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Characterization of Mucilage Produced from the Solid-state Fermentation of Soybean Grit by *Bacillus firmus*

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Abstract Mucilage containing γ-polyglutamic acid (PGA) was efficiently generated by the solid-state fermentation (SSF) of soybean grit by *Bacillus firmus* NA-1. *B. firmus* NA-1 was shown to be a glutamate-dependent strain for PGA production. The SSF of soybean grit was optimized in order to produce mucilage with a fortification of 5% glutamate, resulting in higher levels of mucilage production (6.14%) and a higher consistency index (1.1 Pa·sec^n). The sticky mucilage was comprised of 38% PGA, 7% levan, and some biopolymers. With regard to the viscoelastic properties of the mucilage solution, the viscous modulus (G") obtained from soybean grit fortified with 5% glutamate was approximately 64 times higher than that of the mucilage solution obtained without glutamate. Although the addition of glutamate in the SSF of soybean grit influenced the rate of PGA production, the molecular weight of PGA remained unaltered, and was detected in a range between 1,400-1,440 kDa.

Keywords: γ-polyglutamic acid, soybean grit, *Bacillus firmus*, solid-state fermentation

Introduction

Biopolymers are usually produced by microorganisms that are abundant in nature. Biopolymers are a diverse and versatile class of materials that have many potential applications (1). Among the biopolymers, γ-polyglutamic acid (PGA) is an unusual anionic, naturally occurring homo-polyamide comprised of D-and L-glutamic acid units connected by amide linkages between the α-amino and γ-carboxylic acid groups. PGA is a water-soluble, biodegradable polymer, and is non-toxic to humans and the environment. PGA is an edible functional biomaterial which has been applied to the production of foods, cosmetics, and medicines (2).

There have been many reports regarding the mechanism underlying the production of PGA from microorganisms (3-6). PGA is efficiently generated by strains of *Bacillus* during the fermentation of *natto* or *cheonggukjang*, both of which are traditional fermented soybean foods. PGA, a viscous material, evidences a particular set of viscoelastic properties. Its production is dependent upon microorganisms and environmental factors, including temperature, and nutrient composition (2, 7).

The microorganisms involved in PGA production are generally divided into two groups; one group requires L-glutamic acid as a nitrogen source for cell growth and PGA production, whereas the other group requires no L-glutamic acid as a nitrogen source. *Bacillus* strains independent of L-glutamic acid utilize citric acid and glucose as primary carbon sources for the production of PGA. On the other hand, *B. licheniformis* is dependent on L-glutamic acid for PGA production, although it utilizes citric acid and glycerol as a carbon source, thereby indicating that L-glutamic acid activates the enzymes involved in PGA biosynthesis (2, 8, 9).

PGA production is dependent on the carbon and nitrogen sources, as well as ionic strength, pH, and aeration. Submerged fermentation (SmF) has been previously applied to PGA production, but the technology utilized for PGA fermentation remains problematic, in that the significant increase in the viscosity of the media results in uncontrollable foaming and limitation of volumetric oxygen mass transfer, which in turn results in insufficient cell growth and a reduction in PGA yield (6, 10). Furthermore, a relatively high expenditure for SmF media limits the production of PGA as a prevalent commercial valuable product. However, solid-state fermentation (SSF) using soybeans represent a simple and economical process, owing primarily to the simplicity of the cultivation equipment and the lower energy requirements. In addition, it avoids the problems inherent to SmF using a fermentor (11-13). In the solid-state fermentation of soybeans, the *Bacillus* strain normally generates the sticky mucilage comprised of PGA and levan as a biopolymer (14-16). Considering the efficient production of mucilage, soybean grits with small particle size have many advantages as a raw material for SSF. The small particle size allows for increases in the surface area. Additionally, the efficient flow behavior evidenced by the soybean grits facilitates the easy mixing and transport of raw materials during food processing. It has been recently reported that the solid-state fermentation of soybean grits and soybean milk cake resulted in the enhanced production of biologically active components, including mucilage, peptides, and digestive enzymes (17, 18).

