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Characterization of γ-Polyglutamic Acid Produced from the Solid-state Fermentation of Soybean Milk Cake Using *Bacillus* sp.

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Abstract In this study, we optimized the production of γ-polyglutamic acid (PGA) in soybean milk cakes (SMC) fermented with *Bacillus subtilis* GT-D and *B. subtilis* KU-A, to be utilized as a functional food ingredient. PGA production was dependent upon the glutamate content, fermentation time, and type of *Bacillus* sp. The consistencies of the SMCs fermented by *B. subtilis* GT-D and *B. subtilis* KU-A were highest after 36 hr of fermentation, and then decreased gradually. The SMC fermented by *B. subtilis* KU-A had a higher consistency than the SMC fermented by *B. subtilis* GT-D. In the presence of 10% defatted soy flour (DFS), 5% glutamate in the SMC was efficiently converted into polyglutamic acid (PGA) for 24 hr, indicating a conversion yield above 96%, but its conversion then decreased with higher concentrations of glutamate. The soluble solid content (mucilage) of the SMC fermented with *B. subtilis* KU-A was 9.5%(w/w), and composed of 65.6% PGA (Mw 1,536 kDa) and some polysaccharides. However, the SMC fermented with *B. subtilis* GT-D had a mucilage content of 7.8%(w/w), and was composed of 66.4% PGA (Mw 1,409 kDa), 11.5% levan, and some polysaccharides. The viscoelastic values of the mucilage obtained using *B. subtilis* KU-A were much higher than those of mucilage obtained using *B. subtilis* GT-D. Also, the G′-value (elastic modulus) was higher than the G″-value (viscous modulus).

Keywords: soybean milk cake, *Bacillus* sp., polyglutamic acid, glutamate, mucilage

Introduction

Soybeans are a major protein source for the Oriental people. In particular, legumes have been eaten as processed foods, including soybean curd and bean sprouts, and used for fermented foodstuffs (1, 2). Soybean curd, which is the concentrate of soybean protein, is eaten as a principal food in Korea and Japan. During soybean curd processing, soluble proteins are aggregated with calcium and magnesium, followed by pressing of the soybean curd. As a waste product, soybean milk cake (*biji*) is widely produced in the local and industrial productions of soybean curd (3). Soybean milk cake (SMC) is generally used as feed for livestock (4). Now, with a more modernized soybean curd industry, SMC with less microflora contamination could be obtained under the HACCP system by the utilization of heat sterilization and sanitary processing equipment.

Recently, the potential economic profit from this waste product has been considered. The successful utilization of SMC could provide great economic gains to soybean processing companies. Early research on SMC has focused on the preservation of raw SMC (5, 6); and dried SMC has been applied in the manufacture of formulated soybean curd (7). Also, SMC was used as an added ingredient in the manufacture of bread (8), and in Japan, the polysaccharide from SMC was extracted and purified as a functional ingredient (9). Others have previously reported on changes in the carbohydrate composition of SMC during solid-state fermentation (10), and a lactic acid bacterium isolated from raw SMC was applied in the bioconversion of SMC (11). Recently, various *Bacillus* strains were isolated from Korean fermented foods, in order to select a poly-γ-glutamate producer (12). Alkaline fermentation of SMC has been performed to determine the biochemical and microbiological changes that occur during solid-state fermentation (13). Also, it was reported that a fermented soybean paste of SMC could be prepared by mixing with wheat bran (14). Very little, however, has been reported about the efficient bioconversion of SMC by solid-state fermentation (SSF).

One of the best means for converting SMC from a waste product to a valuable food ingredient quickly is the alkaline fermentation of SMC with *Bacillus* sp. The SSF of SMC is greatly dependent upon the strain of *Bacillus*, and there are many reports on isolating novel strains from traditional fermented foods (15-17). Furthermore, the production of biologically active components such as enzymes and PGA has been carried out by alkaline fermentation (18-20).

In this study, to efficiently convert SMC into a functional food ingredient containing high amounts of PGA, *Bacillus subtilis* KU-A and *B. subtilis* GT-D, which are novel strains isolated from Korean traditional foods, were used for the SSF of SMC. Overall, the production of PGA in fermented SMC was optimized, and its physicochemical properties were characterized.

