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Characterization of the Functional Properties of Soy Milk Cake Fermented by *Bacillus* sp.

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Abstract The mucilage production and tyrosine content in soy milk cake (SMC) fermented by *Bacillus firmus* NA-1, *Bacillus subtilis* GT-D, and *B. subtilis* KU-A was improved by fortification with 10% defatted soybean flour. The fibrinolytic activity and consistency of the SMC were drastically increased by solid-state fermentation for 1 day. However, the consistency of the fermented SMC gradually decreased during fermentation for 3 days. Furthermore, the tyrosine content of the freeze-dried powder of SMC fermented by three *Bacillus* sp. was 9 times higher than that of unfermented SMC. The soybean proteins, including the 7S and 11S subunits, were partially digested during alkaline fermentation, producing lower molecular-weight peptides. The fibrinolytic enzyme produced in SMC fermented by *B. firmus* NA-1 and *B. subtilis* KU-A exhibited higher thermal stability than that of *B. subtilis* GT-D fermentation. The powder obtained from *B. subtilis* GT-D fermentation had an α-amylase activity and lower consistency compared to those of *B. firmus* NA-1 and *B. subtilis* KU-A. In addition, this powder contained 6.3% moisture content, 27% crude protein content and 9 units of fibrinolytic activity and proteolytic activity.

Key words: soy milk cake, *Bacillus* sp., fibrinolytic enzyme, defatted soybean flour, consistency

Introduction

Traditionally, soybeans have been used in making Korean fermented foods, such as soybean paste and soy sauce. In addition, legumes are often eaten as processed foods, including soybean curd and bean sprouts, and used for various foodstuffs (1). In particular, soybean curd has been a major protein source and is one of the principal foods in Korea. During soybean curd processing, most of the soybean proteins are aggregated, and the soy milk cake (also known as *biji*) and whey are generated as waste products (2). Soy milk cake (SMC) has been utilized as both soil fertilizer and animal feed (3, 4). Recently, better-quality SMC has been obtained from the modernized soybean curd industry equipped with heat sterilizers and other sanitary processing equipment under the hazard analysis and critical control point (HACCP) system. Now, SMC has relatively lower moisture content and less contaminating microflora than SMC produced in the past. The successful utilization of this higher quality SMC in the food industry will have a great impact on soybean processing companies in both economical and environmental aspects.

A great deal of research has been conducted, both to characterize and to further utilize SMC. Some of the research regarding SMC has focused on the preservation of raw SMC (5). The physicochemical properties of dried SMC have also been characterized, and formulated soybean curd has been manufactured by adding dried SMC (6, 7). In another study, the carbohydrate composition of SMC during solid-state fermentation was monitored in the presence of a commercial enzyme (8). SMC was also used as an additional ingredient in the bread manufacturing process (9). In Japan, it has been reported that a polysaccharide was extracted and purified from SMC for use as a functional ingredient (10).

Recently, a lactic acid bacterium isolated from raw SMC was applied to the bioconversion of SMC (11). In addition, alkaline fermentation of SMC has been performed to determine the biochemical and microbiological changes that occur during solid-state fermentation (12). Also, it has been reported that fermented soybean paste was prepared by mixing SMC and wheat bran (13). Rhee *et al.* (14) reported that a novel *Bacillus subtilis* could reduce off-flavor by suppressing the indigenous microflora in SMC. Moreover, it was recently reported that selected *B. subtilis* from *cheonggukjang* were able to reduce unpleasant *cheonggukjang* odors (15), and the mucosal immune activity in gastrointestinal tract of rats increased with *cheonggukjang* intake (16). Although there are numerous reports about biologically active metabolites produced from the *Bacillus* strain, the solid-state fermentation of SMC for enhancing their functional properties has only recently been reported (17).

