Journal of Marine Science and Technology

Volume 22 | Issue 4 Article 15

PROTEOLYSIS AND LACTOBACILLUS FERMENTATION EFFECTS ON THE ISOFLAVONES BIOTRANSFORMATION AND REMOVAL OF ANTI-NUTRITIONAL FACTORS OF SOY BEAN

Li-Jung Yin
Department of Seafood, National Kaohsiung Marine University, Kaohsiung, Taiwan, R.O.C

Hsueh-Ming Tai
Department of Food and Nutrition, Providence University, Taichung, Taiwan, R.O.C.Nugen Bioscience (Taiwan) Co., Ltd., Taichung Industry Park, Taichung,Taiwan, R.O.C

Hui-Hung Lee
Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.

Shann-Tzong Jiang
Department of Food and Nutrition, Providence University, Taichung, Taiwan, R.O.C Department of Food Science, National Taiwan Ocean University, Keelung,Taiwan, R.O.C., stjiang@pu.edu.tw

PROTEOLYSIS AND LACTOBACILLUS FERMENTATION EFFECTS ON THE ISOFLAVONES BIOTRANSFORMATION AND REMOVAL OF ANTI-NUTRITIONAL FACTORS OF SOY BEAN

Acknowledgements

This study was supported by a grant (NSC 102-2221-E126-001) from the National Science Council, Taiwan.

Journal of Marine Science and Technology, Vol. 22, No. 4, pp. 525-530 (*2014*)
DOI: 10.6119/JMST-014-0613-1

PROTEOLYSIS AND *LACTOBACILLUS* FERMENTATION EFFECTS ON THE ISOFLAVONES BIOTRANSFORMATION AND REMOVAL OF ANTI-NUTRITIONAL FACTORS OF SOY BEAN

Li-Jung Yin[1], Hsueh-Ming Tai[2, 3], Hui-Hung Lee[4], and Shann-Tzong Jiang[2, 4]

Key words: isoflavones, *Lactobacillus* fermentation, soybean, anti-nutritional factors

ABSTRACT

To investigate the effects of proteolysis and *Lactobacillus johnsonii* (LAB) fermentation on the removal of anti-nutritional factors and isoflavones biotransformation, soy bean was hydrolyzed by *Bacillus subtilis* YJ1 proteases and cellulases and further fermented with *Lactobacillus johnsonii*. Increases in soluble proteins, peptides, free amino acids, daidzin and genistin ($p < 0.01$) were observed after 10 hr hydrolysis at 50°C. Although no further increases in soluble proteins and peptides ($p < 0.01$) were observed after 24 hr *Lactobacillus johnsonii* fermentation in both 5-L and 120-L fermentors, substantial transformation from daidzin and genistin to daidzein and genistein was obtained ($p < 0.01$). Most of the anti-nutritional factors such as stachyose, raffinose, glycinin and β-conglycinin were not detected, suggesting that the hydrolysis and further *Lactobacillus johnsonii* fermentation could effectively remove the anti-nutritional factors and transform most of isoflavones into aglycones.

I. INTRODUCTION

Soybeans are an abundant and relatively inexpensive source of proteins that are widely recognized for their high nutritional value and excellent functional properties. Their major proteins (greater than 85%) are β-conglycinin and glycinin. The latter does not contain any carbohydrates, whereas conglycinin is a glycoprotein containing approximately 4% carbohydrate (mainly mannose moieties). Heating, acidic and enzymatic hydrolysis have been extensively applied to improve the solubility and other functional properties of soy protein products [22]. Their protein hydrolysates are physiologically better than the intact proteins because their intestinal absorption appears to be more effective [35]. The enzymatic process involves commercial enzymes or enzymes from microorganism such as *Mucor* sp., *Apergillus oryzae*, *Rhizopus* sp., *Bacillus natto* and *B. subtilis* [31]. One of the simplest ways of producing food grade of hydrolyzed proteins is to use *Lactobacillus johnsonii*, which are generally recognized as safe and are traditionally used to ferment raw materials of vegetable and animal origin. The use of *Lactobacillus johnsonii* in fermented soy products has attracted researcher's attention with study on bacterial growth, end-product formation and taste [11]. Several studies on metabolism of α-galactosyl oligosaccharides by *Lactobacillus johnsonii* and *Bifidobacterium* strains have been reported [10, 11], but there is lack of detailed information in the literature about the proteolytic activity of *Lactobacillus johnsonii* on soybean proteins.

The presence of raffinose oligosaccharides (RO), particularly raffinose and stachyose, in soybean might cause gastric distress in humans, since these sugars are utilized with extensive gas production by microbial flora in the lower intestinal tract [14, 29]. Consequently, demands for α-galactoside-free soybean products are rapidly rising. Among the techniques proposed to reduce RO contents in soy products, the enzymatic processing by α-galactosidases has been considered to be the most effective [30].

