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Characterization of Exopolysaccharide (EPS) Produced by *Weissella hellenica* SKkimchi3 Isolated from Kimchi

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***Weissella hellenica* SKkimchi3 produces the higher exopolysaccharide (EPS) on sucrose than lactose, glucose, and fructose at pH 5 and 20°C. Sucrose was exclusively used to cultivate SKkimchi3 in all experiments base on the EPS production tests. The molecular mass of EPS, as determined by gel permeation chromatography, was 203,000. ^{1}H and ^{13}C NMR analysis indicated that the identity of EPS may be a glucan. When EPS, starch, and cellulose was treated with α-amylase, glucoamylase, glucosidase, and cellulase, glucose was produced from starch and cellulose but was not produced from EPS. Based on HPLC analysis, elemental analysis, ^{1}H and ^{13}C NMR analysis, and enzymatic hydrolysis tests, EPS was estimated to be a glucan. EPS suspension was not precipitated even by centrifugation at 10,000×g for 60 min, and EPS made the fermented milk and bacterial culture viscous.**

***Keywords*:** exopolysaccharide, β-glucan, NMR-spectroscopy, lactic acid bacteria, *Weissella hellenica*

Various polysaccharides have been known to be produced by plants (cellulose, pectin, and starch), seaweeds (alginate and carrageenan), and bacteria (alginate, gellan, and xanthan). Some of these polysaccharides have been applied in food and nonfood industries as viscosifying, stabilizing, emulsifying, gelling, or water-binding agents (Sutherland, 1972; Souw and Demain, 1979; Whitfield, 1988; Sutherland, 1990; Sutherland, 1998). In particular, the exopolysaccharides (EPSs) produced by lactic acid bacteria are generally recognized as safe for consumption, and may contribute to the textures of fermented dairy products and other foods (Dunican and Seeley, 1965; Cerning, 1990; Roller and Dea, 1992; Van Geel-Schutten *et al*., 1999). Lactic acid bacteria (LAB) are widely used for the production of numerous fermented foods and some extracellular polysaccharides (Korakli *et al*., 2003). Some EPS-producing LAB such as *Lactobacillus bulgaricus*, *Lactococcus lactis*, and *Streptococcus thermophilus* have been isolated from fermented dairy products, particularly from yoghurt (Cerning *et al*., 1991; De Vuyst *et al*., 2001). The EPSs produced by LAB are heteropolysaccharides that are composed of repeating units consisting of two or more monosaccharides, mainly galactose, glucose, and fructose, or homopolysaccharides that are composed of repeating units consisting of a single monosaccharide, mainly glucose or fructose (Salminen *et al*., 1998; Van Geel-Schutten *et al*., 1998; De Vuyst and Degeest, 1999; Van Geel-Schutten *et al*., 1999; Korakli *et al*., 2000; Tieking *et al*., 2003). The respective homopolysaccharides consisting of glucose and fructose are glucan and fructan. The microbial production of EPS is known to be influenced by sugar composition and culture conditions. In addition, EPS production may be stimulated by excess sugars in the growth medium and by low temperatures (Cerning *et al*., 1986; Arad *et al*., 1988; Shu and Yang, 1990; Cerning *et al*., 1991; Kojic *et al*., 1992). Some *Weissella* species that were isolated from kimchi have been physiologically and biochemically studied (Choi *et al*., 2002; Kang *et al*., 2004; Park *et al*., 2004). However, research on EPSs produced by *Weissella* species that were isolated from kimchi has rarely been reported.

In the present study, we isolated *Weissella hellenica* SKkimchi3 from kimchi and purified EPS from the culture fluid of SKkimchi3 grown in medium containing sucrose. The EPS was biochemically and structurally characterized in order to determine its identity.

Materials and Methods

Optimal condition for exopolysaccharide (EPS) production

The EPS production by SKkimchi3 was compared in MRS medium containing glucose, fructose, sucrose, lactose, or a mixture of glucose and fructose at different pH from 3 to 9 and temperature from 10 to 40°C for 48 h. Sugars were separately autoclaved and then added to the sterilized medium.

