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Short Communication

Exopolysaccharide produced by *Bacillus licheniformis* strains isolated from Kimchi

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Exopolysaccharides (EPSs) derived from various sources have been applied in the food industry as food additives such as natural viscosifiers, stabilizers, emulsifiers, and texturizers (Duboc and Mollet, 2001). In addition, EPSs recently began to serve as functional materials in pharmaceutical applications beyond the food industry due to their activities such as anti-tumor, anti-oxidant and anti-ulcer (Asker et al., 2009). Meanwhile, synthetic antioxidants often used in the food industry, including butylated hydroxyanisole, butylated hydroxytoluene and propyl gallate, are associated with significant problems in human health (Valentão et al., 2002). Therefore, the use of functional natural materials such as polymers that are produced by microorganisms has been investigated across diverse industries. Polymers produced by mushrooms have received a great deal of attention due to their functionalities (Rhee et al., 2008). However, polysaccharides derived from fungal species are problematic for industrial applications because it is difficult to maintain a steady growth rate and suitable fermentation conditions, making production at the industrial level challenging despite advances in fermentation technology. Many studies indicated a very low EPS production yield of below 5 g/L during approximately 10-day fermentation periods.

Environmental stresses can cause microorganisms to secrete EPS for survival (Decho and Lopez, 1993). Kimchi, a traditional fermented food in Korea, constitutes a complex, severe environment for microorganisms (Park et al., 2001) but then typically contains a complex and varied bacterial consortium (Min et al., 2003). For these reasons, Kimchi might be a good source to isolate EPS-producing strains. To the best of our knowledge, studies of EPS producing strains derived from Kimchi are lacking. The objective of this study was to screen strains to produce high amount of EPS from Kimchi and evaluate antioxidant activities of the obtained EPS.

A Kimchi sample acquired from a local supermarket was decimally diluted and then the suspensions were inoculated onto modified MRS agar media containing 2% (w/v) $CaCO_3$. After incubation for 2 days at 37°C, 80 strains were initially selected with the naked eye as putative EPS production strains among all isolates (Jung et al., 2009). Then, twenty-four strains showing the mucoid phenotype were again purified by streaking onto sucrose agar medium (5% sucrose, 1% tryptone, 0.5% yeast extract, 0.5% dipotassium phosphate

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and 0.5% diammonium citrate, adjusted to pH 7.0). The final screening followed after cultivating the selected strains in sucrose liquid media for 48 h at 37°C by comparing EPS volumes based on dry weight (Shivakumar and Vijayendra, 2006). As a result, strain KS-17 and strain KS-20 were ultimately selected (Fig. 1).

The two isolates were identified using 16S rDNA sequences and biochemical methods. The crude total genomic DNA of each isolate was extracted as the PCR template. PCR reactions were performed with *Taq* DNA polymerase and the universal primers proF (5′-AGA GTT TGA TCC TGG CTC AG-3′), 534r (5′-ATT ACC GCG GCT GCT GG-3′) and 9Rev (5′-AAG GAG GTG ATC CAG CC-3′) using a PCR cycler (Primus 25, MWG Biotech, UK) under the following conditions: 95°C for 2 min; 30 cycles of 95°C for 1 min, 54°C for 30 s and 72°C for 1 min; and a final extension of 72°C for 5 min. To confirm PCR amplification, PCR products were separated on 1.2% agarose gel in 1× TAE buffer by gel electrophoresis. The agarose gel was stained with 1 mM ethidium bromide and the bands were visualized using the ChemiDoc XRS Imaging System (Bio-Rad, USA). The confirmed PCR products were directly sequenced on an ABI PRISM 3700 DNA analyzer and analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). The strains were characterized further in accordance with the biochemical method by an automated microbe identification analyzer (VITEK 2 Compact, bioMerieux, France) using a BCL card. In the results of 16S rDNA sequence analysis, the strains showed the highest sequence similarity of 99.0% in KS-17 and 99.1% in KS-20 to *Bacillus licheniformis*. These strains were also characterized by a biochemical method with VITEK 2, resulting in 85% and 92% sequence similarity with *B. licheniformis*, respectively (data not shown). The strains were therefore identified as *B. licheniformis* and named *B. licheniformis* KS-17 and *B. licheniformis* KS-20, respectively.