Isoflavones, abundant in soybeans, have been reported to have various health benefits related to their estrogenic activities, including reduced risk of cardiovascular disease, lower

Paper submitted 08/30/13; revised 04/08/14; accepted 06/13/14. Author for correspondence: Shann-Tzong Jiang (e-mail: stjiang@pu.edu.tw).
[1] *Department of Seafood, National Kaohsiung Marine University, Kaohsiung, Taiwan, R.O.C.*
[2] *Department of Food and Nutrition, Providence University, Taichung, Taiwan, R.O.C.*
[3] *Nugen Bioscience (Taiwan) Co., Ltd., Taichung Industry Park, Taichung, Taiwan, R.O.C.*
[4] *Department of Food Science, National Taiwan Ocean University, Keelung, Taiwan, R.O.C.*

rates of prostate, breast and colon cancers and improved bone health. In soybeans, they exist predominantly in glucoside forms, rather in aglycon forms [13]. Their content and composition vary with types of soybean-based foods, depending upon processing techniques such as heat treatment, de-foaming, water soaking, enzymatic hydrolysis and microbial fermentation [33]. The major dietary isoflavones, daidzein and genistein, have estrogen-like activity and are classified as phytoestrogens [12]. Recent research has shown that isoflavone aglycones are absorbed faster and in higher amounts in humans than their respective glucosides [25]. Tsangalis *et al.* [27] reported that β-glucosidase from Bifidobacteria in soy milk was capable of converting glycosides to their aglycons. Attempts have been made to increase purposely the aglycone forms mediated by β-glucosidase or microbial fermentation [28]. Recent studies have shown that soybean-derived peptides may have anti-hypertension [32], anti-cancer [9], lowering cholesterol [4] and immune-stimulation activities [5]. Compared with many studies of the enzymes on the functional and biological properties of soybean proteins, there is limited information of the proteolysis and subsequent *Lactobacillus johnsonii* fermentation on isoflavones that are associated with many soybean products and ingredients.

This study aimed to investigate the effects of hydrolysis by microbial proteases and cellulases and *Lactobacillus johnsonii* fermentation on the elimination of anti-nutritional factors and isoflavones biotransformation of soybean.

II. MATERIALS AND METHODS

1. Experimental Design

The soybean, purchased in Taichung, Taiwan, was stored at room temperature. It was immersed in water overnight, ground with a grinder and boiled. The resulted samples were subjected to proteolysis and further *Lactobacillus johnsonii* fermentation (Fig. 1).

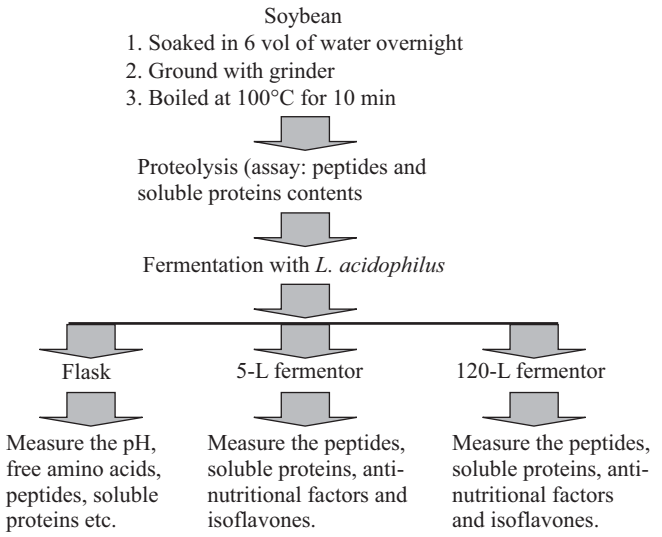

Fig. 1. Experimental design.

2. Protease, Bacteria and Media

Protease (from *Bacillus subtilis* YJ1) was purchased from Nugen Bioscience (Taiwan) Company (Taichung, Taiwan). *Lactobacillus johnsonii* BCRC 17010 was obtained from Bioresouce Collection and Research Center (BCRC) at Food Industry Research and Development Institute (Hsinchu, Taiwan). The bacterial strains were stored in 50% glycerol at -80°C. When activated, it was transferred twice using MRS medium before use.

3. Proteolysis of Soybean

After being immersed in distilled water (1:6) for 10 hr, the soaked soybean was ground by using a grinder (PB1B Penguin Easy Adjustment Power Grinder, Global Bear Co., Taiwan) and then boiled for 10 min. Samples were hydrolyzed with 2% of protease powder at 50°C for 10 hr. The degree of hydrolysis (DH) was evaluated by measuring the contents of soluble proteins, peptides, and/or free amino acids of hydrolysates or *Lactobacillus johnsonii* fermented hydrolysates.