EPS purification

The bacterial cells were discarded following centrifugation at 4°C and 5,000×g for 30 min. Supernatant (bacterial culture fluid) was incubated at 4°C for 5 h; two volumes of 99% cold ethanol (4°C) were added to the supernatant, which was then incubated at 4°C for 12 h. EPS coagulated by ethanol was then separated by centrifugation at 4°C and 10,000×g for 60 min. The precipitant (EPS) was resus-

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pended in 4% trichloroacetic acid and incubated at 4°C for 5 h, and was then centrifuged at 4°C and 12,000×g for 40 min in order to remove the proteins denatured by trichloroacetic acid. The supernatant was mixed with two volumes of 99% ethanol and incubated at 4°C for 12 h. EPS coagulated by ethanol was then separated by centrifugation at 4°C and 10,000×g for 60 min and then resuspended in pure water. Trichloroacetic acid contamination in EPS was removed using a dialysis bag (MW cut-off 10,000, Spectrum Lab., USA). The EPS resuspended in pure water was fractionated by gel-filtration chromatography with Sephacryl S-200 HR (Amersham Biosciences, Sweden) in order to remove other contaminants; pure water was used as elution buffer. Finally, the EPS fraction was lyophilized, yielding purified EPS.

Identification of SKkimchi3

Weissella hellenica SKkimchi3 was isolated using MRS agar medium from relatively fresh kimchi that had been fermented at 10°C for 7 days. The SKkimchi3 strain was identified using the 16S rDNA sequence. 16S ribosomal RNA-coding gene was amplified by direct PCR using universal primers: forward; 5'-GAGTTGGATCCTGGCTCAG-3' and reverse; 5'-AAGGAGGGGATCCAGCC-3'. The reaction mixture consisted of 300 mM Tris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 20 pM of each primer, 20 mM of each dNTP, 2 U *Taq* polymerase (Genenmed, USA), and a 20 ng template. Amplification was conducted for 30 cycles of 1 min at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C using a PCR machine (T Gradient model, Biometera, German). The PCR products were directly sequenced with an ABI Prism 3700 genetic analyzer upon request to a professional company (Macrogen Inc., Korea). The 16S rDNA sequences were analyzed using the GenBank database, and identification was performed on the basis of 16S rDNA sequence homology. The SKkimchi3 strain was registered in the GenBank database system, from which it was given accession number (EU099614).

Determination of molecular mass

The molecular mass of the purified EPS was determined by gel-filtration chromatography (Superose HR, Amersham Pharmacia, Sweden). Pure water was used as the elution buffer at a flow rate of 0.2 ml/min; the flow rate was controlled by an HPLC pumping system (Eyela 214 dual pump, Japan) and fractions were detected by a UV detector (Young Lin, UV730D, Korea) at 210 nm. Standard dextran (Fluka, molecular mass, 25,000, 50,000, 150,000, 410,000) was used to calibrate for the relationship between retention time and molecular mass.

Analysis of sugar composition

The sugar composition of EPS was analyzed by HPLC equipped with an RI detector (Young Lin Acme 9000, Korea) and an HPX-87H ion-exchange column (BioRad, USA), in which the flow rates and column temperature were adjusted to 0.6 ml/min and 35°C, respectively. Pure water containing 0.008 N-sulfuric acid was used as the mobile phase. Samples were prepared by centrifugation at 12,000×g and 4°C for 30 min, and were then filtrated with a membrane filter (pore size 0.22 μm). The filtrate was used as a sample for injection into the HPLC injector. The injection volume was automatically controlled with a 20 μl loop. The concentration was calculated by using the peak area obtained with standard materials. Galactose, fructose, glucose, and mannose were used as standard sugars, the retention times of which were 9.555, 9.688, 8.787, and 9.333 min, respectively. EPS was hydrolyzed with 2 N HCl at 121°C for 40 min, and was then neutralized with NaOH. To remove salt, EPS hydrolyzate was passed through two chromatography columns packed separately with anion (DEAE-cellulose, Bio-Rad, USA) and cation exchange resin (Carboxy methyl-cellulose, Bio-Rad).