Materials and Methods

Materials and strains The SMC was obtained from a local soybean curd manufacturing company. Samples of SMC were packed into 1 kg vinyl bags and kept at -20°C in a freezer. The compositional analysis and titratable acidity of the raw SMC were determined by AOAC methods (21). The water activity of the raw SMC was

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determined using a water activity meter (TH-500; Novasina, Switzerland). The defatted soybean flour (DSF) was obtained from Archer Daniels Midland Co. (Decatur, IL, USA). Glutamate was obtained from Yakuri Pure Chemicals Co., Ltd. (Kyoto, Japan). Dextran was purchased from Sigma Co. (St. Louis, MO, USA). All chemicals were of the highest grade. The *B. subtilis* GT-D and *B. subtilis* KU-A were confirmed by API kit analyses (22), and identified by 16S rDNA sequencing.

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

Solid-state fermentation of SMC Fifty g of SMC were thoroughly mixed with 10% DSF in a 250 mL glass beaker and then sterilized at 121°C for 15 min. The starter cultures of *B. subtilis* KU-A and *B. subtilis* GT-D were transferred to the sterilized SMC at a 1% level, mixed thoroughly, and allowed to incubate at 42°C for 3 days.

Mucilage content of the fermented SMC Fermented SMC (5 g) was mixed with 100 mL of distilled water and shaken at 150 rpm for 20 min (22). The filtrate was prepared by passage through a stainless steel sieve (0.99 mm), and the supernatant was obtained after centrifugation at 24,900×g for 20 min. The supernatant was mixed with two volumes of absolute isopropanol, and the mucilage aggregate that formed was recovered by centrifugation at 9,740×g for 20 min. The solid content of the mucilage was determined by drying at 60°C overnight, or by freeze-drying.

Effect of glutamate on the production of mucilage Fifty g of SMC were fortified with 10%(w/w) DSF in a 250 mL glass beaker, and then sterilized at 121°C for 15 min. The starter cultures of *B. subtilis* KU-A and *B. subtilis* GT-D were inoculated at a 1% level. The sterilized glutamate solution (35%, w/v) was then thoroughly mixed to an adjusted content of 0-7%(w/w), followed by fermentation at 42°C for 24 hr.

Rheological properties of the mucilage To measure the consistency of the fermented SMC, 5 g of fermented SMC were mixed with 45 mL of distilled water and shaken at 150 rpm for 20 min, followed by filtration using a steel wire sieve. The filtrate (13 mL) from the fermented SMC was loaded into 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) in the shear rate range of 1-100/sec at 20°C. The flow behavior and consistency indices were determined by a power law equation (23). The viscoelastic properties were determined using a cone plate device (Platte PP35 Ti; 3.5 cm diameter, 2°). Each sample (1.4 mL) was loaded onto the rheometer plate, and dynamic shear data were obtained from frequency sweeps over the range of 0-4.6 rad/sec at constant strain amplitude (2%). G′ is a measure of the energy that was stored in the material, or was recoverable; G″ is a measure of the energy that was lost as viscous dissipation.

Isolation of PGA from the mucilage The fermented SMC (5 g) was mixed with 100 mL of distilled water and then shaken at 150 rpm for 20 min, followed by filtration using a stainless steel sieve. The supernatant was obtained after removing the remaining cells and debris by centrifugation at 24,900×g for 20 min. The supernatant was kept at 4°C for 16 hr after adjusting it to pH 4.0. After removing the insoluble debris by centrifugation at 24,900× g for 20 min, the supernatant containing PGA was mixed with 2 volumes of isopropanol. The polymer aggregate was recovered and used for the determination of PGA (24).

Determination of glutamate content in the fermented SMC To determine the glutamate content present in the fermented SMC, the polymer aggregate was removed and the isopropanol solution was concentrated using a vacuum evaporator (H-3000; Hanshin, Korea), which was then passed through a 0.45 mm syringe filter (Minisart RC 15; Sartorius, Germany) after centrifugation. The concentration of glutamate was determined by HPLC (equipped with a Young Lin M930 solvent delivery system and a Young Lin M720 absorbance detector) on a reverse phase column (Synergi 4u Fusion-RP 80; 250×4.60 mm, Phenomenex, Torrance, CA, USA). The sample was eluted with a mobile phase consisting of methanol and 0.2% aqueous phosphate, pH 2.4 (1:9, v/v). The flow rate was set at 0.8 mL/min, and the eluant was monitored at 220 nm. The glutamate conversion yield was determined based on the glutamate content remaining in the SMC fermented with various glutamate concentrations.

