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Metabolic and biochemical evaluation of *Weissella hellenica* SKkimchi3 that produces different amount of exopolysaccharide (EPS) according to kind of sugars

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ABSTRACT

Growing cells of *W. hellenica* SKkimchi3 produced significantly higher exopolysaccharide (EPS) from sucrose than glucose, fructose, and glucose-fructose mixture. Crude enzymes isolated from SKkimchi3 grown on sucrose, glucose, and fructose commonly catalyzed higher EPS synthesis from sucrose than glucose and fructose. The affinity and activity (K_m and V_{max}) of crude enzymes for sucrose were relatively higher than that for glucose and fructose. The pattern of 2D-SDS-PAGE for total soluble protein isolated from SKkimchi3 grown on sucrose was similar to that grown on glucose, but the expression of some proteins differed. Sucrose may not be a factor to induce metabolic activity of SKkimchi3 for EPS production but may be an optimum substrate to induce enzymatic activity for EPS synthesis.

KEYWORDS

Weissella hellenica;
Exopolysaccharide;
□-Glucan;
2D-SDS-PAGE;
MALDI-TOF.

INTRODUCTION

In a previous study, the EPS produced by *W. hellenica* SKkimchi3 was biochemically and chemically characterized to be the homopolysaccharide that is □-1,3-glucan consisted of glucose[1]. In that research, we found that SKkimchi3 produced much more EPS from sucrose than glucose, fructose, and lactose. Sucrose has been employed as a substrate for EPS production in a number of studies using various lactic acid bacteria because EPS production was relatively higher on sucrose than other sugars[2]. The variation of EPS yields caused by the use of different sugars may be a general phenomenon in the EPS-producing bacteria, but this has not been metabolically and biochemically studied.

EPS is generally synthesized from galactose-1-phosphate and glucose-1-phosphate, which are biochemically derived from various sugars by the catalysis of enzymes responsible for EPS synthesis[3,4]. However, production of EPS may vary due to both the biocatalysts (bacterial strains) and substrates (sugars) used. *Lactobacillus sanfranciscensis* requires sucrose for the biosynthesis of EPS, but requires fructose for growth[5-8]. Lactic acid bacteria originating from dairy products produced more EPS from lactose than from other sugars[9,10]. *Lactibacillus casei* CG11 produced much more

EPS on glucose than galactose, lactose, sucrose, maltose, and melibiose, and *Lactococcus lactis* subsp. *cremoris* strain NIZO B40 produced about 8 times more EPS when grown on glucose than when grown on fructose[11,12]. Accordingly, it is possible that a specific sugar may be a factor to induce or activate the metabolism of a specific species of lactic acid bacteria to synthesize EPS.

In this research, we investigated the reasons as to why *Weissella hellenica* SKkimchi3 produced significantly higher yields of EPS from sucrose than from glucose or fructose. The EPS production by growing cells of SKkimchi3 and the EPS synthesis by crude enzymes were analyzed, and K_m and V_{max} of the crude enzymes for different sugars were evaluated. We then compared patterns of 2D-SDS-PAGE for total soluble proteins isolated from bacterial cells grown on sucrose and glucose.

MATERIALS AND METHODS

EPS production by growing cells

SKkimchi3 was cultivated in MRS medium containing 200mM of sugars that are sucrose, glucose, fructose, or glucose-fructose mixture at 20°C for 72 h. Bacterial cells were separated from culture fluid by centrifugation at 5,000xg and 4°C for 30min. EPS was isolated from the culture fluid using cold alcohol precipitation and was purified according to the method of Kim *et al*.[1] as modified by Shimamura et al.[13]. The purified EPS was lyophilized prior to comparison on the basis of dry weight.

EPS synthesis by catalysis of crude enzyme

Crude enzyme was isolated from SKkimchi3 grown on MRS medium containing 200 mM of glucose, fructose, or sucrose, for 48 hr. Bacterial cells were harvested by centrifugation at 5,000xg and 4°C for 30min. The harvested cells were washed three times with 25 mM phosphate buffer, each time being centrifuged at 5,000xg and 4°C for 30min and disrupted by a bead beater at 4°C and 3,000 strokes per min for 30min. Cell debris were discarded through centrifugation at 10,000xg and 4°C for 60min, and the supernatant was used for determination of the crude enzyme produced. The reaction mixture was composed of crude enzyme (20mg/ml of protein), 25 mM of phosphate buffer (pH 7.2) and 200 mM of sugars, and being incubated at 20°C and 120 strokes per min for 6 h. EPS was isolated from the reaction mixture by cold ethanol precipitation and was then purified by chromatography[1].

