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Structural Characterization and Anticancer Activity of Cell-Bound Exopolysaccharide from *Lactobacillus helveticus* MB2-1

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Supporting Information

ABSTRACT: A novel cell-bound exopolysaccharide (c-EPS) was isolated from *Lactobacillus helveticus* MB2-1 by ultrasonic extraction, anion exchange, and gel filtration chromatography before being structurally characterized. The c-EPS is a heteropolysaccharide with an average molecular weight of 1.83×10^5 Da and is composed of glucose, mannose, galactose, rhamnose, and arabinose at a molar ratio of 3.12:1.01:1.00:0.18:0.16. Methylation analysis and nuclear magnetic resonance analysis revealed that the c-EPS is a linear glucomannogalactan containing repeating units of $\rightarrow 6)$ - β -D-Manp-(1 \rightarrow 3)- β -D-Glcp-(1 \rightarrow 3)- β -D-Glcp-(1 \rightarrow 3)- β -D-Glcp-(1 \rightarrow 4)- α -D-Galp-(1 \rightarrow 4)-Arap residues. Complex formation with Congo red demonstrated a triple-strand helical conformation for the c-EPS. Scanning electron microscopy of the c-EPS revealed many regular feather-like structural units. Topographical examination of c-EPS by atomic force microscopy revealed that the c-EPS formed rounded-to-spherical lumps with different sizes and chain formations. Furthermore, preliminary in vitro tests revealed that c-EPS significantly inhibited the proliferation of HepG-2, BGC-823, and especially HT-29 cancer cells.

KEYWORDS: *Lactobacillus helveticus* MB2-1, cell-bound exopolysaccharide (c-EPS), structural characterization, anticancer activity

INTRODUCTION

For hundreds of years, lactic acid bacteria (LAB) have been an important part of a nutritious diet and a source of medicine. Extensive studies have revealed that many species of LAB have promise for improving human health and preventing disease.^{1,2} Exopolysaccharide (EPS), one of the most important biomacromolecules isolated from LAB, is reported to have unique physical and rheological properties that facilitate its application in the dairy industry as a thickener, emulsifying agent, and texturizer.^{3–5} Additionally, there has been increasing interest in exploiting EPS-producing LAB for their numerous biological activities, such as their antioxidant, anticancer, probiotic, immunomodulatory, and cholesterol-lowering activities.^{6–10} Among these, the anticancer activity of EPS has received particularly intense interest due to the high mortality of an increasing number of patients suffering from cancer. Though the anticancer agents currently used in chemotherapy practice possess strong activity, considerable doubt has been raised about their safety and side effects, which include nausea, vomiting, and fatigue and hence disturb the quality of patients' lives.^{11,12} Therefore, seeking new alternative strategies for the prevention and treatment of cancer is essential. EPS from safe, natural sources such as LAB may serve as a good substitute for synthetic anticancer agents.

EPS is a general term that refers to two types of secreted polysaccharides. The first type of EPS is attached to the bacterial surface as cell-bound exopolysaccharide (c-EPS), whereas the other is released into the surrounding medium as released exopolysaccharide (r-EPS).¹³ Most EPS-producing LAB strains reported to date produce r-EPS, but some LAB

strains can simultaneously produce both c-EPS and r-EPS. The EPS produced by LAB can also be subdivided into two groups, homopolysaccharides (HoPSs) and heteropolysaccharides (HePSs).¹⁴ HoPSs are composed of one type of monosaccharide (e.g., glucose or galactose), whereas several different types of monosaccharides constitute HePSs. HePSs have great variability in their structures. The EPS backbones of LAB have repeated units composed of seven monosaccharides, where glucose, galactose, rhamnose, and mannose are the principal sugar residues. HePSs are often linear or branched with different types of linkages. Their structures are also complex, and they are named depending on their principal monosaccharides; for example, galactoglucan or glucogalactan differ in their ratios of each sugar, as do galactoglucomannan or glucomannogalactan. The structural properties of EPS molecules determine their technological applications and biological activities.^{15–17} Therefore, elucidation of the chemical composition and structure of a novel EPS is relevant for predicting its potential applications.

The r-EPSs from LAB have recently become attractive as food additives and as sources for the development of novel drugs. Several r-EPSs from LAB exhibit strong antioxidant and anticancer effects that are relevant to their health-protecting functions.^{18–21} Several studies have emphasized the potential role of LAB in probiotic mechanisms that are mediated by the

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c-EPS from their cell wall.²² However, there have been few studies focusing on the anticancer activity and chemical characterization of c-EPS produced by LAB.²³ Recently, the *Lactobacillus helveticus* MB2-1 strain, which produces abundant EPS, was isolated in our laboratory from traditional Sayram rropy fermented milk (SRFM) in the southern Xinjiang region of China.² In vitro antioxidant activity assays demonstrated that the c-EPS and three r-EPS fractions (r-LHEPS-1, r-LHEPS-2, and r-LHEPS-3) had strong scavenging activities against four free radicals.^{24,25} In addition, we found that all of the r-EPS fractions, especially r-LHEPS-2, exerted high inhibitory activity in vitro against BGC-823 human gastric cancer cells. Furthermore, we elucidated the fine structure of purified r-LHEPS-2, which showed a →4,6-D-Manp-(1 → 6)-D-Manp-(1 → 4)-D-Galp-(1 → backbone with a single T-D-Glcp-(1 → 4)-D-Manp-(1 → 6)-D-Glcp-(1 → side-branch on the O-6 of the → 4,6-D-Manp-(1 → residue.²⁶

In the present work, the c-EPS from *L. helveticus* MB2-1 was prepared and characterized by gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), nuclear magnetic resonance (NMR), atomic force microscopy (AFM), and scanning electron microscopy (SEM). Furthermore, the in vitro anticancer activity of c-EPS against human HepG-2 liver cancer, BGC-823 gastric cancer, and HT-29 colon cancer cells was evaluated.

MATERIALS AND METHODS

Microorganism and Reagents. The *L. helveticus* MB2-1 strain was isolated from SRFM. This strain was grown in liquid medium (1000 mL) containing 80 g of whey and 5 g of yeast extract and adjusted to pH 6.7 with 2.0 M NaOH. HepG-2, BGC-823, and HT-29 cancer cells and human colonic epithelial HCoEpiC cells were obtained from the Cell Bank of the Shanghai Institute of Cell Biology (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were purchased from Gibco/Invitrogen (Gibco BRL, Grand Island, NY). Dimethyl sulfoxide (DMSO), methyl iodide (CH_3I), 5-fluorouracil (5-FU), penicillin, streptomycin, rhamnose, arabinose, mannose, fucose, glucose, galactose, xylose, and inositol were obtained from Sigma Chemical Co. (St. Louis, MO). DEAE-cellulose 52 and Sephadex G-100 were purchased from Whatman Co. (Maidstone, Kent, UK), and T-series dextrans were purchased Pharmacia Co. Ltd. (Uppsala, Sweden). All other reagents were of analytical grade.

Observation of *L. helveticus* MB2-1 Capsules. The presence of capsules on the cell surface of *L. helveticus* MB2-1 was confirmed by observation by transmission electron microscopy (TEM) using an H-7650 TEM (Hitachi High Technologies Corporation, Tokyo, Japan) operating at 80 kV. The sample was prepared by mounting the cells onto a copper grid, immersing the samples in 2% phosphotungstic acid (pH 7.0) and drying at room temperature.