From sucrose broth culture of *B. licheniformis* KS-17 and *B. licheniformis* KS-20, EPS KS-17 and EPS KS-20 were purified and then characterized according to monosaccharide composition, structure, molecular weight, and antioxidant activities. First, the monosaccharide compositions of EPS KS-17 and EPS KS-20 were determined by TLC and HPLC-ELSD analysis after EPS was hydrolyzed by heating with 2 M H_2SO_4 at 110°C for 5 h. TLC was performed on silica gel 60 F254 (Merck, Germany) plates with *n*-butanol/2-propanol/water/acetic acid (7 : 5 : 4 : 2, v/v/v/v) as a developing solvent system. After the development process was performed twice, the developed spots were visu-

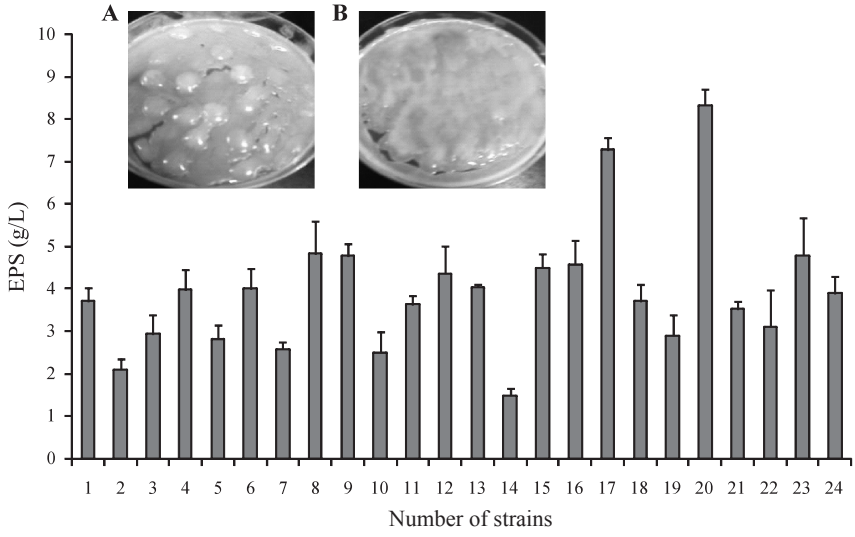

Fig. 1. The EPS producing strain KS-17 (A) and KS-20 (B) were isolated from the selected 24 strains.

Each strain was first confirmed on a plate, and then grown on sucrose liquid medium for 48 h at 37°C with shaking. EPS production yield was assessed by comparisons of dry weight. A: The EPS producing strain KS-17 was isolated from the selected 24 strains. B: The EPS producing strain KS-20 was isolated from the selected 24 strains.

alized by spraying the plates with ethanol/sulfuric acid/*p*-anisaladehyde solution (18 : 1 : 1, v/v/v) following heating of the plates at 120°C for 1 min. The hydrolysates were also analyzed using HPLC with a Supelcosil^{TM} LC-NH_2 column (Supelco, 5 μm, 4.6×250 mm) at room temperature and evaporative light scattering detector (Alltech 500 ELSD) at 55°C . HPLC grade water/acetonitrile (25 : 75, v/v) was used as the mobile phase for isocratic elution at a flow rate of 1.0 ml/min. The sulfuric acid-hydrolysates of both KS-17 and KS-20 showed the same single apparent spot on TLC (*Rf* = 0.551), presenting the most similar *Rf* value to glucose (*Rf* = 0.548) compared to *Rf* values of other monosaccarides (*Rf* of galactose = 0.481, *Rf* of fructose = 0.563, *Rf* of mannose = 0.6) (Fig. 2A). The results corresponded with HPLC analysis showing a strong peak that was observed only with the same retention time as glucose (8.4 min) as shown in Fig. 2B and 2C. From these results, it appears that the EPS KS-17 and EPS KS-20 consist of only the glucose monomer. Previously, it was reported that EPS synthesized by a *B. licheniformis* strain isolated from ropy cider was mainly composed of mannose (80.5%), glucose (6.5%) and other unknown components (13%) (Larpin et al., 2002), while EPS synthesized by *B. licheniformis* 8-37-0-1 isolated from soil was reported as fructose and glucose in a molar ratio of 33 : 1 (Liu et al., 2010). However, the EPS from isolated from our strains of *B. licheniformis* KS-17 and KS-20 showed only a single constituent glucose molecule, indicating that these must be different from those previously reported in *B. licheniformis* strains derived from other sources.