4. Determination of Soluble Proteins

The soluble proteins were determined according to Lowry *et al.* [20]. After 20 min centrifugation at 4°C, 5000 × g, 0.3 mL distilled water and 5 mL alkaline reagent (containing 0.1 N NaOH and 2% Na_2CO_3) were added to 0.2 mL of supernatants and then stood at room temperature for 10 min. Finally, 0.5 mL Folin reagent was added and reacted at room temperature for 30 min. The absorbance at 660 nm was measured. The soluble proteins were calculated based on the standard curve constructed by using bovine serum albumin as standard.

5. Determination of Peptides

Peptides were determined according to o-phthaldialdehyde method [7]. The content of peptides was calculated, based on the standard curve constructed by using Leu–Gly as standard.

6. Determination of Free Amino Acids

Free amino acids were analyzed by Amino Acid Analyzer (Hitachi, Japan) after samples were pretreated according to Konosu *et al.* [15].

7. Lactobacillus Johnsonii Fermentation

The resulted hydrolysate was sterilized at 121°C for 20 min and then fermented with 1% *Lactobacillus johnsonii* at 37°C for 24 hr. After adjusting the pH of fermented hydrolysate to 7.2, it was spray-dried with 6% of dextrin at an inlet temperature of 130°C and outlet temperature of 75-85°C. The spray-dried powder was subjected to the following assays.

8. Extraction of Isoflavones

One g of spray-dried sample was dissolved in a mixture of

10 mL 0.1 N HCI and 40 mL methanol/water (85/15). The resulted sample was then refluxed in a cooling-condensing system for 2 hr and filtered through a Whatman's filter paper. It was neutralized with 3.0% NaOH and filtered again using the same type of filter paper. The volume of extract was set to 100 mL with methanol/water (85/15). After 5 min centrifugation at 3,000 × g, supernatant was passed through a PTFE 4 mm, 0.45 μm membrane and subjected to Liquid Chromatography (LC) analysis.

9. Liquid Chromatography

The extraction of isoflavones, including daidzin, genistin, daidzein, and genistein was performed according to two references [3, 17]. Isoflavones standards were purchased from Sigma Chemicals Co. (St. Louis, MO) and prepared using HPLC grade ethanol.

Isoflavones quantification was carried out on a high performance liquid chromatography (HPLC - Shimadzu®), in a system provided with auto sampler (SIL-10AF), diode array ultraviolet (UV) visible detector (SPD-10MA), quaternary pump, vacuum degasser, and Hypersil ODS C18 (250 mm × 4.6 mm) reverse-phase column (Supelco®). All reagents used in isoflavones extraction and HPLC analyses were filtered through a 0.22 μm membrane (PTFE – Millipore).

HPLC isocratic elution [acetic acid-water 60% (2:98 v/v - solvent A) and methanol-acetonitrile 40% (80:20 v/v - solvent B)] was used to isolate the isoflavones in 40 min with a flow rate of 1.1 mL/min. The diode array UV-visible detector was set at a wavelength of 262 nm to detect daidzin, genistin, daidzein and genistein. The identification of isoflavones was confirmed by HPLC retention time.

10. Extraction of Raffinose and Stachyose

Five grams of spray-dried sample were placed in Erlenmeyer 100 and added 25 mL of 60% ethanol. The resulted samples were shaken in water bath at 50°C for 15 min. After cooling down to room temperature, it was centrifuged at 10,000 × g for 5 min at room temperature. The supernatant was evaporated and re-dissolved twice and then cleaned up by sequentially filtering through a Sep-Pak plus C18 cartridge and 0.45 μm membrane filter before injection onto HPLC.

11. HPLC System

The HPLC system (Agilent 1100 series, Agilent Technologies, Wilmington, DE, USA) with automatic sampler and RID detector (Agilent Model 1362A) was used in this experiment. A Zorbax carbohydrate column (4.6 × 100 mm, Diameter: 5 μm particle size, Agilent Technologies, Wilmington, DE, USA) was employed to separate raffinose and stachyose using a mobile phase of acetonitril (CAN):H_2O (70:30) with a flow rate of 1.2 mL/min (in a 92 bar isocratic system) at 35°C. Various concentrations of raffinose and stachyose (6, 12 and 18 mg/mL) from Sigma (St. Louis, MO, USA) were used as working standards.

12. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Sadeghi *et al.* [23]. Gels were stained with Coomassie Brilliant Blue R-250 (0.05%, w/v) in methanol-acetic acid-water and destained in the same solution without Coomassie Brilliant Blue. Conglycinin α' (MW 57,000~72,000), conglycinin α (MW 57,000~68,000), conglycinin β (MW 42,000~52,000), glycinin acidic polypeptides (MW 38,000), glycinin basic polypeptides (MW 20,000) were used as standards to estimate the molecular weight ranges of polypeptides in each fraction, as well as to identify the subunits of the major soybean proteins.