Elemental analysis of EPS

The elemental composition of the purified EPS was analyzed by using an elemental analyzer (Flash EA-1112, Thermo Finnigan, USA) without acid hydrolysis at the Center for Collaborative Instruments (Inha University, Korea). Nitrogen, carbon, hydrogen, and oxygen were analyzed in order to estimate the elemental composition of EPS.

Measurement of viscosity

The viscosity of EPS was measured with the Ostwalt viscometer at 10, 20, and 30°C following purification. The EPS concentration was controlled at levels from 0.2 to 1.0% (w/v). The effect of sucrose on the viscosity of the culture fluid (MRS) of *W. hellenica* SKkimchi3 was measured by the same method as that of EPS after bacterial cells had been removed by centrifugation. The concentration of sucrose added to the bacterial culture of MRS was controlled at levels from 1 to 5%. Viscosity was calculated as follows:

Viscosity (CP, centipoise) $=(\gamma_{EPS} \times t_{EPS} \times \mu_{water}) \div (\gamma_{water} \times t_{water})$

γ_{EPS} (g/cm^3) : density of EPS suspension

γ_{water} (g/cm^3) : density of water

t_{EPS} (sec) : flow time of starch suspension through capillary tube of viscometer

t_{water} (sec) : flow time of pure water through capillary tube of viscometer

μ_{water} (CP, centipoise) : viscosity of pure water.

NMR spectroscopy

The ^1H and ^{13}C-NMR spectroscopy were analyzed with an FT-NMR spectrometer (500 MHz, Varian model UI580, USA) in the Korea Basic Science Institute (Seoul, Korea). For NMR measurements, the >99% pure EPS was lyophilized and then dissolved in DMSO (dimethylsulfoxide), the hydrogen atom of which had been substituted with deuterium. The ^1H and ^{13}C-NMR spectra were recorded at 25°C.

Enzymatic hydrolysis of EPS

In order to estimate the glycosidic bond character of the purified EPS, enzymatic hydrolysis was tested using glucosidase (α-D-glucoside glucohydrolase, 1.0 μM glucose/min/unit), α-amylase (1,4-α-D-glucan gluconohydrolase, 0.33 mg maltose/min/unit), cellulase [1,4-(1,3:1,4)-β-D-glucan glucanohydrolase, 1.0 μM glucose/min/unit], and glucoamylase (exo-α-1,4-glucosidase, 1.0 μM glucose/min/unit). Glucosidase catalyzes hydrolysis of α(1→4) glycosidic bond of starch

and α(1→6) glycosidic branch of dextrin. 5 g/L of EPS, soluble starch, or cellulose was suspended in 50 mM phosphate buffer (pH 7.0), to which final 100 units of commercial enzymes (Sigma-Aldrich, USA) were added. The enzyme-substrate mixture was incubated at 30°C for 3 h, and the products were then quantitatively analyzed by HPLC.

Effect of sucrose on solidification of fermented milk
Three, six, nine, and twelve percent (w/v) of sucrose was added to the 10% (w/v) skim milk broth after autoclaving. The skim milk broth without sucrose was used for the control test. Five percent (v/v) of the SKkimchi3 pre-cultivated on MRS medium was inoculated into skim milk broth. Solidification of fermented milk was compared with that of the control after cultivation at 20°C for 48 h.

Results

Determination of sugar species and conditions for EPS production
The EPS produced by SKkimchi3 grown on glucose, fructose, sucrose, lactose, and mixture of glucose and fructose at pH 5 and 20°C was 2.23, 1.56, 5.12, 0.95, and 2.05 g/L, respectively, which were the highest values in all experimental groups (data not shown) for 48 h. On the basis of these results, sucrose, pH 5, and 20°C was exclusively applied to the culture for EPS production.

