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Physicochemical Properties of Roasted Soybean Flour Bioconverted by Solid-State Fermentation Using *Bacillus subtilis* and *Lactobacillus plantarum*

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Abstract

To produce novel cheese-like fermented soybean, the solid-state fermentation of roasted soybean flour (RSF) was performed using 1.0% inoculum *Bacillus subtilis* HA and *Lactobacillus plantarum*, with the initial 60% substrate moisture for 10 hr at 42°C, resulting in pH 6.5, 0.82% acidity, 3.5% mucilage, 14.3 unit/g protease activity, 7.6 unit/g fibrinolytic activity, 216 mg% tyrosine content and 1.7×10^{10} CFU/g of viable cell counts. After the second lactic acid fermentation with 10～30% skim milk powder, the fermented RSF resulted in an increase in acidity with 1.64～1.99%, tyrosine content with 246～308 mg% and protease activity in the range of 5.2～17.5 unit/g and 0.966 water activity. Viable cell counts as probiotics indicated 1.6×10^{8} CFU/g of *B. subtilis* and 7.3×10^{10} CFU/g of *L. plantarum*. The firmness of the first fermented RSF with 2,491 g·ømm^{-1} greatly decreased to 1,533 g·ømm^{-1} in the second fermented RSF, although firmness was slightly increased by adding a higher content of skim milk. The consistency of the second fermented RSF also decreased greatly from 55,640 to 3,264～3,998 in the presence of 10～30% skim milk. The effective hydrolysis of soy protein and skim milk protein in the fermented RSF was confirmed. Thus, the second fermented RSF with a sour taste and flavor showed similar textural properties to commercial soft cheese.

Key words: solid-state fermentation, *Bacillus subtilis*, *Lactobacillus plantarum*, peptides, probiotic

INTRODUCTION

Soybean is an important source of inexpensive vegetable protein and has been used as an ingredient for processed and fermented foods in oriental countries. Since the functional properties of soybean have been intensively investigated and are widely recognized, the consumption of soybean has increased in oriental and Western countries (1). Wide uses of soybean in fermented products include soybean paste and sauce, red pepper paste, sprouts, and many processed products such as tofu, soymilk and soybean oil. Furthermore, soybean is utilized as a raw ingredient in cosmetic and medical products (2). Recently, soybean has been used for making imitation surumi meat (3,4) and as an ingredient in the production of imitation cheeses in food industries (5).

Fermented soybean products generally take a long period of time to complete due to the fermentation process; however, soybean fermented by *Bacillus subtilis* can be completed in a very short period and is recognized as an important health food because of its nutritional fortification and enhanced functional properties (6). Soybean proteins during fermentation can be efficiently hydrolyzed and converted into polypeptides and peptones, such as soluble nitrogen, resulting in the increase of digestion and absorption.

Generally, *Bacillus subtilis* can produce the mucilage containing polyglutamic acid and fructan which provide a particular consistency and taste (7). Particularly, fermented soybean has superior nutritional value, compared to soybean paste, consisting of various functional properties for protecting the liver and enhancing colon microflora by probiotics (8,9). Fermented soybean has positive effects on chronic diseases such as hypertension (10), together with anti-cancers (11), anti-oxidants (12), biological activities (13,14), fibrinolytic activities and prevention of osteoporosis (15), and therefore studied intensively as a healthy food. In spite of the highly nutritional and functional properties of fermented soybean, the indigenous odor produced by *Bacillus subtilis*, accompanied by the restriction as a food ingredient in food industries, resulted in fewer consumption by younger generations and foreigners (16). Soybean natto, fermented by a novel *Bacillus subtilis* and produced in sanitary and safety certified conditions, contains nutritional and functional properties previously reported (17).

The addition of natural ingredient such as Yucca was

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able to reduce the off-flavor in fermented soybean (18). The quality enhancement of fermented soybean has been accomplished by the fortification of oriental herbs (19), chitosan (20), and seaweed (21). Considering the consumer trends for the preference of healthy foods, the development of cheese-like products using soybean protein could be expected to increase the consumption of new products in health food markets.

Soybean protein is a typical raw material used for the production of imitational products, such as cheese-like products with similar tastes and textures. Traditionally fermented cheese is produced with milk protein, which is concentrated and fermented by lactic acid bacteria or fungi, and has been developed in western countries, providing indigenous flavor, taste and textural properties (5). Generally, various cheeses are classified based on the region of production, raw materials used and aging method. Fermented cheese has superior nutritional and storage properties and is a popular representative fermented food in western regions (22). In the case of Korea, the infrastructure for dairy agriculture is not industrialized to develop milk and cheese products. Furthermore, the dairy industry is shrinking due to frequent occurrences of mad-cow disease and costly livestock feed.