Fermented soybean paste generally includes indigenous mixed microflora, including *Bacillus* sp., and contains various biologically active compounds such as mucilage, enzymes and peptides (17). Therefore, the alkaline fermentation of SMC will be one of the best means to convert SMC to functional ingredients in a short period, providing new functional ingredients for the food and bio-industries. However, the solid-state fermentation of SMC is greatly dependent upon the *Bacillus* strain used. Therefore, novel *Bacillus* strains were isolated from traditional Korean foods and used for the alkaline fermentation.

In this study, *B. firmus* NA-1 and *B. subtilis* isolated from *natto* and traditional Korean foods, respectively, were

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used for the novel solid-state fermentation of SMC, and the production of biologically active compounds such as mucilage, peptides, and hydrolytic enzymes in fermented SMC was optimized.

Materials and Methods

Materials and strains Soy milk cake (SMC) was obtained from a local soybean curd manufacturing company. The SMC was packed into 1 kg vinyl bags and kept at -20°C. The defatted soybean flour (DSF) was obtained from Archer Daniels Midland Co. (Decatur, IL, USA). Plasmin, fibrinogen and thrombin were purchased from Sigma Co. (St. Louis, MO, USA). Folin-phenol reagent was purchased from Junsei Chemical Co. (Tokyo, Japan). All other chemicals and reagents were high-grade. *B. firmus* NA-1 was isolated from *natto* and confirmed by API kit analysis (18). *Bacillus* strains were isolated from Korean traditional fermented soybean and identified by the16S rDNA sequencing.

Preparation of starter culture Fifty mL of 5% DSF solution was transferred into a 250 mL culture flask and then sterilized at 121°C for 15 min. *Bacillus* sp. was inoculated and then grown at 42°C for 1 day using a shaking incubator (SI-900R; Jeio Tech Co., Ltd., Korea) at 150 rpm. The viable cell counts in the starter culture were determined by plating on MRS agar.

Solid-state fermentation of SMC Fifty g of SMC was thoroughly mixed with 10-30% DSF in a 250-mL glass beaker and then sterilized at 121°C for 15 min. The starter culture (1%) of *B. firmus* NA-1, *B. subtilis* GT-D, or *B. subtilis* KU-A was transferred to the sterilized SMC was mixed thoroughly, followed by incubation at 42°C for 3 days.

Physicochemical properties of fermented SMC The fermented SMC (10 g) was mixed with 90 mL of distilled water and then homogenized at 5,000 rpm with a homogenizer (Jeil Scientific Ind. Co., Ltd., Korea) for 2 min, followed by filtration using a steel wire sieve. The filtrate from the fermented SMC was then loaded in a cylinder-type measuring cup (DG43), and its consistency was measured using a Viscometer (Haake RheoStress 1; Thermo Electron Co., Karlsruhe, Baden Württemberg, Germany) attached to a spindle (Rotor DG43 DIN 53544 Titan) with a shear rate range of 1-100/sec. The flow behavior and consistency indexes were determined by the power law equation (19). The insoluble solids in the filtrate were removed by centrifugation (22,250×*g* for 15 min), and the tyrosine content of the resulting supernatant was determined using Folin-phenol reagent (20). The absorbance of the reaction mixture was determined at 660 nm using a Spectrophotometer (UNION; Kontron Instruments, St. Quentin Yvelines, France).

Hydrolytic enzymes The fermented SMC (5 g) was diluted with 95 mL distilled water and mixed for 20 min. After centrifugation at 22,250×*g* for 15 min, the supernatant was used as a crude enzyme mixture for determining fibrinolytic, α-amylase and protease activities. Fibrinolytic enzyme was determined by the Astrup and Müllerz method (21). A plasmin was used as the standard to generate a fibrinolytic enzyme standard curve, and the relative activity of fibrinolytic enzyme from fermented SMC was calculated from the activity (5 units/mL) of the standard plasmin.

Relative activity (%)
= (area of lysis from crude extract/ area of lysis from plasmin)×100

For determination of α-amylase activity, 1% soluble starch suspension (1 mL) was mixed with 2.6 mL Mc buffer and 0.2 mL $CaCl_2$ (0.1%), and then incubated with 0.2 mL crude enzyme. After incubation at 37°C for 30 min, 0.1 mL of the reaction mixture was mixed with 5 mL of iodine solution, and the absorbance was determined at 660 nm. One unit of enzyme activity was defined as a 10% decrease in absorbance from that of a blank sample (22, 23).