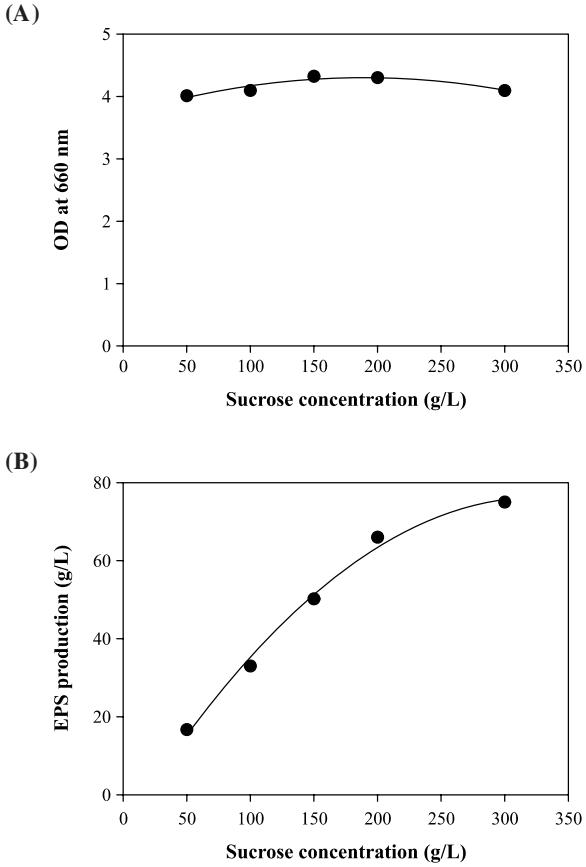

Fig. 1. Growth (A) of and EPS production (B) by the strain SKkimchi3 in MRS medium containing different sugar for 48 h.

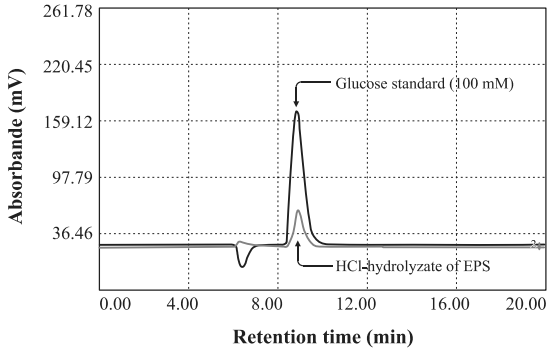

Fig. 2. Chromatogram of EPS and EPS hydrolyzate which was analyzed with HPLC equipped with HPX-87H ion-exchange column and RI detector.

Effect of sucrose concentration on EPS production
Variable concentrations of sucrose, ranging from 50 g/L to 300 g/L, were used to test the relationship between EPS production and sucrose concentration at pH 5 and 20°C. As shown in Fig. 1, EPS production was proportional to the amount of sucrose added to the culture. Meanwhile, the bacterial growth was slightly inhibited at 300 g sucrose/L.

Determination of molecular mass
The molecular mass of EPS was determined by gel permeation chromatography. The relative molecular mass of

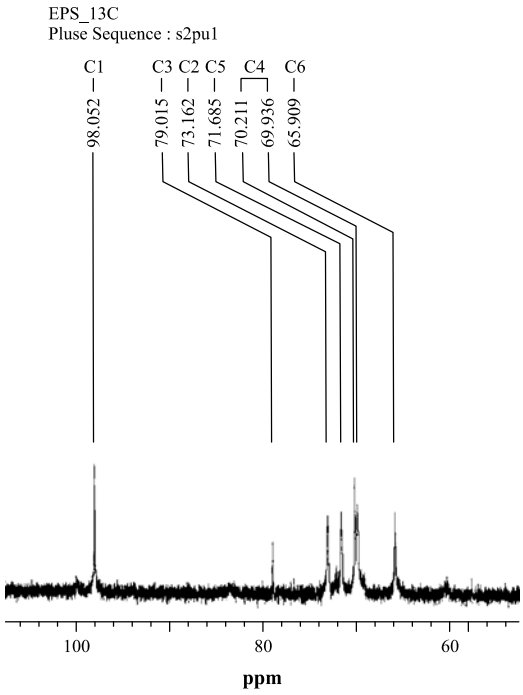

Fig. 3. ^{13}C NMR spectrum of exopolysaccharide synthesized by *Weissella hellenica* SKkimchi3. The carbon resonance from C-1 to C-6 was determined based on the spectra reported by other researchers (Table 3).