Considering the production process of cheese using milk protein, the acids and proteases produced by novel microorganisms may play an important role to determine the quality of aged cheeses, which is determined by moisture content and aging process. The moisture content of cheese is a basis for classifying soft and hard cheese (23). In particular, milk protein in cheese can be converted into various peptides by protease enzymes during aging, affecting the functional properties and qualities and indigenous taste (24). Thus, protein-rich soybean can be transformed into the novel cheese-like product through controlled fermentation by *Bacillus subtilis* and lactic acid bacteria. Soybean is expected to be bio-converted into a novel fermented soybean by *Bacillus subtilis*, supplying necessary protease activity for aging and then providing indigenous acid, taste and flavor by the lactic acid fermentation.

In this study, a novel *Bacillus subtilis* isolated from fermented soybean was applied to roasted soybean flour to produce wholesome fermented soybean paste with reduced off-flavor and good textural properties. To produce a cheese-like soybean product, the second fermentation of fermented soybean was carried out by *L. plantarum* with the fortification of skim milk powder. The physicochemical properties of the final mixed fermented paste and its qualities were determined during storage.

MATERIALS AND METHODS

Materials

Roasted soybean flour and skim milk powder were purchased from Chunho Co. (Daegu, Korea) and Seoul Milk Co. (Seoul, Korea), respectively. MRS broth was bought from Difco (Detroit, MI, USA). Ricotta cheese, as soft cheese, was purchased from Lemons Foods Pty Ltd. (Dandenong South, Australia). Absolute grade chemicals were used in all experiments. Standard proteins for SDS-PAGE were purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA).

Starter culture

The solid-state fermentation of roasted soybean was performed with *Bacillus subtilis* HA, deposited in Korean Culture Collection Microorganism (KCCM), as KCCM 10775P. *Lactobacillus plantarum* was isolated from fermented vegetables such as Kimchi and used for lactic acid fermentation. *B. subtilis* HA was cultured with 5% defatted soybean powder suspension by inoculating a single colony of *B. subtilis* grown on MRS agar at 42°C for 24 hr. The starter culture of *B. subtilis* was prepared by cultivation at 180 rpm using a shaking incubator (SI-900R, Jeio Tech Co. Ltd., Seoul, Korea) at 42°C for 24 hr. Starter culture of *L. plantarum* was prepared by static culture with MRS broth at 37°C for 24 hr.

Production of fermented RSF fortified with skim milk

Fermentation was conducted in sterile stainless vessels (9×29 cm) containing 400 g substrate (roasted soybean flour) with a final moisture content of 62.9% and 1% starter culture at 42°C for 10 hr. Different percentages of skim milk powder (10, 20 and 30%) were mixed and the second fermentation was continued for the fermented RSF at 30°C for 24 hr by inoculating 1% of *L. plantarum* starter culture.

pH and acidity

The pH of fermented soybean was determined with a pH meter (model 420A$^+$, Thermo Orion, Washington, DC, USA). The titratable acidity was measured by determining the 0.1 N NaOH content necessary for adjusting to pH 8.3 and then expressed with lactic acid content (%, v/v).

Analysis of moisture, water activity and particle size of RSF

The carbohydrate, crude protein, crude fat and moisture content of the RSF were determined by AOAC (Association of Official Analytical Chemist) method (25). Using 1 g of the samples, water activity was determined using a moisture tensiometer (Labmaster-Aw, Novasina, Lachen, Switzerland). Micro spheres of RSF

were analyzed, in triplicate, for their size distribution using laser diffraction in a particle size analyzer (CILAS 1064, Orleans, France). Particle size was expressed as the equivalent volume diameter.

Viable cell counts

Serial dilutions were performed using 1 g of the samples and spread on Lactobacilli Martin Rogosa Sharped MRS and Nutrient Agar (NA) plates. The plates were incubated at 42 and 30°C for 24 hr, after which colony counts were carried out (CFU/g).

Measurements of tyrosine content and protease activity

To measure the crude tyrosine content, the water extract obtained from fermented RSF was reacted with Folin phenol reagent (26). The blue color of the reaction mixture was determined using a spectrophotometer (UVIKON Kontron Co., Milano, Italy) at 660 nm. Proteolytic activity was determined by modifying the method from Anson (27). One unit of enzyme activity was defined as the generation of 1 μg tyrosine for 1 min at the same reaction condition.