The production and characterization of PGA in a liquid culture has been previously accomplished and extensively

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described. On the other hand, as a means to efficiently produce PGA, the SSF of soybean grits has been somewhat underreported. In the current study, we attempted to optimize the production of mucilage containing PGA, using soybean grits as a raw material. The effects of glutamate on PGA production were evaluated in order to optimize the SSF of soybean grits by *Bacillus firmus* NA-1 isolated from fermented soybeans, and the physicochemical properties of mucilage containing PGA were assessed.

Materials and Methods

Material and reagents Soybean grit was obtained from the DooWon Food Co. (Gyeongbuk, Korea). Glutamate was purchased from the Yakuri Pure Chemicals Co., Ltd. (Kyoto, Japan). Micronized full-fat soy flour (MFS) was obtained from the Perican Co. (Toyko, Japan). Dextran-polysaccharide standards were purchased from American Polymer Standards Co., (Mento, OH, USA). All other reagents used in this study were of the highest available grade.

Strains *B. firmus* NA-1 isolated from Japanese fermented soybean food (*natto*) was utilized (19). The *Bacillus* strain was grown for 24 hr on MRS agar plates at 42°C.

Starter culture preparation The MFS solution (5%) was used for culture broth after 15 min of sterilization at 121°C. *B. firmus* NA-1 was inoculated and grown for 24 hr at 42°C using an incubator (SI-900R; Jeio Tech Co., Ltd., Gyeonggi, Korea) at 180 rpm.

Fermentation of soybean grits Soybean grits (25 g) were mixed with an identical weight of distilled water, and then sterilized for 15 min at 121°C. The starter culture (1%) was transferred to the sterilized soybean grits and was thoroughly mixed, followed by 24 hr of incubation at 42°C. In order to determine the particle size of the soybean grits, the soybean grits were suspended in absolute ethanol and then analyzed using a laser difference particle size analyzer (LS13320; Beckman Coulter Inc., Fullerton, CA, USA).

Mucilage content of fermented soybean grit Fermented soybean grits (5 g) were mixed with 45 mL of distilled water and shaken for 20 min at 150 rpm (17). The filtrate was prepared by passing it through a stainless steel sieve (0.99 mm), and the supernatant was subsequently obtained after 10 min of centrifugation at 24,900×g. The supernatant was mixed with 3 volumes of absolute ethanol, and the mucilage aggregate formed was recovered via 10 min of centrifugation at 9,940×g. The solid content of the mucilage was determined by drying at 60°C or freeze-drying overnight.

Effects of glutamate on mucilage production Soybean grit (25 g) fortified with 0-10%(w/w) glutamate was mixed with 25 mL of distilled water in a 250 mL glass beaker, then sterilized for 15 min at 121°C. The starter culture of *B. firmus* NA-1 (1%) was inoculated and then the sterilized glutamate solution (35%, w/v) was mixed thoroughly to adjust to 0-10%(w/w) content, followed by 24 hr of fermentation at 42°C.

Rheological properties of mucilage Fermented soybean grits (5 g) were mixed with 45 mL of distilled water, shaken for 20 min at 150 rpm, then filtered through a steel sieve (0.9 mm). The consistency of the filtrate (13 mL) was determined with a controlled stress Haake viscometer (Haake RheoStress 1; Thermo Electron Co., Karlsruhe, Baden Württemberg, Germany) equipped with a measuring cup and spindle (Rotor DG43 DIN 53544 Titan). The flow behavior was determined in the shear rate (1-100/sec) at 20°C, and was evaluated using the Power law model (20).

The dynamic rheological measurements were conducted using a cone-plate device (Platte PP35 Ti, 3.5 cm diameter, 2°). Each sample (1.4 mL) was loaded on the rheometer plate. The dynamic shear data were obtained from frequency sweeps over a range of 0.63-62.1 rad/sec at a constant strain amplitude (2%). Dynamic strain sweeps were conducted prior to the frequency sweeps in order to ensure operation within the linear viscoelastic region. All experiments were performed at 20°C. G′ is a measure of the energy stored in the material or recoverable; G″ is a measure of the energy lost as viscous dissipation. Dynamic rheological measurements were conducted in triplicate.