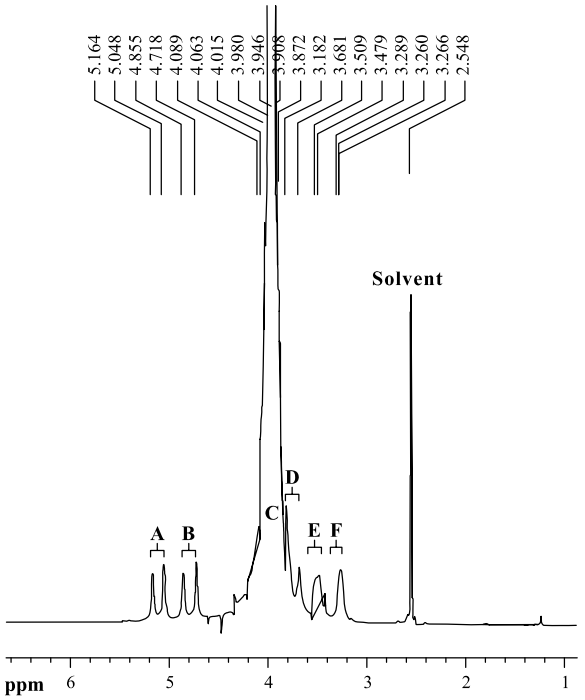

Fig. 4. A 500 MHz ^{1}H NMR spectrum of EPS produced by the strain SKkimchi3 grown on sucrose.

EPS was 203,000, as determined based on the calibration curve obtained according to the relationship between molecular mass and the retention time of standard dextran (data not shown).

Elemental component of EPS

Based on the elemental analysis, EPS was found to be composed of carbon, oxygen, and hydrogen, as shown in Table 1. No other sugars or sugar derivates except hexose could be inferred from the analytical balance. The deviation between the analytical balance and the theoretical balance was 5.5869%, which is an acceptable error range. According to the chromatographic retention time of sugars used as a standard, the acid hydrolyzate of EPS was clearly glucose as shown in Fig. 2. From the results of HPLC and elemental analysis, the constituent sugar species of EPS was confirmed to be glucose.

NMR spectroscopy

As shown in Fig. 3, six carbon signals were observed in the 1D ^{13}C-NMR spectrum of the EPS. Comparison of the ^{13}C-NMR spectrum of the EPS with published chemical shifts of α-glucose, β-glucose, α-1,4-glucan, and β-1,6-glucan demonstrate that the EPS may be a glucan as shown in Table 2. The signal at δ98.1 ppm is assigned to anomeric carbon signals for the (1→4)-D-Glucoside or (1→3)-D-Glucoside, but not for the (1→6)-D-Glucoside. As shown in Fig. 4, the 1D ^{1}H-NMR spectrum of the EPS showed two specific signals (δ=∼5.1 and ∼4.8) in the anomeric regions (δ=5.3 to 4.3). Comparison of the spectrum with glucan synthesized by *L. reuteri* grown on sucrose demonstrates that the EPS is a glucopyranose (Van Geel-Schutten *et al*., 1999). The ^{13}C and