K_m and V_{max} of crude enzyme

Reaction mixture, composed of sugars, 25 mM of phosphate buffer (pH 7.2), and crude enzyme (20mg/ml of protein), was prepared on ice water to control the reaction, and was then incubated in shaking water bath at 20°C and 120 strokes per min for 30 min. Concentration of sugar contained in each reaction mixture was gradationally increased from 0 to 20 mM at intervals of 2 mM. After the reaction was finished, the reactant was cooked in boiled water for 2 min to stop the enzyme reaction. Residual sugars were analyzed by HPLC, and K_m and V_{max} was determined by Lineweaver-Burk plotting based on the sugar consumption in each reactant per unit time.

2D-SDS-PAGE of total soluble protein

Two-dimensional gel electrophoresis (2D-SDS-PAGE) was performed according to procedures and methods used by Wilkins *et al*.[14] with the reagents, kits and apparatuses provided by Bio-Rad (Bio-Rad Laboratories, Hercules, USA). SKkimchi3 cultivated on sucrose and glucose for 48 h was harvested and washed twice with a 50 mM Tris-HCl (pH 7.5), and was then disrupted by a bead beater cell disruptor. Cell debris was discarded by centrifugation at 10,000xg and 4°C for 60 min, and the protein concentration in the soluble extract was determined with Bradford reagent (Bio-Rad). Proteins in the soluble extract were first separated based on their isoelectric point using an IPG strip (Readstrip, 170 mm, pH 3-10, Bio-Rad) and an isoelectricfocusing system (Protean IEF Cell, Bio-Rad). The isoelectrically focused proteins were then separated based on molecular weight by SDS-PAGE. SDS-PAGE was carried out using a precast gel (8-14% gradient, Bio-Rad) and an electrophoresis system (Protean II XL cell, Bio-Rad). Protein spots on 2D-SDS-PAGE gel were visualized by silver staining (Silver staining kit, Bio-Rad).

In-gel digestion

Protein spots of interest were manually excised from the gel and placed in Ependorf tubes. Gel pieces were destained in a 1:1 mixture of 30 ml potassium ferricya-

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nide and 100 mM sodium thiosulfate, washed with 50% Acetonitrile (ACN)/25mM ammonium bicarbonate at pH 7.8, and incubated in 50% ACN for 5 min. Gel pieces were dehydrated in a vacuum centrifugal concentrator and were incubated in 10□L of trypsin (0.02□g/□L) solution on ice for 45 min. After replacing with 20 mM ammonium carbonate, gel pieces were digested at 37°C overnight. On the following day, 0.5% (v/v) trifluoroacetic acid (TFA) in 50% ACN was added, and the extraction was conducted twice in an ultrasonic water bath. Peptides were extracted in 0.1% formic acid in 2% ACN for further MALDI-TOF MS analysis.

MALDI-TOF MS analysis

Mass of proteins isolated from 2D-gel was analyzed using a PerSeptive Biosystem Voyager-DE STR™ MALDI-TOF MS (Applied Biosystems, CA, USA) under condition for positive ion detection. Peptide extracts were mixed with a matrix solution consisting of 10 mg/ml -cyano-4-hydroxycinnamic acid, 0.1% TFA and 50% ACN. Angiotensin 1 (monoisotopic mass, 1296.6853), rennin substrate (1758.9331) and adrenocorticotropic hormone (2465.1898) were employed for mass calibration. Autolytic fragments of trypsin were employed for internal calibration. Proteins were identified by peptide mass fingerprinting with MS-FZI supplemented with the option for Bacteria in the NCBI database. The criteria for positive identification of proteins were set as follows: (i) at least 4 matching peptide masses, (ii) 50 ppm or better mass accuracy, and (iii) matching of MW and PI of identified proteins with the values estimated from image analysis.

Analysis of sugars

Sugars were analyzed by HPLC equipped with an RI detector (Young Lin Acme 9000) and an HPX-87P column (Bio-Rad), in which the flow rates and column temperature were adjusted to 0.6 ml/min and 85°C, respectively. Deionized water was used as the mobile phase. The concentration was calculated through comparison of the peak area with that obtained from standard solutions.