13. Statistical Analysis

One-way analysis of variance (ANOVA) was run using the Statistical Analysis System ([SAS/STAT], Release 8.0; Carry, NC, USA). Duncan's multiple range test was used to determine the significance of differences within treatments. For each treatment, three replicates were measured and the mean values were calculated. Values were considered to be significantly different when $p < 0.01$.

III. RESULTS AND DISCUSSION

1. Changes in Soluble Proteins and Peptides during 10 hr Proteolysis at 50°C and Further 24-hr *Lactobacillus johsonii* Fermentation at 37°C

As shown in Table 1A, significant increases in soluble proteins, peptides and free amino acids during 10 hr proteolysis at 50°C (increased to 23.9, 24.2, 8.6 mg/mL, respectively) were observed ($p < 0.01$), while the pH decreased to 5.8. The decrease of pH was obviously due to the production of lactic acid by *Lactobacillus johnsonii*. These phenomena suggested that the breaking down of the macromolecular proteins into small molecules peptides and free amino acids occurred during 10 hr proteolysis.

For further investigating the effects of the combination use of proteolysis with LAB fermentation (5-L and 120-L fermentors) on the pilot scale's hydrolysis of soy proteins, the soy proteins were hydrolyzed at 50°C for 10 hr and then subjected to *Lactobacillus johnsonii* fermentation at 37°C for another 24 hr. Significant decrease in pH and increases in soluble proteins and peptides ($p < 0.01$) were observed after 24 hr fermentation in 5-L and 120-L fermentors at 37°C (Table 1B and 1C). Further decrease in pH was considered to be due to the formation of lactic acid and other organic acids during *Lactobacillus johnsonii* fermentation.

During 10 hr hydrolysis at 50°C in flask, 5-L and 120-L fermentors, soluble proteins increased from 3.1, 4.1 and 8.3 mg/mL to 23.9, 25.4, 27.9 mg/mL, while the peptides increased from 5.2, 6.1 and 5.8 to 24.2, 21.4 and 25.3 mg/mL, respectively (Table 1A-1C). No significant differences in both soluble proteins and peptides ($p < 0.01$) were observed among flask scale, 5-L and 120-L fermenters (Table 1A-1C).

Table 1. Changes in degree of hydrolysis (DH) and pH of soybean, A: with various hydrolysis times at 50°C; B: before/after 10 hr hydrolysis at 50°C and 24 hr *L. johnsonii* fermentation in 5-L fermentor at 37°C; and C: before/after 10 hr hydrolysis at 50°C and 24 hr *L. johnsonii* fermentation in 120-L fermentor at 37°C.

A

	0	5	10
Soluble proteins (mg/mL)	$3.1 \pm 0.3^{c**}$	17.8 ± 1.3^{b}	23.9 ± 1.5^{a}
Peptides (mg/mL)	5.2 ± 0.2^{c}	9.5 ± 0.8^{b}	24.2 ± 0.5^{a}
Free amino acids (mg/mL)	1.6 ± 0.2^{c}	4.5 ± 0.2^{b}	8.6 ± 0.2^{a}
pH	7.6 ± 0.2^{a}	6.3 ± 0.2^{b}	5.8 ± 0.3^{c}

B

Parameters	Before hydrolysis	After hydrolysis	After fermentation*
Soluble proteins (mg/mL)	$4.1 \pm 0.6^{c**}$	25.4 ± 2.8^{b}	29.8 ± 1.5^{a}
Peptides (mg/mL)	6.1 ± 0.8^{c}	21.4 ± 1.6^{b}	27.9 ± 2.5^{a}
pH	6.8 ± 0.4^{a}	5.7 ± 0.2^{b}	3.8 ± 0.2^{c}

C

Parameters	Before hydrolysis	After hydrolysis	After fermentation*	Spray-dried
Soluble proteins (mg/mL)	8.3 ± 0.8^{d}**	27.9 ± 2.1^{c}	29.4 ± 1.8^{b}	349.0 ± 4.8^{a}
Peptides (mg/mL)	5.8 ± 0.8^{d}**	25.3 ± 1.2^{c}	27.5 ± 1.4^{b}	198.3 ± 3.4^{a}
pH	7.0 ± 0.3^{a}	5.9 ± 0.2^{b}	3.9 ± 0.2^{d}	4.5 ± 0.4^{c}

*: After 10 hr proteolysis at 50°C and 24 hr *Lactobacillus johnsonii* fermentation at 37°C.