Activity of fibrinolytic enzyme

The fibrinolytic enzyme activity was determined by the method of Astrup and Mullertz (9). The 0.1 M sodium phosphate buffer (pH 7.5) extract (20 μL) of fermented product was spotted on the fibrin plate, followed by incubation at 37°C for 2 hr. The diameter of the clear zone was measured and its activity was determined by comparing the activity of a standard plasmin enzyme.

Texture analysis of fermented RSF

Texture profile analysis of the fermented RSF was determined using a texture analyzer (TA-XT plus, micro stable system, Godalming, UK). The fermented RSF was loaded in the cylinder cup (φ 21 mm) and compressed by the cylinder plunger (φ 30 mm) moving at constant speed (2 mm/s), and measured for 3 sec at 5 g trigger force and 45% of deformation. The texture parameters such as hardness, springiness, chewiness, adhesiveness, and cohesiveness of fermented RSF were evaluated.

The firmness and consistency of fermented RSF were evaluated using mayonnaise back extrusion (MBE) analysis program. The fermented RSF was loaded in the forward extrusion cell (plastic device, φ30 mm × height 38 mm, extrusion die hole Φ 7 mm). The compression was carried out in the condition of cylinder plunger (φ 35 mm) moving down 30 mm at constant speed (1 mm/sec), and measuring for 3 sec at 10 g trigger force.

Analysis of protein hydrolysis and Hunter color value

To analyze the patterns of protein hydrolysis during fermentation, SDS-PAGE was performed using 10% separating gel (Biorad, PA, USA) (28). Freeze dried samples (250 mg) were dissolved in 1 mL of SDS-sample buffer (0.15 M Tris-HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol) and then sonicated for 5 min, followed by mixing for 1 hr. After samples were separated, gel was stained with instant blue solution (coomassie dye, ethanol, phosphoric acid, solubilizing agent) and protein bands were compared to the standard ladder proteins (Protein multicolor marker, Abnova Co., Ltd., Taipei city, Taiwan).

The Hunter color value was determined by measuring fermented RSF in a test tube with a Choma meter (CR-400, Minolta, Japan) to determine lightness (L), redness (R), and yellowness (B).

Biochemical properties of fermented RSF during storage period

The fermented RSF (20 g) was placed in a petri dish and sealed with a parafilm. The samples were incubated at 20°C for 4 weeks. Samples were collected every week and analyzed for tyrosine content, protease activity, viable cell counts and Hunter color value.

RESULTS AND DISCUSSION

Physicochemical properties of RSF fermented by *B. subtilis*

The proximate composition of roasted soybean powder used as a substrate for solid-state fermentation consists of 37% carbohydrate, 35% protein, 19% fat, and 7.94% moisture. Its particle size was about 46.33 μm. As shown in Table 1, the first fermentation was carried out using *B. subtilis* HA for 10 hr to produce odor-free fermented paste with 62.04% moisture and 0.977 water activity. Fig. 1 showed pH 6.47 and 0.82% acidity. The tyrosine content and protease activity of the first fermented RSF were 216 mg% and 14.3 unit/g, respectively (Fig. 2). According to Son and Lee, RSF contained 310 mg% tyrosine after 24 hr of fermentation (29). Generally, tyrosine content in fermented soybean may indicate the degree of hydrolysis of soybean protein. Thus, high tyrosine content indicated the effective hydrolysis of protein, resulting in the exposure of peptide residues including tyrosine.

During the short period of RSF fermentation by *B. subtilis* HA, the fibrinolytic activity and mucilage content were 7.56 unit/g and 4.0%, respectively (Table 1). However, RSF fermented by *B. subtilis* for 24 hr showed 6 unit/g fibrinolytic activity and 7.8% mucilage content according to a previous study (29). Thus, the fibrinolytic activity was almost similar in RSF fermented by *B. subtilis* within 24 hr. However, mucilage content in fer-

Table 1. Comparison of water content and activity, viable cell counts, fibrinolytic activity, and mucilage content of RSF fermented with various amount of skim milk

	First fermentation	Second fermentation skim milk (%)		
		10	20	30
Water content (%)	62.04	61.72	59.81	57.97
Water activity (%)	0.977	0.973	0.971	0.966
Fibrinolytic activity (unit/g)	7.56	10.01	12.64	12.64
Mucilage content (%)	4	3.8	4	4.2
Viable cell count of MRA (CFU/g)[1)]	–	4.6×10^{10}	5.6×10^{10}	7.3×10^{10}
Viable cell count of *Bacillus* (CFU/g)[2)]	1.7×10^{10}	4.2×10^{8}	2.2×10^{8}	1.6×10^{8}

[1)]MRS agar was used for determining *Lactobacillus* sp in first and second fermented RSF, respectively.
[2)]NB-agar was used for determining *Bacillus* sp in second fermented RSF.

