

## Production of Angiotensin I Converting Enzyme Inhibitory Peptides from Peanut Meal Fermented with Lactic Acid Bacteria and Facilitated with Protease

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**Abstract:** The aim of this study was to develop a simpler and cheaper technique using a combination of Lactic Acid Bacteria (LAB) fermentation and protease hydrolysis to accelerate the production of bioactive peptides in peanut meal. Firstly, peanut meal was fermented with a total of 14 LAB strains to assay *in vitro* Angiotensin I Converting Enzyme (ACE) inhibitory activity and Degree of Hydrolysis (DH). Among the strains used, *Lactobacillus plantarum* Lp6 was selected for further studies because of its highest ACE inhibitory activity (47.83±4.92%) and DH of protein (2.29±0.51%). The addition of protease during the fermentation could highly improve the soluble protein, DH and ACE inhibitory activity of peanut meal. Molecular weight distribution analysis revealed the Extracts from Fermented Peanut Meal (EFPM) was mainly composed of oligopeptides. The ACE inhibitory activity of EFPM remained stable after pepsin and pancreatin treatments simulating an *in vitro* gastrointestinal digestion. Furthermore, the EFPM exerted potent antihypertensive effect in Spontaneously Hypertensive Rats (SHR) after oral administrations at a dose of 200 or 500 mg/kg body weight. In conclusion, the peanut meal fermented with *L. plantarum* Lp6 and facilitated with protease could serve as a useful antihypertensive product in the prevention and treatment of hypertension.

**Keywords:** ACE inhibitory activity, antihypertensive, fermentation, lactic acid bacteria, peanut meal, protease addition

### INTRODUCTION

Hypertension is a major risk factor for the development of cardiovascular diseases that threatens human health. In human blood, Angiotensin I Converting Enzyme (ACE, EC 3.4.15.1) 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. Synthesized ACE inhibitors, such as captopril, alacepril and lisinopril, are widely used in the treatment of hypertensive patients, but these substances may provoke undesirable side effects (Kapel *et al.*, 2006). Therefore, research and development to find safer, innovative and economical ACE inhibitors is necessary for the prevention and remedy of hypertension.

Peptides derived from food proteins are recognized as functional food ingredients in preventing lifestyle-related diseases. Among them, ACE inhibitory peptides have been shown to reduce blood pressure at a healthy level (Murray and Fitzgerald, 2007). Currently, the most widely used method of preparing ACE inhibitory

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). Another method of preparing ACE inhibitory peptides is fermentation. Various ACE inhibitory 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.

Peanut meal is a co-product from the processing of peanut to produce dietary oil and a high quality source of protein. Peanut protein is also a good source of ACE inhibitory peptides and can be used as a health enhancing ingredient in functional foods (Guang and Phillips, 2009). The purpose of this study is to develop a method to produce ACE inhibitory peptides from peanut 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. Furthermore, the stability of peptides after *in vitro* gastrointestinal digestion which produced during the fermentation and its antihypertensive effect in Spontaneously Hypertensive Rats (SHR) were evaluated.

## MATERIALS AND METHODS

**Microorganisms and materials:** The strains *Lactobacillus reuteri* 62, *Lactobacillus fermentum* B45, *Lactobacillus helveticus* C14, *Bifidobacterium bifidum* B04, *Lactobacillus plantarum* Lp1, *Lactobacillus plantarum* Lp2, *Lactobacillus plantarum* Lp3, *Lactobacillus plantarum* Lp6, *Lactobacillus delbrueckii* subsp. *bulgaricus* L2, *Streptococcus thermophilus* S5, *Lactobacillus casei* LC35, *Lactobacillus casei* LC2, *Lactobacillus acidophilus* LA1, *Lactobacillus acidophilus* LA4 used in this study were obtained from the Culture Collection of Jiangnan University (Wuxi, China). Commercial defatted peanut meal (crude protein concentration 48.3%) was provided by Eastocean Cereals and Oils Co., Ltd. (Zhangjiagang, China). Prior to further use, peanut 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). Pepsin (100000 U/g) and pancreatin (250000 U/g) were purchased from the Shanghai Biochemical Company of China National Pharmaceutical Group (Shanghai, China). ACE from rabbit lung and the ACE synthetic substrate hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma Chemicals Co (St. Louis, MO). All other reagents and chemicals used in this study were of analytical grade.

**Fermentation:** Peanut meal 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 mm×200 mm). The bags were vacuum sealed and incubated at 37°C for 72 h.

