Production and Physicochemical Characterization of β-Glucan Produced by *Paenibacillus polymyxa* JB115

Hee-Kyoung Jung[1,2], Joo-Heon Hong[1], Seung-Chun Park[3], Byung-Kwon Park[4], Doo-Hyun Nam[5], and Sang-Dal Kim[2]*

[1] Bio Industry Center, Daegu New Technology Agency, Daegu 704-801, Korea
[2] Department of Applied Microbiology, College of Natural Resources, Yeungnam University, Gyeongsan 712-749, Korea
[3] College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea
[4] JEONJINBIO, Daegu 704-948, Korea
[5] Department of Pharmacy, Yeungnam University, Gyeongsan 712-749, Korea

Abstract This study was conducted to develop a bacterial glucan as an animal feed additive. A novel glucan-producing bacterium, *Paenibacillus polymyxa* JB115, was isolated from Korean soil. The glucan, JB115-BG, produced by *P. polymyxa* JB115, was confirmed by TLC to be composed of glucose only. By examining FT-IR, ^{1}H NMR, and ^{13}C NMR spectra, it was proven that JB115-BG has a β-(1→3)- and β-(1→6)-linked glucan structure. The particle size of JB115-BG was distributed in the range of 4~800 μm, with a mean value of 149.1 μm, and its molecular distribution ranged from 6.9~3,103.7 kDa. It was also observed that 80% of the purified JB115-BG had a molecular distribution above 100 kDa. The obtained results suggest that the glucan JB115-BG can be used as an animal feed additive for the purpose of enhancing immunity. © KSBB

Keywords: β-D-glucan, exopolysaccharide, polysaccharide structure, Paenibacillus polymyxa, *feed additive*

INTRODUCTION

Microbial exopolysaccharides (EPSs) are the primary or secondary metabolites produced by a variety of microorganisms. The rheological and physiological properties of EPS are quite different from those of natural gums or synthetic polymers [1], and its physicochemical characteristics vary depending on the microbial source and culture conditions. Microbial EPSs have been widely used within bioindustries as foods [2], medicines [3], and cosmetics [4], as well as for the removal of metal ions from waste water [5,6], because the production cost of microbial EPS is lower than that of algal or plant polysaccharides [7]. Additionally, bacterial EPS is non-toxic, biodegradable, and environmentally friendly [8].

Generally EPSs have various physiological functions, including antiviral and immunoregulatory properties [9]. Specifically, β-glucan, a glucose homopolymer, has shown high anticancer and immunomodulating effects [10-15], thus promoting its utility in the food and pharmaceutical Industries. In addition to human health benefits, recent studies on feeding glucan to fish and shrimp [16,17], as well as on the application of non-degradable inulin-type fructan in animal feed [18,19] to enhance natural immunity, have expanded its use spectrum to the aquaculture and livestock industries.

Even though several bacteria, including *Agrobacterium* spp. [20] and *Bacillus* spp. [21], are reported to produce glucans, very little information on the bacterial production of glucan has been provided. Therefore, in industry, glucans from mushrooms or yeast lysate are widely used, rather than bacterial glucans. However, plant and yeast glucans are low in purity and yield, and research on bacterial glucans is needed.

In order to develop a bacterial glucan as an animal feed additive instead of antibiotic feeding, a novel bacterium, *Paenibacillus polymyxa* JB115, was isolated from Korean soil, and the physicochemical characteristics of its produced glucan were determined in this study.

***Corresponding author**
Tel: +82-53-810-2395 Fax: +82-53-810-4663
e-mail: sdkim@ynu.ac.kr

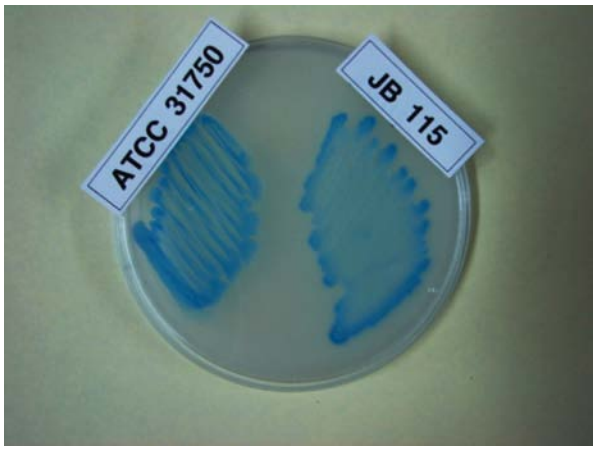

Fig. 1. Glucan production by the strain JB115 and *Agrobacterium* sp. ATCC 31750 on aniline blue agar. *Agrobacterium* sp. ATCC 31750 was used as a positive control for glucan-producing bacteria [20]. Both strains were streaked on aniline blue agar and cultured at 30°C for 3 day.

MATREIALS AND METHODS

Isolation of Bacterial Strains Producing Glucans

A soil extract was prepared by adding 9 mL of sterilized distilled water to 1 g of soil, which was collected from the Gyoungbuk Province area of Korea, and then diluted serially. The diluted solution was plated onto aniline blue agar (per L; 20 g sucrose, 5 g yeast extract, 20 g agar, 0.05 g aniline blue, 3 g $CaCO_3$) [22] for the isolation of glucan producers.