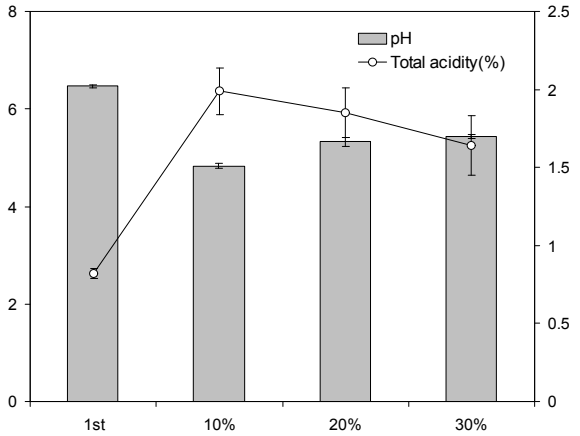

Fig. 1. Comparison of pH and total acidity in RSF fermented by mixed culture with various amounts of skim milk compared to RSF fermented by *B. subtilis*. Mixed culture consisted of *L. plantarum* and *B. subtilis*. RSF: roasted soybean flour.

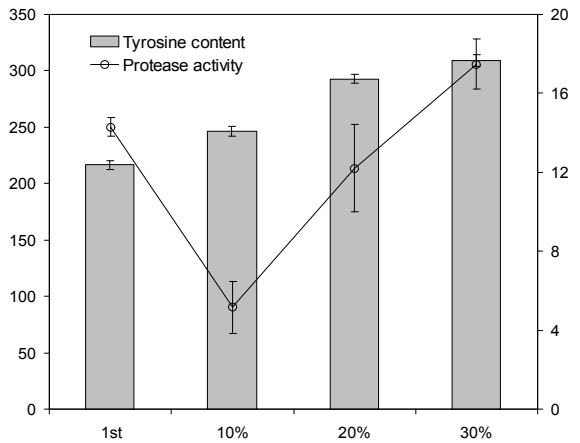

Fig. 2. Comparison of tyrosine content and protease activity in RSF fermented by mixed culture with various amounts of skim milk compared to RSF fermented by *B. subtilis*. Mixed culture consisted of *L. plantarum* and *B. subtilis*. RSF: roasted soybean flour.

mented RSF highly increased with longer fermentation time.

The solid-state fermentation of RSF fortified with barley and carrot juice enhanced the fibrinolytic activity with 31 unit/g (29). The production of bioactive compounds by solid-state fermentation is affected by raw materials and fermentation time (30,31). Thus, low mucilage content in fermented RSF is due to the short fermentation period (10 hr). In particular, the solid-state fermentation time by *B. subtilis* plays an important role in the production of various biological active compounds without off-flavor. Thus, although RSF fermented for a short period contained less amounts of bioactive compounds, solid-state fermentation was also performed for a short period to produce the fermented product with good consistency and flavor to be used as a raw ingredient for making a cheese-like product.

Physicochemical properties and probiotics in RSF fermented by mixed culture

The RSF fermented by *B. subtilis* was subjected to the second fermentation using *L. plantarum* in the presence of skim milk powder. As shown in Fig. 1, compared to the first fermented RSF, the second showed acidic pH range of 4.8～5.4. Adding more skim milk showed slightly higher pH values. Generally, pH of cheese curd affected the textural property such as extensibility of cheese (11). Commercial cheese products such as cottage and cream cheese showed the pH range of 4.8～5.2 (1). Thus, the second fermented RSF resulted in a similar pH value compared to those of commercial cheeses, providing taste and texture of imitation cheese.

In particular, the acid produced during the second fermentation of RSF decreased in pH, as well as the consistency of mucilage including γ-polyglutamic acid. γ-Polyglutamic acid consists of glutamic acid residues with a carboxyl group which are weekly ionized in acidic conditions. In acidic conditions, γ-polyglutamic acid can form a tightly compacted α-helix conformation due to strong hydrogen bonding. As pH rises, the α-helix conformation changes into a linear random-coil conformation, resulting in an increase in viscosity (32). Generally, soluble carbohydrates such as arabinogalactans and acidic polysaccharides, cellulosic material as well as raffinose, and stachyose in soybean can not be utilized by

lactic acid bacteria, thereby, not affecting acid production (32). The RSF, fermented by the mixed culture fortified with skim milk, could produce the acid by converting lactose into lactic acid, resulting in the decrease of pH.