**Preparation of Extracts from Fermented Peanut Meal (EFPM):** Fermented peanut 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 filtrate was then lyophilized and used for chemical analyses or fed to SHR.

**Chemical analysis:** The Degree of Hydrolysis (DH) was calculated by determination of free amino groups (h) by reaction with TNBS (Adler, 1979). The total number of amino groups (ht) was determined in samples 100% hydrolysed by treatment with 6 mol/l HCl at 110°C for 24 h. The DH was calculated using the formula  $DH = (h/ht) \times 100$ .

The soluble protein was defined as the protein concentration in the EFPM divided by that in the original peanut meal. Crude protein content ( $N \times 6.25$ ) was determined using the micro-Kjeldahl method.

**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 (EFPM 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:  $ACE \text{ inhibitory activity (\%)} = [(A-B) / (A-C)] \cdot 100$ , 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 EFPM needed to inhibit 50% of the original ACE activity.

**Determination of molecular weight distribution:** Molecular weight distribution of EFPM 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.

**In vitro gastrointestinal digestion:** Simulation of gastrointestinal digestion of EFPM was carried out according to the method of Zhang *et al.* (2009). Briefly

- Samples were incubated with pepsin (5 mg/g of EFPM) for 180 min at a pH of 2.5 and 37°C.

- Samples were incubated with pancreatin (5 mg/g of EFPM) for 240 min at a pH of 8.0 and 37°C.
- Samples were hydrolysed with pepsin (5 mg/g of EFPM) for 180 min at a pH of 2.5 and 37°C followed by hydrolysis with pancreatin (5 mg/g of EFPM) at a pH of 8.0 and 37°C for 240 min. The reactions were then heated in boiling water for 5 min to inactivate the enzyme. After enzymatic treatment, each sample was centrifuged and the supernatant was adjusted to pH = 8.3 and used for the ACE inhibitory activity determination.

**Measurement of antihypertensive activity in SHR:**

Fifteen SHR (12 weeks old, male, 295.3±5.2 g body weight) with tail Systolic Blood Pressure (SBP) over 170 mmHg were obtained from Shanghai SLAC Laboratory Animal Co. Ltd, China. Rats were kept in a room with a 12-h light/dark cycle. Temperature and humidity were controlled at 24±1°C and 50±10%, respectively. The rats were fed a standard laboratory diet (M02, Shanghai SLAC Laboratory Animal Co. Ltd) and tap water was available *ad libitum*. After a one week adaptation, all SHR were divided into three groups: two EFPM groups (200 or 500 mg/kg body weight, dissolved in distilled water) and control group (the same volume of distilled water), with each group of 5 rats. SBP of the rats was measured at 0, 2, 4, 6, 8 and 12 h after administration by the tail-cuff method using an electro-sphygmomanometer (model BP-98A; Softron Co., Tokyo, Japan).

**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:** The ACE inhibitory activity and DH of peanut meal fermented with different LAB are given in Table 1. The capacity of LAB to generate ACE inhibitory peptides in peanut meal during fermentation is strain-dependent, as previously reported in milk by many researchers (Yamamoto *et al.*, 1994; Ahn *et al.*, 2009; Pihlanto *et al.*, 2010). Among the strains used, *L. plantarum* Lp6 showed the highest ACE inhibitory activity (47.83±4.92%) and DH of protein (2.29±0.51%). Therefore, this strain was selected for further studies.

When used as starter, LAB is able to synthesise cell-surface proteinases, which can hydrolyse proteins and release several kinds of peptides in the medium. Since the peptides are not all used by bacteria for their growth, a large amount of peptides can accumulate during fermentation (Leclerc *et al.*, 2002). Many studies have reported that the ACE inhibitory activity of milk

Table 1: Angiotensin I Converting Enzyme (ACE) inhibitory activity and Degree of Hydrolysis (DH) of peanut meal fermented with different Lactic Acid Bacteria (LAB) for 72 h

Bacteria strain	DH (%)	ACE inhibitory activity (%) <sup>1</sup>
1. <i>L. reuteri</i> 62	0.92±0.41	11.53±2.50
2. <i>L. fermentum</i> B45	1.83±0.19	36.62±3.74
3. <i>L. helveticus</i> C14	1.75±0.74	32.35±5.91
4. <i>B. bifidum</i> B04	0.89±0.23	19.32±3.79
5. <i>L. plantarum</i> Lp1	1.47±0.24	27.64±2.82
6. <i>L. plantarum</i> Lp2	2.11±0.62	41.75±8.75
7. <i>L. plantarum</i> Lp3	1.04±0.10	12.45±6.07
8. <i>L. plantarum</i> Lp6	2.29±0.51	47.83±4.92
9. <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> L2	1.57±0.14	15.81±1.37
10. <i>S. thermophilus</i> S5	1.38±0.37	18.56±2.86
11. <i>L. casei</i> LC35	2.04±0.26	20.75±4.18
12. <i>L. casei</i> LC2	0.87±0.09	17.61±1.72
13. <i>L. acidophilus</i> LA1	1.15±0.32	9.02±4.35
14. <i>L. acidophilus</i> LA4	0.76±0.13	5.76±3.41