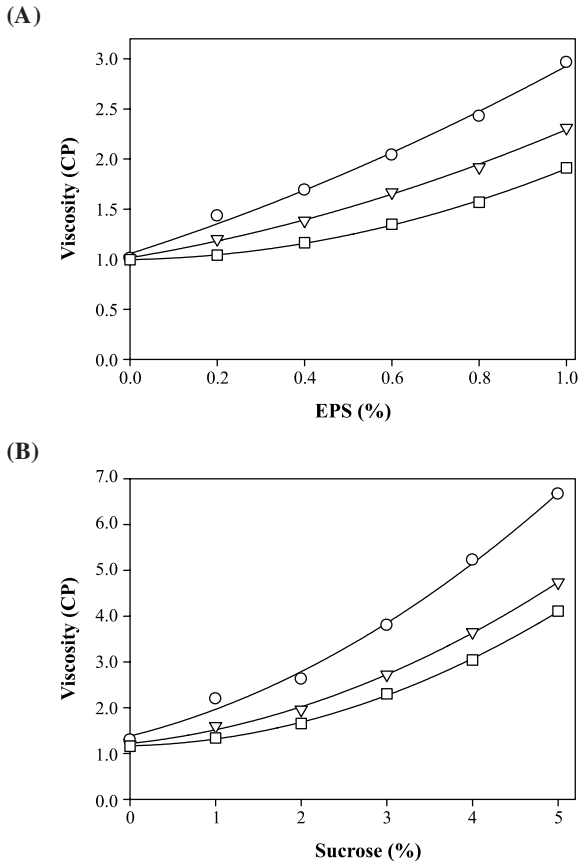

Fig. 5. Viscosity variation of exopolysaccharide suspended in water (A) and effect of sucrose on viscosity of culture fluid of the strain SKkimchi3 grown in MRS (B) at 10℃ (○), 20℃ (▽) and 30℃ (□).

^{1}H-NMR spectra did yield any clue to determine the glycosidic polymer of EPS. In order to determine the bond char-

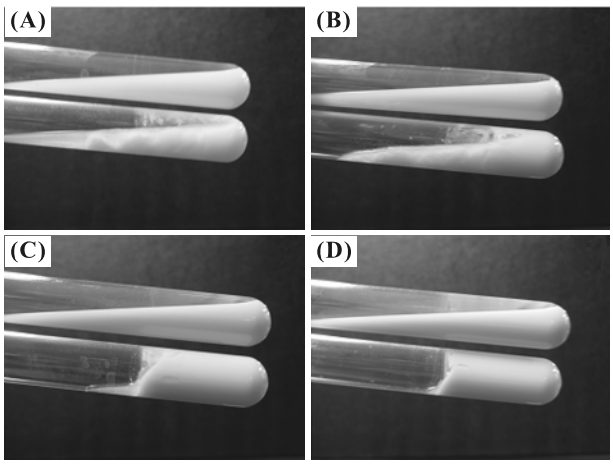

Fig. 6. Effect of exopolysaccharide on curd-forming of 10% skim milk medium in which the strain SKkimchi3 was cultivated for 48 h. 3% (A), 6% (B), 9% (C), and 12% (D) sucrose was added to the skim milk medium (lower culture tube).

Table 1. Elemental composition of the purified EPS was analyzed by an elemental analyzer. Molecular weight determined by gel filtration chromatography is 203,000. Theoretical balances of elemental composition were calculated as follows: (180×n)-[(n-1)×18]=203,000, in which n=1,253. Molecular mass of C, H, and O calculated on the basis of elemental composition was 90,216 (6×12×1,253), 12,530 (10×1×1,253) and 100,240 (5×16×1,253), respectively

Elements	Theoretical balances[a]	Analytical balances	Balance deviation between a and b
Carbon	0.4439	0.384418	+0.05948
Hydrogen	0.0617	0.065181	-0.00348
Oxygen	0.4938	0.493571	+0.000229
Total balance	0.9998	0.943931	0.055869

[a] It was calculated based on follow data: 90,126/203,000 for carbon, 12,530/203,000 for hydrogen, and 100,240/203,000 for oxygen

Table 2. ^{13}C NMR chemical shifts of EPS isolated from the strain SKkimchi3, which was compared with other sugars and sugar polymers

Samples	^{13}C NMR chemical shift (ppm)						References
	C1	C2	C3	C4	C5	C6	
α-Glucose	96.7	74.9	76.7	70.4	76.5	61.6	Kang *et al*. (2005)
β-Glucose	92.8	72.3	73.6	70.4	72.3	61.6	Kang *et al*. (2005)
α-1,4-Glucan	98.35	71.81	70.62	75.3	69.26	61.16	Charkaraborty *et al*. (2004)
β-1,6-Glucan	103.42	73.48	76.01	69.94	73.78	65.98	Charkaraborty *et al*. (2004)
β-2,6-Fructan	61.7	105.0	78.1	76.6	81.2	64.3	Van Geel-Schutten *et al*. (1999)
EPS	98.1	71.6	79.0	70.2/69.9	73.9	65.9	This study