Isolation and Purification of c-EPS. The c-EPS was isolated and purified according to our previous method, with minor modifications.²³ After growth at 40 °C for 24 h, the cultures were centrifuged at 12 000g and 4 °C for 15 min. The viscous cell precipitates were washed twice with 0.85% NaCl and then centrifuged as above. The centrifuged precipitates were resuspended in 1 M NaCl and treated with a JV92-IIIN cell sonicator (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) at 40 W and 4 °C for 3 min. After centrifugation to eliminate insoluble material, the supernatants were combined and then concentrated using a Laborota 4000 efficient rotary evaporator (Heidolph Instruments GmbH & Co, Schwabach, Germany). The final concentrated supernatant was mixed with three volumes of ice-cold ethanol and stirred vigorously. After standing overnight at 4 °C, the precipitate was collected by centrifugation and dialyzed using a dialysis bag (8000–14 000-Da molecular weight (MW) cutoff) in distilled water for 48 h at 4 °C before being lyophilized using a Heto

Power Dry LL3000 freeze-dryer (Thermo Electron Co., Bath, UK). The freeze-dried crude c-EPS was fractionated with a DEAE-cellulose column (2.6 × 30 cm) and was eluted stepwise with distilled water and 0.1, 0.3, and 0.5 M NaCl solutions at a flow rate of 60 mL/h. The fractions were assayed for carbohydrate content by the phenol–sulfuric acid method.²⁷ The peak fractions containing c-EPS were pooled, dialyzed, and lyophilized. Further purification of c-EPS was performed using a Sephadex G-100 column (2.6 × 100 cm), which was eluted with distilled water at a flow rate of 12 mL/h. The fraction containing purified c-EPS was collected, dialyzed, and lyophilized.

Purity and Molecular Weight Estimation of c-EPS. The purity and MW of the c-EPS were determined by high-performance size-exclusion chromatography (HPSEC) with an Agilent 1200 series system (Palo Alto, CA) equipped with a TSK gel G4000 PW XL column (300 × 7.8 mm, Tosoh Corp., Tokyo, Japan) and an evaporative light-scattering detector (ELSD).²⁵ Twenty microliters of purified c-EPS (1 mg/mL) was injected and was eluted with distilled water at 50 °C at a flow rate of 0.7 mL/min. The linear regression was calibrated using the T-series dextran standards (T-500, T-200, T-100, T-50, and T-10).

Basic Component Analysis of c-EPS. The total sugar content was measured by the phenol–sulfuric acid method mentioned above.²⁷ Protein, uronic acid, and sulfate group quantities were determined according to our previous method.²⁵

Analysis of Monosaccharide Composition. Five milligrams of purified c-EPS was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h.⁵ The hydrolysates were then subjected to aldononitrile acetate precolumn derivatization for GC determination of the monosaccharide composition. GC was performed on an Agilent 6890N GC (Palo Alto, CA) equipped with a flame ionization detector (FID) and a HP-5 capillary column (30 m × 0.32 mm i.d., 0.25 mm, J&W Scientific Inc., Folsom, CA). N₂ was used as the carrier gas at a flow rate of 1 mL/min, and the temperatures of the injector and detector were set to 250 °C and 280 °C, respectively. The following chromatographic conditions were used: the initial column temperature was held at 100 °C for 5 min, increased at a rate of 5 °C/min to 150 °C, held at 150 °C for 5 min, and then subsequently increased at 5 °C/min to 240 °C, where it was held at 240 °C for 2 min. Arabinose, mannose, rhamnose, fucose, glucose, galactose, and xylose standards were prepared for comparison, and inositol was used as an internal standard.

Ultraviolet–Visible (UV–vis) and Fourier-Transform Infrared (FT-IR) Spectroscopy. UV–vis spectroscopy analyses were conducted with a U-4100 spectrophotometer (Hitachi Ltd, Japan). The c-EPS solution was prepared by suspending the sample in distilled water at a concentration of 1.0 mg/mL for UV–vis measurement over a wavelength range of 190–700 nm. FT-IR was recorded from a sample in a KBr pellet on a FT-IR spectrophotometer (Nicolet Nexus470, Thermo Nicolet Co., WI), and the spectrum from 400 to 4000 cm^{-1} was recorded.²⁵

Methylation Analysis and Mass Spectrometry. Five milligrams of purified c-EPS was completely methylated using methyl iodide and solid sodium hydroxide in dimethyl sulfoxide, as described in our previous report.²³ Briefly, the permethylated c-EPS was hydrolyzed with 2 M TFA at 120 °C for 2 h. Next, the partially methylated monosaccharides were reduced with NaBH₄ for 2 h at room temperature and acetylated with pyridine–acetic anhydride (1:1, v/v) at 100 °C for 1 h. The resulting methylated alditol acetate derivatives were analyzed by GC–MS. GC–MS analysis was performed on an Agilent 5975MSD-6890 GC–MS (Agilent Technologies, Santa Clara, CA) equipped with a HP-5 capillary column and using He as the carrier gas. The initial oven temperature was 100 °C, which was then increased by 6 °C/min to 250 °C, where it was held for 5 min.

Nuclear Magnetic Resonance Spectroscopy Analysis. The NMR spectrum of the c-EPS solution was recorded with D₂O as the solvent using a Bruker AVANCE AV-500 spectrometer (Bruker Group, Fällanden, Switzerland) operating at 500 MHz.²⁸ The residual solvent signal was used as an internal standard. Both one-dimensional (1D) ¹H and ¹³C NMR experiments were performed, and the

operating temperature was 323 K. The delay (D_1) and acquisition (AQ) times were 4.00 and 2.92 s for the ^1H NMR spectra and 1.08 and 2.00 s for ^{13}C NMR, respectively. The chemical shift (δ) values are given in parts per million (ppm), and coupling constants (J) are given in hertz. Two-dimensional (2D) ^1H – ^1H correlated spectroscopy (COSY), ^1H – ^{13}C heteronuclear single quantum coherence (HSQC), ^1H – ^{13}C heteronuclear multiple quantum coherence (HMBC), and nuclear Overhauser effect spectroscopy (NOESY) measurements were used to assign signals and to determine the sequence of sugar residues.

Colorimetric Triple Helix Determination with Congo Red.

The conformational structure of the purified c-EPS was established by helix–coil transition analysis according to Villares's procedure.²⁹ Briefly, the purified c-EPS was dissolved at 1 mg/mL in various NaOH solutions (0.05–0.4 M), and these samples were added to a Congo red solution (40 μM). Spectra were recorded at room temperature (25 °C) with a UV-vis spectrophotometer (U-4100, Hitachi Ltd., Japan).

Atomic Force Microscopy. A c-EPS solution (1 mg/mL) was prepared by adding purified c-EPS to distilled H_2O . The aqueous solution was stirred at 50 °C for approximately 1 h in a sealed bottle under N_2 until the c-EPS dissolved completely. After cooling to room temperature, the solution was diluted to a final concentration of 0.01 mg/mL. Approximately 5 μL of the diluted c-EPS solution was dropped onto the surface of a mica sample carrier and allowed to dry at room temperature. Later, the AFM image was collected using a Bruker Dimension Icon AFM (Bruker Biosciences, Billerica, MA) equipped with Bruker ScanAsyst-Air PeakForce tapping-mode AFM tips. The scan resolution and rate were 512 \times 512 and 1 Hz, respectively.

Scanning Electron Microscopy. The morphology of the purified c-EPS was observed using an S-3000 scanning electron microscope (Hitachi Science Systems, Ltd., Hitachinaka, Japan) at an accelerating voltage of 30 kV. For SEM analysis, the sample was mounted on a metal stub and sputtered with gold. Micrographs were recorded at 2000 \times magnification to ensure clear images.