<sup>1</sup>: The concentration of Extracts from Fermented Peanut Meal (EFPM) in the assay was 5 mg/mL

Table 2: Effect of protease addition on the Degree of Hydrolysis (DH), soluble protein and IC<sub>50</sub> of Angiotensin I Converting Enzyme (ACE) of peanut meal fermented with *L. plantarum* Lp6

Protease addition (%) <sup>1</sup>	DH (%)	Soluble protein (%)	IC <sub>50</sub> (mg/mL)
0	2.49±0.23	5.05±0.33	4.85±0.17
0.1	5.92±0.37	7.26±1.21	1.47±0.21
0.3	9.08±0.35	11.35±2.08	0.82±0.13
0.5	11.79±0.43	16.37±3.21	0.79±0.09
0.7	12.1±0.12	18.62±1.66	0.64±0.11
0.9	12.3±0.09	19.74±2.76	0.62±0.06

<sup>1</sup>: Mean the ratio of weight (w/w) of acid protease and peanut meal

fermented with LAB was correlated to the DH of protein (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 the present study, we found that the ACE inhibitory activity of peanut meal was positively correlated with DH value (r = 0.837, p<0.01) based on Pearson's correlation analysis, suggesting that, for peanut meal proteins, it is essential to reach a certain level of DH to allow maximum release of active peptides from inactive protein precursors.

**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 (Table 1). Therefore, in this study, an acid protease was added into the medium at the beginning of fermentation. As shown in Table 2, after 72 h fermentation, protease addition could highly increase the content of water soluble protein and DH of peanut meal. At the same time, fermentation with protease resulted in lowered IC<sub>50</sub> of ACE from 4.85 to 0.62 (0.9% acid protease) mg powder/mL, indicated that more ACE inhibitory peptides were produced during the fermentation.

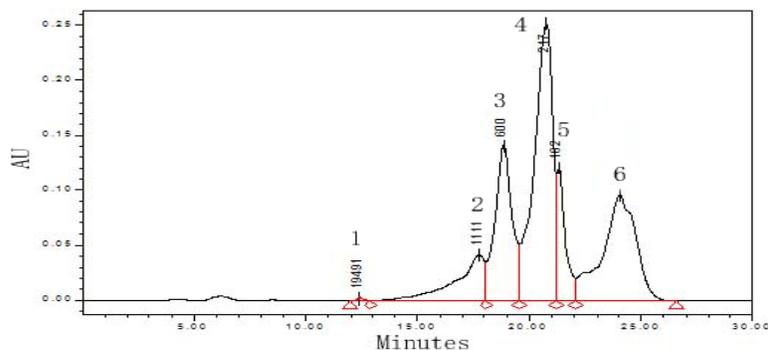


Fig. 1: HPLC chromatogram of EFPM. The absorbance peaks are numbered as 1, 2, 3, 4, 5 and 6

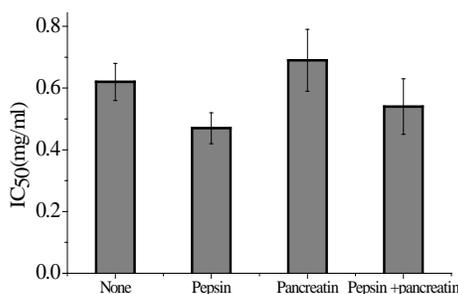


Fig. 2: Effect of gastrointestinal proteases treatments on ACE inhibitory activities of EFPM

Table 3: Molecular weight distribution of EFPM

Fraction	Retention time (min)	Molecular weight (Da)	Ratio (%)
1	12.373	19491	0.15
2	17.718	1111	9.84
3	18.868	600	19.16
4	20.761	217	36.96
5	21.314	162	8.22
6	24.062	<100	25.67

**Molecular weight distribution of EFPM:** 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 EFPM determined by HPLC method is shown in Fig. 1 and Table 3. It can be seen that many low molecular weight peptides were formed after fermentation. To date, the length of most of ACE inhibitory peptides which have been reported was between 2 and 15, with molecular weight ranging from 200-1500 Da (Li, 2005). Guang and Phillips (2009) reported a small peptide (Lys-Ala-Phe-Arg) derived from peanut protein hydrolysates showed high ACE inhibitory activity. In this study, peptides with a low molecular weight successfully obtained by fermentation and protease hydrolysis probably associated with higher ACE inhibitory activity.

