1 M HCl. Sodium, potassium, calcium, magnesium, iron, zinc, manganese and copper were measured by atomic absorption Spectrophotometry (Garcia *et al.*, 1972) using a Varian spectra atomic absorption spectrophotometer (Varian SpectrAA220, Varian, Palo Alto, CA).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE of samples carried out using discontinuous system described by Laemmli (1970) with 4% stacking and 12% separating gel. Separating gel was run at a constant current of 20 mA for about 3 h. The gel was stained in Coomassie Brilliant Blue R-250. Subunit Molecular Weight (MW) was estimated using low MW calibration kit (Shanghai Institute of Biochemistry, Shanghai, China) consisting of the following proteins: phosphorylase (97.4), bovine serum albumin (66.2), rabbit actin (43.0), bovine carbonic anhydrase (31.0), trypsin inhibitor (20.1) and hen egg white lysozyme (14.4) kDa.

Amino Acid Analysis

The freeze-dried samples were digested with HCl (6 M) at 110°C for 24 h under nitrogen atmosphere. Reversed phase high performance liquid chromatography (RP-HPLC) analysis was carried out in an Agilent 1100 (Agilent Technologies, Palo Alto, CA, USA) assembly system after precolumn derivatization with o-phthalaldehyde (OPA). Each sample (1 µL) was injected on a Zorbax 80 A C18 column (i.d. 4.6×180 mm, Agilent Technologies, Palo Alto, CA, USA) at 40°C with detection at 338 nm. Mobile phase A was 7.35 mmol L⁻¹ sodium acetate/ triethylamine/tetrahydrofuran (500:0.12:2.5, v/v/v), adjusted to pH 7.2 with acetic acid, while mobile phase B (pH 7.2) was 7.35 mmol L⁻¹ sodium acetate/methanol/acetonitrile (1:2:2, v/v/v). The amino acid composition was expressed as gram of amino acid per 100 g of protein.

In vitro Trypsin Digestibility

The *in vitro* trypsin digestibility of protein samples was determined according to the method of Yin *et al.* (2008); 5 mL of protein dispersions (1%, w/v) in 10 mM phosphate buffer (pH 8.0) was mixed with 1 mg of trypsin powder and the mixtures were incubated at 37°C for 30, 60, 90 and 120 min. The reactions were stopped by adding an equal volume of 20% (w/v) trichloroacetic acid and the protein precipitates were removed by centrifugation at 10,000 g for 20 min. The trichloroacetic acid TCA-soluble nitrogen in the supernatants was determined by micro-Kjeldahl method (N×6.25). The % N release during the digestion was calculated as:

$$\text{N release (\%)} = \frac{(N_s - N_0) \times 100}{N_{\text{tot}}} \quad (1)$$

where, N_s is TCA-soluble nitrogen in supernatant phase, N_0 (mg) is TCA-soluble nitrogen at 0 min and N_{tot} (mg) total nitrogen of protein.

DPPH Radical-Scavenging Activity

The scavenging effect of FSPM extract on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured according to the method of Shimada *et al.* (1992) with little modification. Two milliliters of each sample solution (3, 5, 7, 10 and 15 mg mL⁻¹) were added to 2 mL of 0.1 mM DPPH dissolved in 95% ethanol. The mixture was shaken and left for 30 min at room temperature and the absorbance of resulting solution was read at 517 nm. A lower absorbance represents a higher DPPH scavenging activity. The scavenging effect was expressed as shown in the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{\text{Blank absorbance} - \text{Sample absorbance}}{\text{Blank absorbance}} \times 100 \quad (2)$$

Statistics Analysis

All experiments were conducted at least in triplicate. Data are reported as Means±SD. Analysis of Variance (ANOVA) was performed and differences in mean values were evaluated by Tukeys test at p<0.001 using SPSS version 13.0 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Minerals Composition

