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**Research Article**

# Production of Bioactive Peptides from Soybean Meal by Solid State Fermentation with Lactic Acid Bacteria and Protease

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**Abstract:** In this study, soybean meal was first solid state fermented with different strains of Lactic Acid Bacteria (LAB). Among the strains used, *Lactobacillus plantarum* Lp6 was selected for further studies because of its highest Degree of Hydrolysis (DH) of protein (2.49±0.08%) in soybean meal after 72 h fermentation. Soybean meal fermented with *L. plantarum* Lp6 can also improve its DPPH radical scavenging and Angiotensin Converting Enzyme (ACE) inhibitory activities. The addition of protease into soybean meal during the fermentation resulted in lowered $IC_{50}$ of DPPH radical scavenging and ACE inhibitory activities, indicating more bioactive peptides were produced during fermentation. Molecular weight distribution analysis revealed the Extracts from Fermented Soybean Meal (EFSM) was mainly composed of oligopeptides. These results indicated that soybean meal fermented with *L. plantarum* Lp6 and protease could be an easy and cheap method to produce functional food.

**Keywords:** Bioactive activity, lactic acid bacteria, protease, solid state fermentation, soybean meal

## INTRODUCTION

Peptides derived from food proteins with bioactive activities, such as anti-hypertension, immunomodulation, antioxidation, antimicrobial action and antithrombosis are recognized as functional food ingredients in preventing lifestyle-related diseases (Inoue *et al*., 2011). These peptides can be released from protein sequences by digestive proteases, microbial or plant enzymes or by fermentation. Currently, the most widely used method of preparing bioactive peptides is adding outer proteases to hydrolyze protein, which has the advantage of conveniently controlling the concentration of enzyme and substrate. However, removal of salts and bitter peptides from enzyme hydrolysate mixture on large scale is very difficult and relatively inefficient (Wang *et al*., 2010).

Fermentation is one of the oldest techniques in food production and preservation. Especially, some bioactive substances may be produced during fermentation, thus the functionalities of foods can be improved. Among the starters used for fermentation, Lactic Acid Bacteria (LAB) are thought to be safe bacteria that have been widely used in food or drink products for many years. Proteases of LAB can hydrolyze various proteins, producing a large number of different oligopeptides (Wang *et al*., 2008). Various bioactive peptides have been produced from different protein sources fermented by LAB, such as milk (Ahn *et al*., 2009; Pan and Guo, 2010), soymilk (Tsai *et al*., 2006) and marine shrimp (Wang *et al*., 2008). However, Compare to the proteases from fungi or plants, the proteolytic activity of LAB is low and proteins could not reach a high DH after fermentation when only LAB was used as starters.

Soybean meal is a co-product from the processing of soybean to produce dietary oil and a high quality source of protein. Soybean protein is also a good source of bioactive peptides and can be used as a health enhancing ingredient in functional foods (Yu *et al*., 2008). The purpose of this study is to develop an easy and cheap method to produce bioactive peptides from soybean meal using lactic acid fermentation and protease, which can not only avoid the adverse factors such as high ash content but also accelerate the production of bioactive peptides and reduce the cost.

## MATERIALS AND METHODS

**Microorganisms and materials:** The strains *Lactobacillus fermentum* B45, *Lactobacillus helveticus* C14, *Lactobacillus plantarum* Lp1, *Lactobacillus plantarum* Lp2, *Lactobacillus plantarum* Lp3, *Lactobacillus plantarum* Lp5, *Lactobacillus plantarum* Lp6, *Lactobacillus delbrueckii* subsp., *bulgaricus* L2,

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*Streptococcus thermophilus* S5, *Lactobacillus casei* LC35, *Lactobacillus acidophilus* LA1, used in this study were obtained from the Culture Collection of Jiangnan University (Wuxi, China). Commercial defatted soybean meal (crude protein concentration 47.6%) was provided by Eastocean Cereals and Oils Co., Ltd. (Zhangjiagang, China). Prior to further use, soybean meal was cleaned and ground into fine flour to pass a 0.4 mm screen. Acid protease (from *Aspergillus niger*, 60000 U/g, working pH: 2.5-6.0, working temperature: 10-55°C) was provided by Sunson Bio-Technology Co., Ltd. (Yinchuan, China). All other reagents and chemicals used in this study were of analytical grade.

