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Characterization and antibacterial activity of a novel exopolysaccharide produced by *Lactobacillus kefiranofaciens* DN1 isolated from kefir

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ABSTRACT

Kefir is fermented milk produced by co-cultures of lactic and acetic acid bacteria and yeast that are encapsulated by an exopolysaccharide (EPS) called kefiran. Probiotic kefir provides many benefits, including antimicrobial effects against many pathogens. The mechanisms underlying these effects, however, are not yet completely understood. In this study, a total of 22 strains of lactic acid bacteria (LAB), including eight strains of *Lactobacillus kefiranofaciens*, two strains of *Lactobacillus kefiri*, seven strains of *Lactococcus lactis*, and five strains of *Leuconostoc mesenteroides*, were isolated from kefir and classified by 16S ribosomal RNA gene sequencing. The ability of each strain to produce EPS was determined, and *Lactobacillus kefiranofaciens* DN1 was found to have the highest EPS yield among all isolates. *Lactobacillus kefiranofaciens* DN1 produced EPS, using glucose and lactose, and EPS yield rose to 2.2 g/L in modified MRS broth (60 g/L glucose). The antimicrobial activities of the EPS produced by *Lactobacillus kefiranofaciens* DN1 (EPS_DN1) against *Listeria monocytogenes* and *Salmonella* Enteritidis were assessed by growth curve analysis. We found that EPS_DN1 at a concentration of at least 1% was able to exert bactericidal effects against both pathogens. Notably, results of high-performance size-exclusion chromatography (HPSEC) analysis indicated that EPS_DN1 was not kefiran, the major EPS of kefir grains, suggesting that EPS_DN1 represents a novel bioactive compound in kefir.

1. Introduction

The beneficial health effects of fermented foods arise not only from probiotic microorganisms themselves but also from their metabolites (Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2006). These include exopolysaccharides (EPS) and proteins produced during fermentation (Vinderola et al., 2006). EPS is a polymer produced by some lactic acid bacteria (LABs), and may be produced either on the cell surface or in the surrounding medium as a muco-substance (Ramchandran & Shah, 2009). Although EPS has been recognized as a natural biothickener owing to its smoothing and stabilizing effects on fermentation products, it has also received attention owing to its bioactive functions such as its immunostimulatory, antitumor, and cholesterol-lowering effects (Kim, Chon, Kim, & Seo, 2015a; Shiomi, Sasaki, Murofushi, & Aibara, 1982; Vinderola et al., 2006). However, information is limited regarding the EPS produced by LABs originating from kefir and its antimicrobial activity.

Kefir is fermented milk produced by the co-culture of LABs, yeasts, and acetic acid bacteria trapped in a complex matrix of kefir grains containing microorganisms and their metabolites such as EPS and peptides (Zajšek, Kolar, & Goršek, 2011). Kefir is known to have beneficial properties, including antimicrobial, antitumor, immunomodulatory, cholesterol-lowering, and antiallergenic effects, as well beneficial effects on the gut microbiota (Guzel-Seydim, Kok-Tas, Greene, & Seydim, 2011; Kim et al., 2015a). However, the mechanisms underlying these effects remain unclear.

Early research regarding the structure of kefir grains determined that bacteria and yeasts were encapsulated by an EPS called kefiran (Arihara, Toba, & Adachi, 1990). Kefiran is produced by various kefir microorganisms, and its wound-healing properties and antitumorigenic activity have been reported (Rodrigues, Caputo,

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Carvalho, Evangelista, & Schneedorf, 2005). However, there is still a lack of knowledge concerning the antimicrobial activities of other EPSs that may be produced by individual kefir strains.

The aims of the current study were to: (i) isolate the various LAB strains from kefir; (ii) identify and screen EPS-producing strains and select the strain with the highest yield of EPS; (iii) maximize EPS production by optimization of glucose concentration; (iv) purify the EPS from the selected strain and test its antimicrobial activity against two major foodborne pathogens, *Listeria monocytogenes* and *Salmonella* Enteritidis; and (v) identify the monosaccharide composition of EPS_DN1 following hydrolysis by high-performance size-exclusion chromatography (HPSEC).

2. Material and methods

2.1. Isolation of LABs from kefir

Kefir was prepared by adding 50 g of viable kefir grains to 1 L of sterilized milk (5% w/v) and fermenting the mixture for 24 h at 25 °C. For isolation of LABs, a loop of kefir milk was streaked onto de Man, Rogosa, and Sharpe (MRS) agar (Difco, Detroit, MI, USA) and incubated anaerobically at 30 °C for 72–96 h. Colonies were subject to Gram staining and catalase test using 3% hydrogen peroxide (Sigma, St. Louis, MO). Therefore, total 22 different colonies that were gram-positive and catalase-negative were considered LABs, and were sub-cultured. After subculturing colonies twice anaerobically on MRS agar at 30 °C for 72–96 h, the following four colony parameters were assessed for each strain: shape (round or punctiform), texture (ropy, dry, or moist), pigmentation (transparent, white, cream-white, or grayish), and margin (entire or smooth).