The mineral compositions of unfermented soybean protein meal and fermented soybean protein meal with or without the addition of protease was found to contain between 1135.3-1776 µg g⁻¹ calcium and 28.4-44.05 µg g⁻¹ Zinc (Table 1). In general, the results showed that fermentation increased the concentration of almost all the mineral elements investigated in this study except copper. The difference when compared with the unfermented sample was significant (p<0.001). Similarly, addition of protease to the fermentation medium brought about a significant (p<0.001) increase in elemental composition of the fermented samples. The increase may be attributed to the activities of the fermenting microorganisms as well as the composition of the fermentation medium. These results were in agreement with the findings of Oladele and Oshodi (2008), who reported an increase in the contents of phosphorus, potassium, calcium and magnesium in fermented physic nut (*Jatropha curcas*) and berlandier nettle spurge (*Jatropha cathartica*). Similar work was also reported by Ibrahim and Antai (1986) in their study on chemical changes during the fermentation of African locust-bean (*Parkia filicoidea* Welw) seeds for production of Daddawa.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE electrophoregrams of fermented and unfermented soybean protein meal samples under reducing conditions is shown in Fig. 1. The control sample as well as fermented soybean protein meal without added protease (FSPM) showed similar banding patterns containing about seven polypeptides with estimated MWs ranging between 14.4 to 82.6 kDa. However, five polypeptides with estimated MWs of 14.4, 39.0, 54.6, 66.2 and 82.6 kDa were the major polypeptides in the control sample and fermentation affected the width and intensity of the bands in FSPM sample (Hong *et al.*, 2004). On the other hand, FSPMe showed only one band with estimated MW of 14.4 kDa. The absence of high molecular weight polypeptides in FSPMe may be attributable to the degradation of polypeptide chains by the proteolytic enzymes. This resulted in the formation of low molecular weight polypeptides.

Table 1: Minerals composition of control (unfermented soybean meal), FSPM (fermented soybean protein meal without protease added) and FSPMe (fermented soybean protein meal with protease added)

Minerals (µg g ⁻¹)	Control	FSPM	FSPMe
Zinc (Zn)	28.44±0.03a	33.11±1.74a	44.05±0.04b
Iron (Fe)	76±0.00a	116.67±1.53b	128±1.00c
Copper (Cu)	10.17±1.02c	9.1±1.55b	8.45±1.04a
Magnesium (Mn)	9.63±0.08a	9.90±0.11ab	10.25±0.02b
Sodium (Na)	5250±1.53a	14204±1.83b	14300±1.00c
Potassium (K)	1641±1.00a	2177±2.52b	2395±0.60c
Calcium (Ca)	1135.3±0.58a	1475±2.38b	1776±1.00c
Manganese (Mg)	1219±3.50a	1203±4.00b	1221±1.50c
Phosphorus* (P)	0.01405±0.00a	0.01417±0.0002ab	0.01425±0.00b

*g/100 g. Values are Mean±SD of three determinations. Rows with different letter(s) indicate statistical differences (p<0.001)

