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Isolation, structural characterization and immunological activity of an exopolysaccharide produced by *Bacillus licheniformis* 8-37-0-1

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ABSTRACT

A strain of *Bacillus licheniformis* 8-37-0-1 with high exopolysaccharide (EPS) production ability was isolated and identified based on morphological and physiological characteristics and phylogenetic analysis of 16S rDNA sequences. A new type of EPS was isolated from the strain fermentation broth by enzymolysis, isopropanol precipitation, anion-exchange, and gel-filtration chromatography. The new EPS was determined as homogeneous, with a molecular weight of 2.826×10^4, as determined by High-Performance Size-Exclusion Chromatography Multi-Angle Laser Light Scattering analysis. Its structural characteristics were investigated and elucidated by methylation analysis, partial acid hydrolysis, gas–liquid chromatography mass spectrometry, Fourier transform infrared, and nuclear magnetic resonance spectroscopy. Based on obtained data, the EPS was found to be a levan containing a $(2 \rightarrow 6)$-linked backbone with a single β-D-fructose at the C-1 position every seven residue, on average, along the main chain. Preliminary *in vitro* tests revealed that EPS could significantly stimulate the proliferation of spleen lymphocyte.

1. Introduction

Microbial exopolysaccharides (EPS) are soluble or insoluble polymers secreted by microorganisms (Kumar et al., 2007). The diversity in chemical composition of microbial polysaccharides results in a variety of properties that cannot be found in plant polysaccharides (Kambourova et al., 2009). Due to their characteristic physical and rheological properties, EPS are widely used in the food industry as viscosifying, stabilizing, gelling, or emulsifying agents (De Vuyst and Degeest, 1999; Laws et al., 2001). New areas for the action application of microbial polysaccharides include their use as bioflocculants, bioabsorbents, heavy metal removal agents, drug delivery agents, and others (Wang et al., 2008). In recent years, there has been increasing interest in their biological activities. These include antitumor, antiviral, immunostimulatory, and anti-inflammatory activities (Arena et al., 2006; De Stefano et al., 2007; Weiner et al., 1995).

Bacillus sp. and strains produce a variety of EPS such as levan (Han, 1989), β-1,3-Glucan (Gummadi and Kumar, 2005), and heteropolymers mainly composed of neutral sugar (Larpin et al., 2002), uronic acid (Corsaro et al., 1999), uncommon sugar (Kodali et al., 2009), or sugar–protein conjugate (Zheng et al., 2008). Some EPS from *Bacillus* have shown excellent emulsifying, flocculating, heavy metal removal capacity, or pharmaceutical activity (Arena et al., 2006; Kodali et al., 2009; Salehizadeh and Shojaosadati, 2003; Zheng et al., 2008). The aim of the present study was the identification of a strain that produces the highest amount of EPS, to chemically characterize the EPS and to evaluate its *in vitro* immunological activity.

2. Methods

2.1. Screening and identification

Soil samples (10 g) collected from an orchard near Jinan, China were suspended in 90 mL sterile water. Serial dilution and the spread-plate method were used for obtaining the different microorganisms. The screening medium contained 30 g sucrose, 1.0 g beef extract, 0.5 g $(NH_4)_2SO_4$, 2.5 g $K_2HPO_4{\cdot}3H_2O$, 2.5 g KH_2PO_4, 1.0 g NaCl, 0.2 g $MgSO_4{\cdot}7H_2O$, and 0.001 g $FeSO_4{\cdot}7H_2O$ in 1000 mL of water (pH 7.0). The first screening was conducted by selection of a smooth, humid, and mucoid colony on solid plates. Afterwards, the isolates were cultured in liquid screening medium at

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30 °C with shaking at 180 rpm for 48 h. After centrifugation at 17,000*g* for 10 min, the supernatant was mixed with three volumes of chilled isopropanol. The precipitate was collected by centrifugation at 17,000*g* for 10 min and the pellets were dried at 50 °C under vacuum. EPS production was determined by quantifying the carbohydrate content of the pellets as D-glucose equivalents using the phenol–sulfuric acid method (Dubois et al., 1956). Strain 8-37-0-1, which produces high amounts of EPS, was identified based on morphological and physiological characteristics (Holt et al., 1994) combined with the 16S rDNA sequence analysis. The nearly full-length 16S rDNA gene sequence was amplified by polymerase chain reaction (PCR) using the universal primers 27f [5′-AGAGTTTGATCMTGGCTCAG-3′] and 1541r [5′-AAGGAGGTGATCCAGCC-3′]. Sequence analysis was performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic analysis was performed using a *MEGA* version 4.1 program by neighbor joining (NJ)/maximum parsimony (MP) (Kumar et al., 2004).