Purification of EPS

Cold alcohol-precipitated EPS was rehydrated in pure water and was purified 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 a HPLC pumping system (Eyela 214 dual pump, Tokyo, Japan) and fractions were monitored by a UV detector (Young Lin, UV730D, Seoul, Korea) at 210 nm. EPS was selectively fractionated based on retention time, as determined in previous research[1].

RESULTS

EPS production by growing cells

EPS metabolically produced by grown on different sugars was quantitatively compared. Approximate 4.9g/L, 2.1g/L, 1.5g/L, and 2.1g/L of EPS from sucrose, glucose, fructose, and glucose-fructose mixture, respectively, was produced (TABLE 1). The yields of EPS produced from SKkimchi3 grown on glucose and on a mixture of glucose and fructose were similar to each other, and were greater than that of fructose. The glucose-fructose mixture was not effective as a substitute for sucrose in the metabolism of SKkimchi3 for EPS production.

TABLE 1 : Effects of sugar species on growth and EPS production of *W. helenica* SKkimchi3.

Substrates	Sucrose	Glucose	Fructose	Glucose+ Fructose
Growth (OD)	3.10 ± 0.21	2.53 ± 0.13	2.87 ± 0.19	2.91 ± 0.25
EPS (g/L)[a]	4.89 ± 0.16	2.06 ± 0.38	1.45 ± 0.23	2.12 ± 0.43
EPS/OD	0.158	0.081	0.051	0.073

a, dry weight

EPS synthesis by catalysis of crude enzyme

In order to determine whether different sugars affect the synthesis of enzymes responsible for EPS synthesis, or affect EPS-synthesis, a crude enzyme isolated from SKkimchi3 cultivated on sucrose, glucose, and fructose was used as catalyst for EPS synthesis from different sugars. Approximate 50% of fructose, 65% of glucose, and 78% of sucrose was converted to EPS, as shown in TABLE 2. However, the tendency of EPS production from different sugars was the same for growing cells. Glucose and fructose produced from sucrose by catalysis of crude enzyme remained in the reaction mixture in inverse proportion to EPS produced from glucose and fructose in reaction mixture.

Affinity of crude enzyme for different sugars

The difference of EPS production by catalysis of the crude enzyme was determined by kind of sugars used as substrates. EPS production may be influenced by affinity and reaction rates between substrate and enzyme. Affinity and reaction rates were estimated based on K_m and V_{max} values. The K_m and V_{max} of crude enzyme for glucose was 4.31 mM/L and 0.124 mM/L/min, fructose was 5.13 m/ML and 0.039 mM/L/min, and sucrose was 3.7 mM/L and 0.22 mM/L/min, respectively, as shown in Figure 1 These results are presented in the supplementary data, and indicate that sucrose is a more suitable substrate for the crude enzyme catalyzing EPS synthesis, compared to glucose or fructose.

TABLE 2 : EPS production from different sugars by crude enzyme isolated from SKkimchi3 cultivated on different sugars.

Growth substrates	Reaction substrates	Consumed substrates (g/L)	Products (g/L)		
			Glucose	Fructose	EPS
	Fructose	9.4±1.2	-	-	4.8±0.3
Glucose	Glucose	12.2±0.6	-	-	8.2±0.5
	Sucrose	19.2±1.1	1.5±0.1	2.6±0.2	15.1±1.1
	Fructose	8.8±0.7	-	-	4.6±0.6
Fructose	Glucose	11.8±0.8	-	-	7.2±0.4
	Sucrose	18.2±1.3	1.2±0.1	2.3±0.2	14.1±0.8
	Fructose	9.2±0.7	-	-	4.7±0.5
Sucrose	Glucose	12.4±0.9	-	-	7.5±0.4
	Sucrose	18.8±1.2	1.3±0.1	2.4±0.1	14.8±1.2

2D-SDS-PAGE and MALD-TOF

Total soluble proteins isolated from SKkimch3 cultivated on sucrose and glucose were compared by 2D-SDS-PAGE, as shown in Figure 2 Most protein spots on 2D gel for glucose were from the same as those for sucrose; however, some protein spots on 2D gels were more weakly or strongly expressed, or were not mutually expressed at all. All of the protein spots on the 2D-gel were not totally identified with the proteins released in the MALD-TOF MS database when compared based on the matching peptide mass (MW), pI, and protein sources. This result shows that both sucrose and glucose may not be a factor to induce significant variation of SKkimchi3 metabolism for EPS synthesis.