**Fermentation:** Soybean flour, freeze-dried cells (De Valdez *et al*., 1983) of each strain to give approx. 7 log cfu/g in flour after inoculation and protease dispersed in distilled water in the amount to adjust moisture content of the mixtures to 55% were mixed in a food processor mixer (DW-25, Better Boiler Ltd., Shanghai, China). After thorough mixing, the mixtures were placed in polyethylene bags (140×200 mm). The bags were vacuum sealed and incubated at 37°C for 72 h.

**Preparation of Extracts from Fermented Soybean Meal (EFSM):** Fermented soybean meal (10 g) was homogenized with 100 mL of distilled water at normal temperature for 20 min. The suspensions were then heated at 98°C for 10 min to inactivate the protease and LAB. The insoluble material was removed by centrifugation at 6000 g for 30 min and filtered through a 0.45 μm membrane filter. The filtrates were used for the determination of pH and Degree of Hydrolysis (DH) of protein. The filtrates were then were freeze-dried and stored at 4°C before further analysis. The lyophilized hydrolysates were named as EFSM.

**Chemical analysis:** The Degree of protein Hydrolysis (DH), defined as the percentage of peptide chain cleaved, was determined by an amino nitrogen analysis using trinitrobenzenesulfonic acid (Adler-Nissen, 1979). pH values were determined with a pH meter (model Delta 320, Mettler-Toledo International Inc).

**DPPH radical scavenging activity:** The scavenging activity of EFSM on DPPH radical was measured according to the method of Shimada *et al*. (1992). Each extract (4 mL, 0.5-8 mg/mL) in methanol was mixed with 1 mL of methanolic solution containing DPPH radical (final concentration of DPPH was 0.2 M). The mixture was shaken vigorously and left in the dark at room temperature for 20 min. The absorbance of the resulting solution was then measured at 517 nm. The scavenging effect on DPPH radical was calculated using the following equation: Scavenging effect (%) = (1-Absorbance of sample at 517 nm/Absorbance of control at 517 nm) ×100. $IC_{50}$ value (mg EFSM/mL) is the effective concentration at which DPPH radicals are scavenged by 50% and was obtained from the plotted graph of scavenging activity against the concentration of EFSM.

**Ferrous ion-chelating activity:** The chelating activity of the extracts on $Fe^{2+}$ was measured according to the method of Decker and Welch (1990). Each EFSM (1 mL) at different concentrations in methanol was mixed with 1.75 mL of deionized water. The mixture was left for reaction with 0.05 mL of $FeCl_2$ (2 mM) and 0.2 mL of ferrozine (5 mM) for 10 min at room temperature and then the absorbance was measured at 562 nm. The capability to chelate $Fe^{2+}$ was calculated by the following equation: Chelating activity (%) = (1-Absorbance of sample at 562 nm/Absorbance of control at 562 nm) ×100.

**Determination of ACE inhibition activity:** ACE inhibitory activity was determined according to the method of Zhang *et al*. (2009). A 50 μL aliquot of a sample solution (EFSM dissolved in distilled water) and 50 μL of a 5 mU ACE solution were added to 50 μL of a 5 mmol/L substrate (HHL) solution in 1 mol/L phosphate buffer at pH 8.3. After incubation at 37°C for 30 min, the reaction was stopped by adding 150 μL of 1 mol/L HCl. The liberated hippuric acid was extracted with 1 mL of ethyl acetate. The mixture was centrifuged and 0.5 mL of the organic phase (ethyl acetate) was transferred to a fresh test tube and evaporated to dryness in a water bath at 100°C. The residue containing hippuric acid was dissolved in 3 mL deionised water and the solution was measured using a UV visible spectrophotometer (UV-1700; Shimadzu Co., Kyoto, Japan) at 228 nm against deionised water as the blank. Inhibition was calculated from the equation: ACE inhibitory activity (%) = [(A-B) ⁄ (A-C)]·100, where A is the absorbance with ACE and HHL without ACE inhibitory sample, B is the absorbance with ACE, HHL and ACE inhibitory sample and C is the absorbance with HHL without ACE and ACE inhibitory sample. The ACE inhibitory activity was also expressed as $IC_{50}$, which was expressed as the amount of EFSM needed to inhibit 50% of the original ACE activity.

**Determination of molecular weight distribution:** Molecular weight distribution of EFSM was determined using a Waters 600 High Performance Liquid Chromatography (HPLC) system, with TSK gel column (2000 SWXL, 300 mm 9×7.8 mm), in combination with 2,487 UV detector and M32 work station. Elution was acetonitrile/water/trifluoroacetic acid (45/55/0.1) at the flow rate of 0.5 mL/min at 30°C. The wavelength of detection was at 220 nm and results were processed with Waters M32 GPC Software.