In Vitro Anticancer Activity. The inhibitory effects of purified c-EPS on the HepG-2, BGC-823, and HT-29 cancer cell lines and on human colonic epithelial HCoEpiC cells in vitro were evaluated using the MTT assay on a 96-well plate.²⁰ Briefly, 100 μL of cancer cells (2×10^5 cells/mL) was cultured at 37 °C for 24 h in DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/L) under 5% CO_2 . The cells were then treated with various concentrations of purified c-EPS (0, 50, 100, 200, 400, and 600 $\mu\text{g}/\text{mL}$) and 5-FU (50 $\mu\text{g}/\text{mL}$) for 24, 48, and 72 h. At the end of each treatment, 10 μL of MTT (5 mg/mL) was added, and the cells were incubated for another 4 h. The liquid was then removed, and 100 μL of DMSO was added to the well. After dissolving the formazan crystals that formed, the absorbance was measured in a Synergy-2 microplate reader (BioTek Instruments, Inc., Burlington, VT) at 570 nm. The inhibitory rate was expressed as follows:

$$\text{inhibitory rate (\%)} = [1 - (A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}})] \times 100$$

where A_{control} and A_{blank} are the absorbance values of the system without the addition of c-EPS and without cells, respectively.

Statistical Analysis. The obtained results were analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL). All data were analyzed by one-way analysis of variance (ANOVA). Significant differences were determined by Tukey's honest significant difference (Turkey's HSD) post hoc test. Half-maximal (50%) inhibitory concentration (IC_{50}) values were calculated by probit regression analysis. Statistical significance was set to $p < 0.05$. In all cases, there were three replicates ($n = 3$).

RESULTS AND DISCUSSION

Visualization of Capsules. When cells of *L. helveticus* MB2-1 were stained using a negative dyeing method and examined with an optical microscope under an oil-immersion lens, the capsules appeared as unstained, colorless halos

surrounding the *L. helveticus* MB2-1 cells against a light violet-gray background, while the bacterial cells stained distinctly violet (Figure 1a). Further visualization by TEM of

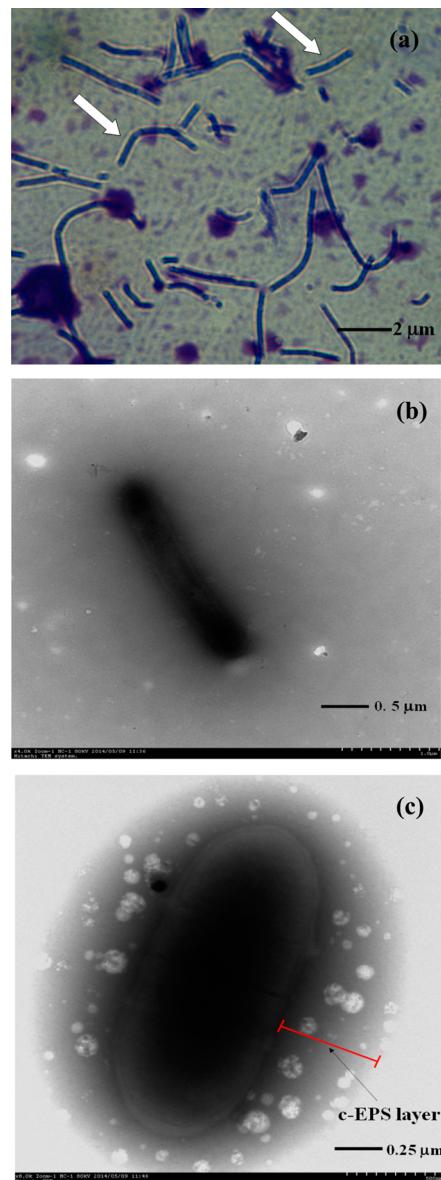


Figure 1. Micrographs showing the capsules of *L. helveticus* MB2-1 cells grown in whey medium. The capsules appear as unstained, colorless halos surrounding the bacterial cells when observed by optical microscopy (a) and as fibrillose materials attached to the bacterial surface when observed by TEM (b and c).

the stained cells revealed a clearly visible cell wall with fibrillose materials on the bacterial surface, confirming the formation of capsules by *L. helveticus* MB2-1 (Figures 1b and 1c).

Isolation and Composition Analysis of c-EPS. The crude c-EPS of *L. helveticus* MB2-1 was obtained by ultrasonic extraction; the supernatant fraction was subsequently extracted with cold ethanol and fractionated first on a DEAE-cellulose 52 anion exchange chromatography column (Figure 2a) and then subjected to chromatography on a Sephadex G-100 gel filtration column (Figure 2b) for further purification. The corresponding chromatogram showed only one peak for c-EPS, indicating that the ultrasonically extracted c-EPS may be a

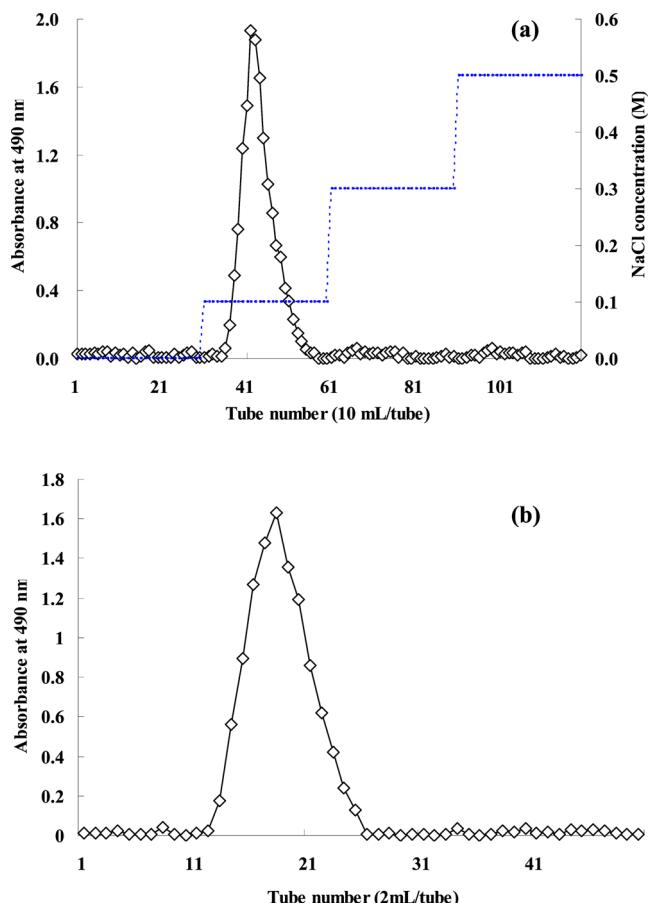


Figure 2. DEAE-cellulose 52 anion exchange chromatogram (a) and Sephadex G-100 gel filtration chromatogram (b) of crude c-EPS.

homogeneous polysaccharide. The recovery rate of c-EPS was 88.75%. Further HPSEC analysis of the purified c-EPS fraction showed a single sharp, symmetrical peak (Figure 3a), confirming that the purified c-EPS was homogeneous. According to the elution curve of the standards, the MW of the purified c-EPS was estimated to be 1.83×10^5 Da, which was less than those of the three r-EPS fractions isolated from *L. helveticus* MB2-1 (approximately 2.00×10^5 Da).²⁵ This MW was also lower than those of c-EPS from *L. pentosus* LPS26 (2.00×10^6 Da),³⁰ *L. plantarum* EP56 (8.50×10^5 Da),³¹ and *L. rhamnosus* JAAS8 (5.50×10^5 Da).¹³ The total carbohydrate content of the c-EPS was 95.45%. The uronic acid and sulfated group contents were 1.48% and 0.89%, respectively (Table 1). The lack of absorption by the purified c-EPS at 280 and 260 nm in the UV-vis spectrum demonstrated the absence of both proteins and nucleic acids in the polysaccharide (Figure 3b). Additionally, the c-EPS showed the same optical rotation, $[\alpha]_D^{20} = -56.8^\circ$ (*c* 0.5, water), in different low concentrations of ethanol using an analytical AUTOPOL IV automatic polarimeter (RUDOLPH Research, Flanders, NJ) at room temperature.