Determination of levan in the mucilage The levan content of the mucilage was determined by sugar analysis using HPLC. To transform the levan polymer into fructose selectively, the mucilage solution (1%) was mixed with the same volume of 1% oxalic acid, and heated at 100°C for 15 min (25). The supernatant was obtained after centrifugation at 22,250×g for 5 min, and passed through a 0.45 μm syringe filter (Minisart RC 15; Sartorius). The filtrate (20 μL) was injected into HPLC (Waters, Milford, MA, USA) that was connected to a sugar analysis column (Asahipak NH2P 50; Shodex Co., Tokyo, Japan) at 40°C. Acetonitrile (75%), as the mobile phase, was applied at a flow rate of 1.0 mL/min, and the sugar content was determined by measuring the refractive index (Waters).

Quantification and molecular weight of the PGA The concentration and molecular weight of the PGA were measured at 40°C using an Autochro-GPC system (Young In Scientific Co., Seoul, Korea) equipped with a Waters 410 Refractometer and a Shodex OHpak SB 800 HQ series column (SB 802.5, SB 805). Dextrans were used as the polysaccharide standards to construct a calibration curve for calculating the unknown molecular weight of the PGA. An eluant containing 0.1 M Na_2SO_4 and 0.05%(w/v) NaN_3 was brought to pH 4.0 using glacial acetic acid;

the flow rate was set at 1.0 mL/min (26). The amount of PGA was calculated using the peak area of the GPC measurements, with purified PGA as the standard.

Results and Discussion

Identification of *Bacillus* sp. *Bacillus* sp. isolated from traditionally fermented soybeans was identified by 16S rDNA sequencing. *B. subtilis* GT-D was previously identified (27). *B. subtilis* KU-A showed 99% homology with *B. subtilis* Z99104. When the *Bacillus* strains were grown on MRS agar plates, *B. subtilis* KU-A showed a dome shaped morphology and had sticky mucilage. The morphology of this strain was similar to a previously reported jelly-like type that formed a thick, wrinkled layer (17). *B. subtilis* GT-D, however, had a flat shape without mucilage. When both *Bacillus* sp. were grown on nutrient broth agar plates, they covered the surface of plates without sticky mucilage. Therefore, in order to isolate candidates of novel *Bacillus* strains that produce much higher amounts mucilage, it may be useful to use MRS agar plates.

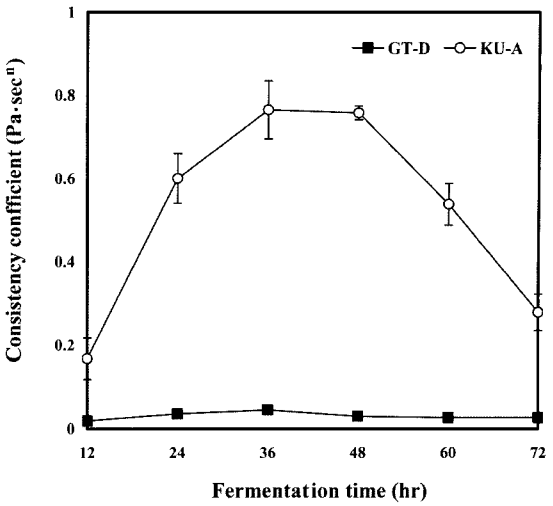

Fig. 1. Changes in consistency of the SMCs fermented by *B. subtilis* KU-A and *B. subtilis* GT-D according to fermentation time. The SMC fortified with 10% DSF was fermented at 42°C.

Solid-state fermentation of SMC In soybean curd manufacturing, SMC is produced via hot processes, including heat-sterilization and hot screw pressing. The general composition of SMC, including its acidity and water activity value, is shown in Table 1. Because the solid-state fermentation (SSF) of SMC is generally affected by the initial moisture content, the moisture content of SMC was crucial for an efficient alkaline fermentation using *Bacillus* strains. The raw SMC with 74.1% moisture content had a water activity value of 0.94, which was sufficient for microbial growth. In addition, the raw SMC was sterilized before the solid-state fermentation, because it contained indigenous *Bacillus* sp. (about 1×10^5 viable cells) in spite of a heat treatment during the separation of SMC.

For efficient solid-state fermentation, the SMC was fortified with 10% DSF, followed by heat-sterilization. The moisture content of SMC was progressively reduced from 74.1 to 68%. The SSF of SMC enhanced the consistency of the fermented SMC. Ultimately, addition of the 10% DSF allowed for increases in the consistency. We found that the SMC fermented by *B. subtilis* KU-A contained higher amounts of crude protein (8.7%), crude lipids (3.2%), and dietary fiber (13.6%) compared to the raw SMC. The SMC fermented by *B. subtilis* GT-D showed similar results, including a dietary fiber content of 12.4%.