2.2. Extraction and purification of polysaccharide

The EPS sample was prepared from strain 8-37-0-1 culture in the fermentation medium containing 100 g sucrose, 1 g beef extract, 0.6 g yeast extract, 3 g $K_2HPO_4{\cdot}3H_2O$, 3 g KH_2PO_4, 1 g NaCl, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.001 g $FeSO_4{\cdot}7H_2O$ in 1000 mL of water (pH 7.0). The fermented broth was collected and centrifuged at 12,000 rpm at 4 °C for 10 min. The supernatant was incubated at 37 °C for 1 h after adding 1.0% (v/v) pepsin (20 U/ml) and then incubated at 100 °C for 10 min. The deproteinated supernatant was collected after centrifugation at 17,000*g* for 10 min. The crude polysaccharide fraction was obtained from the deproteinated solution through precipitation with 3 vol chilled isopropanol and desiccation *in vacuo*. The precipitate was re-dissolved in distilled water and sequentially applied to a DEAE Sepharose Fast Flow (Amersham, US) column (3.5 × 30 cm) eluted with a gradient of 0 → 2 mol/L NaCl, and a Sepharose 4B (Amersham, US) column (1.1 × 35 cm) eluted with 0.1 mol/L NaCl. The resulting fractions were combined according to the carbohydrate content quantified by the phenol–sulfuric acid method. The main fraction was dialyzed against flowing tap-water using dialysis tubing (MWCO 2000) for 24 h, and then dialyzed against flowing distilled water for at least 3 d. The dialyzate was then lyophilized to obtain white purified EPS.

2.3. General analysis methods

The specific rotation was determined at 20 ± 1 °C using an automatic polarimeter (Kernchen, Germany). Ultraviolet and visible (UV–Vis) absorption spectra were recorded with a Varian Cary100 spectrophotometer. The Fourier transform infrared (FT-IR) spectra (KBr pellets) were recorded on a Nicolet 470 FT-IR spectrophotometer. Elemental analysis was conducted on an Elementar Vario EL III instrument. Total carbohydrate content was determined as D-glucose equivalents using the phenol–sulfuric acid method (Dubois et al., 1956). Uronic acid content was determined according to an m-hydroxydiphenyl colorimetric method in which neutral sugars do not interfere (Filisetti-Cozzi and Carpita, 1991). Protein was analyzed using the Bradford method (1976).

2.4. HPSEC–MALLS analysis

The homogeneity and molecular weight of EPS were determined as described by Liu et al. (2008) on a Waters High-Performance Size-Exclusion Chromatography (HPSEC) apparatus equipped with Two TSK columns, G3000 PWXL and G4000 PWXL in series, coupled to a refractive index detector (RID), and a Wyatt Technology Dawn-EOS Multi-Angle Laser Light Scattering detector (MALLS). The carrier solution was 0.2 M $NaNO_3$, and the samples were dissolved in 0.2 M $NaNO_3$ with stirring. The carrier and sample solutions were rendered dust-free by passing through a 0.22 μm Millipore filter, and degassed before use. The injection volume was 200 μl and the flow rate was 0.6 ml/min. The normalization of RID was conducted with bovine albumin monomer. The specific RI increment (dn/dc) at 658 nm and 25 °C was determined using an interferometric refractometer (Optilab/DSP, Wyatt Technology, USA). The dn/dc value was averaged to 0.135 ml/g and was assumed to be constant during the sample elution. ASTRA software (http://www.wyatt.com/) was utilized for data acquisition and analysis.

2.5. Monosaccharide analysis

The polysaccharide sample was hydrolyzed with 2 M CF_3COOH at 110 °C for 2 h in an oven. The resulting hydrolysates were trimethylsilylated (1:1:5 hexamethyldisilazane–trimethylchlorosilane–pyridine; 30 min, room temperature) and quantitatively analyzed by gas–liquid chromatography (GLC) as described by Sánchez-Medina et al. (2007). The absolute configurations of the monosaccharide were investigated according to the method described by Gerwig et al. (1978), which uses (+)-2-butanol.

2.6. Methylation analysis

The EPS were methylated three times by the Needs and Selvendran (1993) method. The pre-methylated products were depolymerized with 90% HCOOH at 100 °C for 6 h and further hydrolyzed with 2 M CF_3COOH at 110 °C for 3 h. The partially methylated residues were reduced and acetylated. The resulting products were analyzed by gas–liquid chromatography mass spectrometry (GLC–MS). The GLC temperature program was isothermal at 150 °C, followed by a 3 °C/min gradient up to 220 °C and 30 °C/min up to 280 °C. Methylated alditol acetates were identified by their fragment ions in GLC–MS and by relative retention time on GLC while the molar ratios were estimated from the peak areas and the response factors.

2.7. Partial acid hydrolysis with trifluoroacetic acid (TFA)

The purified EPS (100 mg) was partially hydrolyzed with a solution adjusted to pH 2.0 (20 ml) using aqueous TFA at 100 °C for 18 h. After TFA was removed by evaporation *in vacuo*, the hydrolysate was fractioned on a Superdex 30 column (1.6 × 80 cm) and eluted with 0.15 M NaCl at a flow rate of 0.5 ml/min. All the fractions were collected and subjected to monosaccharide analysis and methylation analysis as mentioned above (Sun et al., 2009).

2.8. Nuclear Magnetic Resonance (NMR) spectroscopy

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and dissolved in 99.96% D_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX Avance 600 MHz spectrometer (operating frequencies 600.13 MHz for ^1H NMR and 150.92 MHz for ^{13}C NMR) at 27 °C. Chemical shifts were reported relative to internal 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) at δ H 0.00 ppm for ^1H spectra and δ 21.777 ppm for ^{13}C spectrum. Standard homo- and heteronuclear correlated two-dimensional (2D) techniques were used for general assignments of EPS: correlation spectroscopy (COSY), nuclear overhauser effect spectroscopy (NOESY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC) (Liu et al., 2007). The total correlation spectroscopy (TOCSY) was recorded at mixing time of 150 ms. Complete assignment required several