Determination of the monomer composition was conducted after acid hydrolysis (Figure 3c). Quantitative monosaccharide analysis of the purified c-EPS revealed the presence of glucose, mannose, galactose, rhamnose, and arabinose at a molar ratio of 3.12:1.01:1.00:0.18:0.16 (Table 1). Glucose represented the greatest proportion compared to the other monosaccharides, corroborating previous reports for c-EPS or r-EPS from LAB of food origin, where glucose was often found in equal or greater

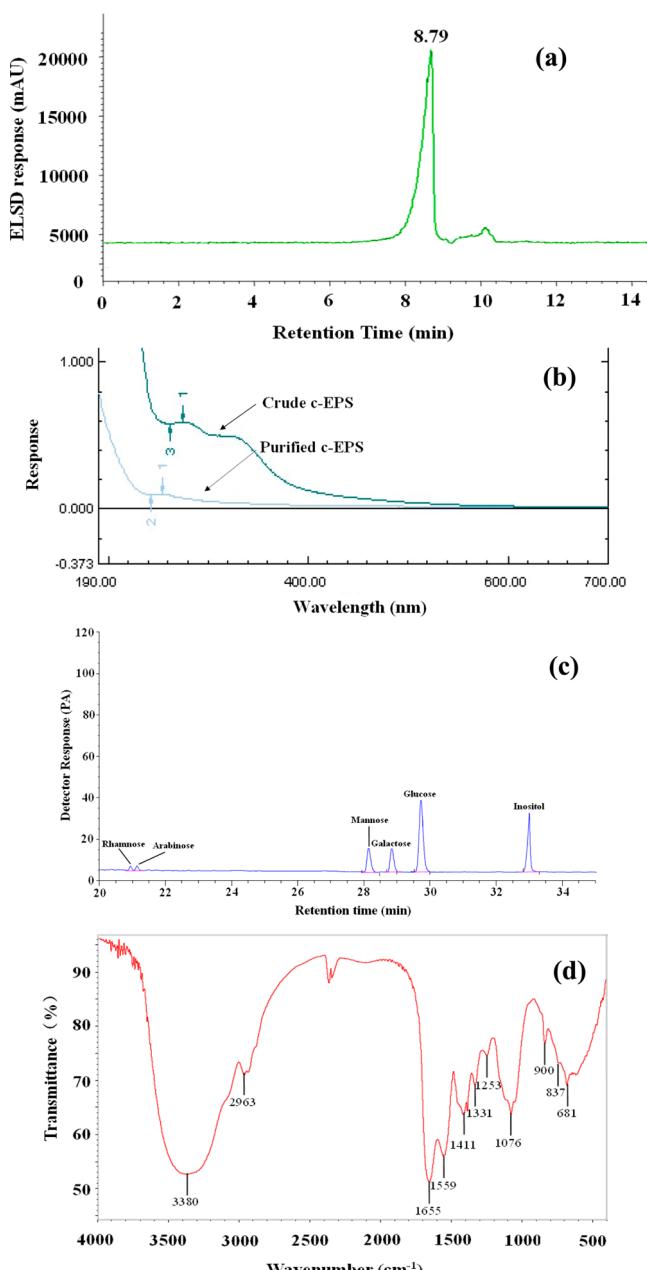


Figure 3. An HPSEC chromatogram of purified c-EPS (a), a UV-vis spectrum of purified c-EPS (b), a GC chromatogram of the monosaccharide composition (c), and a FT-IR spectrum (d) of purified c-EPS.

proportions than other monosaccharides.^{14,15} The presence of different sugar moieties suggested that the polysaccharide was a HePS. A similar result was found by Rodríguez-Carvajala et al.,³⁰ who reported that glucose and rhamnose were the predominant components of c-EPS produced by *L. pentosus* LPS26. Tallon, Bressollier, and Urdaci³¹ reported that the c-EPS from *L. plantarum* EP56 was composed mainly of glucose and galactose. Moreover, *N*-acetylglucosamine, *N*-acetylgalactosamine, and phosphoric acid have also commonly been reported as constituents of EPS produced by LAB.^{13,14} However, this result was different from that obtained by Svensson, Zhang, Huttunen, and Widmalm,³² who reported that the c-EPS from *Leuconostoc mesenteroides* ssp. *cremoris* PIA2 was a HoPS containing repeating units of linear galactose

Table 1. Monosaccharide Composition and the Carbohydrate, Protein, Uronic Acid, and Sulfate Group Contents of the Crude and Purified c-EPSs

sample	carbohydrate (%)	protein (%)	uronic acid (%)	sulfate (%)	sugar component (molar ratio) ^a				
					Gal	Glc	Man	Rha	Ara
crude c-EPS	84.09	6.49	3.46	1.79	1.00	3.36	1.22	0.12	0.15
purified c-EPS	96.23	— ^b	1.48	0.89	1.00	3.12	1.01	0.18	0.16

^aIndividual components were identified and quantified based on the elution of known standards, and the data are presented as the molar ratio for each sugar. ^bNot detected.

residues. Taken together, these results suggest that the monosaccharide constituents and molar ratios of c-EPS from LAB may be affected by the strain type.

Structural Analysis of Purified c-EPS. To investigate the functional groups of the purified c-EPS, an FT-IR spectrum was recorded over an absorbance range of 4000 to 500 cm⁻¹. As shown in Figure 3d, the spectrum of purified c-EPS displayed an intense, broad band at approximately 3380 cm⁻¹, which was assigned to the hydroxyl stretching vibration. The absorption band at 2963 cm⁻¹ was due to the C-H stretching vibration.³³ The band at approximately 1655 cm⁻¹ was due to bound water.³⁴ Peaks at 1559, 1411, and 1331 cm⁻¹ were characteristic of carboxyl groups or carboxylates, indicating that c-EPS was an acidic polysaccharide. This result was consistent with its uronic acid content, which was quantified using the carbazole-sulfuric acid method using D-glucuronic acid as a standard.²³ The absorption peaks at 1253 and 1076 cm⁻¹ were attributed to the stretching vibrations of the pyranose ring. Moreover, the band at 900 cm⁻¹ indicated the β-pyranose form of the glucosyl residue, and the band at 837 cm⁻¹ suggested the β-pyranose form of the mannose residue.⁵ Finally, a characteristic absorption peak at 681 cm⁻¹ indicated the existence of α-configurations in the purified c-EPS.²⁰

The fully methylated c-EPS was hydrolyzed with TFA, converted into alditol acetates, and analyzed via GC-MS (Table 2). The analysis revealed the presence of five

(1 → 4)-linked galactopyranose, (1 →)-linked rhamnopyranose and (1 → 4)-linked arabinopyranose residues was 3.29:1.21:1.00:0.12:0.09. No branched structures were found in c-EPS, suggesting that c-EPS may be a linear glucomannogalactan. The trace amounts of Rhap-(1 →) and (1 → 4)-Arap linkages are also most likely randomly located within the c-EPS chains.