Culture Conditions for Glucan Production

The seed cells were cultivated on nutrient broth (Difco, USA) at 30°C for 12 h. Ten milliliters of seed culture was inoculated into 1 L of mineral salt medium (MSM; per L; 1.74 g KH_2PO_4, 0.015 g $CaCl_2 \cdot 2H_2O$, 0.49 g K_2HPO_4, 0.01 g $MnCl_2 \cdot 4H_2O$, 3.7 g $Na_2SO_4 \cdot 10H_2O$, 0.21 g citrate, 0.25 g $MgCl_2 \cdot 6H_2O$, 1.5 g NH_4Cl , 0.024 g $FeCl_3 \cdot 6H_2O$) [20] that was supplemented with 10% sucrose as a carbon source to induce glucan production, and cultured at 30°C for 3 day with shaking at 180 rpm.

Identification of the Bacterial Strain

To identify the isolated bacterium, its microscopic morphological characteristics were examined. For molecular identification, the 16S rDNA region of chromosomal DNA was amplified using the primer set of 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-ACCTTGTTACGACTT-3′). The PCR product was sequenced by Solgent Co. (Daejeon, Korea), and the sequence homology was examined using the BLAST program (http://www.ncbi.nlm.nih.gov). The alignment of the determined partial 16S rDNA sequence with those of closely related bacteria was performed using the ClustalX and Bioedit programs, and the phylogenetic tree was constructed by the Mega2 program.

Purification of Glucan from the Culture Broth

To separate the glucan produced by *P. polymyxa* JB115, the culture broth was centrifuged at 5,000 rpm for 15 min to collect the culture supernatant. From the supernatant, the glucan was precipitated by incubating at 4°C for 12 h after adding 3 volumes of ice-cold ethanol. The pellet recovered from the mixture by centrifugation was washed 3 times with sterilized distilled water, and then lyophilized to obtain the crude glucan. The harvested crude glucan was dissolved in 0.1 M NaCl solution, loaded onto a Sephadex G-100 column, and fractionated with 0.1 M NaCl at a flow rate of 0.1 mL/min. The glucan content of each fraction was determined by measuring the total carbohydrate content, using the phenol-sulfuric acid method [23]. The glucan fractions having a high content of carbohydrate were pooled and lyophilized to give the purified glucan JB 115-BG.

Physicochemical Analysis of Glucan

For the thin layer chromatographic (TLC) analysis of the purified JB115-BG, 50 mg of glucan were dissolved in 5 mL of 2 N sulfuric acid and hydrolyzed at 100°C for 5 h. The glucan hydrolysate was loaded onto a TLC plate, developed in a solvent system of acetone-butanol-water (4:5:3 vol.), and then visualized by treating with an α-naphthol solution [0.5% (w/v) α-naphthol and 5% (v/v) sulfuric acid in ethanol] at 120°C for 10 min. The chemical characterization of the produced glucan was confirmed by Fourier-transform infrared (FT-IR) spectrometry and nuclear magnetic resonance (NMR) spectrometry. The FT-IR spectra were determined at 400 to 4,000 cm^{-1} using a Galaxy 7020A spectrophotometer (Mattson Instruments, USA), and ^{1}H and ^{13}C NMR was run on a VNS600 spectrometer (Varian, USA). The particle size of the glucan was measured with a particle analyzer (LS 13320, Beckman, USA), and the molecular weight was determined by gel permeation chromatography (GPC; Waters, USA) at the Korean Polymer Testing and Research Institute (Seoul, Korea).

RESULTS AND DISCUSSION

Isolation and Identification of Glucan-producing Bacteria

Four bacterial strains, JB1, JB115, JB135, and JB144, which stained blue on aniline blue agar (Fig. 1), were isolated from soil that was collected in the Gyoungbuk Province area of Korea. Because this type of staining is highly specific for β-1,3-glucans [22], these strains were considered glucan producers. Among them, strain JB115 was found to have the highest glucan productivity, producing up to 10 g/L (Table 1), and was selected as the glucan producer in developing an animal feed additive.

Table 1. Glucan production by bacteria strains isolated from soil

Strain number[a]	Dry cell weight[b] (g/L)	Production of glucan[b] (g/L)
JB1	1.2 ± 0.1	3.0 ± 1.5
JB115	1.3 ± 0.2	10.6 ± 2.3
JB135	1.8 ± 0.2	7.3 ± 0.8
JB144	2.1 ± 0.4	4.1 ± 0.9
Agrobacterium sp. (ATCC 31750)	1.8 ± 0.3	6.2 ± 0.3

[a]These strains presented as blue-colored bacteria on aniline blue agar after incubation for 48 h.
[b]The glucan producing bacteria were cultured in MSM broth containing 10% sucrose at 30°C for 3 day. Dry cell weight was determined by direct weighting of the cell, after drying to a constant weight at 105°C. The glucan productivity was determined by measuring the total carbohydrate content, using the phenol-sulfuric acid method. Each sample was assayed in triplicate. Data are shown as mean ± SD of three measurements.