Isolation of PGA from mucilage Soybean grits (5 g) were mixed with 100 mL distilled water and shaken for 20 min at 150 rpm, followed by filtration using a stainless steel sieve. The supernatant was acquired after the removal of the remaining cells and debris via 10 min of centrifugation at 24,900×g. The supernatant was maintained for 16 hr at 4°C after adjusting it to a pH of 4.0. The insoluble debris was then removed via 10 min of centrifugation at 24,900×g, after which the resultant supernatant was mixed with 3 volumes of ethanol. The polymer aggregate was recovered and subsequently utilized for PGA determination (15).

Determination of glutamate in fermented soybean grit In order to determine the glutamate content in the fermented soybean grits, the ethanol solution used to remove the polymer aggregate was concentrated using a vacuum evaporator (H-3000; Hanshin, Korea), and passed through a 0.45 μm syringe filter (Minisart RC 15; Sartorius, Göttingen, Germany) after centrifugation. In addition, in order to visualize the glutamate quantitatively, a sample (2 μL) and a standard glutamate were applied to the thin-layer chromatography (TLC). TLC was conducted on a cellulose plate (Merck, Darmstadt, Germany) with solvent systems using butanol-acetic acid-water (3:1:1, w/w) and 96% ethanol-water (63:37, w/w) then detected via spraying with 0.2% ninhydrin in acetone (21).

The glutamate concentration was also determined via high performance liquid chromatography (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, at a pH of 2.4 (1:9, v/v). The flow rate was set

to 0.8 mL/min, and the eluant was monitored at 220 nm. The conversion yield of glutamate was determined on the basis of the glutamate content remaining in the soybean grits fermented with various glutamate concentrations.

Determination of levan in mucilage The levan content in the mucilage was determined via sugar analysis using HPLC. In order to selectively transform the levan polymer into fructose, the mucilage solution (1%) was mixed with an identical volume of 1% oxalic acid, then heated for 15 min at 100°C (22). The supernatant was acquired after 5 min of centrifugation at 24,900×g, and then passed through a 0.45 μm syringe filter (Minisart RC 15; Sartorius). The filtrate (20 μL) was injected into HPLC (Waters, Milford, MA, USA), and connected to a sugar analysis column (Asahipak NH2P 50; Shodex Co., Tokyo, Japan) at 40°C. The mobile phase (75% acetonitrile) was applied at a flow rate of 1.0 mL/min, and the sugar content was determined via measurements of the refractive index (Waters 410; Waters).

PGA Quantification and molecular weight determination The concentration and molecular weight of 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 as polysaccharide standards were employed in the construction of a calibration curve, from which the unknown molecular weights of PGA were calculated. The eluant containing 0.1 M Na_2SO_4, 0.05%(w/v) NaN_3 was brought to a pH of 4.0 using glacial acetic acid, and the flow rate was set to 1.0 mL/min (23). The quantity of PGA was calculated using the peak area of the GPC measurements, using purified PGA as a standard.

Results and Discussion

Strain A novel *B. firmus*, which efficiently produces mucilage, was isolated from Japanese fermented soybean foods (19). The strain, designated *B. firmus* NA-1, was also utilized for the alkaline fermentation of soybean grits. A *Bacillus* strain evidenced a particular morphology an irregularly shaped mucous colony when it was grown on an MRA plate.

Effect of glutamate on the mucilage production Compared to the whole soybeans, the soybean grits as raw materials evidenced an average particle size of 851.4 mm and a bulk density of 0.65 g/cm^3. Soybean grits were converted efficiently into functional ingredients via SSF (17). Particularly for alkaline fermentation with soybean grits, the process of steeping the raw materials can be omitted, thereby minimizing nutrition loss and water use. Previously, sticky mucilage was produced efficiently from the SSF of soybean grits by *Bacillus* sp. isolated from traditional fermented soybean foods (unpublished results).