**Statistical analysis:** Unless otherwise indicated, all results in this study were means of three independent trials±S.D. Data were analyzed using SPSS version 19 (SPSS, Chicago, IL, USA). Results were considered statistically significant at $p<0.05$.

## RESULTS AND DISCUSSION

**Selection of strains:** Bioactive peptides are inactive in the amino acid sequence of original proteins, their bioactivity can be released by proteolytic enzymes during gastrointestinal digestion or food processing (Cheng *et al*., 2008). Many studies have reported that the bioactive activities of milk fermented with LAB, such as antioxidant or ACE inhibitory activities were correlated to the DH of protein (Virtanen *et al*., 2007; Ramchandran and Shah, 2008; Pan and Guo, 2010; Pihlanto *et al*., 2010; Gonzalez-Gonzalez *et al*., 2011). Donkor *et al*. (2005) also found there was a slight positive correlation ($r = 0.60$) between the free amino groups content and ACE inhibition in soymilk fermented with LAB. In our previous study, we found that the ACE inhibitory activity of peanut meal was positively correlated ($r = 0.837$, $p<0.01$) with DH value (Zeng *et al*., 2013). These reports suggest that, for lactic acid fermented soybean meal proteins, it is essential to reach a certain level of DH to allow maximum release of active peptides from inactive protein precursors.

The growth performance of different strains of LAB in soybean meal after 72 h of solid state fermentation was assessed by measuring pH and DH (Fig. 1). Among the strains used, *L. plantarum* Lp6 showed the highest DH of protein (2.49±0.08%). Therefore, this strain was selected for further studies.

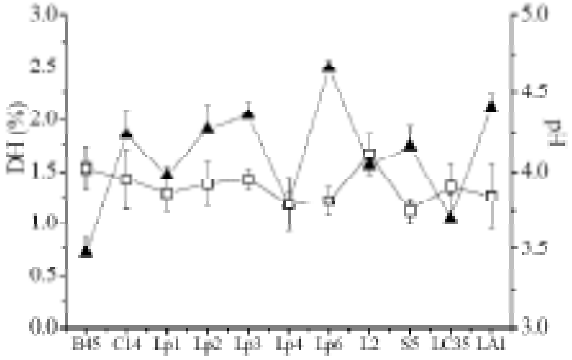


Fig. 1: pH (□) and DH (▲) of soybean meal fermented with different LAB for 72 h
B45: *Lactobacillus fermentum* B45; C14: *Lactobacillus helveticus* C14; Lp1: *Lactobacillus plantarum* Lp1; Lp2: *Lactobacillus plantarum* Lp2; Lp3: *Lactobacillus plantarum* Lp3; Lp5: *Lactobacillus plantarum* Lp5; Lp6: *Lactobacillus plantarum* Lp6, L2: *Lactobacillus delbrueckii* subsp., *bulgaricus* L2; S5: *Streptococcus thermophilus* S5; LC35: LA1: *Lactobacillus casei* LC35, *Lactobacillus acidophilus* LA1

Table 1: Effect of *L. plantarum* Lp6 fermentation on the antioxidant and ACE inhibitory activity of soybean meal

| Fermentation time (h) | DPPH radical scavenging activity[a] (%) | $Fe^{2+}$ chelating activity[a] (%) | ACE inhibitory activity[b] (%) |
|---|---|---|---|
| 0 | 23.43±1.12 | 14.75±0.83 | 13.42±0.07 |
| 12 | 52.39±2.31 | 15.29±0.72 | 37.96±3.21 |
| 24 | 64.15±3.45 | 14.81±1.03 | 59.63±4.15 |
| 36 | 69.30±2.65 | 14.56±0.98 | 73.10±1.87 |
| 48 | 71.07±1.74 | 15.07±0.57 | 81.16±3.11 |
| 60 | 72.12±3.41 | 14.68±0.95 | 84.32±2.34 |
| 72 | 75.46±2.06 | 14.94±1.77 | 85.37±1.95 |