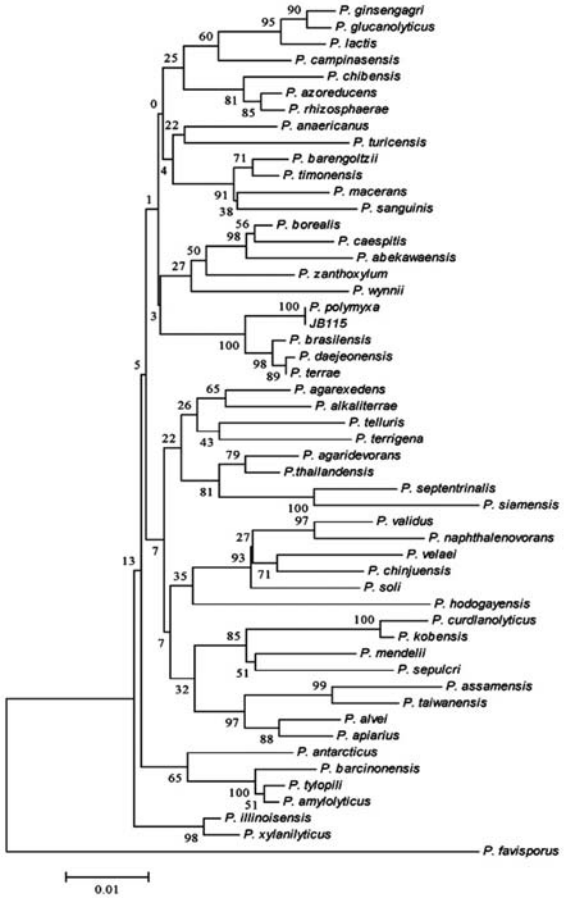

Fig. 2. Phylogenetic tree of *Paenibacillus* spp. including the strain JB115 based on its 16S rDNA sequence. The sequence homology of the partial 16S rDNA sequence of strain JB115 was evaluated via the BLAST search program (http://www.ncbi.nlm.nih.gov). The alignment of the 16S rDNA sequences of *Paenibacillus* spp. were performed by ClustalX and Bioedit programs, and a phylogenetic tree was constructed by the neighbor-joining method using the Mega2 program. The marker bar denotes the relative branch length. Bootstrap values, expressed as percentage of 1,000 replications, are given at the branch points.

The strain JB115 is a Gram-positive, rod-shaped bacterium capable of producing a high yield of glucan. Based on its 16S rDNA sequence, the strain was confirmed as *P. polymyxa* through nucleotide homology analysis and a phylogenetic tree of *Paenibacillus* spp. (Fig. 2).

To date, several bacterial strains producing glucan have been reported, including *Agrobacterium* sp. [24], *Alcaligenes* sp. [25], and *Bacillus* sp. [9,21]. Although *P. polymyxa* was previously reported to produce EPS [1], this is the first report of glucan production by this species. Since *P. polymyxa* is known to produce bacteriocin [26], the direct use of *P. polymyxa* JB115 culture broth as a feed additive could suppress the growth of harmfully intestinal bacteria in animals, as well as provide immune-enhancing benefits.

Chemical Characterization of Glucan JB115-BG

The sugar composition of glucan JB115-BG produced by *P. polymyxa* JB115 was examined by TLC after hydrolyzing with acid. The hydrolysate of JB115-BG gave one spot with an Rf value of 0.41, which is the same value as glucose. This implied that glucan JB115-BG is composed of glucose only (Fig. 3).

In order to confirm the glycosidic pattern, glucan JB115-BG was further analyzed by FT-IR, and compared with the β-(1,3) linkage of curdlan. In the FT-IR spectrum of JB115-BG, absorption peaks were observed at 3,420, 2,938, and 1,634 cm^{-1}, indicating O-H stretching, a C-H bond, and a C=O bond, respectively (Fig. 4). Additionally, a major characteristic peak for the β-configuration of D-glucopyranosyl residues was detected at 890 cm^{-1} [27]. However, the peak at 920 cm^{-1} that was observed in JB115-BG was not found in curdlan, which corresponds to β-(1,6) linkage [28]. This suggests that JB115-BG has both β-(1,3) linkage as well as β-(1,6) linkage. Furthermore, specific peaks for protein-conjugated glucan at 1,242 cm^{-1} [29], and for α-D-glucan at 850 cm^{-1} [30], were not found in the JB115-BG spectrum. Thus, one can conclude that glucan JB115-BG is a polysaccharide composed of glucose with a β-configuration having β-(1,3) and β-(1,6) linkages.