The signals in the ¹H and ¹³C NMR spectra of the purified c-EPS were assigned based on the component and methylation analyses and on values reported in the literature.^{20,23,26,32} As shown in Figure 4a, three clear chemical shift signals from anomeric protons were found at δ 4.80, 4.50, and 4.37 ppm in the ¹H NMR spectrum. The signals at δ 3.40–4.20 represent the signal peak of the remaining proton, which is formed of multiple overlapping signal peaks. The signal at δ 4.71 was from the hydrogen of D₂O. This spectrum indicated that the purified c-EPS contained three residues (named A, B, and C). Based on their chemical shifts (δ) and the values of the coupling constant (J) for the anomeric signals in the ¹H NMR spectrum, residue A was determined to have an α-configuration (the J_{1,2} values were 3–4 Hz), while residues B and C had β-configurations (the J_{1,2} values were 7–8 Hz). The molar ratio of A, B, and C was approximately 1.03:1.00:3.15 when estimated using the ratio of the peak areas from the integration of the H-1 signals for residues A, B, and C. Based on the results of the aforementioned methylation analysis, the signal at δ 4.80 ppm corresponded to 2,3,6-Me₃Galp, and the signals at 4.50 and 4.37 ppm corresponded to 2,3,4-Me₃Manp and 2,4,6-Me₃GlcP, respectively. The ¹³C NMR spectrum (Figure 4b) showed that the C-1 signals of the three residues were detectable at δ 105.96, 105.83, and 102.28 ppm, respectively. The ¹³C NMR chemical shifts in the anomeric carbon region also suggested three types of linkages for the galactose, glucose, and mannose residues.

Complete assignments for the ¹H and ¹³C chemical shifts of c-EPS are shown in Supporting Information and were completed using 2D ¹H-¹H COSY, ¹H-¹³C HSQC, ¹H-¹³C HMBC, and NOESY experiments based on methylation analysis and data from the literature.^{3,4,26,35} The single-bond correlations between the protons and the corresponding carbons obtained from the HSQC spectrum (Figure S1, Supporting Information) of c-EPS in D₂O enabled all of the ¹³C NMR signals to be assigned. The signal at δ105.96 ppm (C) was attributed to the C-1 of →3)-β-D-GlcP-(1 → , δ105.83 ppm (B) to the C-1 of →6)-β-D-Manp-(1 → , and δ102.28 ppm (A) to the C-1 of →4)-α-D-Galp-(1 → . These signals cross-correlate to the proton signals at δ4.37, 4.50, and 4.80, respectively. The results from the COSY spectrum (Figure S2, Supporting Information), based on stepwise magnetization transfers from the anomeric protons, aided in the assignment of the chemical shifts of other protons. The linkage of the residues was obtained from the HMBC spectrum (data not shown). The sequence of the glycosyl residues of c-EPS was also confirmed

Table 2. Methylation Analysis of Purified c-EPS from *L. helveticus* MB2-1

retention time (min)	methylated sugar	primary mass fragments (<i>m/z</i>)	molar ratio	deduced linkage
14.024	2,3,6-Me ₃ Arap	43.1, 45, 71, 87, 101.1, 117, 128.9, 161.1	0.09	(1→4)-D-Arap
14.835	2,3,4,6-Me ₄ Rhap	43.1, 45, 72, 89, 101.1, 115, 131, 161.1	0.12	T-D-Rhap-(1→
15.457	2,4,6-Me ₃ GlcP	43.1, 71.1, 101.1, 128.9, 161.1, 205.1, 244.9, 280.9, 341	3.29	(1→3)-D-GlcP
15.813	2,3,4-Me ₃ Manp	43.1, 65, 85, 117, 139, 159.1, 201.1, 231, 261.1, 281.9	1.21	(1→6)-D-Manp
18.052	2,3,6-Me ₃ Galp	43.1, 71, 101.1, 125, 145.1, 173.1, 205.1, 245.2, 281.1	1.00	(1→4)-D-Galp

components. The c-EPS showed three major peaks of 2,4,6-Me₃GlcP, 2,3,4-Me₃Manp, and 2,3,6-Me₃Galp, which originated from (1 → 3)-linked glucopyranose, (1 → 6)-linked mannopyranose, and (1 → 4)-linked galactopyranose residues, respectively. Trace amounts of 2,3,4,6-Me₄Rhap and 2,3,6-Me₃Arap were also detected, indicating the presence of D-Rhap-(1 →)-linked rhamnopyranose and (1 → 4)-linked arabinopyranose residues, respectively. The molar ratio of the (1 → 3)-linked glucopyranose, (1 → 6)-linked mannopyranose,

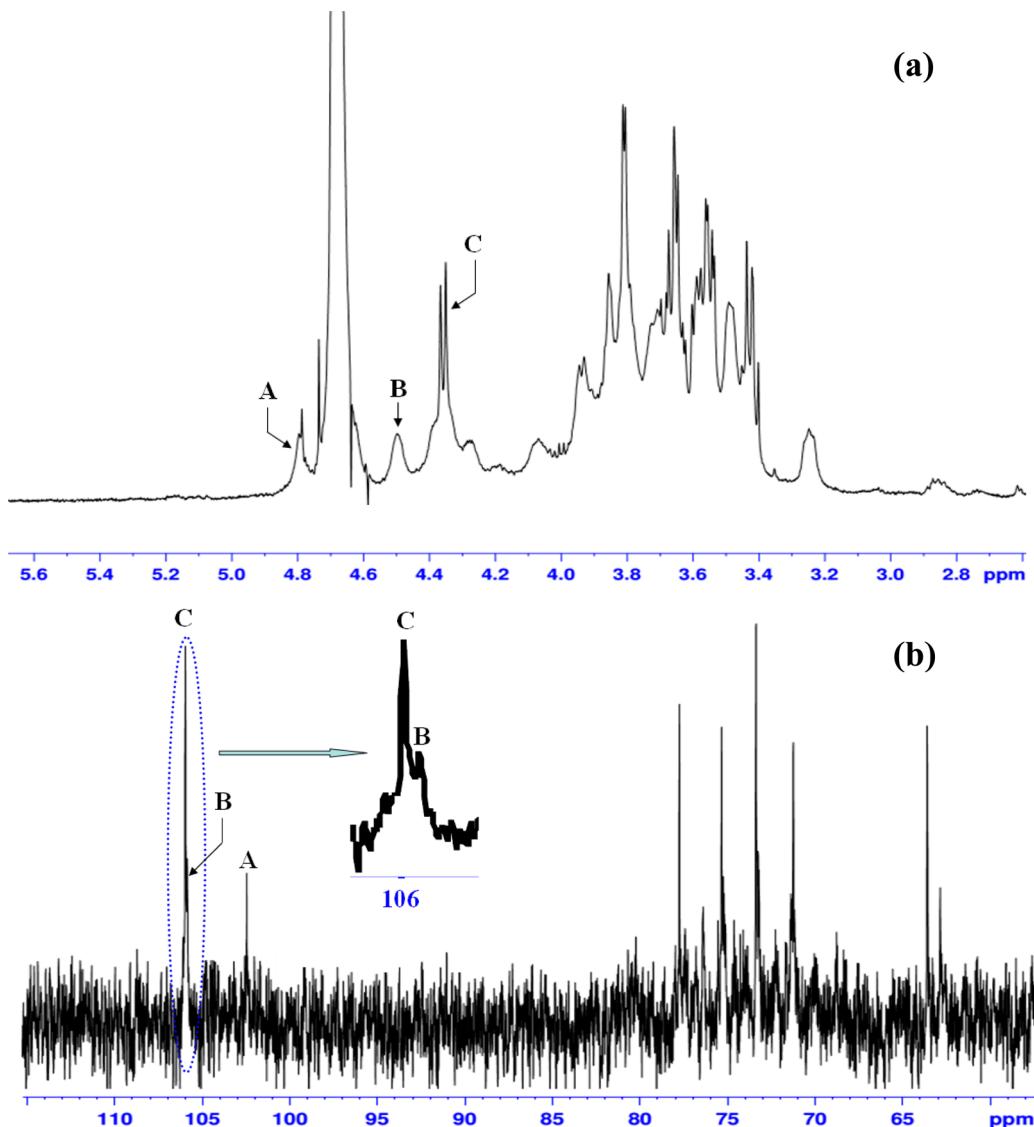


Figure 4. 500-MHz 1D ^1H (a) and ^{13}C (b) NMR spectra of purified c-EPS from *L. helveticus* MB2-1. Anomeric protons and carbon atoms are labeled with A, B, and C according to their decreased and increased chemical shifts, respectively.