To estimate the mucilage production of the SMCs fermented by *B. subtilis* KU-A and *B. subtilis* GT-D, the consistencies of the water extracts were determined. As shown in Fig. 1, consistency of the water extract depended greatly on the *Bacillus* strain type and fermentation time. For both of the *Bacillus* strains used in the alkaline fermentation, consistency increased for the first 36 hr of fermentation, and then decreased with longer fermentation times. Particularly, the SMC fermented with *B. subtilis* KU-A showed a greater increase in consistency compared to that using *B. subtilis* GT-D. This implies that *B. subtilis* KU-A is a potent strain for producing biopolymers in SMC with 10% DSF. In addition, one can conclude that *B. subtilis* KU-A is a glutamate dependent strain for the production of PGA. On the other hand, *B. subtilis* GT-D didn't have a positive effect on consistency in the presence of glutamate, showing a consistency value below 0.1 (Pa·sec^n). In the SMCs fermented with *B. subtilis* KU-A and *B. subtilis* GT-D, mucilage extract consistency decreased with extended fermentation times. This may have been due to the partial enzymatic hydrolysis of the mucilage late in fermentation. Such a hypothesis was supported for *natto* mucilage, which was hydrolyzed by depolymerase produced by *Bacillus* sp., resulting in decreases of viscosity (28). When considering a high consistency and wholesome flavor for the fermented SMC, it is most suitable to perform the alkaline fermentation within 24 hr.

Effect of glutamate on mucilage production For efficient PGA production in the solid-state fermentation of SMC, the effect of glutamate concentration was determined over 24 hr of fermentation. Without glutamate, the SMC was converted into a sticky mass by *B. subtilis* KU-A. Furthermore, increasing the glutamate content during fermentation caused the consistency of the mucilage to gradually increase, indicating a maximal glutamate content value of 5% (Fig. 2). This, it was

Table 1. Physicochemical properties of unfermented SMC

Moisture content (%)	Crude protein (%)	Crude lipids (%)	Crude ash (%)	Carbohydrate (%)	Crude fiber (%)	Acidity (%)	Water activity value
74.1	6.9	0.9	1.0	14.8	2.3	0.01	0.938

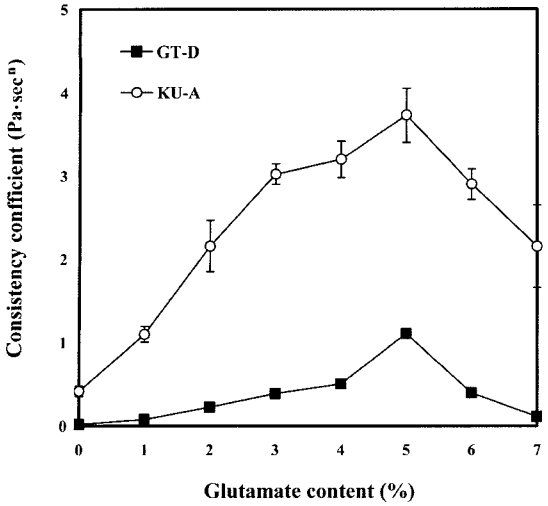

Fig. 2. Effect of glutamate content on the consistency of SMCs fermented by *B. subtilis* KU-A and *B. subtilis* GT-D. The SMC fortified with 10% DSF and various glutamate concentrations was fermented for 24 hr.

concluded that *B. subtilis* KU-A is dependent upon glutamate for mucilage production, including PGA. In particular, the glutamate concentration is a crucial factor in optimizing the production of mucilage via complete conversion of the glutamate. The mucilage consistency decreased with glutamate concentrations above 5%, and then drastically dropped when the SMC was fortified with 7% glutamate. *B. subtilis* GT-D showed a similar pattern according to glutamate content, but had a lower consistency compared to *B. subtilis* KU-A.

In the SMC fermented with 5% glutamate, the conversion yield of the glutamate was dependent upon the fermentation time. As shown in Table 2, both *Bacillus* strains had greater than 96% conversion yields after fermentation for 24 hr. The TLC analysis showed similar results, with trace amounts of glutamate present in the fermented SMC after 24 hr of fermentation (unpublished result). Furthermore, the conversion of glutamate was nearly complete after fermentation for 36 hr. However, we concluded that the SSF of SMC with 10% DSF is suitable when performed for 24 hr because the fermentation of SMC for 36 hr resulted in the production of unpleasant flavors. Conclusively, fermented SMC consisting of a high amount of mucilage and wholesome flavor could be utilized as a food ingredient.