It was reported that the ^{1}H chemical shift of the anomeric region is observed around 4~6 ppm [29], and that the anomeric signals for (1→3)-β-glucan and (1→6)-β-glucan ap-

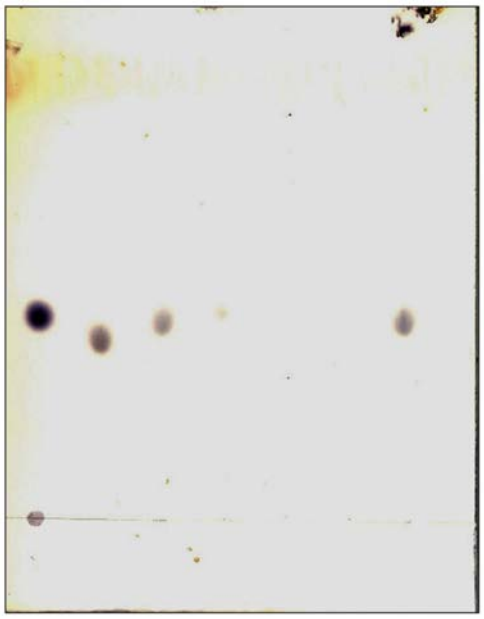

Fig. 3. Thin layer chromatogram of purified JB115-BG after hydrolyzing with 2 N sulfuric acid at 100°C for 5 h. The hydrolyzed JB115-BG was neutralized with 5 N NaOH, and then filtered through a 0.45 μm filter. The chromatogram was developed in acetone-butanol-water (4:5:3 vol.), and the spot was visualized by treating with α-naphthol solution at 120°C for 10 min. A, fructose (0.1 mg/mL); B, galactose (0.1 mg/mL); C, glucose (0.1 mg/mL); D, JB115-BG hydrolysate.

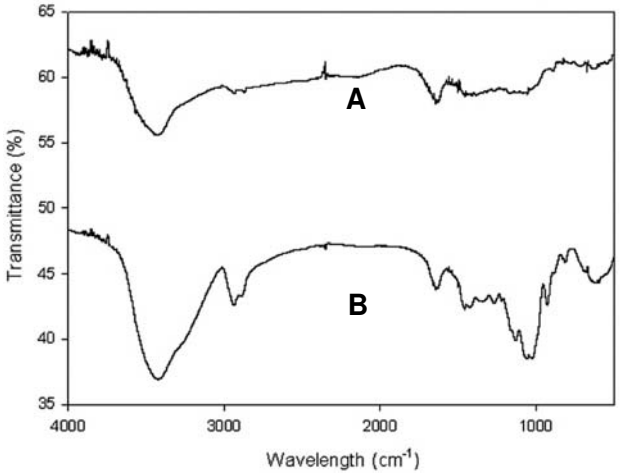

Fig. 4. Fourier-transform infrared spectroscopy (FT-IR) spectrum of curdlan (A) and the purified JB115-BG (B). For FT-IR analysis, the β-(1,3)-linked cudlan (Sigma, USA) and purified JB115-BG were made as KBr pellets.

pear at 4.7~4.8 and 4.5~4.6 ppm, respectively [31]. In the ^{1}H NMR spectra of JB115-BG dissolved in D_2O (Fig. 5A), a doublet peak at 4.51 and 4.53 ppm indicated the presence of β-(1→6)-linked glucopyranosyl residues. However, a peak characteristic to β-(1→3)-glucopyranosyl residues overlapped at 4.7 ppm with a D_2O solvent peak (4.6 ppm). In the ^{1}H NMR spectra of JB115-BG in d_6-DMSO, however, a doublet peak around 4.7 ppm showing a (1→3)-β-glucopyranosyl unit chain was clearly observed, similar to a previous report [32] (Fig. 5B). It was also observed that the ^{1}H chemical shifts in the d_6-DMSO/D_2O (6:1) solvent system were almost similar to the results of a β-D-(1→3, 1→6) glucan from a marine diatom [33] (Fig. 5C), confirming the presence of β-(1→3) and β-(1→6) linkage in JB115-BG. In particular, a β-(1→3)-linked backbone was found at 4.58 ppm, and the nonreducing end group and β-reducing resonances in the β-(1→3) chain appeared at 4.42 and 4.47 ppm, respectively. Furthermore, the signal peaks at 4.23 and 4.36 ppm represented the presence of β-(1→6)-linked residues [34]. Finally, the absence of peaks in the range of 0.5~3.0 ppm implied that JB115-BG is a pure glucan [35].

For ^{13}C NMR spectra, it was previously reported that the anomeric carbon (C-1) of a glycoside generally appears at 100~104 ppm, and that C-2, C-3, C-4, C-5, and C-6 of the