[a]: The concentration was 10 mg/mL; [b]: The concentration was 5 mg/mL

**Development of antioxidant and ACE inhibitory activities during fermentation:** DPPH is a stable free radical and shows maximum absorbance at 517 nm in methanol. When DPPH encounters a proton-donating substance such as an antioxidant, the radical would be scavenged and the absorbance at 517 nm is reduced. Therefore, DPPH is widely used to evaluate the free radical scavenging activity of natural antioxidants (Zhu *et al*., 2006). In Table 1, soybean meal fermented with *L. plantarum* Lp6 could highly improve the DPPH radical scavenging activity. After 72 h fermentation, at 10 mg/mL, the DPPH radicals scavenging activity of EFSM increased from 23.43 to 75.46%. This result is similar with the findings of soybean or cowpea fermented with LAB (Yang *et al*., 2000; Pyo *et al*., 2005), indicating that some metabolites with superior radical scavenging activity might be produced during fermentation.

Iron is an extremely reactive metal and plays a significant role in the oxidation of biological molecules. Chelating agents may serve as secondary antioxidants and stabilize the oxidized form of the metal ions (Gordon, 1990), consequently reducing free radical damage. To better estimate the potential antioxidant potential of EFSM, $Fe^{2+}$ chelating activity of each extract was investigated. As the Table 1 shown, the EFSM from fermented soybean meal did not exhibit higher chelating capacity than soybean meal. Yang *et al*. (2000) reported that soybean broth fermented with LAB could significantly improve its chelating capacity on $Fe^{2+}$. In contrast, Liu *et al*. (2005) found that milk or soymilk fermented with kefir grains did not improve their chelating capacity. Further studies are required in order to know the mechanism responsible for the changes of chelating capacity during the lactic acid fermentation.

In human blood, ACE plays an important role in blood pressure regulation. It raises blood pressure by converting the inactive decapeptide angiotensin I to its active form, angiotensin II, resulting in narrowing of small blood vessels and an increase in blood pressure (Parris *et al*., 2008). Thus, inhibition of ACE can result in a lowering of blood pressure. Lactic acid fermentation can increase the ACE inhibitory activity

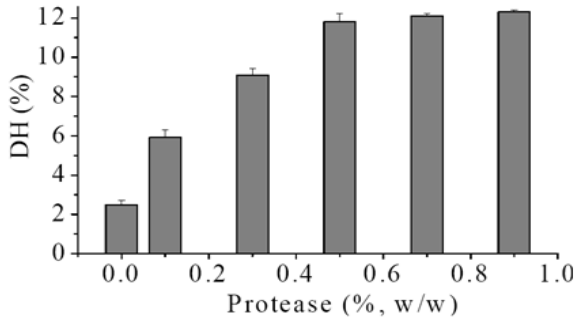


Fig. 2: Effect of protease addition on the Degree Hydrolysis (DH) of protein in soybean meal fermented with *L. plantarum* Lp6 for 72 h

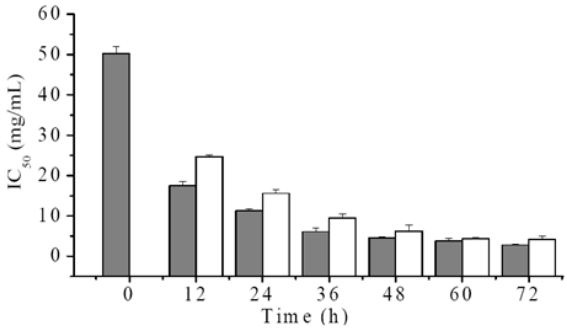


Fig. 3: Effect of protease addition (0.5%, w/w) on the DPPH radical scavenging activity of soybean meal fermented with *L. plantarum* Lp6 during 72 h fermentation

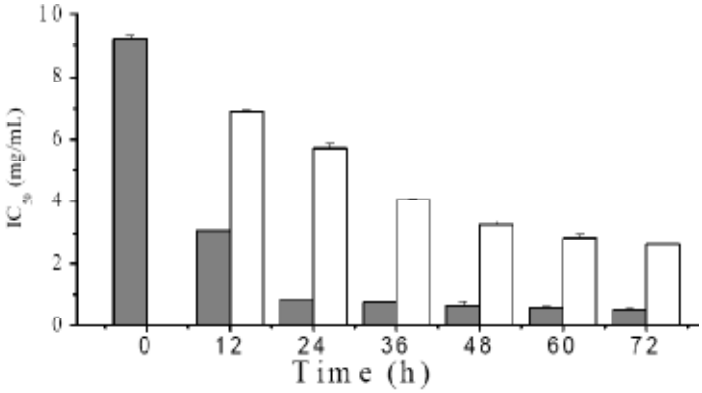


Fig. 4: Effect of protease addition (0.5%, w/w) on the ACE inhibitory activity soybean meal fermented with *L. plantarum* Lp6 during 72 h fermentation