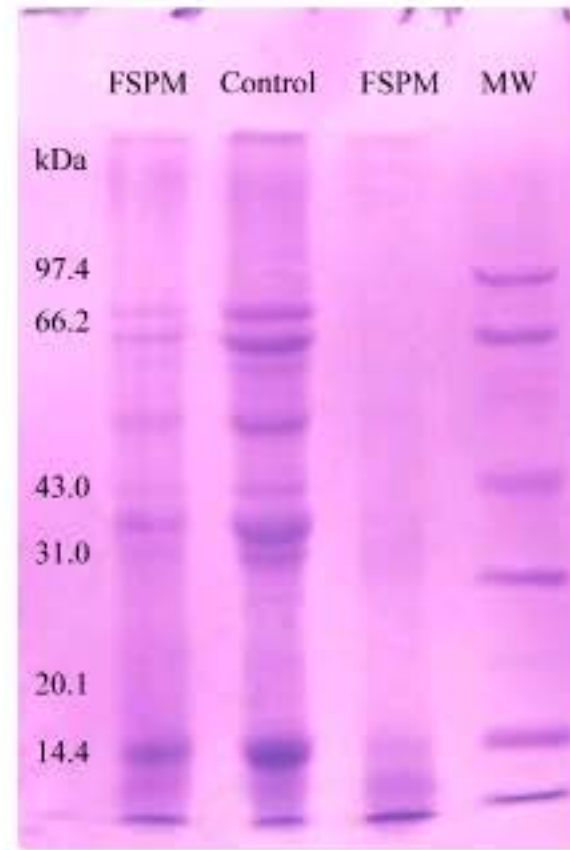


Fig. 1: SDS-PAGE profile of soybean protein meal before and after solid state fermentation. MW: standard molecular-weight marker; FSPM: Fermented soybean protein meal, FSPMe: Fermented soybean protein meal with protease added and Control: Unfermented soybean protein meal

Table 2: Amino acid scores of Fermented Soybean Protein Meal (FSPM) with and without protease added (g/100 g sample)

Amino acid	Total amino acid		
	^a Control	FSPM	^b FSPMe
Aspartic acid	8.7392c	7.1733b	6.3153a
Glutamic acid	16.3089c	12.7648b	11.3741a
Serine	3.6577b	3.4868b	1.8622a
Histidine	1.9691c	1.5891b	1.3759a
Glycine	3.2773c	2.7201b	2.3163a
Threonine	2.7712c	2.3507b	1.7761a
Arginine	6.0683c	4.4274b	3.8653a
Alanine	3.3250c	2.7923b	2.3368a
Tyrosine	2.5187c	1.8647b	1.4548a
Cysteine-s	0.3874b	0.3898b	0.2045a
Valine	4.3192c	2.8850a	3.2382b
Methionine	1.5411b	0.7779a	0.8216a
Phenylalanine	4.2961c	3.3587b	3.1002a
Isoleucine	4.0152c	2.7146a	2.9863b
Leucine	6.3005c	5.0026b	4.5769a
Lysine	5.2246c	3.6944b	3.4558a
Proline	2.7794b	3.3690c	2.0988a
Tryptophan	0.8253b	0.5602a	0.5454a
Sum	78.3201c	61.9212b	53.6833a
^c TFAA	0.2523a	0.7702b	4.8467c

^aUnfermented SPM. ^bFSPMe with protease added. ^cTotal free amino acid. Values are the means of three determinations. Rows with different letters indicate statistical differences (p<0.001)

Amino Acid Analysis

The results showed a significant (p<0.001) decrease in total amino acid after fermentation of the soybean protein meal. The difference in composition of meal and decomposition and subsequent utilization of amino acids by fermenting micro-organisms and proteolytic enzymes may be responsible (Table 2). It was observed that the contents of sulphur

containing amino acid residues were low (FSPM, 1.88 g/100 g sample; FSPMe, 1.91 g/100 g sample) in fermented sample compared to the control sample. There was no significant difference ($p < 0.001$) in the total acidic amino acid contents (control, 31.98 g/100 g sample; FSPM, 32.19 g/100 g sample; FSPMe, 32.93 g/100 g sample) among all the samples. Total essential amino acids increased significantly ($p < 0.001$) in FSPMe when compared to FSPM and control samples. Figure 2a-c show representative HPLC chromatogram of free amino acid in control, FSPM and FSPMe. The free amino acid content of fermented soybean meals increased significantly ($p < 0.001$) from 0.2523 to 4.8467 g/100 g sample. The amino acid patterns of the FSPM (Table 2) were in close agreement with the values reported by Hong *et al.* (2004), who investigated food soybeans and feed soybean meals from Korea. It was previously reported that lactic acid fermentation of soybean meal resulted in protein hydrolysis (Yu *et al.*, 2008) and increased liberation of free amino acids. The addition of enzyme protease in the fermentation medium contributed significantly in the release of free amino acids (Table 2).