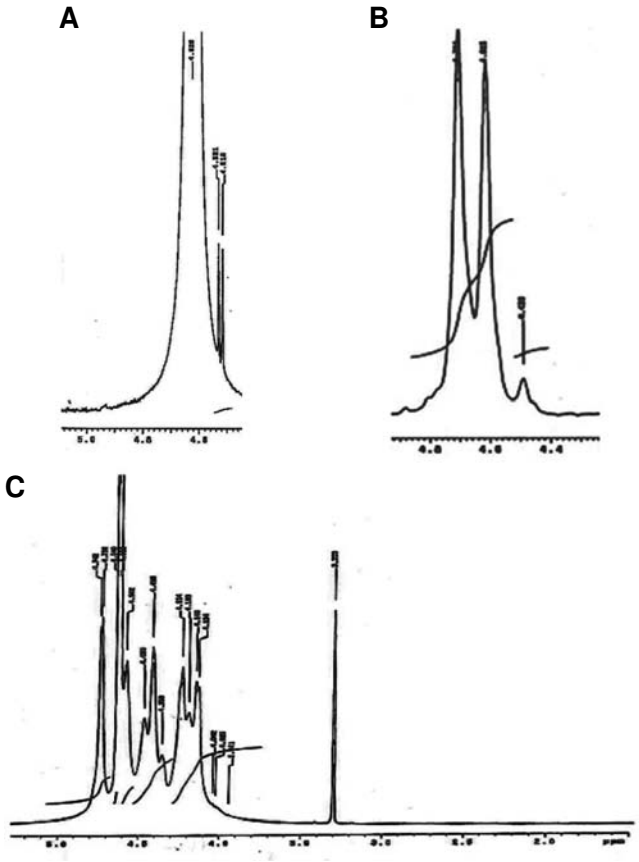

Fig. 5. ^{1}H NMR spectrum of the purified JB115-BG. A, ^{1}H NMR spectrum of the purified JB115-BG (20 mg/mL) dissolved in D_2O; B, in d_6-DMSO; C, in d_6-DMSO/D_2O (6:1 vol.). ^{1}H NMR was run on a VNS600 spectrometer (600 MHz, Varian, USA) at 30°C.

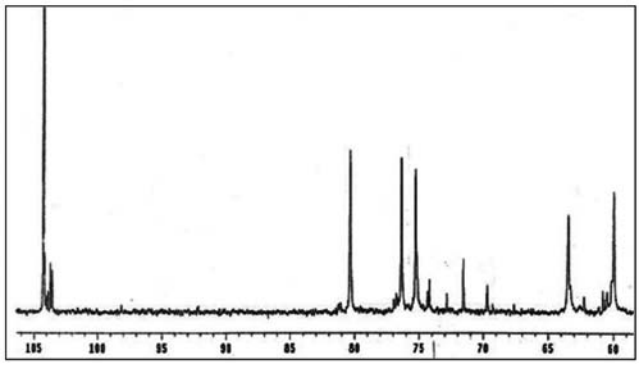

Fig. 6. ^{13}C NMR spectrum of the purified JB115-BG. The purified JB115-BG (20 mg/mL) was dissolved in d_6-DMSO and analyzed by a VNS600 spectrometer (600 MHz, Varian, USA) at 30°C.

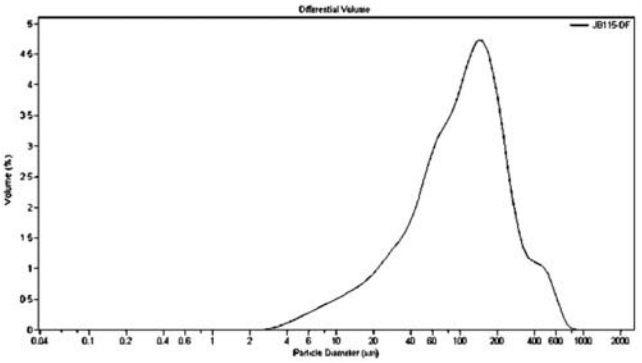

Fig. 7. Molecular size distribution of the purified JB115-BG. Glucan JB115-BG was dissolved in isopropyl alcohol and analyzed using a particle analyzer (LS 13320, Beckman, USA) at a range of 0.040~2,000 μm.

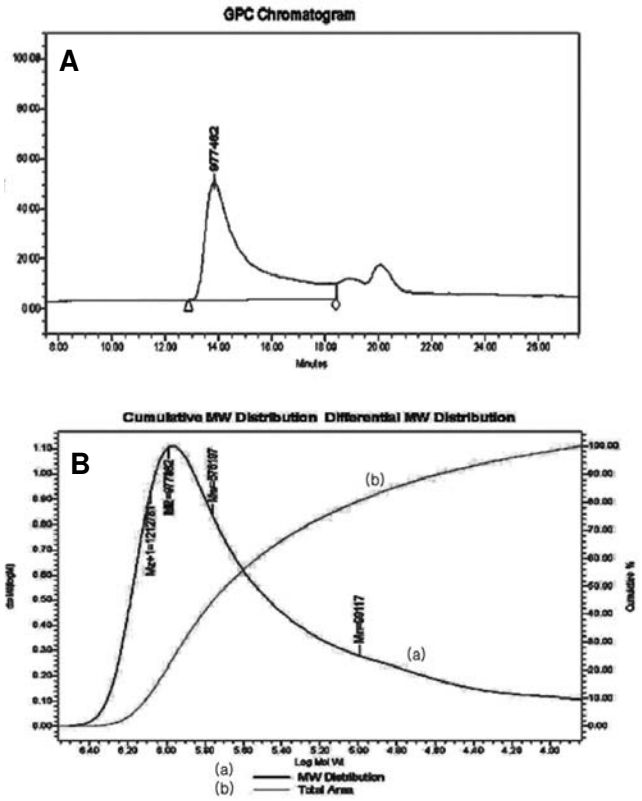

Fig. 8. Gel permeation chromatogram of the purified JB115-BG for the determination of molecular weight (A) and molecular distribution (B). The purified JB115-BG (3 mg/mL) was dissolved in distilled water, separated on a PL-aquagel mixed-hydroxy column (7.8 × 300 mm) with a flow rate of 1.0 mL/min at 35°C, and detected with a Waters 2410 differential refractometer.