Table 3. Test of glucose production from starch and cellulose hydrolyzed by α-Amylase, glucosidase, glucoamylase and cellulase. No glucose was produced from EPS in same enzyme reactions

Enzymes substrates	Glucose production (mM)			
	α-Amylase (α-1,4)	Glucosidase (α-1,4, α-1,6)	Glucoamylase (exo-α-1,4)	Cellulase (β-1,4)
Starch	3.9	-	18.9	-
Cellulose	-	-	-	6.8
EPS	-	-	-	-

acter, hydrolases capable of degrading specific glycosidic bond were applied to EPS, starch, and cellulose.

Enzymatic hydrolysis of EPS

EPS was treated with α-amylase, glucoamylase, glucosidase, and cellulase. As shown in Table 3, no monosaccharide was produced from EPS treated with the four enzymes when analyzed by HPLC. However, glucose was produced from starch through the enzyme reactions of α-amylase and glucoamylase, and from cellulose through the reaction of cellulase. From these results, EPS produced by SKkimchi3 was confirmed not to be a α-glucoside, but instead is a β-glucoside polymer.

Effect of EPS on viscosity of bacterial culture

EPS did not precipitate naturally from the viscous EPS suspension. The viscosity of EPS was proportional to increases of EPS concentration and decreases of as shown in Fig. 5. The viscosity of the culture fluid was increased in proportion to the concentration of sucrose added to the MRS medium, as shown in Fig. 5B. On the basis of 0.25∼0.3 g EPS production per gram sucrose (Fig. 1), the concentration of sucrose contained in MRS medium was steadily controlled (Fig. 5A); nevertheless, the culture fluid viscosity was much higher than that of the pure EPS suspension. This shows that ingredients contained in MRS medium may have synergistic effects on changes in viscosity.

Effect of sucrose on solidification of fermented milk

Skim milk can be solidified at pH lower than 3.5. The solidification is proportional to the skim milk concentration and lactic acid concentration. In general, 10% skim milk is difficult to solidify through the fermentation of lactic acid bacteria. When *W. hellenica* SKkimchi3 was cultivated in 10% skim milk medium for 48 h, solidification was not observed. However, solidification was activated by the addition of sucrose to the skim milk medium, as shown in Fig. 6.

Discussion

The production of EPS may be influenced by environmental factors and sugar species (Shu and Yang, 1990; Cerning *et al*., 1994; Lloret *et al*., 1998; Looijesteijn *et al*., 1999; Korakli *et al*., 2003). The maximal EPS was produced by SKkimch3 grown on sucrose and at 20°C and pH 5, which may be caused by the original habitat (kimchi) of SKkimch3. EPS

production was distinctly higher on sucrose, whereas bacterial growth was not influenced by the sucrose concentration. This is consistent with that EPS production is the substrate concentration-dependent but not being correlated with growth (Bryan *et al*., 1986; Cerning *et al*., 1986; Sutherland, 1990). *L. casei* CG11 was reported to show the highest EPS production on glucose, but the lowest production on galactose and lactose (Cerning *et al*., 1994), while *Streptococcus thermophilus* was reported to produce EPS on lactose (Levander and Rådström, 2001; Levander *et al*., 2002). This information indicates that glucose may not be the most efficient carbon source for EPS production in spite of plentifulness in nature (Lloret *et al*., 1998).