In an effort to optimize the production of mucilage from the SSF of soybean grits, the effects of glutamate concentration were determined. Without glutamate, the soybean grits were converted into a sticky mass by *B. firmus* NA-1. The water extract of fermented soybean grits evidenced a consistency of 0.045 (Pa·sec^n) and a mucilage content of 3.25%. Its mucilage content was relatively higher than that of whole soybeans fermented via the general method. This implies that the soybean grit is suitable for mucilage production by SSF. In soybean grits fermented with increasing glutamate content, the consistency of mucilage increased gradually, evidencing a maximal glutamate content of 5% (Fig. 1). However, the consistency of mucilage obtained from fermented soybean grits decreased at a glutamate content level of above 5%, and then declined drastically when glutamate was fortified to a level of 8% in the soybean grits.

As is shown in Fig. 1, the soluble mucilage content of soybean grits fermented with 5% glutamate was 6.14%(w/w). In a pattern similar to that of the consistency, the mucilage content of fermented soybean grits increased up to 5% glutamate, and then decreased slightly after the addition of more than 5% glutamate.

In PGA production from the SSF of soybean grits by *B. firmus* NA-1, the addition of glutamate enhanced the production of mucilage, thereby resulting in higher viscosity. The defined medium without glutamate proved unsuccessful in the production of PGA (unpublished data). Thus, it was concluded that *B. firmus* NA-1 is dependent upon glutamate for the production of mucilage containing PGA. In particular, glutamate concentration is the most crucial factor in the optimization of the production of mucilage via the complete conversion of glutamate.

The dynamic viscoelastic properties of the mucilage solution obtained from fermented soybean grits were dependent on the glutamate concentration and frequency sweep. As is shown in Fig. 2, with increasing glutamate content both moduli of the mucilage solution increased over the tested frequency range. However, both moduli of the mucilage solution decreased at a glutamate content of above 5%. This result coincides with the patterns of production and consistency of mucilage from the fermented soybean grits. The viscous modulus (G″) for the mucilage solution exceeded that of the elastic modulus (G′). In particular, the G″ obtained from the soybean grits

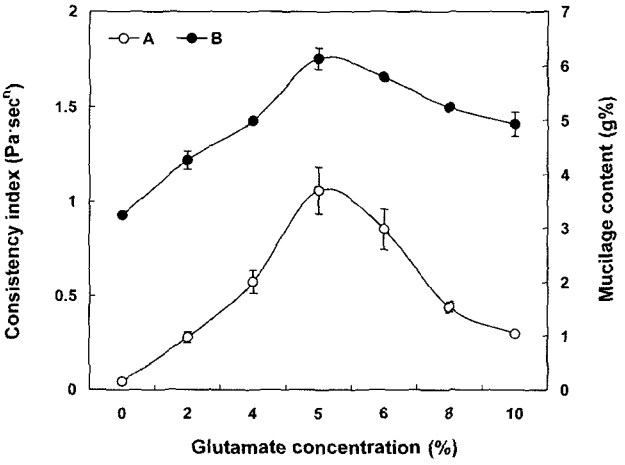

Fig. 1. Effects of glutamate concentrations on consistency index and mucilage content in soybean grits fermented by *B. firmus* NA-1. A, consistency index; B, mucilage content.

fortified with 5% glutamate was approximately 64 times higher than that of mucilage solution obtained from the soybean grits fermented without glutamate. This implies that the mucilage obtained from soybean grits fermented by *B. firmus* NA-1 evidences more profound viscous properties according to the fortification of glutamate in soybean grits for solid-state fermentation. In the assessed mucilage solution, the G′ was increased profoundly as compared to that of G″ when the frequency is increased. Ultimately, both moduli evidenced similar values at the highest frequency, and a cross-over point of G′ and G″ was observed in the soybean grits fermented with 5% glutamate (Fig. 2).