glycosidic ring appear at 60~80 ppm [29]. The ^{13}C NMR spectra of JB115-BG showed not only the characteristic peak for C-1 with β-(1→6) linkage at 104.55 ppm, but also the typical peak for C-1 with β-(1→3) linkage at 103.70 ppm (Fig. 6), similar to previous reports [32,36]. The signals around 72.80, 80.37, 69.67, 76.36, and 60.10 ppm, which assigned to C-2, C-3, C-4, C-5, and C-6, respectively, were identical to those of a β-(1→3)-linked glucopyranoside [33,36,37]. The signals at 74.20, 76.01, 71.55, 76.36, and 63.40 ppm were also assigned to C-2, C-3, C-4, C-5, and C-6, respectively, with β-(1→6)-linkage, based on previous reports [33,36]. The typical peaks for a protein-conjugated glucan at 20 and 40 ppm [29] were not found in the ^{13}C NMR spectrum of JB115-BG. From the results of the FT-IR, ^{1}H NMR, and ^{13}C NMR spectra, it was concluded that JB115-BG is a β-(1→3) and β-(1→6)-linked glucan.

Physical Characterization of Glucan JB115-BG

The particle size and molecular weight of microbial EPS are central factors in the separation of the glucan as well as its biological activity. The particle size of glucan JB115-BG, as determined by a particle analyzer, ranged from 4~800 μm, with a mean value of 149.1 μm (Fig. 7). In GPC analysis, the molecular distribution of JB115-BG ranged from 6.9~3,103.7 kDa, with a number-average molecular weight (M_n) of 99.1 kDa, a weight-average molecular weight (M_w) of 576.2 kDa, and a polydispersity index (P_i) of 5.81. In order to reduce the P_i value for glucan consistency in industrial production, it is necessary to remove low molecular substances from the *P. polymyxa* JB115 culture broth by ultrafiltration (removing substances of 8~10 kDa) in the purification step [7].

It was reported that polysaccharides having molecular weights higher than 100~200 kDa are good immunogens and exhibit immunomodulating activity [38], and that the biological response modifying capabilities of polysaccharides are increased depending on their molecular weight [39]. Since 80% of glucan JB115-BG showed a molecular distribution above 100 kDa, it could be used as a potential immunostimulant in livestock feed.

CONCLUSION

Polysaccharides, including β-glucans, have been produced not only in the food and pharmaceutical industries as anticancer and immunomodulating agents, but also in the aquaculture and livestock industries for enhancing the natural immunity of animals. In order to develop a bacterial glucan as an animal feed additive instead of antibiotic feeding, various bacterial strains were screened from Korean soil. One novel strain, JB115, was isolated as a glucan producer for the development of animal feed additives. Based on microscopic examination and 16S rDNA analysis, this strain was confirmed as *P. polymyxa* JB115. The glucan, JB115-BG, produced by *P. polymyxa* JB115 was identified by TLC to be composed of glucose only. By examining the FT-IR, ^{1}H NMR, and ^{13}C NMR spectra, it was proved that JB115-BG has a β-(1→3)- and β-(1→6)-linked glucan structure. The particle size of JB115-BG was distributed in the range of 4~800 μm, with a mean value of 149.1 μm, and its molecular distribution ranged from 6.9~3,103.7 kDa, with a M_n of 99.1 kDa, M_w of 576.2 kDa, and P_i of 5.81. It was also observed that 80% of glucan JB115-BG had a molecular distribution above 100 kDa.

To date, β-(1→3, 1→6) glucans are known as biological response modifiers (BRMs) and natural immunomodulators [40], and the β-(1→3) backbone is essential for antitumor activity [41]. If low molecular glucans can be removed from the *P. polymyxa* JB115 culture broth by ultrafiltration during purification, most of the glucan is high molecular weight, above 100 kDa, and can be used as an animal feed additive for immune-enhancement and as a potential antitumor agent for livestock.