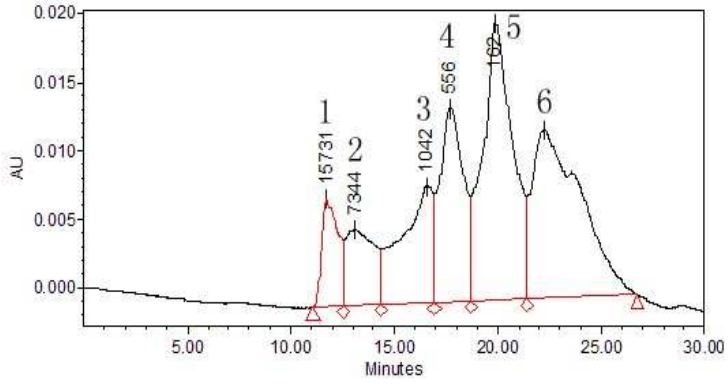


Fig. 5: HPLC chromatogram of EFSM
Addition of protease: 0.5%; The absorbance peaks are numbered as 1, 2, 3, 4, 5 and 6, respectively; EFSM was obtained by addition of protease: 0.5% (w/w) and fermentation for 72 h

of many kinds of foods, such as milk (Ahn *et al*., 2009; Pan and Guo, 2010), sourdough (Rizzello *et al*., 2008), marine shrimp (Wang *et al*., 2008) and soymilk (Donkor *et al*., 2005). In this study, *L. plantarum* Lp6 fermentation could highly improve ACE inhibitory activity of soybean meal, indicating this product may potentially be used as a functional food in prevention and/or treatment of hypertension in human.

**Protease addition:** Compare to the proteases from fungi or plants, the proteolytic activity of LAB is low. We could not obtain a high DH of protein when only LAB was used as starters (Fig. 1). Therefore, in this study, an acid protease was added into the medium at the beginning of fermentation. As shown in Fig. 2, after 72 h fermentation, protease addition could highly increase the DH of soybean meal. At the same time,

Table 2: Molecular weight distribution of EFSM[a]

| Fraction | Start time (min) | End time (min) | Mw (Da) | Area (%) |
|---|---|---|---|---|
| 1 | 11.083 | 12.550 | 14206 | 6.10 |
| 2 | 12.550 | 14.367 | 6377 | 7.54 |
| 3 | 14.367 | 16.933 | 1719 | 12.66 |
| 4 | 16.933 | 18.700 | 543 | 16.03 |
| 5 | 18.700 | 21.383 | 161 | 28.76 |
| 6 | 21.383 | 26.750 | 37 | 28.90 |

[a]: EFSM was obtained by addition of protease: 0.5% (w/w) and fermentation for 72 h

fermentation with protease resulted in lowered $IC_{50}$ of DPPH radical scavenging (Fig. 3) and ACE inhibitory (Fig. 4) activities, indicating that more bioactive peptides were produced during the fermentation. Tsai *et al*. (2006) reported soymilk fermented with LAB and protease could highly increase its ACE inhibitory activity. In our study, we used solid state fermentation. It can be easier and cheaper than their study.

**Molecular weight distribution of EFSM:** Molecular weight is an important parameter reflecting the hydrolysis of proteins. A lot of studies have already shown that the bioactivities of peptides were depended on their molecular weight distribution (Niu *et al*., 2013). The molecular weight distribution of EFSM determined by HPLC method is shown in Fig. 5 and Table 2. It can be seen that many low molecular weight peptides were formed after fermentation. In this study, peptides with a low molecular weight successfully obtained by fermentation and protease hydrolysis probably associated with higher ACE inhibitory activity.

## CONCLUSION

The results obtained in this study indicated that soybean meal is suitable for the growth of LAB during solid state fermentation. LAB have different abilities to hydrolyze the protein in soybean meal. Among the strains used, *L. plantarum* Lp6 has the highest proteolytic activity. The ACE inhibitory and antioxidant activities of sobean meal were also increased after fermentation with *L. plantarum* Lp6. The addition of protease during the fermentation could highly improve DH and bioactive activity of soybean meal. In conclusion, solid state fermentation with LAB could be a cheaper and effective method to improve the nutritional value of soybean meal.

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