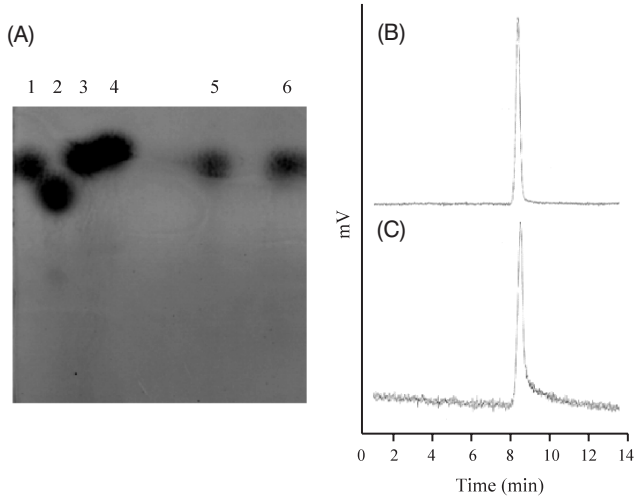

Fig. 2. Results of TLC and HPLC-ELSD analysis of purified EPS KS-17 and KS-20.

A: TLC analysis: Lane 1, glucose; Lane 2, galactose; Lane 3, fructose; Lane 4, mannose; Lane 5, hydrolysis product of EPS KS-17; Lane 6, hydrolysis product of EPS KS-20. Chromatogram of the hydrolysis product of EPS KS-17 (B) and EPS KS-20 (C) by HPLC-ELSD: lactose, 6.6 min; mannose, 6.9 min; galactose, 7.8 min; glucose, 8.4 min; fructose; 12.5 min as retention times of standards.

FT-IR (Fourier transform infrared) spectrum analysis was used to examine basic structures of EPS. The EPS samples were ground with spectroscopic-grade potassium bromide (KBr) powder and then pressed into 1 mm pellets. FT-IR spectrum of the composite was recorded using a Bruker IFS 66v FT-IR spectrophotometer in the absorbance mode from 4000 to 500 cm^{-1} with 32 accumulated scans. As shown in Fig. 3, the FT-IR spectra of both EPS KS-17 and KS-20 showed almost identical patterns, indicating that they have the same structure. The specific absorption band at the 3410 cm^{-1} region indicates a strong O-H band and the band at the 2936 cm^{-1} region indicates a C-H stretching band of EPS. The band at the 1456 cm^{-1} region indicates a CH_2 band and the bands at the 1127 cm^{-1}, 1061 cm^{-1} and 1015 cm^{-1} regions indicate strong C-O-C stretching bands by glycosidic bond vibration of EPS. Thus, we concluded that EPS KS-17 and EPS KS-20 are best assigned to glucose residues (Richert et al., 2005).

The molecular weight of EPS was determined by both gel filtration chromatography and MALDI-TOF MS (matrix-assisted laser desoption/ionization mass spectrometry) analysis. We first tried to obtain more purified EPS through three purification steps of ion exchange with a linear gradient of 0 to 1 M NaCl (Uno-Q1 anion-exchange column, flow rate 1.0 ml/min) and gel filtra-

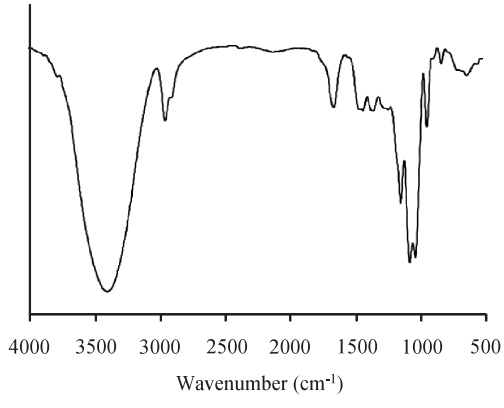

Fig. 3. Structural analysis of EPS by FT-IR.