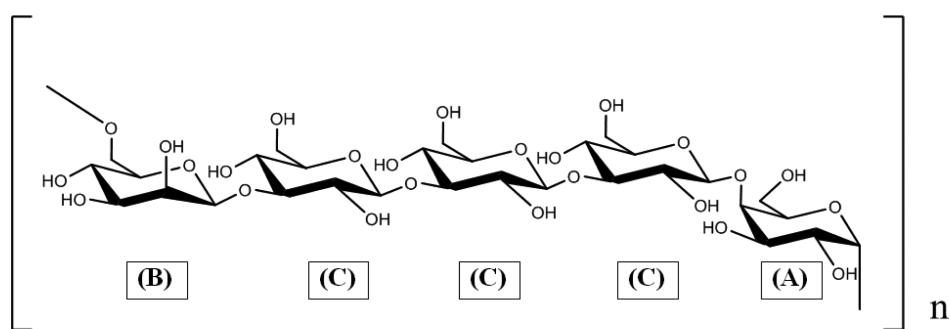


Figure 5. Proposed structure of purified c-EPS from *L. helveticus* MB2-1.

by the NOESY experiment. Inspection of the NOESY spectrum (Figure S3, Supporting Information) showed that the A H-1 to B H-6, B H-1 to C H-6, C H-1 to C H-3, and C H-1 to A H-4 were in agreement with the A-(1 → 6)-B, B-(1 → 3)-C, C-(1 → 3)-C, and C-(1 → 4)-A linkages, respectively. These results suggest that c-EPS is a linear glucomannogalactan containing repeating units of →6)- β -D-Manp-(1 → 3)- β -D-Glcp-(1 → 3)-

β -D-Glcp-(1 → 3)- β -D-Glcp-(1 → 4)- α -D-Galp-(1 → . In summary, the predicted repeating unit of c-EPS was determined (Figure 5), and the value of “n” for the number of repeating structural units in c-EPS was approximately 231.

Conformational and Morphological Characterization.

Polysaccharides are considered to exist in an ordered three-dimensional structure, generally a triple-stranded helical

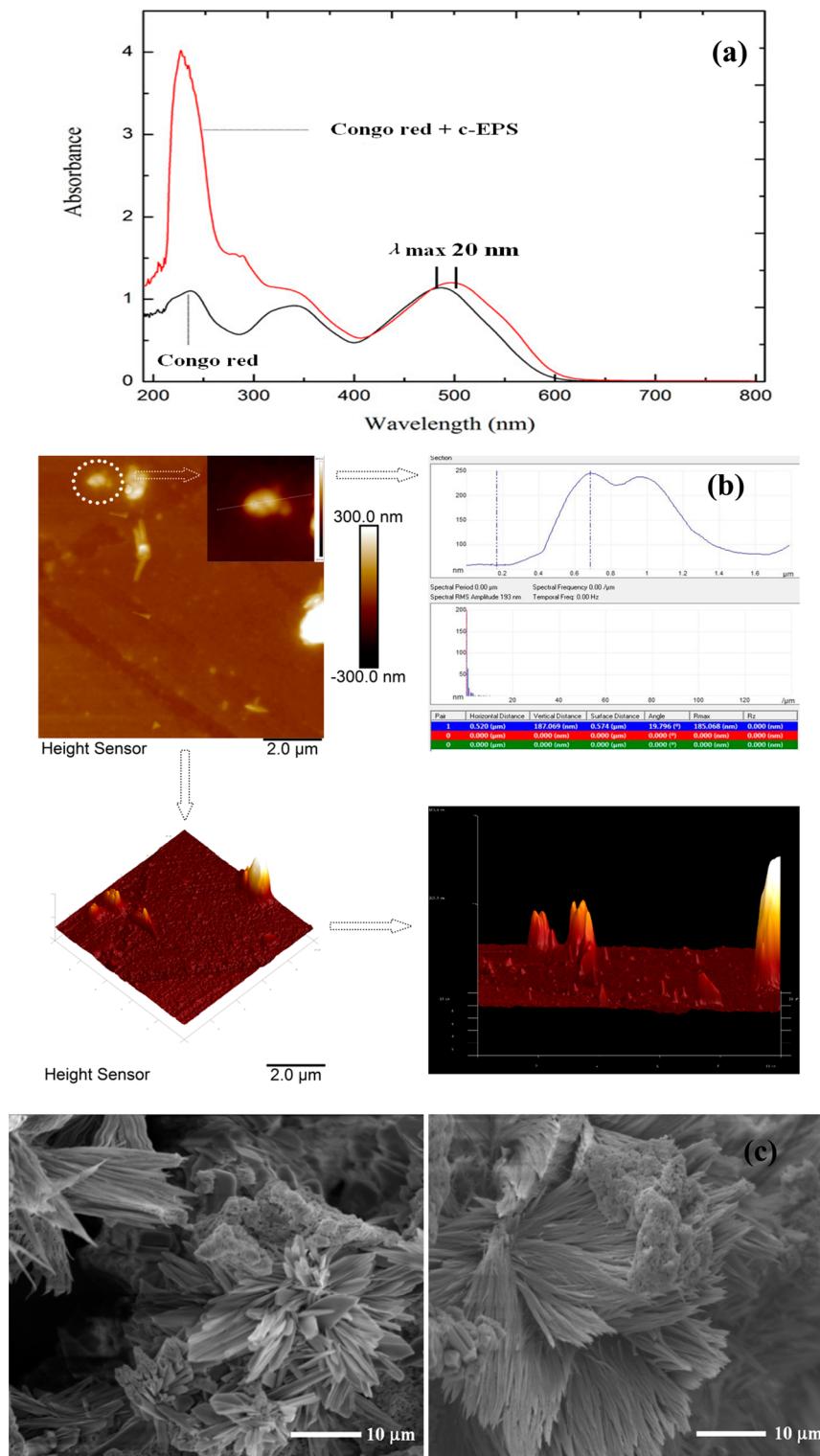


Figure 6. Absorption spectra of Congo red (control) and of Congo red with purified c-EPS (a), AFM (b), and SEM (c) images of purified c-EPS.

conformation, and they form a complex with Congo red in dilute NaOH solutions (less than 1 M). The complexation of polysaccharides with Congo red is denoted by a bathochromic shift in the visible absorption maxima (λ_{max}) of the Congo red spectrum. Hence, a polysaccharide displaying a triple-stranded helical conformation exhibits a shift in the UV absorption spectrum, whereas no shift should be observed for other conformations. Purified c-EPS from *L. helveticus* MB2-1 was

complexed with Congo red at different NaOH concentrations. Laminarin and dextran were used as triple-stranded helical and random-coil controls (data not shown), respectively, and Congo red in NaOH was evaluated as the negative control. The absorption spectra of Congo red in NaOH and the c-EPS complexes with Congo red are shown in Figure 6a. The c-EPS solution exhibited a significant bathochromic shift of approximately 20 nm in the λ_{max} between 0.05 and 0.4 M

NaOH. These results suggested that purified c-EPS formed a complex with Congo red, which could be ascribed to its arrangement in a triple-stranded helical conformation.