Proteolytic activity was determined by the modified method of Anson (24). A 0.6% casein suspension (0.35 mL) was mixed with crude enzyme (0.35 mL) and then incubated at 37°C for 10 min. The reaction was stopped by adding 0.7 mL of 0.44 M TCA solution, followed by incubation at 37°C for 30 min. The reaction mixture was subsequently centrifuged (22,250×*g*, 15 min), and the supernatant (1 mL) was mixed with 2.5 mL of 0.55 M Na_2CO_3 and 0.5 mL of Folin-phenol reagent, followed by incubation at 37°C for 30 min. The absorbance was determined at 660 nm, and one unit of proteolytic activity was defined as the amount of enzyme producing 1 μg tyrosine per min.

Thermal stability of fibrinolytic enzyme The supernatant from the water extract of freeze-dried, fermented SMC was heat-treated at 60°C for 45 min. Samples were then collected every 15 min, and the thermal stability of the fibrinolytic enzyme was determined by the fibrin plate method. The relative amount of fibrinolytic enzyme activity was compared to that of the filtrate without heating.

Physicochemical properties of freeze-dried SMC fermented SMC containing 10% DSF was fermented by *B. firmus* NA-1, *B. subtilis* GT-D and *B. subtilis* KU-A, and then freeze-dried using the freeze-dryer (Modulyo-220; Thermo Savant, NY, USA). The dried samples were milled using a Cutting Mill (Type 3, Micro Hammer Mill; Eisenach, Thüringen, Germany). The crude protein was determined by AOAC method (25), and mucilage content was determined by drying the aggregate obtained by alcohol precipitation.

SDS-PAGE analysis Freeze-dried samples were dissolved in SDS-sample buffer (0.15 M Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, SDS, 5% β-mercaptoethanol) and heated at 100°C for 3 min (26). The analysis of total soluble proteins in fermented SMC was performed using 12.5% SDS polyacrylamide gels in a vertical electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA) (27). Gels were stained with Coomassie

Brilliant Blue R-250 (0.05%, w/v) in methanol-acetic acid-water (15:10:65 v/v/v) and de-stained in the same solution without the dye. The defatted soybean protein was used as a marker for relevant soybean proteins.

Results and Discussion

Identification of *Bacillus* sp. *Bacillus firmus* NA-1 was previously isolated from Japanese *natto* (18). *Bacillus* strains were isolated from traditionally fermented soybean using MRA plates, and then identified by 16S rDNA sequencing. *B. subtilis* GT-D and *B. subtilis* KU-A had 99% homology to *B. subtilis* AY583216 and *B. subtilis* Z99104, respectively. When these *Bacillus* strain were grown on MRS agar plates, both *B. firmus* NA-1 and *B. subtilis* KU-A exhibited a dome-shaped morphology with sticky mucilage. However, *B. subtilis* GT-D had a flat shape without mucilage. In contrast, all *Bacillus* sp. grew quickly on nutrient broth agar plates, covering the surface of plate. Therefore, MRS agar plates could be a useful means to isolate novel *Bacillus* strains that produce much higher mucilage.

Effect of DSF on fermented SMC In the soybean curd manufacturing process, SMC is produced by either a cold or hot process, which is differentiated by the means of isolating soymilk from crushed soybean. As mentioned, SMC can be obtained from a hot process that includes heat-sterilization and SMC pressing. Oh *et al.* (17) reported the compositional analysis of this SMC; which contained 72% moisture content. On the other hand, SMC obtained by centrifugation (cold process) contains much higher moisture content. Because the solid-state fermentation of SMC could be generally affected by its initial moisture content, the source of SMC is crucial for alkaline fermentation with *Bacillus* strains. Indigenous microorganisms, including *Bacillus* sp. and lactic acid bacteria, were dominant in poor quality SMC so that the raw SMC could easily be putrefied. Therefore, the fermentation of SMC could also be strongly dependent on the indigenous microflora present in the SMC, depending on its source.