**In vitro gastrointestinal digestion:** The ACE inhibitory activity is not the only criterion for select

antihypertensive peptides. To exert an antihypertensive effect, the peptides should pass the intestinal barrier to arrive in the blood in an active form (Balti *et al.*, 2010). Simulated gastric digestion *in vitro* provides a practical and easy process to imitate the resistance of ACE inhibitory peptides during gastrointestinal enzymes digestion (Zhang *et al.*, 2009). Treatments of EFPM by pepsin and pancreatin are shown in Fig. 2. The ACE inhibitory activity of EFPM increased markedly by decreased  $IC_{50}$  from  $0.62 \pm 0.06$  to  $0.47 \pm 0.05$  mg/mL by hydrolysis with pepsin. ACE inhibitory activity of EFPM decreased by the increase of  $IC_{50}$  from  $0.62 \pm 0.06$  to  $0.69 \pm 0.10$  mg/mL for pancreatin and to  $0.54 \pm 0.09$  mg/mL for pepsin and pancreatin by hydrolysis. The enzyme digested EFPM still exhibited high ACE inhibitory activity, indicating this peptide may potentially be used as a functional food in prevention and/or treatment of hypertension.

**Antihypertensive activity of EFPM:** Milk fermented with LAB has been widely reported to reduce blood pressure of SHR or hypertensive humans after oral administration (Tsai *et al.*, 2008). To the best of our knowledge, no study has been conducted on the antihypertensive activity of peanut meal fermented with LAB. Some peptides with potent ACE inhibitory activity *in vitro* or intravenously are found to be inactive if given orally (Erdmann *et al.*, 2008). For the practical purpose of utilising food materials as a physiological modulator, it is necessary to confirm the antihypertensive effect of orally administrated EFPM in SHR.

Figure 3 shows the changes of SBP measured in SHR during a 12-h observation period following oral administration of two different doses (200 and 500 mg/kg of body weight) of EFPM or distilled water. Compared with the control group, the two doses (200 and 500 mg/kg of body weight) of EFPM were found to cause a significant decrease of SBP in SHR after oral administration. For both doses, the greatest decreases were found at 4 h after oral administration, which were  $15.4 \pm 7.5$  mmHg,  $23.9 \pm 5.8$  mmHg, respectively. This antihypotensive effect was no longer observed at the end of the observation period (12 h). The results

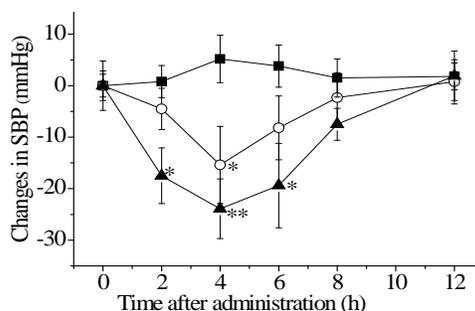


Fig. 3: Effect of single oral administration of EFPM on Systolic Blood Pressure (SBP) of 12-week spontaneously hypertensive rats (SHR, 5 rats per group): distilled water (■); 200 mg/kg body weight of EFPM (○); 500 mg/kg body weight of EFPM (▲); Asterisk indicate significant difference from control at each time interval (\*p<0.05, \*\*\*p<0.01) with Student's t-test

confirm that EFPM exerted potent antihypertensive activities in SHR after oral administration. This same physiological effect on antihypertension for humans is expected.

### CONCLUSION

The results obtained in this study indicate the development of ACE inhibitory activity in peanut meal fermented by LAB was significant correlated with the DH of protein. Among the strains used, *L. plantarum* Lp6 was the most suitable for the fermentation of peanut meal, for its highest ACE inhibitory activity and DH of protein. The addition of protease during the fermentation could highly improve the soluble protein, DH and ACE inhibitory activity of peanut meal. In addition, the ACE inhibitory activity of EFPM remained stable after an *in vitro* gastrointestinal digestion and exhibited antihypertensive activity in SHR after oral administration. In conclusion, this fermented peanut meal was expected to be a useful ingredient in functional food for prevention of hypertension in human. Further detailed studies on isolation and purification of peptide from EFPM as well as the antihypertensive activity in human are needed.

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