The constituent unit of EPS, glucose, was estimated by elemental analysis of EPS and chromatography. The molecular weight, determined by gel-permeation chromatography with the standard dextran, was 203,000; this mass was theoretically determined to be composed of 1,253 glucose molecules. In general, the EPSs synthesized by lactic acid bacteria appear to be high molecular ranging from 4.0×10^4 to 6.0×10^6 (Grobben *et al*., 1997). According to this datum, the EPS produced by SKkimchi3 is not a very high molecular mass, but its mass is large enough for it to function as a viscosifying, gelling, or stabilizing agent (Dunican and Seeley, 1965; Roller and Dea, 1992; Kang *et al*., 2005). The EPS produced by SKkimchi3 is insoluble in water, but could not even be precipitated by centrifugation at 10,000×g for 60 min. This is evidence that the EPS sugar chain may be naturally linear, but not micellar or crystalline in structure, and may have a natural gelatiniform structure different from that of starch or cellulose. However, the gelatiniform structure of EPS does not act as evidence that EPS is not α-1,4 glucoside (starch) or β-1,4 glucoside (cellulose).

^{13}C-NMR spectroscopy confirmed the EPS to be a glucan, and its glycosidic bond character was confirmed not to be a β-1,6-glycosidic bond. In ^{1}H-NMR spectroscopy, two anomeric protons (peak A and B: δ=5.164/5.048 and δ=4.855/4.718) were detected (Fig. 4). These protons are very similar to the β-anomeric protons of β-1,2-glucan (δ=4.9/4.88) and succinylglycan (δ=4.81/4.77), but very different from the α-anomeric protons of galactose and glucuronic acid (δ=5.33), and provide useful information to estimate the glycosidic bond character of EPS (Gray *et al*., 1991). The ^{1}H-NMR signals indicating the presence of β-anomeric protons can be a clue that the glycosidic bond of EPS may be a β-glucan. The EPS hydrolysis tests performed with four hydrolases give us useful information to estimate the glycosidic bond character. The character of EPS was confirmed not to be an α-1,4-, α-1,6-, or β-1,4 glycosidic bond. Papers published by other researchers give us useful information to determine the glycosidic bond of EPS. The glycosidic bond of EPS may not be β-1,2-glucoside, as ^{1}H-NMR spectroscopy of the EPS produced by SKkimchi3 was very different from that of β-1,2-glucan (Johnson and Jankowski, 1978; Gray *et al*., 1991; Charkaraborty *et al*., 2004). Consequently, based on information inferred from all data obtained from ^{13}C-NMR spectroscopy, ^{1}H-NMR spectroscopy, hydrolase reactions, and elemental analysis, the EPS was confirmed not to be a α-1,4-, α-1,6-, β-1,4 or β-1,6-glycoside; however, it may be estimated to be a β-1,3-glucoside.

In general, LAB are inhabitants of the gastrointestinal tract, and they have received special attention because of their long history of safe use in foods, as well as their probiotic effects (Souw and Demain, 1979; Reynold *et al*., 1980; Salminen *et al*., 1998; Rachini *et al*., 2007). *Weissella hellenica* SKkimchi3 is a LAB that inhabits in a fermented vegetable product (kimchi), which is a specific environment for LAB. Kimchi is a typical fermented food different from milk products because it does not contain lactose, but does contain various sugars such as glucose, fructose, mannose, and sucrose that originate from chinese cabbage, radish, onion, garlic, green onion, and artificially added sugars. The reason that SKkimchi3 produced relatively higher amounts of EPS on sucrose may be inferred from the habitat (kimchi) of the SKkimchi3 strain. Solidification of fermented skim milk was activated by the addition of sucrose, which may also activate EPS production in various fermented foods. The physiological function of LAB in EPS production may be a useful tool for the transformation of various sugars to β-glucan in a variety of fermented foods containing sugar.

In short, few studies of EPS produced by LAB isolated from kimchi have been reported. However, we have studied EPS characteristics such as different molecular masses and glycosidic bond according to the various carbon sources for LAB isolated from kimchi. Finally, the β-glucan produced by SKkimchi3 isolated from fermented food (kimchi) can be developed for a safe food additive, but should be further studied for immunological and pathological application as described in other reports (Reynold *et al*., 1980; Holbrook *et al*., 1981; Kim *et al*., 2006; Rachini *et al*., 2007)

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