tion with Sephadex G-25. For gel filtration chromatography, the EPS was loaded into a Sephadex G-25 column with 0.025 M phosphate buffer (pH 6.8) containing 0.15 M NaCl at a flow rate of 1 ml/min. As shown in Fig. 4 (A), EPS KS-17 and EPS KS-20 showed approximately 2.4 kDa and 2.2 kDa molecular weight, respectively by gel filtration, but it was very difficult to discriminate between the two EPSs. Thus, we did MALDI-TOF MS analysis to estimate molecular weight more exactly. The sample was prepared by premixing 1 ml of the purified EPS solution (dissolved in 50% MeCN and 0.1% TFA) with 1 ml of DHB (2,5-dihydroxybenzoic acid) matrix solution (dissolved in MeOH), and allowed to dry at room temperature. The molecular weight was examined and calculated by using MALDI-TOF MS (Voyager-DE STR Biospectrometry workstation, AppliedBiosystems, USA). In Fig. 4(B) and 4(C), major pseudomolecular ions at *m*/*z* 2106.0041 and *m*/*z* 1944.0162 corresponding to EPS sugars Glc13 and Glc12, respectively for EPS KS-17 and 20. The major two ions observed were separated by a 162-atomic mass unit difference, which corresponded to a hexose mass minus a molecule of water for linkage. All together, we concluded that the two EPSs must have at least approximately 13 or 12 glucose units. These molecular weights are almost identical compared to the weights of other EPSs derived from various bacteria from 10 kDa to 2,700 kDa (Vaningelgem et al., 2004).

In order to estimate the glycosidic linkages of the purified EPS, enzymatic hydrolysis tests for EPSs were done by using three different enzymes of α-amylase (1,4-α-D-glucan gluconohydrolase), cellulase [1,4-(1,3:1,4)-β-D-glucan glucanohydrolase] and α-glucoamylase (α-1,4-glucosidase). Five mg/ml of EPS, or cellulose soluble starch as a control was suspended in 50 mM PBS buffer (pH 7.0) with the final 100 units of commercial enzymes and then the mixture was incubated at 37°C for 3 h. The hydrolysis products were then quantitatively analyzed by HPLC-ELSD and TLC. As results, neither EPS KS-17 nor KS-20 was hydrolyzed by α-amylase or α-glucoamylase which is specific for α-linkages. However, both EPSs reacted with only β-cellulase releasing glucose monomers indicating these two EPSs from a *Bacillus* strain isolated from Kimchi must be comprised with β-glucosidic bonds.

Antioxidant and antiaging activity of the EPSs were evaluated on DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, superoxide radical and tyrosinase activity. First, DPPH radical scavenging activities of EPS KS-17 and EPS KS-20 were measured spectrophoto-

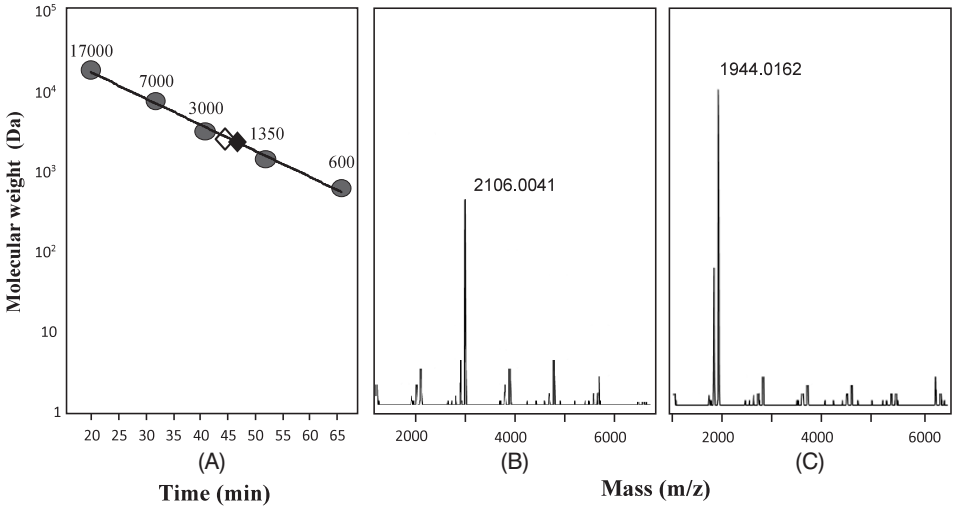

Fig. 4. Molecular weight estimation of EPS by gel filtration and MALDI-TOF MS analysis.