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REFERENCES

1. Ahn, S. G., H. H. Suh, C. H. Lee, S. H. Moon, H. S. Kim, K. H. Ahn, G. S. Kwon, H. M. Oh, and B. D. Yoon (1998) Isolation and characterization of a novel polysaccharide producing *Bacillus polymyxa* A49 KCTC 4648P. *J. Microbiol. Biotechnol.* 8: 171-177.
2. Moon, S. H., J. M. Park, H. Y. Chun, and S. J. Kim (2006) Comparisons of physical properties of bacterial cellulose produced in different culture conditions using saccharified food wastes. *Biotechnol. Bioprocess Eng.* 11: 26-31.
3. Zanchetta, P., N. Lagarde, and J. Guezennec (2003) A new bone-healing material: A hyaluronic acid-like bacterial exopolysaccharide. *Calcif. Tissue Int.* 72: 74-79.
4. Mansel, P. W. A. (1994) Polysaccharides in skin care. *Cosmet. Toilet.* 109: 67-72.
5. Chu, K. H. and E. Y. Kim (2006) Predictive modeling of competitive biosorption equilibrium data. *Biotehchnol. Bioprocess Eng.* 11: 67-71.
6. Shi, F., Z. Xu, and P. Cen (2006) Optimization of γ-polyglutamic acid production by *Bacillus subtilis* ZJU-7 using a surface-response methodology. *Biotechnol. Bioprocess Eng.* 11: 251-257.
7. Kumar, A. S., K. Mody, and B. Jha (2007) Bacterial exopolysaccharides – a perception. *J. Basic Microbiol.* 47: 103-117.
8. Shoda, M. and Y. Sugano (2005) Recent advances in bacterial cellulose production. *Biotechnol. Bioprocess Eng*. 10: 1-8.
9. Arena, A., T. L. Maugeri, B. Pavone, D. Iannello, C. Gugliandolo, and G. Bisignano (2006) Antiviral and immunoregulatory effect of a novel exopolysaccharide from a marine thermotolerant *Bacillus licheniformis. Int. Immunopharmacol.* 6: 8-13.
10. Kawagishi, H., T. Kanao, R. Inagaki, T. Mizuno, K. Shimura, H. Ito, T. Hagiwara, and T. Nakamura (1990) Formolysis of a potent antitumor (1→6) β-D-glucan-protein complex from *Agaricus blazei* fruiting bodies and antitumor activity of the resulting products. *Carbohydr. Polym.* 12: 393-403.
11. Liu, C., Q. Lin, Y. Gao, L. Ye, Y. Xing, and T. Xi (2007) Characterization and antitumor activity of a polysaccharide from *Strongylocentrotus nudus* eggs. *Carbohydr. Polym.* 67: 313-318.
12. Moradali, M. F., H. Mostafavi, S. Ghods, and G. A. Hedjaroude (2007) Immunomodulating and anticancer agents in the realm of macromycetes fungi. *Int. Immunopharmacol.* 7: 701-724.
13. Park, J. H., M. S. Kang, H. I. Kim, B. H. Chung, K. H. Lee, and W. K. Moon (2003) Study on immune-stimulating activity of β-glucan isolated from the cell wall of yeast mutant *Saccharomyces cerevisiae* IS2. *Kor. J. Food Sci. Technol*. 35: 488-492.
14. Seo, H. P., J. M. Kim, H. D. Shin, T. K. Kim, H. J. Chang, B. R. Park, and J. W. Lee (2002) Production of β-1,3/1,6 glucan by *Aureobasidium pollulans* SM-2001. *Kor. J. Biotechnol. Bioeng*. 17: 376-380.
15. Tao, Y., L. Zhang, and P. Cheung (2006) Physicochemical properties and antitumor activities of water-soluble native and sulfated hyperbranched mushroom polysaccharides. *Carbohydr. Res.* 341: 2261-2269.
16. Kumari, J. and P. K. Sahoo (2006) Non-specific immune response of healthy and immunocompromised Asia catfish (*Clarias batrachus*) to several immunostimulants. *Aquaculture* 255: 133-141.
17. Suphantharika, M., P. Khunrae, P. Thanardkit, and C. Verduyn (2003) Preparation of spent brewer's yeast β-glucans with a potential application as an immunostimulant for black tiger shrimp, *Penaeus monodon*. *Bioresour. Technol*. 88: 55-60.
18. Flickinger, E. A. and G. C. Fahey, Jr. (2002) Pet food and feed applications of inulin, oligofructose and other oligosaccharides. *Br. J. Nutr*. 87: S297-S300.