Recently, EPS has been extensively studied by AFM, which is a powerful tool to observe the conformation of individual macromolecules and the molecular structure and dynamics of EPS.^{36–38} Topographical AFM images of c-EPS are shown in Figure 6b. The c-EPS deposited from a 10 µg/mL aqueous solution exhibited rounded-to-spherical lumps with different sizes, and the formation of chains was also visible. However, the tight packing of the molecules suggested that they had a strong affinity for water molecules and had pseudoplastic behavior. Our findings for c-EPS showed rounded lumps with a maximum lump height of 250 nm. SEM is also a very powerful tool to study the three-dimensional and surface morphology of biomacromolecules, and it aids in understanding their common physical properties.³⁹ Micrographs of the microstructure and surface morphology of purified c-EPS are shown in Figure 6c. The c-EPS predominantly appeared as regular, feather-like structural units. This result differed from the work of Yadav, Prappulla, Jha, and Poonia,⁴⁰ who reported that EPS from *L. fermentum* CFR 2195 exhibited flake-like structural units and was highly compact. The different shape and structure, or surface topography, of the polysaccharides was most likely caused by differences in the physicochemical properties of the EPS and by differences in sample extraction, purification, or preparation.⁴¹

In Vitro Anticancer Activity of Purified c-EPS. The inhibitory effects of purified c-EPS from *L. helveticus* MB2-1 on three cancer cell lines and on human colonic epithelial HCoEpiC cells were measured using an MTT assay after the cells were treated with increasing concentrations (0, 50, 100, 200, 400, and 600 µg/mL) of c-EPS or with the positive control (5-FU, 50 µg/mL) for 24, 48, and 72 h. As shown in Figure 7a and 7b, the inhibitory effects of purified c-EPS on HepG-2 and BGC-823 cells significantly increased with increasing concentration and treatment time ($p < 0.05$). At the lowest concentration (50 µg/mL) and with an incubation period of 24 h, the inhibition rates of c-EPS against HepG-2 and BGC-823 cells were $9.03 \pm 0.81\%$ and $8.85 \pm 0.78\%$, respectively. At the highest tested concentration (600 µg/mL), the inhibitory effects of c-EPS increased to $40.98 \pm 0.62\%$ and $38.75 \pm 0.74\%$ for HepG-2 and BGC-823, respectively. However, the inhibitory effects of c-EPS on HepG-2 and BGC-823 cells were all lower than that of the positive control (5-FU) at the same time points and concentrations. The inhibitory effect of c-EPS on HT-29 cells was also demonstrated to be dose- and time-dependent (Figure 7c). The inhibition rate against the HT-29 cells was significantly higher than against the other two cell lines at all c-EPS concentrations; for HT29 cells, the highest inhibition rates of c-EPS were comparable to those of 5-FU ($80.05 \pm 3.04\%$) after 72 h. Moreover, the strong inhibition of the HT-29 cells by c-EPS was evidenced by its IC₅₀ value (250 µg/mL), which was much lower than those for the HepG-2 (1,520 µg/mL) and BGC-823 (2,878 µg/mL) cells. The anticancer activity of c-EPS against the HT-29 cells was also higher than that observed when the cells were treated with two r-EPSs that were isolated from the culture supernatant of *L. plantarum* 70810 at the same concentration and time.²⁰ These results indicated that c-EPS from *L. helveticus* MB2-1 has moderate anticancer activities against HepG-2 and BGC-823 cells and significant inhibitory effects on HT-29 cells. However, the level of c-EPS-mediated cytotoxicity in human colonic

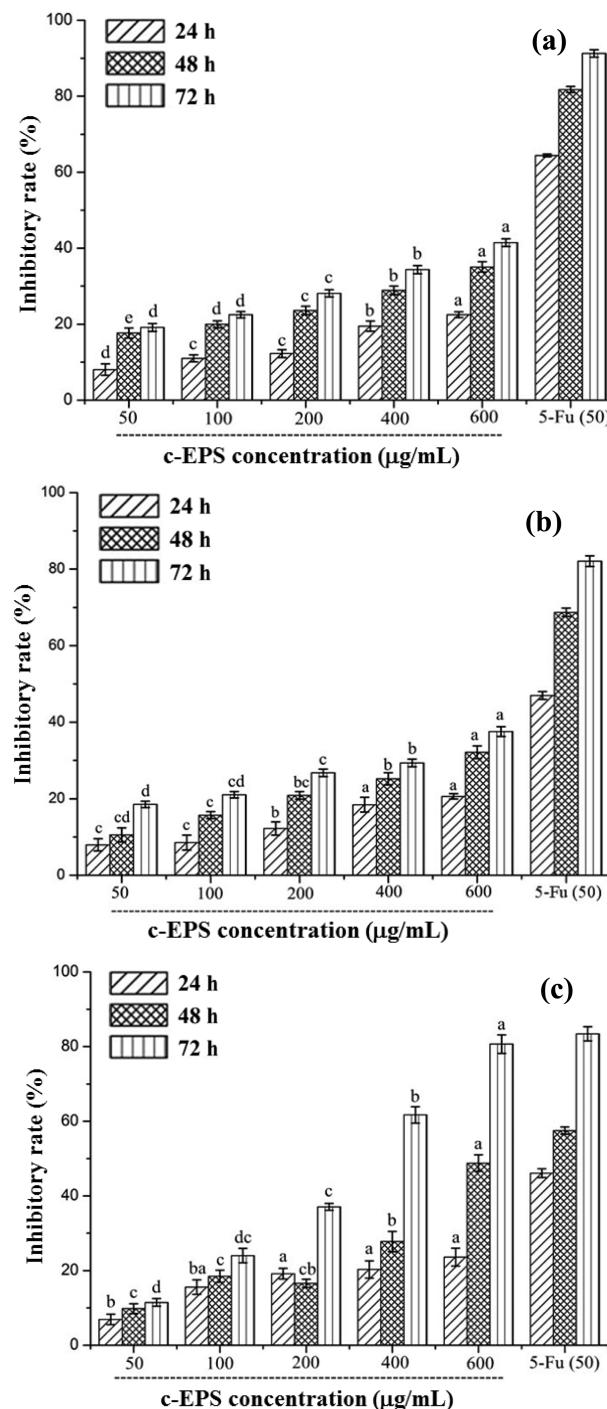


Figure 7. Anticancer effects of purified c-EPS on HepG-2 (a), BGC-823 (b), and HT-29 (c) cells. All values are expressed as the mean \pm the standard deviation (SD) of three replicates. Different alphabets (a–d) in superscript denote significant difference ($p < 0.05$) and are determined by Turkey's HSD using SPSS (version 16.0).

epithelial HCoEpiC cells was not dramatic. Even at the highest dose (600 µg/mL), the level of cytotoxicity induced by a 72-h incubation of normal colonic cells with c-EPS was only $3.50 \pm 0.89\%$ (data not shown). Therefore, c-EPS possessed high anticolon cancer activity in vitro, but it was not cytotoxic to normal colon cells.

The anticancer activity of polysaccharides is related to their chemical composition, MW, chain conformation, and even charge characteristics.^{16,25} Together with our previous study,

although the MW of c-EPS was similar to that of r-LHEPS-2 from *L. helveticus* MB2-1 and to those of r-EPS1 and r-EPS2 from *L. plantarum* 70810, the chemical composition and structure of c-EPS were significantly different from those of the other EPSs obtained. The c-EPS exhibited a triple-stranded helical conformation that is known to be beneficial for the anticancer activity of polysaccharides.^{20,25,26} Other physicochemical properties of polysaccharides, such as the presence of uronic acid, sulfate groups, glucose, and β -type glycosidic linkages, are also conducive to increasing their anticancer activity.^{14,42,43} NMR analysis revealed that r-LHEPS-2, r-EPS1, and r-EPS2 consisted predominantly of α -mannose residues and contained a minor portion of glucose and galactose residues in the β -configuration, whereas c-EPS contained mainly β -glucose residues with a minor portion of mannose and galactose residues in the α -configuration in its repeating units.^{20,26} Such structural variability may profoundly affect the biological activities of EPS molecules. Therefore, certain structural properties of the purified c-EPS, such as a triple-stranded helical conformation, the presence of glucose as the major monosaccharide component, and β -type glycosidic linkages, may contribute to its multiple anticancer activities.