Molecular weights of (◇) EPS KS-17 and (◆) EPS KS-20 were determined by gel filtration chromatography. Equine myoglobin (17,000 Da), heparin polysaccharide (7,000 Da), decasaccharide (3,000 Da), Vitamin B_{12} (1,350 Da), disaccharide (600 Da) were used as standards (Neoparin Inc., USA). For MALDI-TOF MS analysis, the purified EPSs were loaded on the target. The indicated *m*/*z* values correspond to pseudomolecular ions corresponding to the loss of one H and an hexose mass unit minus a molecule of water for the linkage. H is counted as 1 instead of 1.008.

metrically according to the method of Blois (1958) with slight modifications. Briefly, aliquots of 0.1 ml of each sample solution at different concentrations (1−10 mg/ml) were mixed with 0.4 ml of 0.4 mM of DPPH dissolved in methanol. After standing for 30 min at 37°C in the dark, the absorbance was measured at 517 nm. The superoxide radical scavenging activities of KS-17 and KS-20 were measured according to the method of Marklund and Marklund (1974). Aliquots of 1 ml of sample solution, 3 ml of 0.5 mM Tris-HCl buffer (pH 8.2) and 0.2 ml of 7.2 mM pyrogallol were mixed and incubated for 10 min at 25°C. After incubation, the reaction was terminated by adding 0.5 ml of 1 M HCl and then the absorbance of the reaction mixture was measured at 420 nm. Inhibitory effects of KS-17 and KS-20 on L-DOPA autooxidation were measured according to the method of Pomerantzp (1963). Aliquots of 0.2 ml of sample solution were mixed with 0.6 ml of 8.3 mM L-DOPA solution dissolved in 67 mM phosphate buffer (pH 6.8). After mixing the solution well, the absorbance of the blank was measured at 495 nm. Then 0.05 ml of mushroom tyrosinase (100 U/ml) was added to each sample for enzymatic reactions and incubated for 20 min at 37°C . The absorbance of the resulting solution with tyrosinase activity was again measured at 495 nm. All data were analyzed by one-way ANOVA using SPSS version 16.0. All analyses were performed in triplicate and the results are expressed as the mean ± standard deviation (SD). The differences among groups were assessed using Duncan's multiple range tests. Statistical significance was defined as $p<0.05$.

To evaluate the function of EPSs from *B. licheniformis* KS-17 and *B. licheniformis* KS-20, we examined antioxidant activities and compared them to β-glucan extracted from *Inonotus obliquus,* a medicinal mushroom which is known as an excellent antioxidant polysaccharide (Seo and Lee, 2010; Song et al., 2008). As shown in Fig. 5A, the scavenging activities of EPS KS-17 and EPS KS-20 on DPPH radicals increased with increasing EPS concentration ranging from 1 to 10 mg/ml in three test samples. In particular, EPS KS-17 showed significantly stronger scavenging activity than β-glucan over a concentration of 4 mg/ml, while the EPS KS-20 showed significantly stronger activity than β-glucan throughout the tested concentration range ($p<0.05$). In order to confirm the antioxidant activity, we also examined the scavenging activity on superoxide radicals of both KS-17 and KS-20, resulting in increased activity with increasing concentrations ranging from 1 to 10 mg/ml (Fig. 5B). Even though most of the tested samples exhibited almost the same scavenging activities on superoxide radicals at high concentrations of 10 mg/ml, the EPS from *B. licheniformis* KS-20 showed significantly stronger scavenging activity than β-glucan at lower concentrations ($p<0.001$). Moreover, we also examined anti-aging activity by measuring inhibitory activity on tyrosinase, since tyrosinase initiates the oxidation of tyrosine to dihydroxyphenylalanine (DOPA) that in turn stimulates melanin synthesis (Um et al., 1995). As a