19. Verdonk, J. M., S. B. Shim, P. van Leeuwen, and M. W. Verstegen (2005) Application of inulin-type fructans in animal feed and pet food. *Br. J. Nutr*. 93: S125-S138.
20. Kim, M. K., I. Y. Lee, J. H. Ko, Y. H. Rhee, and Y. H. Park (1999) Higher intracellular levels of uridine monophosphate under nitrogen-limited conditions enhance the metabolic flux of curdlan synthesis in *Agrobacterium* species. *Biotechnol. Bioeng*. 62: 317-323.
21. Gummadi, S. N. and K. Kumar (2005) Production of extracellular water insoluble β-1,3-glucan (curdlan) from *Bacillus* sp. SNC07. *Biotechnol. Bioprocess Eng*. 10: 546-551.
22. Nakanishi, I., K. Kimura, T. Suzuki, M. Ishikawa, I. Banno, T. Sakane, and T. Harada (1976) Demonstration of curdlan-type polysaccharide and some other β-1,3-glucan in microorganisms with aniline blue. *J. Gen. Appl. Microbiol*. 22: 1-11.
23. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith (1956) Colorimetric method for determination of sugars and related substances. *Anal. Chem*. 28: 350-356.
24. Harada, T., A. Misaki, and H. Saito (1968) Curdlan: a bacterial gel-forming β-(1,3) glucan. *Arch. Biochem. Biophys*. 124: 292-298.
25. Harada, T., M. Masada, K. Fujimori, and I. Maeda (1966) Production of a firm, resilient gel-forming polysaccharide by a mutant of *Alcaligenes faecalis* var. *myxogenes* 10C3. *Agric. Biol. Chem*. 30: 196-198.
26. He, Z., D. Kisla, L. Zhang, C. Yuan, K. B. Green-Church, and A. E. Yousef (2007) Isolation and identification of a *Paenibacillus polymyxa* strain that coproduces a novel lantibiotic and polymyxin. *Appl. Environ. Microbiol.* 73: 168-78.
27. Xu, F., Z. C. Geng, J. X. Sun, C. F. Liu, J. L. Ren, R. C. Sun, P. Fowler, and M. S. Baird (2006) Fractional and structural characterization of hemicelluloses from perennial ryegrass (*Lolium perenne*) and cocksfoot grass (*Dactylis glomerata*). *Carbohydr. Res.* 341: 2073-2082.
28. Sandula, J., G. Kogan, M. Kacurakova, and E. Machova (1999) Microbial (1→3)-β-D-glucans, their preparation, physico-chemical characterization and immunomodulatory activity. *Cabohydr. Polym.* 38: 247-253.
29. Gonzaga, M. L. C., N. M. P. S. Ricardo, F. Heatley, and S. de A. Soares (2005) Isolation and characterization of polysaccharides from *Agaricus blazei* Murill. *Carbohydr. Polym.* 60: 43-49.
30. Huang, Q., Y. Jin, L. Zhang, P. C. K. Cheung, and J. F. Kennedy (2007) Structure, molecular size and antitumor activities of polysaccharides from *Poria cocos* mycelia produced in fermenter. *Carbohydr. Polym.* 70: 324-333.
31. Sugawara, T., S. Takahashi, M. Osumi, and N. Ohno (2004) Refinement of the structures of cell-wall glucans of *Schizosachharomyces pombe* by chemical modification and NMR spectroscopy. *Carbohydr. Res.* 339: 2255-2265.
32. Kimura, Y., M. Sumiyoshi, T. Suzuki, T. Suzuki, and M. Sakanaka (2007) Inhibitory effects of water-soluble low molecular-weight β-(1,3-1,6) D-glucan purified from *Aureobasidium pullans* GM-NH-1A1 strain on food allergic reactions in mice. *Int. Immunopharmacol*. **7**: 963-972.
33. Storseth, T. R., S. Kirkvold, J. Skjermo, and K. I. Reitan (2006) A branched β-D-(1→3, 1→6) glucan from the marine diatom *Chaetoceros debilis* (*Bacillariophyceae*) characterized by NMR. *Carbohydr. Res.* 341: 2108-2114.
34. Kim, Y. T., E. H. Kim, C. Cheong, D. L. Willams, C. W. Kim, and S. T. Lim. (2000) Structural characterization of β-D-(1→3, 1→6)-linked glucans using NMR spectroscopy. *Carbohydr. Res.* 328: 331-341.
35. Silvestein, R. M., G. C. Bassler, and T. C. Morrill (1994) *Spectrometric Identification of Organic Compounds.* 5th ed. Wiley Interscience, NY, USA.
36. Altabe, S. G., N. Inon, D. de Mendoza, and R. A. Ugalde (1994) New osmoregulated β(1-3), β(1-6) glucosyltransferase(s) in *Azospirillum brasilense*. *J. Bacteriol.* 176: 4890-4898.
37. Ohno, N., M. Uchiyama, A. Tsuzuki, K. Tokunaka, N. N. Miura, Y. Adachi, M. W. Aizawa, H. Tamura, S. Tanaka, and T. Yadomae (1999) Solubilization of yeast cell-wall β-(1→3)-D-glucan by sodium hypochlorite oxidation and dimethyl sulfoxide extraction. *Carbohydr. Res.* 316: 161-172.
38. Bohn, A. and J. N. BeMiller (1995) (1→3)-β-D-glucans as biological response modifiers: a review of structure-functional activity relationships. *Carbohydr. Polym.* 28: 3-14.
39. Leung, M. Y. K., C. Liu, J. C. M. Koon, and K. P. Fung (2006) Polysaccharide biological response modifiers. *Immunol. Lett.* 105: 101-114.
40. Krakowski, L., J. Krzyzanowski, Z. Wrona, and A. K. Siwicki (1999) The effect of nonspecific immunostimulation of pregnant mares with 1,3/1,6 glucan and levamisole on the immunoglobulins levels in colostrums, selected indices of nonspecific cellular and humoral immunity in foals in neonatal and postnatal period. *Vet. Immunol. Immunopathol.* 68: 1-11.
41. Seviour, R. J., S. J. Stasinopoulos, D. P. F. Auer, and P. A. Gibbs (1992) Production of pullulan and other exopolysaccharides by filamentous fungi. *Crit. Rev. Biotechnol*. 12: 279-298.