It has been reported than the G′ of native polymer solution exceeded that of G″ with both moduli evidencing only a slight dependence on frequency (24). It has also been reported that guar gum behaves like a typical macromolecular entangled biopolymer in solution, with G′ predominating over the G″ in the highest ranges of frequency. In contrast to guar gum, the viscous modulus, G″, is greater than the elastic modulus G′ over the entire tested frequency range for freshly prepared locust bean gum solution (25).

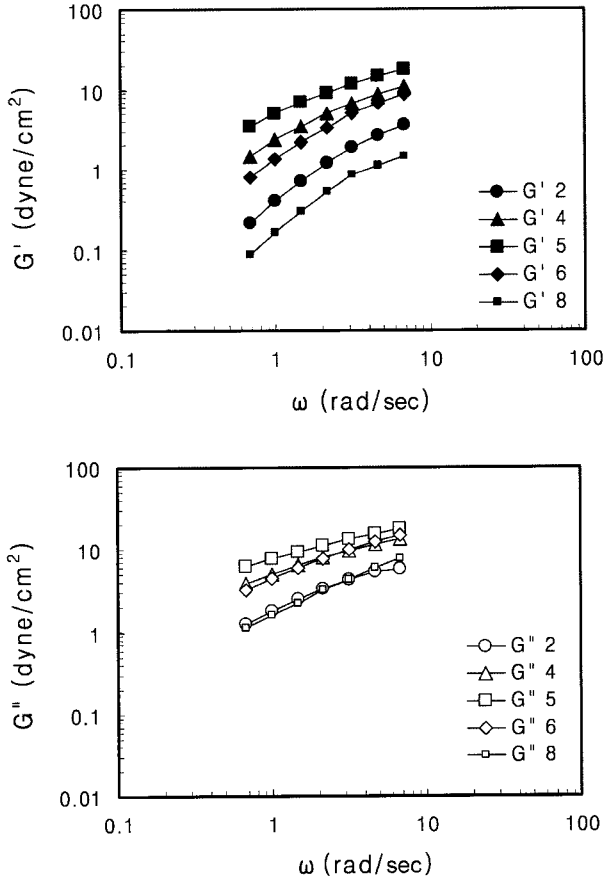

Fig. 2. Effects of glutamate content on elastic (G′) and viscous (G″) moduli of mucilage produced from soybean grits fermented by *B. firmus* NA-1. Numbers indicate glutamate concentration.

Glutamate conversion yield With a glutamate-dependent *Bacillus* strain, although PGA production can be readily controlled by altering the glutamate concentration in the medium, the glutamate content remaining in the medium may impart negative attributes to the edible fermented products. The glutamate content remaining in the fermented soybean grits was determined. As is shown in Fig. 3, the conversion of glutamate by *B. firmus* NA-1 was achieved to a level above 95% in soybean grits fortified with 5% glutamate for SSF. At higher glutamate concentrations, the glutamate conversion yield decreased drastically, indicating higher residual glutamate content (Fig. 3). Therefore, in the SSF of soybean grits, glutamate concentration is crucial for optimal mucilage production. It has been concluded that 5% glutamate is the critical concentration for the efficient conversion of glutamate in the SSF of soybean grits.

In a liquid culture for the production of PGA, the glutamate conversion yield was critically dependent on the strain type: 85-115% by *B. licheniformis* ATCC 9945a (8), 33-66% by *B. subtilis* IFO 3335 (26), 68% by *B. subtilis* F-2-01 (27), and 101% by *B. subtilis* NX-2 (23). This shows that glutamate in a defined medium can be converted efficiently into mucilage, resulting in higher PGA production. However, the effect of glutamate on PGA production in the SSF of soybean grit has yet to be reported.