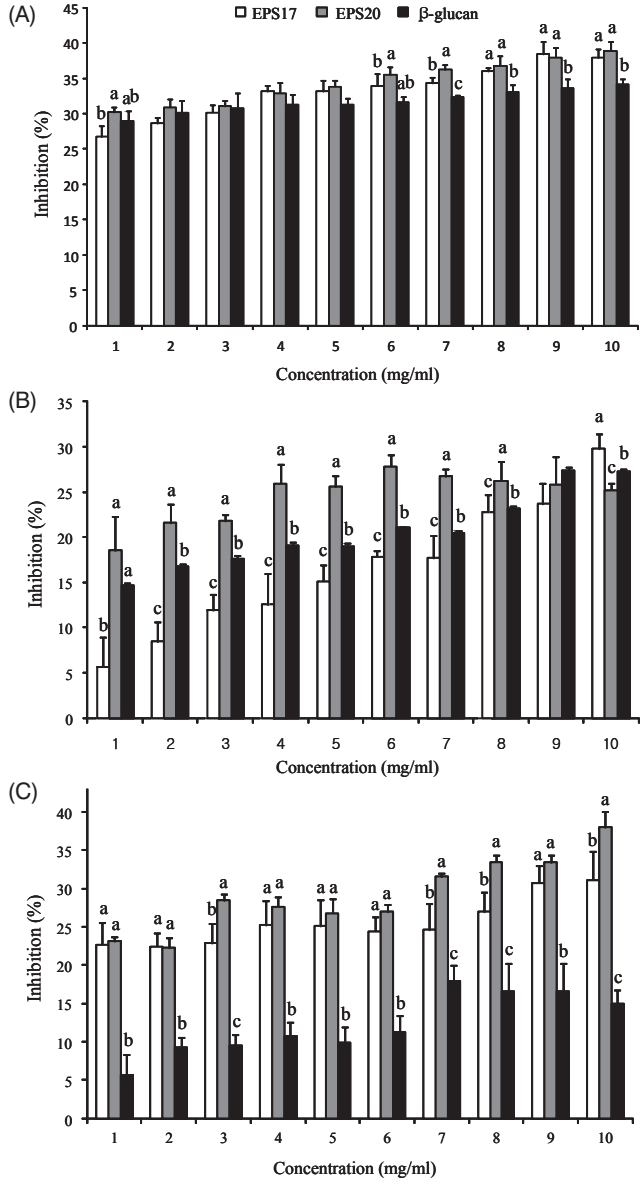

Fig. 5. Antioxidant and antiaging activities of EPS KS-17 and EPS KS-20 on DPPH radicals (A), superoxide radicals (B) and tyrosinase activities (C).

result, both EPSs showed significantly higher tyrosinase inhibitory activity than β-glucan ($p<0.01$) throughout the tested concentration range from 1 to 10 mg/ml (Fig. 5C). In the present study, EPSs produced by *B. licheniformis* KS-17 and *B. licheniformis* KS-20 exhibited very high inhibitory activity on tyrosinase compared to β-glucan for anti-aging effects, and exhibited similar or higher scavenging activity on DPPH radical and superoxide radicals than β-glucan. Both KS-17 and KS-20 exhibit antioxidant activity in terms of free radical scavenging effects and antiaging activity.

In this study, *B. licheniformis* KS-17 and *B. licheniformis* KS-20 strains that produced large amounts of EPS were isolated from a Korean traditional fermented food, Kimchi. EPSs obtained from these two strains were characterized by smaller molecular weights, consisting of a single glucose unit, and relatively high levels of antioxidant and antiaging activity compared to β-glucan. Our results indicate that EPS from *B. licheniformis* KS-17 and *B. licheniformis* KS-20 may be applicable as functional ingredients for a variety of industries such as food, cosmetics, and pharmaceuticals. In addition, functional novel probiotics may be developed from these two strains, since *B. licheniformis* is a useful probiotic (Kim et al., 2005; Sanders et al., 2003). We are currently trying to obtain EPS synthetic related genes from these two strains to compare gene structures and properties to further our understanding of the EPS production mechanisms of these two strains.

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