For the solid-state fermentation of SMC, the SMC was fortified with 10-30% DSF followed by heat-sterilization. As shown in Table 1, the moisture content of SMC was reduced from 72.6 to 54.0% after the addition of 30% DSF. Solid-state fermentation by *B. firmus* NA-1 enhanced both the consistency and flavor of fermented SMC. The tyrosine content of fermented SMC was increased two-fold by fortification with 10% DSF, indicating the increase in soybean peptides. In previous reports, hydrolytic protease activity has been determined by measuring the color formed from tyrosine residues present in peptides derived from intact protein (20).

On the other hand, the fibrinolytic enzyme in fermented SMC decreased slightly. This implies that the addition of DSF affects the production of biologically active components during alkaline fermentation of SMC. It appears that fortification with DSF as a nitrogen source has disadvantages for the production of fibrinolytic enzymes. Ultimately, the addition of 10% DSF allowed an increase in the tyrosine content and consistency of fermented SMC and provided a more wholesome flavor compared to the SMC fermented without DSF.

Interestingly, the solid-state fermentation of non-sterilized SMC resulted in similar patterns in tyrosine content and mucilage, including fibrinolytic enzyme. This implies that it is possible to successfully carry out the alkaline fermentation of non-sterilized SMC by inoculating a starter culture. Alkaline fermentation of SMC on a lager scale without heat-treatment will provide great economical advantages.

In the alkaline fermentation of SMC fortified with 10% DSF, the tyrosine content and fibrinolytic activity were affected by the fermentation time. As shown in Fig. 1, when three *Bacillus* sp. were used for alkaline fermentation, the tyrosine content drastically increased after fermentation for 24 hr, with a tyrosine content of above 451 mg% in contrast to that of non-fermented SMC with 10% DSF (95 mg%). After fermentation for 72 hr, there were no differences in tyrosine content among the three *Bacillus* strains.

Also, the fibrinolytic activity greatly increased after fermentation for 24 hr, and then gradually increased after 72 hr. *B. firmus* NA-1 and *B. subtilis* KU-A showed a similar profile in relative fibrinolytic activity, while *B. subtilis* GT-D displayed lower relative activity. After

Table 1. Effect of DSF on the physicochemical properties of fermented SMC

	DSF (%)	pH	Moisture content (%)	Fibrinolytic activity (%)	Tyrosine content (mg%)	Consistency ($Pa \cdot sec^n$)
S[1)]	0	7.7	72.6	59.4	246.7	0.34
	10	7.7	67.9	54.4	424.6	0.45
	20	7.6	60.7	50.5	504.3	0.45
	30	7.1	54.0	42.9	567.3	0.46
NS[2)]	0	8.5	73.2	44.0	230.3	0.12
	10	8.1	68.1	45.1	424.3	0.24
	20	7.7	61.4	46.2	513.2	0.32
	30	7.6	55.5	45.7	565.1	0.47

[1)]S, SMC sterilized before fermentation.
[2)]NS, Non-sterilized SMC. The SMC was fermented by *B. firmus* NA-1 at 42°C for 18 hr.

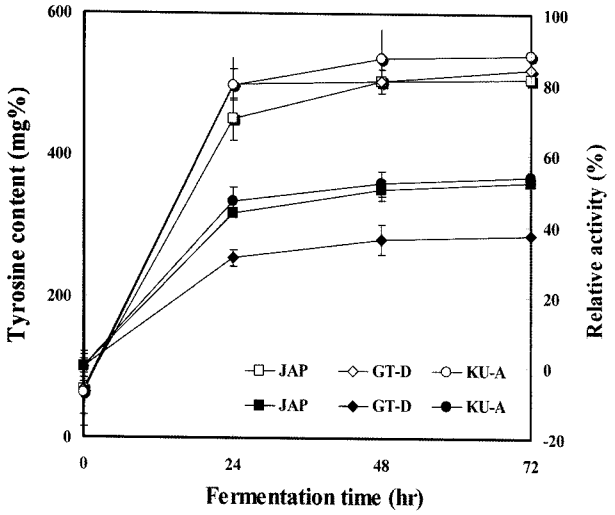

Fig. 1. Changes in tyrosine content and fibrinolytic activity of SMC fermented by three *Bacillus* sp. JAP, *B. firmus* NA-1; GT-D, *B. subtilis* GT-D; KU-A, *B. subtilis* KU-A. Open symbols, tyrosine contents; closed symbols, relative activity.