With glutamate-dependent *Bacillus* strains, even though the production of mucilage containing PGA is enhanced by adding glutamate to the medium, glutamate that remains in the medium may give negative attributes to edible fermented SMC products. Thus, we determined the content of glutamate remaining in the fermented SMCs. As shown in Table 3, the conversion of glutamate by *B. subtilis* KU-A was above 94% in the SSF of SMC fortified with 5% glutamate. However, with higher glutamate fortification, the glutamate that remained in the fermented SMC increased significantly, indicating incomplete conversion of the glutamate. *B. subtilis* GT-D showed similar patterns for its conversion yield of glutamate. It has been reported that endogenous L-glutamic acid directly serves as the precursor to PGA (29, 30). This implies that glutamate, in a limited concentration, may efficiently serve as the precursor for PGA synthesis in the SSF of SMC with 10% DSF. Therefore, glutamate concentration is a critical factor for the optimal production of mucilage in the SSF of SMC, and we concluded that 5% glutamate is the optimal concentration for efficiently converting glutamate in the SSF of SMC with 10% DSF. Previous studies have shown that in a liquid culture for producing PGA, the conversion yield of glutamate was greatly dependent upon the strain type: 85-115% by *B. licheniformis* ATCC 9945a (31), 33-66% by *B. subtilis* IFO 3335 (31), 68% by *B. subtilis* F-2-01 (33), and 101% by *B. subtilis* NX-2 (26). This indicates that glutamate, in a defined medium, can be efficiently converted into mucilage, resulting in a higher production of PGA. However, the effect of glutamate on PGA production in the SSF of SMC has not yet been reported.

Quantification and molecular weight of PGA In general, the production of PGA in a liquid culture is affected by the culture conditions, including the concentrations of the carbon and nitrogen sources (34). To establish the effects of the glutamate content on the production of PGA in the SSF of SMC using *B. subtilis* KU-A, we determined the amount of PGA produced. As shown in Table 4, in the absence of glutamate, the mucilage obtained from the SMC fermented by *B. subtilis* KU-A

Table 2. Conversion yield and remaining glutamate in the SMCs fermented by *B. subtilis* KU-A and *B. subtilis* GT-D according to fermentation time

Fermentation time (hr)	KU-A		GT-D	
	Conversion yield (%)	Remaining glutamate (g/kg)	Conversion yield (%)	Remaining glutamate (g/kg)
12	83.2	8.4	81.0	9.5
24	96.5	1.7	97.6	1.2
36	99.8	0.1	99.9	0.1

Table 3. Effect of glutamate content on the conversion yield of glutamate in the SMCs fermented by *B. subtilis* KU-A and *B. subtilis* GT-D

Glutamate (%)	KU-A		GT-D	
	Conversion yield (%)	Remaining glutamate (g/Kg)	Conversion yield (%)	Remaining glutamate (g/Kg)
1	100	-	100	-
2	100	-	100	-
3	99.6	0.1	100	-
4	93.9	1.2	99.8	0.1
5	94.2	1.5	99.7	0.1
6	86.9	3.9	91.5	5.1
7	85.9	4.9	83.6	11.5

Table 4. Effect of glutamate content on the production of mucilage, PGA, and levan in SMCs fermented by *B. subtilis* KU-A and *B. subtilis* GT-D

Glutamate (%)	KU-A			GT-D		
	Solid content (%)	PGA content (g/kg)	Levan content (%)	Solid content (%)	PGA content (g/kg)	Levan content (%)
0	4.5±0.4	15.4	-	2.6±0.1	2.9	5.3
1	5.6±0.7	19.7	-	3.7±0.3	10.6	4.7
2	7.1±0.4	28.4	-	4.9±0.5	19.7	2.3
3	8.6±0.9	44.6	-	5.8±0.1	28.7	1.9
4	8.6±1.0	53.7	-	6.9±0.7	47.0	1.5
5	9.5±0.7	62.4	-	7.8±0.5	51.8	0.9
6	8.2±0.7	59.3	-	7.9±0.6	37.7	0.9
7	7.2±0.9	41.9	-	7.1±0.6	36.9	0.8

was 4.5%, including PGA (15.4 g/kg). The PGA fraction in the mucilage was increased by increasing the glutamate content of the SMC. The highest PGA content (62.4 g/kg) was obtained from the SSF of SMC fortified with 5% glutamate, and amounted to approximately 65.6%(w/w) of the total soluble mucilage (9.5%) content. In the SMC fermented by *B. subtilis* GT-D, the solid content increased with increasing glutamate concentrations. In the presence of 5% glutamate, the PGA content (51.8 g/kg) was equivalent to 66.4% of the total soluble mucilage content (7.8%).