**: Values with different superscript lowercase letters in the same row differ significantly ($p < 0.01$).

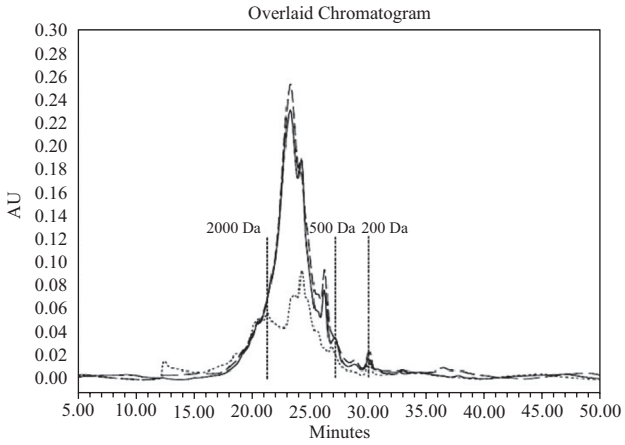

Fig. 2. Profile of the molecular weight distribution on HPLC chromatography (……..: soybean without hydrolysis; ------- : soybean hydrolysate; ——— : 24 hr *Lactobacillus johnsnoii* fermented soybean hydrolysate; AU: Intensity of absorbance).

2. Molecular Weight Distribution on HPLC Chromatography of Soy Bean before/after Proteolysis and after Subsequent *Lactobacillus johnsonii* Fermentation in 120-L Fermentor

According to HPLC profile, the molecular weights of polypeptides after 10 hr proteolysis and 24 hr *Lactobacillus johnsonii* fermentation were between 250 and 2500 Da (Fig. 2), suggesting obvious hydrolysis occurred during proteolysis and further *Lactobacillus johnsonii* fermentation. These results further confirmed the increases of soluble proteins and peptides during proteolysis (Table 1A-1C) and *Lactobacillus johnsonii* fermentation (Table 1B and 1C). The polypeptides digested into oligopeptides (250-2500 Da) might be due to the proteolysis and further LAB fermentation. As we know, many peptides with some specific physiological activities have been identified from the proteolyzed proteins [19]. The physiological activities of these peptides inside the polypeptides are not revealed until they are split out during proteolysis. Some functional peptides isolated from milk, fish proteins, and soybean hydrolysates were found to be able to lower blood pressure [18], regulate serum cholesterol [34] and stimulate calcium absorption [1]. From the data shown in Fig. 2, the peptides contained 2~20 of amino acids and may have the functions mentioned above. This part of study is on-going in our laboratory currently.

3. Effects of Proteolysis and *Lactobacillus johnsonii* Fermentation on the Biotransformation of Isoflavones Into Aglycones

Increases in daidzin (from 26.87 to 31.67 mg/100 g) and genistin (from 41.17 to 53.99 mg/100 g) ($p < 0.01$) were observed after 10 hr hydrolysis at 50°C (Fig. 3A). This might be due to the rupture of polypeptides and consequently releases of the isoflavones embedded inside the protein molecules. However, substantial decreases in daidzin (from 31.67 to 3.08 mg/100 g) and genistin (from 53.99 to 4.49 mg/100 g), and increases in daidzein (from 13.91 to 30.78 mg/100 g) and genistein (from 13.23 to 35.12 mg/100 g) (isoflavone aglycones) ($p < 0.01$) occurred during the further 24 hr *Lactobacillus johnsonii* fermentation at 37°C (Fig. 3A). It is obvious that the isoflavones were biotransformed into aglycone forms. The same biotransformation trend of soy bean isoflavones

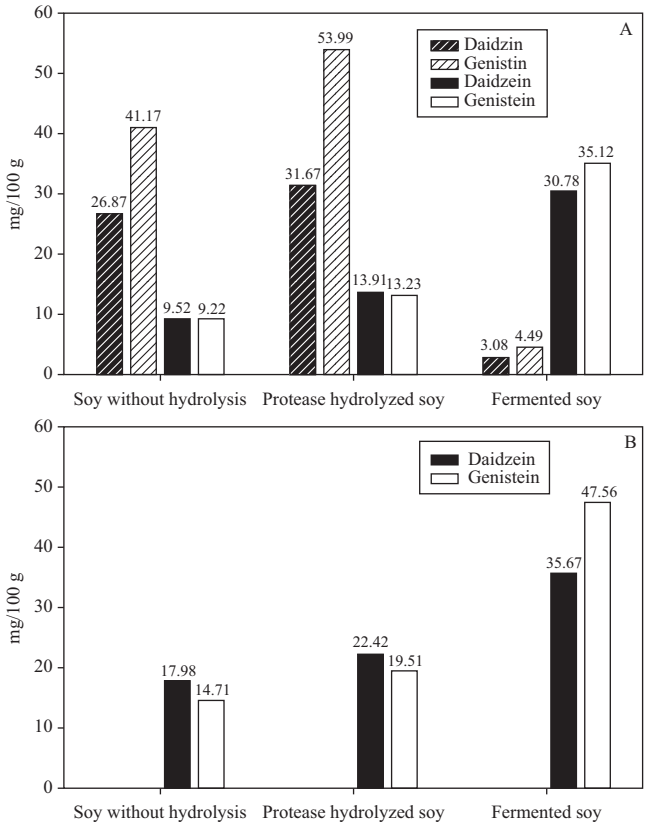

Fig. 3. Contents of isoflavones (mg/100 g) in soybean, proteolyzed soybean and 24 hr *Lactobacillus johnsonii* fermented hydrolysate (A: from laboratory scale, 5-L fermentor; B: data from large scale, 120-L fermentor).