2.2. DNA extraction

Genomic DNA extraction was performed using the NucliSENS easyMAG instrument (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Briefly, each colony was added to 1 mL of lysis buffer, and the mixture was incubated for 10 min at room temperature. The lysed sample was then transferred to the well of a plastic vessel with 50 μL of magnetic silica and subjected to automatic magnetic bead separation. DNA was then resuspended in 50 μL of elution buffer.

2.3. Sequencing and identification of LABs

Partial sequences of the 16S ribosomal RNA gene were amplified from the genomic DNA of LAB strains using the primer pair 27F/1492R (Lane, 1991). PCR products were sequenced using the same primers and the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions. Sequencing was performed in an Applied Biosystems 3730xl DNA Analyzer at Cosmo Genetech (Seoul, Korea). BLAST searches (http://www.ncbi.nlm.nih.gov.blast) against the GenBank DNA database (www.ncbi.nlm.nih.gov/Genbank/) were used to identify LABs isolated from kefir milk. The identities of the isolates were determined on the basis of the highest BLAST score. A neighbor-joining phylogenetic tree of *Lactobacillus kefiranofaciens* DN1 was constructed using the Molecular Evolutionary Genetics Analysis (MEGA) Software Version 6.0 (Kumar, Tamura, & Nei, 1993).

2.4. Screening for EPS production

Each isolate was presumptively screened for EPS production based on its texture on MRS agar. Ropy colonies displaying stickiness or adherence ability after touching with an inoculating loop were assumed to produce large quantities of EPS (Cain, Hanks, Weis, Bottoms, & Lawson, 2009). Next, each isolate was cultured anaerobically on both MRS agar and sucrose agar (1% tryptone, 0.5% yeast extract, 0.5% dipotassium phosphate, 0.5% diammonium citrate, 5% sucrose, pH 7.0) for 48 h at 37 °C (Yun et al., 2013) for evaluation of capsular EPS production ability according to the negative staining methods of Cain et al. (2009), with some modifications. Following growth on MRS and sucrose agar, individual colonies were picked with sterilized loops and transferred to glass slides. Capsules were then observed by staining with crystal violet (for staining bacteria) and 20% $CuSO_4$ aqueous solution (for removing excess stain) (Cain et al., 2009). Strains showing a clear zone around bacterial cells (representing capsule that was unstained by both reagents) were regarded as capsular EPS-producing bacteria.

2.5. Quantification of EPS yield

The content of crude exopolysaccharides from the LABs was determined according to the ethanol precipitation methods of Smitinont et al. (1999) and Yun et al. (2013), with some modifications. To quantify EPS production from screened LABs, each isolate was inoculated (1.0×10^8 CFU/mL) into 200 mL of MRS broth (pH adjusted to 5.5) and incubated at 37 °C for 72 h. Each fermented broth was centrifuged at 10,000 *g* for 25 min at 4 °C, and the cell-free supernatant was precipitated with two volumes of 99% chilled ethanol (−20 °C) and incubated overnight at 4 °C. After overnight precipitation, the sample was centrifuged at 10,000 *g* for 25 min at 4 °C, and the pellet was retained. The remaining ethanol was air-dried, and the remaining pellet was lyophilized using an FD Freeze Dryer (Ilshin Bio, Seoul, Korea) and regarded as crude EPS. The weight of the crude EPS produced by each strain was compared and the strain with the highest EPS yield, *Lactobacillus kefiranofaciens* DN1, was selected for the following experiments. All procedures were repeated three times.

2.6. Optimization of EPS production

To determine the optimal glucose concentration for EPS production by *Lactobacillus kefiranofaciens* DN1, MRS broth was supplemented with different amounts of glucose, providing working concentrations of 20, 40, 60, and 80 g/L. *Lactobacillus kefiranofaciens* DN1 (1.0×10^8 CFU/mL) was inoculated into 200 mL of each broth and incubated at 37 °C for 72 h. EPS produced in each broth was isolated and quantified as described above. All procedures were repeated three times.

2.7. EPS purification

To investigate the antimicrobial activity and determine the monosaccharide composition, the EPS produced by *L. kefiranofaciens* DN1 was purified to remove bacterial cell debris and proteins according to the method of Yun et al. (2013), with some modifications. Crude EPS was purified by dissolving it in an equal volume of 10% (w/v) trichloroacetic acid (TCA) and incubating it for 2 h at 4 °C for bacterial cell and protein precipitation. The protein precipitate was removed by centrifugation at 10,000 *g* for 25 min at 4 °C and then filtered through a syringe filter with a 0.20-μm pore size (Millipore, Bedford, MA, USA). Next, the supernatant was collected and two volumes of 99% chilled ethanol (−20 °C) were added, and the mixture was incubated overnight for precipitation at 4 °C. After centrifugation at 10,000 *g* for 25 min at 4 °C, the remaining pellet was lyophilized using an FD Freeze Dryer (Ilshin Bio). The purified EPS was obtained and regarded as EPS_DN1.