In order to visualize the remaining glutamate in the fermented soybean grits, the alcohol fraction used to remove the mucilage aggregate was used for TLC analysis. As is shown in Fig. 4, the glutamate was utilized efficiently during SSF at 42°C for 24 hr. In soybean grits fermented with 5% glutamate, the residual glutamate content was quite low, thereby indicating complete glutamate utilization. However, in the case of fortification with higher glutamate contents, the glutamate content remaining in the fermented soybean grits proved significant, thereby indicating incomplete glutamate conversion. It has been demonstrated that endogenous L-glutamic acid

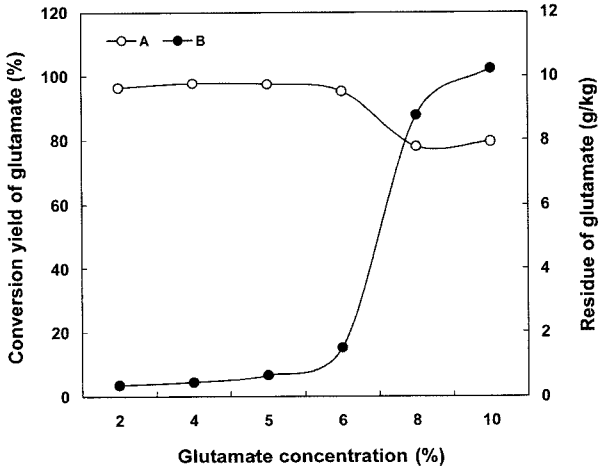

Fig. 3. Changes in the conversion yield and residual content of glutamate in soybean grits fermented by *B. firmus* NA-1. A, conversion yield of glutamate; B, residue of glutamate.

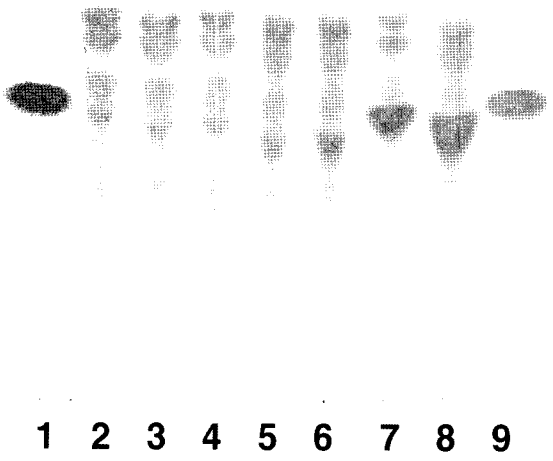

Fig. 4. Thin-layer chromatography of glutamate remaining in the soybean grits fermented by *B. firmus* NA-1 after glutamate fortification. Lane 1 and 9: glutamate marker (10 mg/mL); Lane 2 to Lane 8: 0, 2, 4, 5, 6, 8, 10%(w/w) of glutamate concentration.

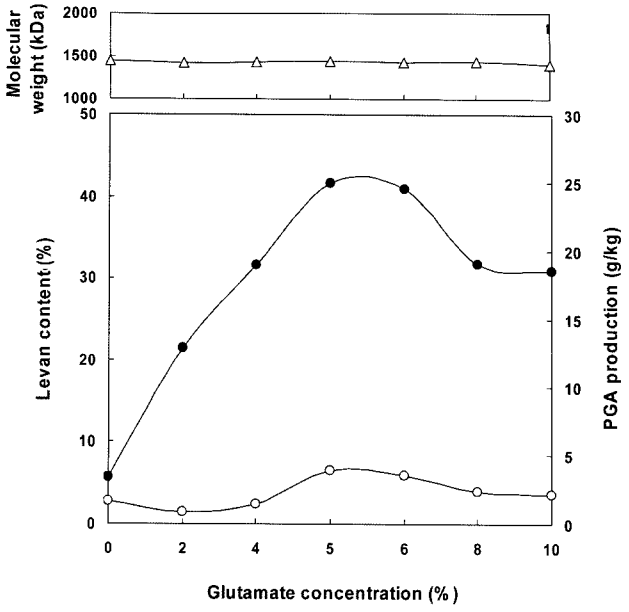

Fig. 5. Changes in the content of levan and PGA, molecular weight of PGAs in mucilage obtained from the soybean grits fermented by *B. firmus* NA-1 after glutamate fortification. △, molecular weight of PGA; ●, PGA production; ○, levan content.

directly serves as the precursor of PGA (10, 28). It has been concluded that glutamate in limited concentrations may serve as a precursor for efficient PGA synthesis in SSF using soybean grits.