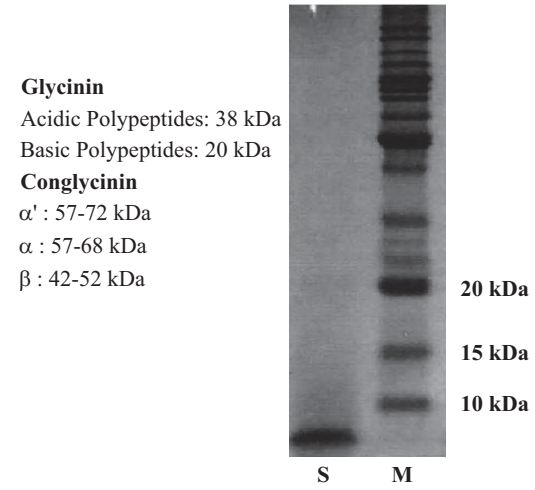

Fig. 4. Components of *Lactobacillus johnsonii* fermented soy bean peptides (no any bands was found on the MW higher than 20 kDa; S: sample; M: marker).

was also found in the 120-L scale fermentation (Fig. 3B). Same results were also observed in the soymilk fermented with *S. thermophilus* alone and fermented with *S. thermophilus* and *B. longum* simultaneously [6].

4. Removal of Anti-Nutritional Factors

According to SDS-PAGE and HPLC analysis, most of the anti-nutritional factors such as glycinins and β-conglycinins were eliminated (Fig. 4). β-conglycinins, a 7S globulin, is a trimeric glycol-protein consisting of three types of subunits (α', α, and β) in seven different combinations [26] with a molecular weight of about 180 kDa [16]. Glycinin consists of an acidic and a basic polypeptide which are linked by a disulphide bridge. It exists as a hexamer (an 11S globulin) with a molecular weight of about 360 kDa [8]. The principal storage proteins of soybean are glycinin (11S) and β-conglycinin (7S) and constitute over 70% of soluble protein [2]. Their content, ratio and dynamics of biosynthesis vary with soybean varieties and environment [2]. Because of compact structure, containing of glycinin and conglycinin which cause them hard to digest, the heating, hydrolysis, or covalent attachment of other constituents are frequently used to modify these soybean proteins [2]. The peptides produced by partial proteolysis have smaller molecular size and less quaternary structure than the original proteins. Several workers have studied limited proteolysis of soy proteins, mainly on pure 7S, 11S fractions and soy protein isolates [14, 21]. However, 7S and 11S proteins expressed different susceptibility to the enzyme-induced hydrolysis. Proteases preferentially hydrolyze β-conglycinin over glycinin [21]. This is due to their compact structure, and makes it more difficult to hydrolyze.

According to HPLC analysis, raffinose and stachyose were below the limits of detection of ≤ 2.00 and ≤ 2.50 mg/g, respectively (data not shown). As we know, raffinose and stachyose are α-galactosides of sucrose comprising three and four monomeric units respectively and are non-digestible in the gut due to the absence of α-galactosidase in the human intestinal mucosa. Consequently, intact oligosaccharides pass directly into the lower intestine where they are metabolized by bacteria that possess this enzyme, resulting in the production of gases [29]. Using a specific enzyme, α-galactosidase or an organism that possesses high α-galactosidase activity could minimize the content of flatulence-causing oligosaccharides in the product [24]. Raffinose and stachyose could not be digested in human intestinal tracts, but decomposed by intestinal bacteria, which produce gas and consequently cause flatulence. According to the present results, the hydrolysis and further *Lactobacillus johnsonii* fermentation could remove these anti-nutritional factors such as stachyose, raffinose, glycinins and β-conglycinins from soy bean (Fig. 4). They are also very helpful for the biotransformation of isoflavone from glucoside into aglycone forms. Therefore, the combination use of proteolysis and *Lactobacillus johnsonii* fermentation might be good way to make soy beans processed into functional foods.

ACKNOWLEDGMENTS

This study was supported by a grant (NSC 102-2221-E-126-001) from the National Science Council, Taiwan.