***In vitro* Trypsin Digestibility**

The *in vitro* digestibility of all the samples increased with increasing time of fermentation. However, fermented SPM with addition of protease exhibited the highest increase in *in vitro* trypsin digestibility followed by fermented SPM without protease (Fig. 3). While the *in vitro* digestibility of the SPM sample ranged between 78.5% at 30 min

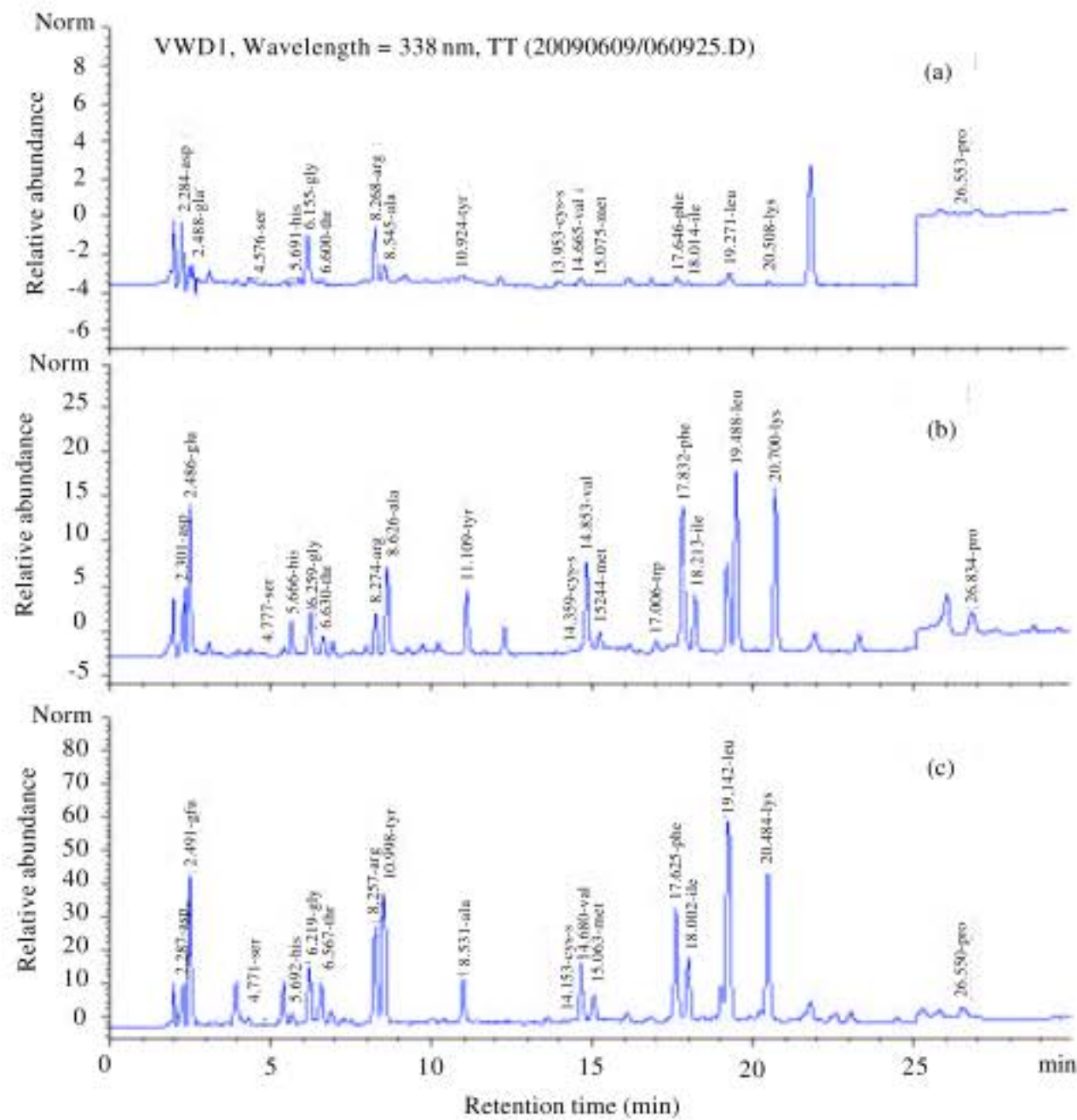


Fig. 2: HPLC chromatograms of free amino acid in (a) control, (b) FSPM and (c) FSPMe

of fermentation to 84% after 2 h of fermentation, the fermented SPM samples ranged between 81% (FSPM) and 82.5% (FSPMe) at 30 min of fermentation to 91.5% (FSPM) and 94% (FSPMe) after 2 h of fermentation. The improvement in *in vitro* protein digestibility caused by fermentation could be attributed to the partial degradation of complex proteins to more simple and soluble products by fermenting microorganisms and proteolytic enzymes (Frias *et al.*, 2008; Chavan *et al.*, 1988).

DPPH Radical-Scavenging Activity

The DPPH radical scavenging activity is a useful and common assay for evaluating the antioxidative ability. At a concentration of 5 mg mL⁻¹, the fermented SPM extracts, regardless of the fermentation medium, exhibited DPPH radical scavenging activity of 69.93% or more (Fig. 4). In addition, extracts of the two fermented samples (FSPM and FSPMe) showed a higher DPPH radical scavenging activity than that of unfermented SPM (Control). The increased DPPH radical scavenging activity observed in this study on fermented SPM