As shown in Fig. 1, the first fermented RSF showed the acidity of 0.82% and then greatly increased to 1.99% in the presence of 10% skim milk. However, fortification of first fermented RSF with 20% and 30% skim milk resulted in a slight decrease of acidity. As expected, the increase of solid content in the first fermented RSF, by adding higher content of skim milk, contributed to the dilution of acid content. The RSF fermented by mixed culture showed higher acidity, 1.64～1.99%, compared to other fermented foods. The acidity of cottage cheese prepared with soy protein isolate and defatted soybean was 1.2%～1.5% (33). Soybean cheese produced by mixing pineapple and soybean oil cream showed the 0.9%～1.3% (34). Soybean cheese prepared by protease treatment of soybean and defatted soybean showed 1.06%～1.44% acidity (1). Thus, the acid production of fermented soybean is dependent upon the type of lactic acid bacteria. *L. plantarum* is a well-known homo- fermentative LAB, which efficiently produces lactic acid from fermentable sugars. Thus, acidity of fermented RSF could be modulated by controlling the type of strain and raw ingredients added.

The moisture content of RSF fermented by a mixed culture was about 62.04% and slightly reduced to 57.97 % by adding 30% skim milk. In addition, water activity of the first fermented RSF indicated 0.977 and then slightly decreased to 0.966 after the second fermentation with 30% skim milk (Table 1). RSF fermented by mixed culture was a sticky paste, causing water activity to remain high even though skim milk was fortified to 30%.

The viable cell counts of *B. subtilis* in the first fermented RSF was about 1.7×10^{10} CFU/g. The second fermented RSF with the fortification of 10%, 20%, and 30% skim milk provided higher viable cell counts of *L. plantarum* at 4.6×10^{10} CFU/g, 5.6×10^{10} CFU/g, and 7.3×10^{10} CFU/g, respectively. The fortification of skim milk in the first fermented RSF may provide lactic acid bacteria with a nutritional source and growth factors. In particular, viable cell counts of *Bacillus subtilis*. The second fermented RSF was performed on the NB agar plates, allowing *Bacillus subtilis* to grow faster compared to MRS agar plates. In the second fermented RSF, although viable cell counts of *Bacillus subtilis* were reduced, the typical white film formed on the surface of fermented products, indicating surface growth of *Bacillus subtilis*. In the RSF fermented with 10%, 20%, and 30% skim milk, viable cell counts of *B. subtilis* as probiotics were 4.2×10^{8}, 2.2×10^{8}, and 1.6×10^{8} CFU/g, respectively. The decrease in *Bacillus* sp viable cell counts is probably due to the active growth of *L. plantarum*, producing metabolites including lactic acid. The fermented RSF showed higher viable cell counts of *Bacillus* sp, 1×0^{10} CFU/g, as well as *L. plantarum*. Choi (33) and Chun (34) have reported that viable cell counts in soybean cream and cottage cheese were 10^{8} and 10^{7} CFU/g, respectively.

Conclusively, RFS fermented with mixed culture contained a high number of viable cell counts for both *B. subtilis* and *L. plantarum.* In particular, the fortification of skim milk provided higher viable cell counts. Considering the role of lactic acid bacteria in the processing and aging of cheese as probiotic, probiotics of *Bacillus subtilis* and *L. plantarum* present in fermented RSF could be essential food factors as functional ingredients in food industry.

Protease and fibrinolytic enzyme activity

The roasted soybean flour contains 146 mg% tyrosine, increasing to 216 and 308 mg% after the first fermentation and second lactic fermentation with 30% skim milk, respectively, as shown in Fig. 2. Roasted soybean after 24 hr fermentation with cereal consisted of 438 mg% tyrosine (29). Wang et al. reported that aqueous extract of Douchi (Chinese fermented soybean) contained 40.7% peptides after 60 hr fermentation (11). The results imply that protein of RSF was partially hydrolyzed by fermentation for 10 hr, indicating lower tyrosine content compared to that of soybean fermented for longer periods.

The protease activity of the first fermented RSF was 14.3 unit/g, slightly higher than the second fermentation. The addition of 10% and 20% skim milk showed decreased protease activities of 5.2 and 12.2 unit/g, respectively. However, 30% skim milk slightly increased the protease activity (17.5 unit/g) compared to the first fermented RSF. The protease activity of the second fermented RSF was mainly derived from *Bacillus subtilis* present in fermented RSF and seems to be dependent on the raw material to be fermented.