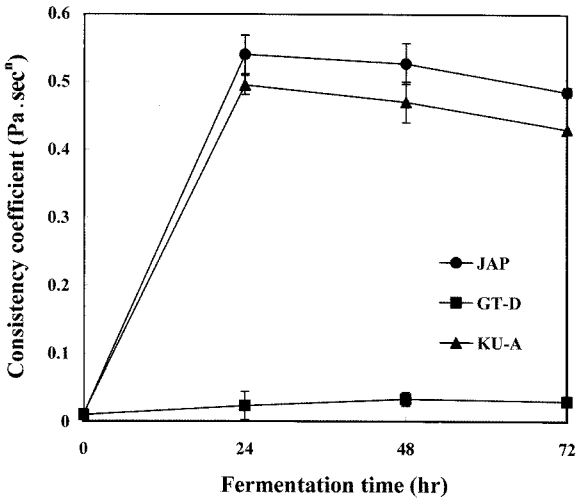

Fig. 2. Changes in consistency coefficient of SMC fermented by three *Bacillus* sp. JAP, *B. firmus* NA-1; GT-D, *B. subtilis* GT-D; KU-A, *B. subtilis* KU-A.

fermentation for 24 hr, the SMC fermented by *B. subtilis* KU-A and *B. firmus* NA-1 showed higher relative activity than SMC fermented by *B. subtilis* GT-D, implying that fibrinolytic enzyme production in fermented SMC is dependent upon the type of *Bacillus* sp. used. In one report, soybean grit fermented by *B. firmus* NA-1 displayed a relative fibrinolytic enzyme activity of approximately 55% (28). Considering the relative fibrinolytic activity produced from soybean grit and SMC, this suggests that solid-state fermentation with *B. firmus* NA-1 resulted in the difference in fibrinolytic activity due to its respective substrates. Ultimately, SMC fortified with 10% DSF was converted into a functional ingredient with wholesome flavor after solid-state fermentation for 24 hr.

To compare the mucilage production from SMC fermented by these three *Bacillus* strains, the consistency of a water extract was determined. As shown in Fig. 2, the consistency of the water extract was greatly dependent upon the type of *Bacillus* strain used. *B. firmus* NA-1, isolated from *natto*, significantly produced mucilage after fermentation for 24 hr, and then decreased slightly. *B. subtilis* KU-A, isolated from Korean traditional fermented soybean, had a similar consistency pattern, but *B. subtilis* GT-D had the lowest consistency with a value below 0.1 Pa·sec^n.

In SMC fermented by *B. firmus* NA-1 and *B. subtilis* KU-A, the consistency of mucilage decreased during extended fermentation time. This may be due to partial enzymatic hydrolysis of the mucilage during the later periods of fermentation. One hypothesis that was supported by these results suggests that the *natto* mucilage was hydrolyzed by depolymerase produced by *Bacillus* sp., resulting in decreases in the viscosity (29).

Physicochemical properties of freeze-dried fermented SMC The drying of raw SMC has generally been performed to improve its shelf-life (5, 6). However, the fermented SMC fortified with DSF has several disadvantages with respect to hot air-drying because it can easily convert to a darker color due to the reaction of amino acids and sugars. Also, the high mucilage content in fermented SMC limits hot air-drying.