Effect of glutamate on levan production In *natto* fermented with *B. subtilis* (*natto*) the mucilage generally consisted of PGA and some levan, and its composition varied (14). The effect of glutamate on the levan content in mucilage obtained from the fermented soybean grits was determined. As is shown in Fig. 5, without glutamate fortification, the mucilage produced by *B. firmus* NA-1 contained 2.8% levan. However, the levan content of mucilage generated by *B. firmus* NA-1 was increased via the addition of glutamate, thereby indicating a higher value (6.5%) at 5% glutamate. The levan content in the mucilage was decreased slightly by the addition of more than 6% glutamate.

The levan content in mucilage depends on the types of *Bacillus* strain used. The levan content in the mucilage of soybean grits fermented by other *Bacillus* strains was approximately 10% (unpublished data). Significantly, the levan content in mucilage decreased gradually with longer fermentation times, and then disappeared completely after 72 hr of fermentation (data not shown). According to the report of Choi *et al.* (29), levan was degraded by an invertase, releasing fructose as a resultant product. Thus, the removal of levan in mucilage can be achieved by controlling the fermentation time in the SSF of soybean grits.

It has been demonstrated that *B. subtilis* generated PGA and levan in a liquid medium including sucrose and L-glutamic acid. PGA and levan production in alkaline fermentation was found to be dependent upon the medium composition, as well as the types of strain. In a liquid medium containing glutamate, mucilage with PGA was produced. On the other hand, in a liquid medium containing 20% sucrose without glutamate, only levan was produced (14). Thus, the biopolymer composition in the mucilage produced by *Bacillus* sp. can be manipulated via the selection of a *Bacillus* strain and the modulation of the culture medium. However, the SSF of soybean grits by *Bacillus* sp. did not produce only PGA, due to its complex nutrient components.

Quantification and molecular weight of PGA In general, PGA production in a liquid culture is influenced by the culture condition, including the concentration of carbon and nitrogen sources (30). In an attempt to determine the effect of glutamate content on the PGA production in the SSF of soybean grits by *B. firmus* NA-1, the PGA production was evaluated. As is shown in Fig. 5, in the absence of glutamate the mucilage obtained from soybean grits fermented by *B. firmus* NA-1 evidenced a PGA content of 3.43 g/kg, approximately 11%(w/w) of the total mucilage content. The PGA fraction in the mucilage was increased by increasing the glutamate content in the soybean grits. A PGA content of 24.9 g/kg was obtained from the SSF of soybean grits fortified with 5% glutamate (Fig. 5), and the PGA content amounts were increased to approximately 38%(w/w) of the total soluble mucilage. Although the PGA content in the mucilage was increased by increasing the glutamate content, it was only slightly decreased at glutamate concentrations of above 5%. It has been reported that 7-18 g/kg of PGA was produced by the SSF, and that its production was dependent upon the moisture content of medium, carbon and nitrogen sources, and fermentation time (13). Therefore, it can be concluded that PGA production in the SSF of soybean grits is significantly affected by the glutamate concentration, resulting in PGA production as much as 7 times higher than normal. On the other hand, PGA production of approximately 10-50 g/L is possible in liquid culture (15,

23, 27, 31, 32).

The molecular weight of PGA obtained from the SSF of soybean grits by *B. firmus* NA-1 evidenced a narrow range of 1,400-1,440 kDa (Fig. 5). It has been reported that the molecular weight of PGA varies in accordance with the culture condition and strain type, ranging between 100 and 2,000 kDa (33). Recently, a super-molecular weight PGA was synthesized by *B. subtilis* in the presence of a high concentration of ammonium sulfate (34). Although the addition of glutamate to the SSF of soybean grits clearly influenced the PGA production, the molecular weight of PGA remained unchanged. This implied that the PGA generated by *B. firmus* NA-1 evidences a similar molecular weight in the SSF, despite glutamate fortification.

Acknowledgments

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