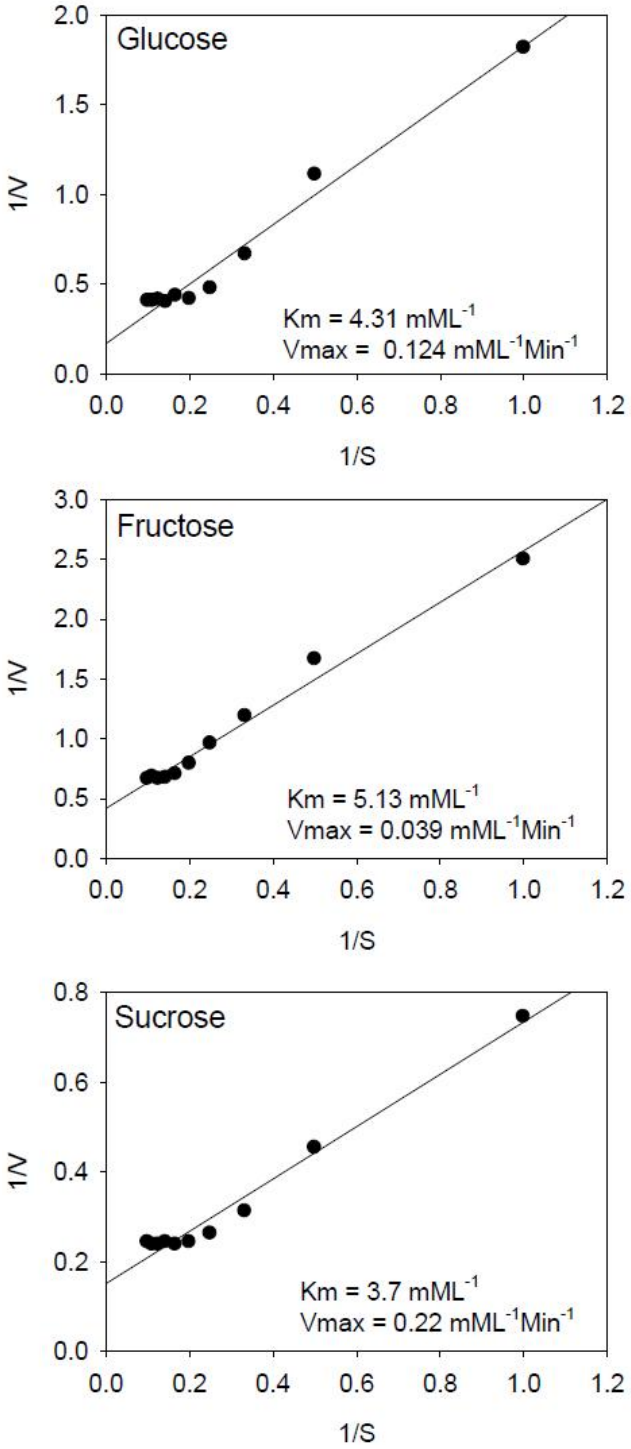

Figure 1 : Lineweaver-Burk plot of kinetic differences for sugar consumption by crude enzyme (20mg/ml) coupled to EPS production. Data are presented as mean of triple tests.

DISCUSSION

EPS produced by lactic acid bacteria responsible for food fermentation may be safe and useful as a food

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additive or ingredient. *W. hellenica* SKkimchi3, a heterofermentative lactic acid bacterium isolated from fermented kimchi, differently produced a homopolysaccharide glucan polymer from sucrose, glucose, fructose, and lactose[1,15]. Various EPS-synthesizing lactic acid bacteria produce relatively high or low concentrations of EPS from a specific sugar, usually sucrose[5,16,17]. Sucrose has been employed as a substrate for glucan production in studies using lactic acid bacteria, because greater yields of EPS were obtained from sucrose than other sugars[2]. Some lactic acid bacteria (e.g. *L. mesenteroids*, *Streptococcus* species, and *Lactobacillus* species) produced structurally and quantitatively different glucans from sucrose[18-20]. Yields, structures, uses, and molecular mass of glucans have been the main subject in most studies related to the EPS-producing lactic acid bacteria. On the other hand, the metabolic relationship between sugars and EPS yield has not been studied. SKkimchi3 is a useful bacterium to study the reasons as to why more EPS was produced from sucrose than glucose and fructose, because crude enzyme isolated from SKkimchi3 catalyzed the splitting of sucrose into glucose and fructose and the synthesis of a homopolysaccharide glucan polymer from sucrose.