The protease played the major role in hydrolysis in fermented RSF, as expected, although lactic acid fermentation was also performed. The protein hydrolysis in fermented foods is greatly affected by a protease secreted from *Bacillus subtilis* (29). Conclusively, in mixed fermentation of RSF during short time periods, the protease activity slightly decreased during the second lactic fermentation in the presence of 10% skim milk. Nevertheless, an overall increase in protease activity was observed with increasing skim milk percentage. Yin et al.

reported the protease activity still increased during the solid-state fermentation of soybean using *Bacillus subtilis* b01 for nine days (18) whereas Choi et al. reported that protease activity greatly increased for 7 days and then drastically decreased during the same experiment (35). Thus, the second lactic fermentation in RSF fermented by *Bacillus subtilis* resulted in the decrease of protease enzyme secretion from *Bacillus subtilis*.

In conclusion, the protease activity in fermented RSF is retained after the second lactic acid fermentation in the presence of skim milk and the alkaline protease from *Bacillus subtilis* has optimum activity in alkaline conditions (36). In spite of the acidic condition of fermented RSF, the role of protease is expected during the aging process.

A previous study reported that protease from *Bacillus subtilis* may become absorbed in the blood through stomachs and intestines of animals and some cultures may be involved in dissolving fibrin or having indirect effects on fibrin hydrolysis (36). Fibrinolytic enzyme, a serine protease, is generally produced by *Bacillus subtilis*. The fibrinolytic enzyme activities in mixed cultures of RSF are shown in Table 1. The fibrinolytic activity of the first fermented RSF showed 7.6 unit/g and then slightly increased, ranging from 10.0～12.64 unit/g, during the second fermented RSF. Son and Lee reported that the fibrinolytic activity was about 6 unit/g in RSF and cereal mixture fermented by *Bacillus subtilis* (29). Kil et al. reported that the fibrinolytic enzyme produced during the initial periods of fermentations increased but then decreased for longer fermentations (37). These results confirm the fibrinolytic enzyme is still active in fermented RSF after the second lactic acid fermentation, where the activity as a protease enzyme may be a valuable factor to be used for functional ingredient.

Texture properties of fermented RSF

The RSF fermented by *Bacillus subtilis* showed peanut butter like texture with enhanced rheological properties compared to that of raw RSF (Table 2); in particular, this RSF indicated excellent consistency without off-flavor compared to a commercial peanut butter. The hardness of the first fermented RSF showed 915 dyne/cm^2, but greatly decreased to 521 dyne/cm^2 in the second fermented RSF fortified with 10% skim milk; however, hardness of the second fermented RSF increased slightly with the increase of skim milk content. A previous study reported that viscosity of mucilage containing γ-polyglutamic acid decreased with decreasing pH due to its molecular characteristic with ionizing residues (38), implying that the mucilage reduced the fermented RSF consistency at acidic conditions, resulting in the higher spreadability. Commercial soft cheese (Ricotta cheese) showed the hardness of 229 dyne/cm^2. The cohesiveness of fermented RSF was 0.62% and then increased to 0.81% when 30% skim milk was added in the second fermentation. The cohesiveness of soft cheese and cream cheese were 0.62% and 0.76%, respectively (24). Also, chewiness of the first fermented RSF was about 616 and then decreased to 287 during the second lactic fermentation with 10% skim milk, showing a slightly higher value with respect to the addition of higher skim milk concentration. The chewiness of soft cheese was about 134. Thus, RSF fermented by *B. subtilis* showed the textural properties with higher hardness and decreased drastically by the second lactic acid fermentation; this change in rheological properties is mainly dependent on the pH drop due to the lactic acid fermentation.

Furthermore, the extrusion test showed similar results compared to results of texture profile analyses. The firmness of the first fermented RSF showed 2,491 g·ømm^{-1} and decreased greatly during the second fermentation. With increasing skim milk concentration, 10, 20 or 30%, the firmness of the second fermented RSF slightly increased, indicating 1,318, 1,511, and 1,533 g·ømm^{-1}, respectively. Considering the consistency, RSF fermented by *B. subtilis* showed 55,640 and drastically decreased to 39,987 after the second fermentation with 30% skim milk. Interestingly, consistency of soft cheese

Table 2. Rheological properties of RSF fermented with various amounts of skim milk

	Control	First fermentation	Second fermentation skim milk (%)		
			10	20	30
Hardness (dyne/cm^2)	229±8	915±35	521±20	534±18	548±17
Springiness	0.94±0.01	0.99±0.01	0.95±0.06	0.96±0.00	0.98±0.01
Cohesiveness (%)	0.62±0.1	0.62±0.1	0.56±0.1	0.62±0.1	0.81±0.1
Gumminess (%)	142±20	620±71	302±33	332±25	431±22
Chewiness	134±18	616±71	287±31	320±25	420±22
Firmness (g·ømm^{-1})	3263±52	2491±23	1318±18	1511±21	1533±15
Consistency	40594±67	55640±72	32643±49	37360±58	39987±46

Control: Ricotta cheese (Lemnos).
First fermentation was performed by *B. subtilis*. Second fermentation was performed by *L. plantarum*.