Therefore, to improve the shelf-life and availability of fermented SMC, the fermented SMC was freeze-dried. After freeze-drying, the fermented SMC was converted into a stable dried product with low moisture content and wholesome flavor. As shown in Table 2, the tyrosine content in SMC fermented by *B. firmus* NA-1, *B. subtilis* GT-D, or *B. subtilis* KU-A greatly increased with a value approximately 9 times higher than that of non-fermented SMC.

In particular, SMC fermented by *B. subtilis* GT-D contained 112 units/g of α-amylase activity, but had the lowest value in consistency. Consequently, the mucilage content recovered by isopropanol precipitation of SMC fermented by *B. firmus* NA-1 (5.8%) and *B. subtilis* KU-A (4.9%) was superior compared to that of *B. subtilis* GT-D

Table 2. Functional properties of fermented SMC after freeze-drying[1)]

	Control	JAP	GT-D	KU-A
Moisture content (%)	3.8±0.4	6.0±0.1	6.2±0.3	6.0±0.3
pH	6.7±0.1	6.5±0.2	6.8±0.1	6.6±0.2
Tyrosine content (mg%)	192±3	1747±30	1859±57	1804±48
α-Amylase activity (unit/g)	-	-	112±16	-
Proteolytic activity (unit/g)	-	447±40	553±48	475±26
Fibrinolytic activity (unit/g)	-	17±2	9±5	17±2
Crude protein (%)	28±0.3	27±0.4	28±0.1	27±0.4
Consistency (Pa·sec^n)	ND[2)]	1.4±0.6	0.2±0.03	1.3±0.4
Mucilage content (%)	-	5.8±0.7	2.9±0.01	4.9±0.5

[1)]JAP, *B. firmus* NA-1; GT-D, *B. subtilis* GT-D; KU-A, *B. subtilis* KU-A.
[2)]ND: not detected.

(2.9%). However, SMC fermented by these three *Bacillus* strains contained similar proteolytic activities, ranging from 475 to 553 units/g. In terms of fibrinolytic enzyme, no enzyme activity was detected in non-fermented SMC, but activity was found in fermented SMC in the range of 9 to 17 units/g. In freeze-dried fermented SMC, the tyrosine content increased approximately three-fold due to the decrease in moisture content in fermented SMC. The tyrosine content in the powder obtained from SMC fermented by *B. firmus* NA-1, *B. subtilis* GT-D, or *B. subtilis* KU-A was 1747, 1859, and 1804 mg%, respectively. Although the fibrinolytic activity also increased, its increase was not proportional to the fold-concentration increase due to the freeze-drying. Therefore, this suggests that the fibrinolytic enzyme in fermented SMC may be partially inactivated during the freeze-drying process.

The fibrinolytic activities of SMC fermented by *B. firmus* NA-1 and *B. subtilis* KU-A gradually decreased after heating at 60°C for 45 min, retaining about 70% of the relative enzyme activity (Fig. 3). However, the fibrinolytic enzyme from SMC fermented by *B. subtilis* GT-D was drastically inactivated by heating, resulting in complete inactivation within 30 min. It was previously reported that the fibrinolytic enzyme from *B. firmus* NA-1 was completely inactivated by heating at 70°C for 10 min (28). Therefore, the fibrinolytic enzyme from *B. firmus* NA-1 was able to maintain its activity below this critical temperature of 70°C. However, the fibrinolytic enzyme from other *Bacillus* strains exhibited different thermal stabilities. Yoo *et al.* (30) reported that the optimal enzyme activity of *B. subtilis* occurred at approximately 65°C, while Kim *et al.* (31) reported that the optimal temperature for fibrinolytic enzyme activity was about 70°C. Taken together, this implies that the thermal stability and optimal activity of fibrinolytic enzyme are greatly dependent upon the *Bacillus* strains used.