Although the PGA content of the fermented SMC increased with increasing glutamate content, it slightly decreased at glutamate concentrations above 5%. This result coincides with previous consistency data. It was reported that PGA amounts of 7-18 g/kg were produced during SSF, and that production was dependent upon the medium moisture content, carbon and nitrogen sources, and fermentation time (35). It was also reported that PGA production was approximately 10-50 g/L in liquid cultures (24, 26, 30, 33, 36, 37).

Therefore, we have concluded that the production of PGA during the SSF of SMC is greatly affected by the glutamate concentration. Interestingly, the mucilage obtained using *B. subtilis* KU-A didn't contain any levan. However, *B. subtilis* GT-D produced some levan, although the amount decreased with increasing glutamate concentrations.

The molecular weights of the PGA obtained from the SSFs of SMC, using *B. subtilis* KU-A and *B. subtilis* GT-D, showed slight differences at 1,536, and 1,409 kDa, respectively. It has been reported that the molecular weight of PGA varies according to the culture conditions and strain type, ranging from 100 to 2,000 kDa (38). Recently, super-molecular weight PGA was synthesized using *B. subtilis* in the presence of a high concentration of ammonium sulfate (18). We conclude that the molecular weight of PGA obtained during the SSF of SMC with glutamate was affected by the type of *Bacillus* strain.

Physicochemical properties of the fermented SMC The viscoelastic properties of the mucilage solution, which was obtained from SMC fermented with 5 % glutamate, were dependent upon the mucilage concentration. As shown in Fig. 3, both moduli of the

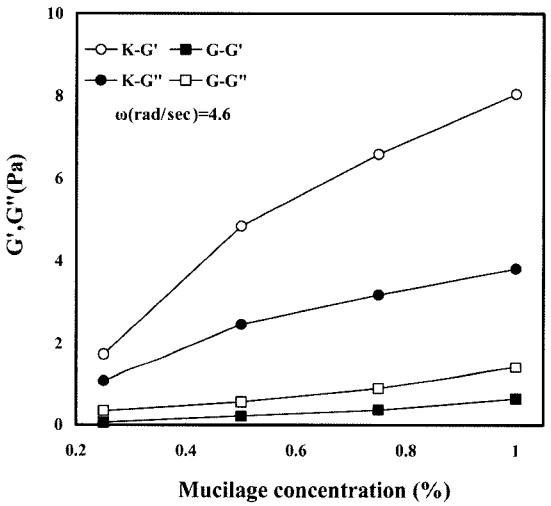

Fig. 3. Changes in viscoelastic properties according to the concentration of mucilage obtained from the fermented SMC. The SMC fortified with 10% DSF and 5% glutamate was fermented for 24 hr. The elastic properties (G′) and viscous properties (G″) were determined at a frequency of 4.6 rad/sec.

mucilage solution increased with increasing mucilage content. In spite of similar mucilage concentrations, the elastic modulus (G′) and viscous modulus (G″) value obtained from the mucilage solution of the SMC fermented by *B. subtilis* KU-A were superior to those of *B. subtilis* GT-D. In the SMC fermented by *B. subtilis* KU-A, the G′ of the mucilage solution exceeded the G″ over the mucilage concentration. However, the G″ was slightly higher than the G′ in the mucilage solution obtained from *B. subtilis* GT-D. This implies that the mucilage obtained from the SMC fermented by *B. subtilis* KU-A had higher viscoelastic properties than the mucilage obtained with *B. subtilis* GT-D. It also implies that the molecular weight of the PGA in the mucilage may affect the viscoelastic properties of the mucilage solution. Thus, it is necessary to elucidate the relationship between the molecular weight and viscoelastic properties of mucilage produced by various *Bacillus* sp.

In conclusion, the production and molecular weight of

PGA obtained during the SSF of SMC was greatly affected by glutamate content and the type of *Bacillus* strain. SMC with a 5% glutamate concentration was efficiently transformed into a functional ingredient containing 9.5% mucilage (65.6% PGA) using a novel *B. subtilis* KU-A strain. Finally, the mucilage obtained by *B. subtilis* KU-A showed higher viscoelastic properties compared to that of *B. subtilis* GT-D.

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