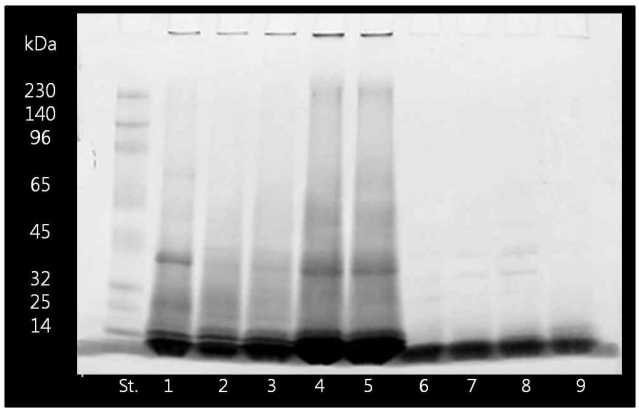

Fig. 3. RSF protein patterns after fermentation by mixed cultures with various amount of skim milk using SDS-PAGE. 1: roasted soybean flour (RSF), 2: first fermented RSF, 3～5: addition of skim milk (10, 20, 30%) to the first fermented RSF, 6～9: second fermented RSF with skim milk (0, 10, 20, 30%).

was about 40,594. Thus, rheological properties of fermented RSF could be modulated by fermentation type as well as fortification of skim milk. In this study, the RSF fermented by *B. subtilis* showed higher textural properties and decreased drastically during the second lactic acid fermentation, owning the expected change in rheological properties of the second fermented RSF mainly to the pH drop due to the lactic acid fermentation.

SDS-PAGE analysis

The protein hydrolysis of fermented RSF was confirmed using SDS-PAGE. As shown in Fig. 3, water soluble proteins extracted from RSF consisted of 7S globulin (β-conglycinin) and 11S globulin (glycinin) as the major soybean proteins. The protein pattern in SDS-PAGE was similar to that of soybean curd (2). In the water extract from RSF, 7S globulin and 11S globulin were determined as major protein bands, but the amount of each soluble protein was relatively low compared to soybean flour without heat denaturation (unpublished results). In the first fermented RSF, soluble major protein was mostly absent. In case of the second fermented RSF, the major proteins of skim milk were determined after mixing with the first fermented RSF; however, major casein protein greatly decreased during the second lactic acid fermentation (Fig. 3). These results suggested that the protein in soybean and skim milk was digested by action of protease derived from *B. subtilis*. In particular, the protein digestion in fermented RSF was greatly accelerated, resulting in the production of small peptides. The textured vegetable protein (TVP) was hydrolyzed to produce peptides during solid-state fermentation using *B. subtilis* (39). In particular, alkaline protease obtained from *B. subtilis* can effectively hydrolyze a 5% soy protein isolate solution, resulting in the production of peptides (unpublished result). Thus, alkaline protease from *B. subtilis* will play an important role in protein hydrolysis in RSF fermentation and aging of fermented products.

Physicochemical properties and viable probiotics of fermented RSF during storage

When storage experiments were carried out for 4 weeks, the pH of the second fermented RSF with 30% skim milk decreased from pH 5.44 to 4.11. Moreover, the acidity of the second fermented RSF drastically increased from initial acidity 1.64 to 5.56% and was then maintained during storage for 4 weeks (Table 3). The acidity of second fermented RSF was maintained at a high value providing acidic taste and flavor.

The tyrosine content of the second fermented RSF was

Table 3. Comparison of biochemical properties in RSF fermented by mixed cultures with various amounts of skim milk during storage period

Storage period (weeks)	Skim milk (%)	pH	Acidity (%)	Tyrosine content (mg%)	Protease activity (unit/g)
0	10	4.83±0.01	1.99±0.00	246±2	5.15±0.03
	20	5.33±0.01	1.85±0.00	292±1	12.20±0.02
	30	5.44±0.01	1.64±0.00	309±2	17.48±0.03
1	10	4.12±0.00	5.79±0.02	242.6±1	4.33±0.02
	20	4.11±0.01	5.57±0.00	202.7±1	5.20±0.01
	30	4.11±0.01	5.56±0.01	211.5±1	2.32±0.00
2	10	4.15±0.01	5.63±0.01	233.0±2	0.34±0.00
	20	4.11±0.01	5.85±0.03	185.6±1	2.90±0.01
	30	4.12±0.00	5.27±0.02	196.6±1	0.57±0.00
3	10	4.16±0.01	5.47±0.01	233.5±1	2.20±0.01
	20	4.12±0.00	5.45±0.01	194.6±1	−
	30	4.12±0.02	5.39±0.02	194.5±1	−
4	10	4.11±0.00	5.5±0.03	228.2±2	1.08±0.00
	20	4.08±0.00	5.44±0.4	196.4±1	−
	30	4.10±0.01	5.41±0.00	196±2	−