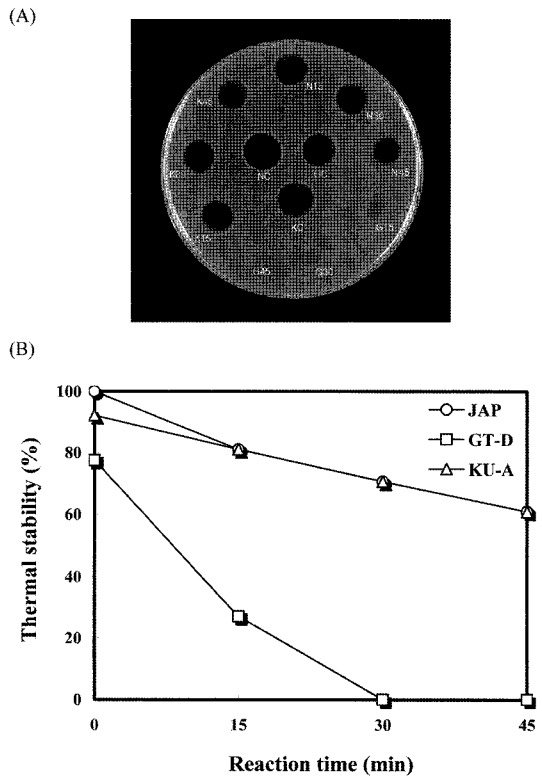

Fig. 3. Thermal stability of fibrinolytic enzyme obtained from SMC fermented by three *Bacillus* sp. (A) digested fibrin plate, (B) residual fibrinolytic activity. NC, *B. firmus* NA-1 (control); GC, *B. subtilis* GT-D (control); KC, *B. subtilis* KU-A (control); N15, *B. firmus* NA-1 (15 min); N30, *B. firmus* NA-1 (30 min); N45, *B. firmus* NA-1 (45 min).

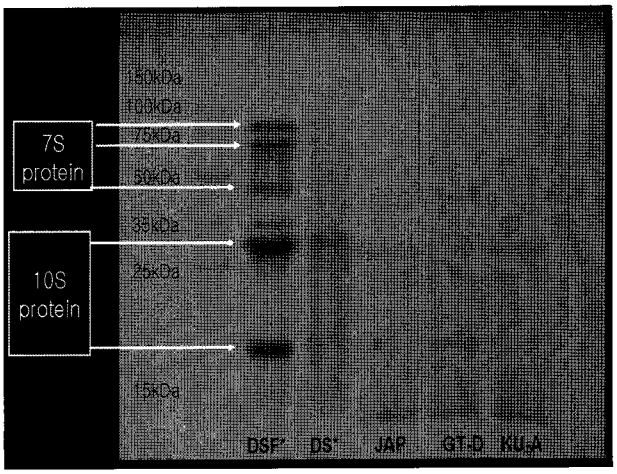

Fig. 4. Digestion patterns of total soluble proteins in SMC fortified with DSF fermented by three *Bacillus* sp. DSF*, defatted soy flour; DS*, SMC fortified with DSF; JAP, *B. firmus* NA-1; GT-D, *B. subtilis* GT-D; KU-A, *B. subtilis* KU-A.

To determine the digestion patterns of soybean protein in fermented SMC, total soluble protein was extracted and subjected to SDS-PAGE. The 11S and 7S proteins were confirmed as major soybean proteins on the SDS-PAGE by comparison with Mujoo's results (26). Soy proteins in fermented SMC with 10% DFS were partially digested. As shown in Fig. 4, *B. firmus* NA-1 and *B. subtilis* KU-A displayed similar digestion patterns, with lower molecular-weight peptides below 15 kDa. The proteolytic enzymes from *B. firmus* NA-1 and *B. subtilis* KU-A digested the 11S polypeptide completely to 15 kDa. In contrast, *B. subtilis* GT-D predominantly hydrolyzed the 11S polypeptide to 34 kDa, producing peptides less than 25 kDa.

Considering the crude protein content, proteolytic and α-amylase activity in the SMC powder fortified with 10% DFS and fermented by *B. subtilis* GT-D, the final product was suitable under the standard for 'enzyme foods' as functional foods in the Korean Food Codex. The powder also contains soybean peptides and mucilage as biologically active compounds, so that it can be utilized as a functional ingredient for processing foods and preparing functional foods in the future.

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