REFERENCES

1. Astawan, M., Wahyuni, M., Tadashi, Y., Yamada, K., Tadokoko, T., and Maekawa, A., "Effects of angiotensin I-converting enzyme inhibitory substances derived from indonesian dried-salted fish on blood pressure of rats," *Bioscience, Biotechnology and Biochemistry*, Vol. 29, No. 2, pp. 425-429 (1995).
2. Barać, M. B., Stanojević, S. P., Jovanović, S. T., and Pešić, M. B., "Soy protein modification - a review," *Acta Periodica Technologica*, Vol. 35, pp. 3-16 (2004).
3. Cavallini, D. C. U., Roselino, M. N., Manzoni, M. S. J., Pauly-Silveira, N. D., de Sylos, C. M., and Rossi, E. A., "Isoflavones supplementation of a probiotic fermentd soy product: effects on quality characteristics and isoflavones profile," *Alimentaçãoe Nutrição Araraquara*, Vol. 21, No. 2, pp. 175-182 (2010).
4. Chen, J. R., Chiou, S. F., Shieh, M., and Yang, S. C., "The effects of soybean protein-derived hydrolysate on lipid metabolism in rats fed a high cholesterol diet," *Food Biochemistry*, Vol. 26, No. 5, pp. 431-442 (2002).
5. Chen, J. R., Suetsuna, K., and Yamauchi, F., "Isolation and characterization of immunostimulative peptides from soybean," *Nutritional Biochemistry*, Vol. 6, No. 6, pp. 310-313 (1995).
6. Chien, H. L., Huang, H. Y., and Chou, C. C., "Transformation of isoflavone phytoestrogens during the fermentation of soymilk with lactic acid bacteria and bifidobacteria," *Food Microbiology*, Vol. 23, No. 8, pp. 772-778 (2006).
7. Church, F. C., Swaisgood, H. E., Porter, D. H., and Catignani, G. L., "Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins," *Dairy Science*, Vol. 66, No. 6, pp. 1219-1227 (1983).
8. Danilenko, A. N., Bikbov, T. M., Grinberg, V. Ya., Leont'eva, A. L., Burova, T. V., Surikov, V. V., Borisov, Y. A., and Tolstoguzov, V. B., "Effect of pH on the thermal stability of 11S-globulin of glycine max seeds as indicated by differential scanning microcalorimetry," *Biophysics*, Vol. 32, No. 3, pp. 434-439 (1987).
9. Galvez, A. F., Chen, N., Macasieb, J., and de Lumen, B. O., "Chemopreventive property of a soybean peptide (Lunasin) that binds to deacetylated histones and inhibits acetylation," *Cancer Research*, Vol. 61, pp. 7473-7478 (2001).
10. Garro, M. S., Aguirre, L., and de Giori, G. S., "Biological activity of *Bifidobacterium longum* in response to environmental pH," *Applied Microbiology Biotechnology*, Vol. 70, No. 5, pp. 612-617 (2006).
11. Garro, M. S., de Valdez, G. F., and de Giori, G. S., "Temperature effect on the biological activity of *Bifidobacterium longum* CRL 849 and *Lactobacillus fermentum* CRL 251 in pure and mixed cultures grown in soymilk," *Food Microbiology*, Vol. 21, No. 5, pp. 511-518 (2004).
12. Hodgson, J. M., Puddey, I. B., Beilin, L. J., Mori, T. A., and Croft, K. D., "Supplementation with isoflavonoid phytoestrogens does not alter serum lipid concentrations: a randomized controlled trial in human," *Nutrition*, Vol. 128, No. 4, pp. 728-732 (1998).
13. Izumi, T., Piskula, M. K., Osawa, S., Obata, A., Tobe, K., Saito, M., Kataoka, S., Kubota, Y., and Kikuchi, M., "Soy Isoflavone aglycones are absorbed faster and in higher amounts than their glucosides in humans," *Nutrition*, Vol. 130, No. 7, pp. 1695-1699 (2000).
14. Kim, S. Y., Park, P. S. W., and Rhee, K. C., "Functional properties of proteolytic enzyme modified soy protein isolate," *Agricultural and Food Chemistry*, Vol. 38, No. 3, pp. 651-656 (1990).
15. Konosu, S., Watanabe, K., and Shimizu, T., "Distribution of nitrogenous constituents in the muscle extracts of eight species of fish," *Bulletin of Japanese Society of Scientific Fisheries*, Vol. 40, No. 9, pp. 909-915 (1974).
16. Koshiyama, I., "Storage proteins of soybean," in: Gottschalk, W. and Müller, H. P. (Eds.), *Seed Proteins: Biochemistry, Genetics, Nutritive Value*, Martinus Nijhoff/Dr. W. Junk Publisher, The Hague, pp. 427-450 (1983).