Table 4. Comparison of Hunter's color values and viable cell counts in RSF fermented by mixture cultures with various amounts of skim milk

Storage period (weeks)	Skim milk (%)	Viable cell (CFU/g)	Color values			
			L	a	b	ΔE
			61.6±0.00	-1.2±0.01	1.9±0.01	
0	10	4.59×10^{10}	46.1±0.6	10.5±0.3	19.1±0.6	25.9±0.4
	20	5.60×10^{10}	46.7±0.8	10.1±0.2	18.7±0.1	25.1±0.3
	30	7.34×10^{10}	47.2±0.6	9.9±0.1	18.4±0.3	24.6±0.2
1	10	3.86×10^{10}	48.8±0.3	10.6±0.1	28.2±0.1	31.5±0.1
	20	3.56×10^{10}	49.4±0.1	10.9±0.1	28.4±0.1	31.6±0.1
	30	3.53×10^{10}	51.7±0.3	10.1±0.1	29.3±0.1	31.2±0.1
2	10	4.05×10^{9}	49.6±0.5	10.8±0.1	27.4±0.2	30.6±0.0
	20	6.12×10^{9}	50.6±0.9	11.1±0.1	28.2±0.1	31.1±0.3
	30	3.17×10^{9}	50.9±0.7	10.8±0.2	27.2±0.1	30.0±0.2
3	10	1.09×10^{9}	47.4±0.3	10.1±0.2	25.1±0.1	29.5±0.1
	20	6.09×10^{8}	50.6±0.8	10.8±0.2	27.2±0.2	30.1±0.3
	30	5.83×10^{8}	52.9±0.4	10.3±0.2	27.6±0.2	29.5±0.1
4	10	4.28×10^{7}	43.8±0.2	10.8±0.1	23.9±0.1	30.7±29.3
	20	6.09×10^{6}	47.8±0.3	10.5±0.1	24.8±0.1	29.3±0.1
	30	1.06×10^{6}	49.1±0.3	10.6±0.1	25.9±0.3	29.5±0.2

Viable cell counts: MRS agar was used for determining *Lactobacillus plantarum* in second fermented RSF.

about 246 mg%, and then slightly increased to 309 mg% in the second fermented RSF fortified with 30% skim milk. However, the tyrosine content of the second fermented RSF decreased to 196 mg% after storage for 4 weeks. The decrease in tyrosine content is due to the growth of LAB, which is utilizing the nitrogen source in a limited nutritional condition during storage.

The protease activity in the second fermented RSF was dependent upon the fortified skim milk content. When the skim milk percentage increased to 30%, the protease activity in the second fermented RSF increased to 17.48 unit/g (Table 3). However, protease activity drastically decreased with increasing storage time, showing 2.32 unit/g (1 week) and 0.57 unit/g (2 weeks). Protease activity was not detected in the second fermented RSF during storage for 3 weeks, concluding that the protease activity was retained only for 3 weeks during storage.

The viable cell counts of the second fermented RSF slightly increased with the fortification of skim milk. The viable cell counts of *L. plantarum* in the second fermented RSF were quite high, $4.59\times10^{10}\sim7.34\times10^{10}$ CFU/g. During the 4-week storage, the viable cell counts decreased to $4.28\times10^{7}\sim1.06\times10^{6}$ CFU/g (Table 4). In particular, the Hunter color value of the second fermented RSF showed less change in lightness (L value), 46.1～47.3, and redness (a value), 9.9～10.5, but, a slight increase in yellowness (b value), 18.4～25.9, during storage. According to Kim, the Hunter color value of traditional fermented paste decreased during the storage period (40). Thus, the second fermented RSF still maintained its initial color value during storage. Particularly, although protease activity and viable cell counts decreased, the physical properties of the second fermented RSF were preserved without any contamination of fungal microorganisms during storage at 20°C. Conclusively, RSF fermented by *B. subtilis* had excellent textural properties without off-flavor, compared with commercial products fermented by *Bacillus* strain. The final RSF fermented by LAB after the addition of skim milk could provide the high number of viable cell counts as probiotics, soft cheese like consistency, and particular taste and flavor to be utilized for food industry.

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