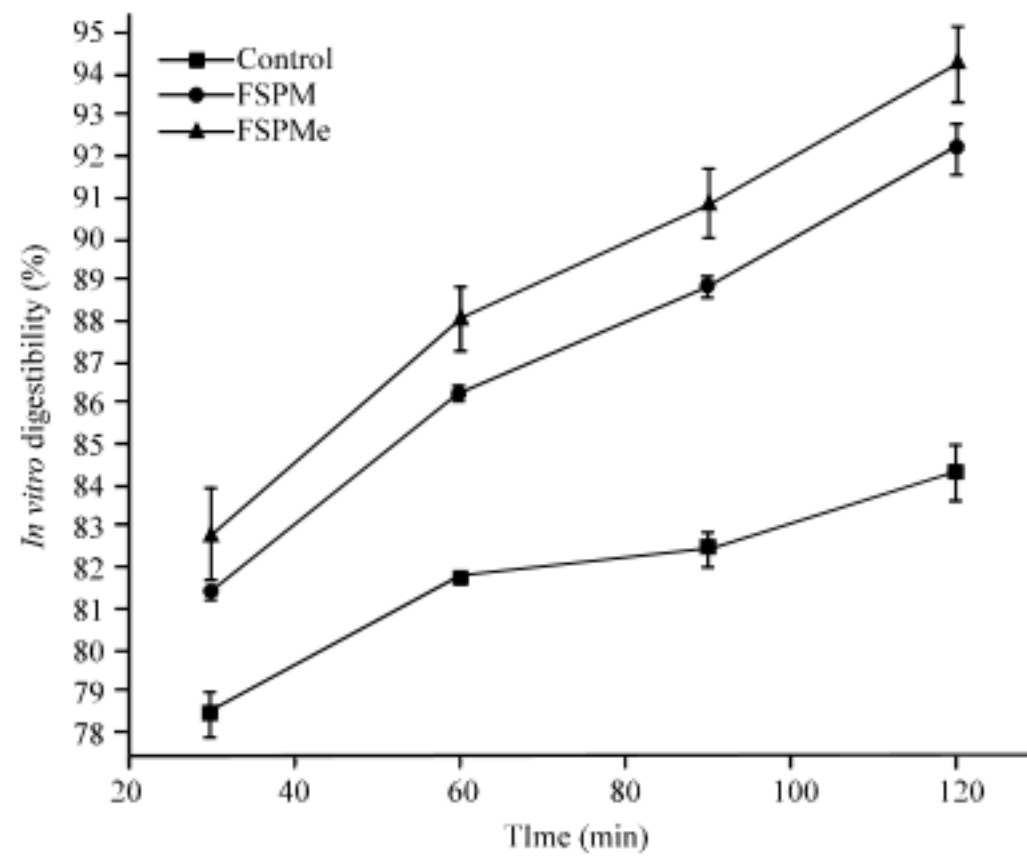


Fig. 3: *In vitro* trypsin digestibility of unfermented and fermented soybean protein meal samples. Values represent the Means±SD of triplicate determinations

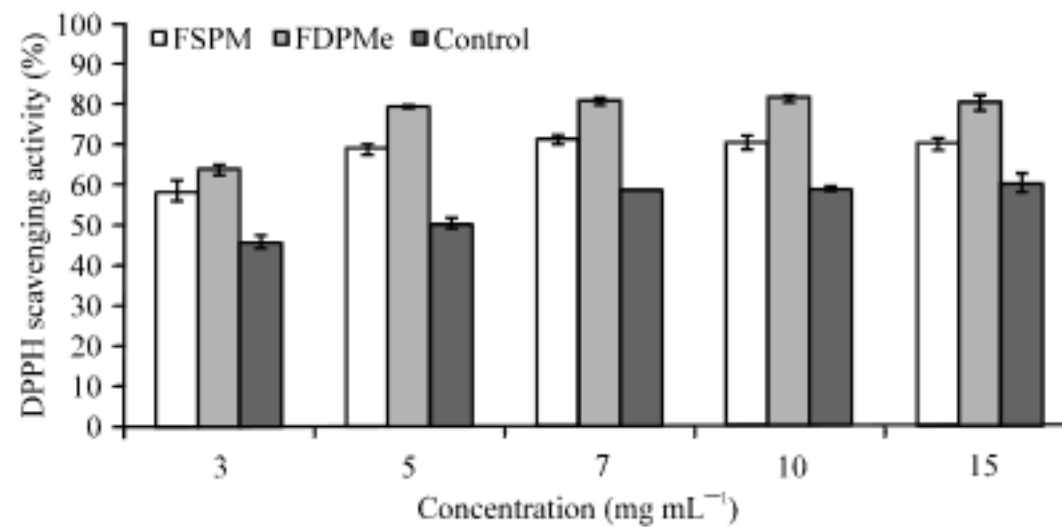


Fig. 4: DPPH radical scavenging activity of unfermented and fermented soybean protein meal samples. Values represent the Means±SD of n = 3 duplicate assays

extracts is in accordance with that observed on fermented soybean products like okara, sufu, miso and Tempeh (Daker *et al.*, 2009; Zhu *et al.*, 2008; Wang *et al.*, 2003; Santiago *et al.*, 1992).

In our study, the FSPMe exhibited the highest DPPH radical scavenging activity and also showed the lowest estimated molecular weight, which signifies a great potential to produce bioactive peptides.

CONCLUSION

Based on the findings obtained from the present study, it can be concluded that enhanced fermentation of soybean meal could improve the nutritional characteristics of soybean meal. The FSPM and FSPMe obtained through fermentation with *Lactobacillus plantarum* Lp6 showed higher mineral contents, improved DPPH radical scavenging activity, enhanced *in vitro* trypsin digestibility, liberation of more free amino acids and formation of low molecular weight peptides. These results have suggested that FSPM and FSPMe were most likely to contain some bioactive peptides with good antioxidant characteristics. Further investigations are going on in our laboratory to optimize the fermentation and identify the active peptides.

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