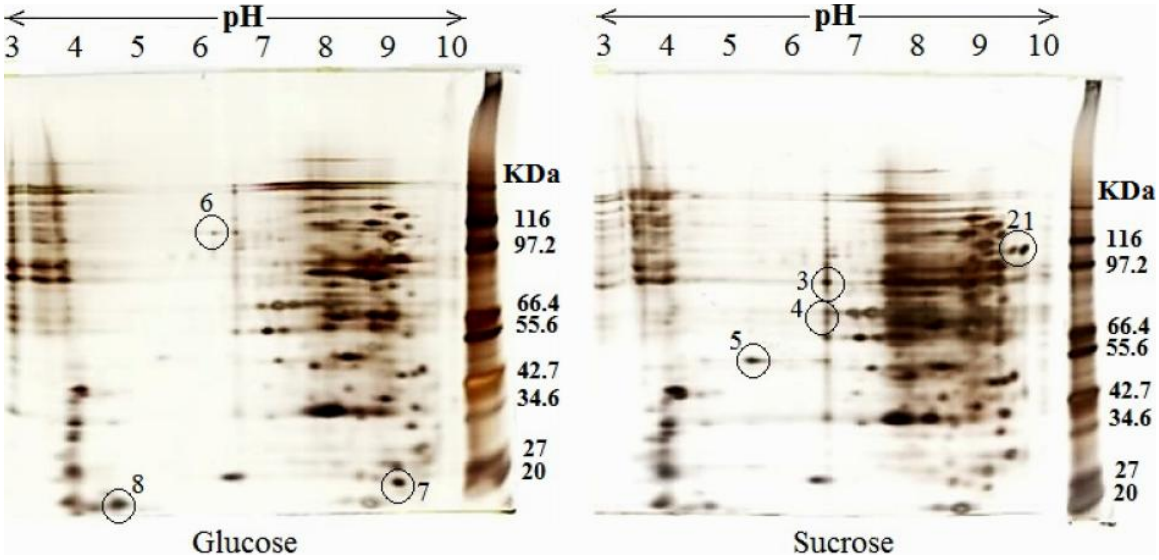

Figure 2 : Silver-stained 2D-SDS-PAGE patterns of total proteins obtained from SKkimchi3 that was cultivated on glucose and sucrose.

Glycansucrase (GS) is a key enzyme which catalyzes the splitting of sucrose into glucose and fructose, as well as the synthesis of polysaccharide from monomeric and dimeric sugars in the EPS-synthesizing lactic acid bacteria[21-24]. Accordingly, SKkimchi3 contains GS based on the experimental data that the crude enzyme of SKkimchi3 catalyzed EPS synthesis from dimeric and monomeric sugars (sucrose, glucose, and fructose) and splitting sucrose into glucose and fructose (TABLE 2). The metabolic synthesis of GS by SKkimchi3 may not be influenced by kinds of sugars used as the substrates for growth, based on the experimental result that crude enzymes isolated from SKkimchi3 grown on sucrose, glucose, and fructose catalyzed the synthesis of nearly the same quantity of EPS from each sugar. GS metabolically synthesized by SKkimchi3 grown on different sugars may not be different in their catalytic activity for EPS synthesis but may be selectively and differently reacted with a specific sugar based on the difference of K_m and V_{max} values for sucrose, glucose, and fructose[25]. This phenomenon observed in EPS-synthesizing metabolism of SKkimchi3 is different from *L. reuteri* LB121, which selectively produces GS when grown on sucrose[2]. Sucrose was an essential factor to induce biosynthesis of GS for *L. reuteri* LB121, but not for SKkimchi3. Specific sugar may not be a factor for the induction of SKkimchi3 to synthesize the enzymatic catalysis of EPS synthesis, or influence the metabolism of SKkimchi3 based on 2D-SDS-PAGE which was not significantly and not differently influenced by sucrose and glucose.

CONCLUSION

It is possible that the EPS synthesis of SKkimch3

may be not metabolically influenced by the kind of sugars employed as a growth substrate, but biochemically (catalytically) influenced by the synthesized enzyme. The chemical energy released by cleavage of the energy-rich glycosidic bond of sucrose may be a cause to produce more EPS from sucrose than other sugars[8]. However, it may be not a general phenomenon based on that relatively more EPS was produced from glucose than fructose or lactose. Accordingly, biochemical EPS production by SKkimch3 may be influenced by the selective reaction between specific sugar and enzyme responsible for EPS synthesis. This study may be the useful information to select bacterial strain and choose kind of sugars to improve enzymatic production of EPS for the safe food additive and ingredient.

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