17. LeBlanc, J. G., Garro, M. S., and de Giori, G. S., "Effect of pH on *Lactobacillus fermentum* growth, raffinose removal, α-galactosidase activity and fermentation products," *Applied Microbiology and Biotechnology*, Vol. 65, No. 1, pp. 119-123 (2004).
18. Lo, W. M. and Li-Chan, E. C., "Angiotensin I converting enzyme inhibitory peptides from in vitro pepsin-pancreatin digestion of soy protein," *Agricultural and Food Chemistry*, Vol. 53, No. 9, pp. 3369-3376 (2005).
19. Loukas, S., Varoucha, D., Zioudrou, C., Streaty, R. A., and Klee, W. A., "Opioid activities and structures of a-casein-derived exorphins," *Biochemistry*, Vol. 22, No. 19, pp. 4567-4573 (1983).
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., "Protein measurement with folin phenol reagent," *Biological Chemistry*, Vol. 193, pp. 265-275 (1951).
21. Ortiz, S. E. M. and An, M. C., "Analysis of products, mechanisms of reaction, and some functional properties of soy protein hydrolysates," *American Oil Chemists' Society*, Vol. 77, No. 12, pp. 1293-1301 (2000).
22. Puski, G., "Modification of functional properties of soy proteins by proteolytic enzyme treatment," *Cereal Chemistry*, Vol. 52, pp. 655-664 (1975).
23. Sadeghi, A. A., Nikkhah, A., Shawrang, P., and Shahrebabak, M. M., "Protein degradation kinetics of untreated and treated soybean meal using SDS-PAGE," *Animal Feed Science and Technology*, Vol. 126, pp. 121-133 (2006).
24. Scalabrini, P., Rossi, M., Spettoli, P., and Matteuzzi, D., "Characterization of *Bifidobacterium* strains for use in soymilk fermentation," *International Journal of Food Microbiology*, Vol. 39, No. 3, pp. 213-219 (1998).
25. Setchell, K. D. R., Brown, N. M., Desai, P., Zimmer-Nechemias, L., Wolfe, B. E., Brashear, W. T., Kirschner, A. S., Cassidy, A., and Heubi, J. E., "Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements," *Nutrition*, Vol. 131, No. 4, pp. 1362S-1375S (2001).
26. Thanh, V. H. and Shibasaki, K., "Heterogeneity of β-conglycinin," *Biochimica Biophysica Acta*, Vol. 439, pp. 326-338 (1976).
27. Tsangalis, D., Ashton, J. F., McGill, A. E. J., and Shah, N. P., "Enzymic transformation of isoflavone phytoestrogens in soymilk by β-glucosidase-producing bifidobacteria," *Food Science*, Vol. 67, No. 8, pp. 3104-3113 (2002).
28. Tsangalis, D., Ashton, J. F., McGill, A. E. J., and Shah, N. P., "Biotransformation of isoflavones by bifidobacteria in fermented soymilk supplemented with D-glucose and L-cysteine," *Food Science*, Vol. 68, No. 2, pp. 623-631 (2003).
29. Tsangalis, D. and Shah, N. P., "Metabolism of oligosaccharides and aldehydes and production of organic acids in soymilk by probiotic bifidobacteria," *International Journal of Food Science and Technology*, Vol. 39, No. 5, pp. 541-554 (2004).
30. Viana, P. A., de Rezende, S. T., Marques, V. M., Trevizano, L. M., Passos, F. M. L., Oliveira, M. G. A., Bemquerer, M. P., Oliveira, J. S., and Guimarães, V. M., "Extracellular α-galactosidase from *Debaryomyces hansenii* UFV-1 and its use in the hydrolysis of raffinose oligosaccharides," *Agricultral and Food Chemistry*, Vol. 54, No. 6, pp. 2385-2391 (2006).
31. Wang, W. and de Mejia, E. G., "A new frontier in soy bioactive peptides that may prevent age-related chronic diseases," *Comprehensive Reviews in Food Science and Food Safety*, Vol. 4, pp. 63-78 (2005).
32. Wu, J. and Ding, X., "Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats," *Agricultural and Food Chemistry*, Vol. 49, No. 1, pp. 501-506 (2001).
33. Wu, J. and Muir, A. D., "Isoflavone during protease hydrolysis of defatted soybean meal," *Food Chemistry*, Vol. 118, No. 2, pp. 328-332 (2010).
34. Yashiro, A., Oda, S., and Sugano, M., "Hypocholesterolemic effect of soybean protein in rats and mice after peptic digestion," *Nutrition*, Vol. 115, No. 10, pp. 1325-1336 (1985).
35. Ziegler, F., Nitenberg, G., Coudray-Lucas, C., Lasser, P., Giboudeau, J., and Cynober, L., "Pharmacokinetic assessment of an oligopeptide-based enteral formula in abdominal surgery patients," *The American Journal of Clinical Nutrition*, Vol. 67, No. 1, pp. 124-128 (1998).