Currently, research on LAB c-EPS is garnering increased attention. Herein, purified c-EPS from *L. helveticus* MB2-1 was prepared and characterized. This novel c-EPS was a linear glucomannogalactan with an estimated MW of 1.83×10^5 Da. Further study revealed that the c-EPS contained repeating units of $\rightarrow 6$ - β -D-Manp-(1 \rightarrow 3)- β -D-GlcP-(1 \rightarrow 3)- β -D-GlcP-(1 \rightarrow 3)- β -D-GlcP-(1 \rightarrow 4)- α -D-Galp-(1 \rightarrow and trace amounts of (1 \rightarrow 3)-Rhap and (1 \rightarrow 4)-Arap residues. Additionally, the c-EPS displayed a triple-stranded helical conformation. Morphological characterization by AFM and SEM showed that the c-EPS formed aggregates of regular feather-like units, where rounded lumps with a maximum height of 250 nm were observed. This morphology indicated good structural stability that may be utilized for film production and for the generation of edible nanostructures for the encapsulation of drug and food additives. In vitro anticancer analysis demonstrated that the c-EPS had significant anticancer activity against HepG-2, BGC-823, and especially HT-29 cancer cells. Therefore, these results suggest that the c-EPS produced by *L. helveticus* MB2-1 may be suitable for use in foods and in natural anticancer drugs.

ASSOCIATED CONTENT

Supporting Information

2D ^1H - ^{13}C HSQC, ^1H - ^1H COSY, and NOESY spectra of c-EPS from *L. helveticus* MB2-1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

REFERENCES

- (1) Axelsson, L. Lactic acid bacteria: Classification and physiology. In *Lactic acid bacteria microbiological and functional aspects*; Salminen, S., Wright, A. V., Ouwehand, A., Eds.; CRC Press: Boca Raton, FL, 2004; pp 1–66.
- (2) Li, W.; Mutuvulla, M.; Chen, X.; Jiang, M.; Dong, M. Isolation and identification of high viscosity-producing lactic acid bacteria from a traditional fermented milk in Xinjiang and its role in fermentation process. *Eur. Food Res. Technol.* **2012**, *235*, 497–505.
- (3) Landersjö, C.; Yang, Z.; Huttunen, E.; Widmalm, G. Structural studies of the exopolysaccharide produced by *Lactobacillus rhamnosus* strain GG (ATCC 53103). *Biomacromolecules*. **2002**, *3*, 880–884.
- (4) Nordmark, E.; Yang, Z.; Huttunen, E.; Widmalm, G. Structural studies of an exopolysaccharide produced by *Streptococcus thermophilus* THS. *Biomacromolecules*. **2005**, *6*, 105–108.
- (5) Li, W.; Ji, J.; Rui, X.; Yu, J.; Tang, W.; Chen, X.; Jiang, M.; Dong, M. Production of exopolysaccharides by *Lactobacillus helveticus* MB2-1 and its functional characteristics in vitro. *LWT-Food Sci. Technol.* **2014**, *59*, 732–739.
- (6) Yang, L.; Lin, W.; Lu, T. Characterization and prebiotic activity of aqueous extract and indigestible polysaccharide from *Anoectochilus formosanus*. *J. Agric. Food Chem.* **2012**, *60*, 8590–8599.
- (7) Liu, C.; Tseng, K.; Chiang, S.; Lee, B.; Hsu, W.; Pan, T. Immunomodulatory and antioxidant potential of *Lactobacillus* exopolysaccharides. *J. Sci. Food Agric.* **2011**, *91*, 2284–2291.
- (8) Hu, J.; Nie, S.; Wu, Q.; Li, C.; Fu, Z.; Gong, J.; Cui, S.; Xie, M. Polysaccharide from seeds of *Plantago asiatica* L. affects lipid metabolism and colon microbiota of mouse. *J. Agric. Food Chem.* **2013**, *62*, 229–234.
- (9) Hu, X. Q.; Huang, Y. Y.; Dong, Q. F.; Song, L. Y.; Yuan, F.; Yu, R. M. Structure characterization and antioxidant activity of a novel polysaccharide isolated from pulp tissues of *Litchi chinensis*. *J. Agric. Food Chem.* **2011**, *59*, 11548–11552.
- (10) Pan, D.; Mei, X. Antioxidant activity of an exopolysaccharide purified from *Lactococcus lactis* subsp. *lactis* 12. *Carbohydr. Polym.* **2010**, *80*, 908–914.
- (11) Adamsen, L.; Quist, M.; Midtgård, J.; Andersen, C.; Møller, T.; Knutsen, L.; Tveteras, A.; Rorth, M. The effect of a multidimensional exercise intervention on physical capacity, well-being and quality of life in cancer patients undergoing chemotherapy. *Support. Care Cancer* **2006**, *14*, 116–127.
- (12) Wagner, A. D.; Grothe, W.; Haerting, J.; Kleber, G.; Grothey, A.; Fleig, W. E. Chemotherapy in advanced gastric cancer: A systematic review and meta-analysis based on aggregate data. *J. Clin. Oncol.* **2006**, *24*, 2903–2909.
- (13) Yang, Z.; Li, S.; Zhang, X.; Zeng, X.; Li, D.; Zhao, Y.; Zhang, J. Capsular and slime-polysaccharide production by *Lactobacillus rhamnosus* JAAS8 isolated from Chinese sauerkraut: Potential application in fermented milk products. *J. Biosci. Bioeng.* **2010**, *110*, 53–57.
- (14) Badel, S.; Bernardi, T.; Michaud, P. New perspectives for *Lactobacilli* exopolysaccharides. *Biotechnol. Adv.* **2011**, *29*, 54–66.
- (15) Jolly, L.; Vincent, S. J. F.; Duboc, P.; Neeser, J. Exploiting exopolysaccharides from lactic acid bacteria. *Antonie van Leeuwenhoek* **2002**, *82*, 367–374.
- (16) Ruas-Madiedo, P.; Hugenholtz, J.; Zoon, P. An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. *Int. Dairy J.* **2002**, *12*, 163–171.
- (17) Kim, S. P.; Park, S. O.; Lee, S. J.; Nam, S. H.; Friedman, M. A polysaccharide isolated from the liquid culture of *Lentinus edodes* (Shiitake) mushroom mycelia containing black rice bran protects mice against a *Salmonella* lipopolysaccharide-induced endotoxemia. *J. Agric. Food Chem.* **2013**, *61*, 10987–10994.

- (18) Li, C.; Li, W.; Chen, X.; Feng, M.; Rui, X.; Jiang, M.; Dong, M. Microbiological, physicochemical and rheological properties of fermented soymilk produced with exopolysaccharide (EPS) producing lactic acid bacteria strains. *LWT-Food Sci. Technol.* **2014**, *57*, 477–485.
- (19) Zhang, S.; Nie, S.; Huang, D.; Huang, J.; Feng, Y.; Xie, M. A polysaccharide from *Ganoderma atrum* inhibits tumor growth by induction of apoptosis and activation of immune response in CT26-bearing mice. *J. Agric. Food Chem.* **2014**, *62*, 9296–9304.
- (20) Wang, K.; Li, W.; Rui, X.; Chen, X.; Jiang, M.; Dong, M. Structural characterization and bioactivity of released exopolysaccharides from *Lactobacillus plantarum* 70810. *Int. J. Biol. Macromol.* **2014**, *67*, 71–78.
- (21) Zhang, S.; Nie, S.; Huang, D.; Feng, Y.; Xie, M. A novel polysaccharide from *Ganoderma atrum* exerts antitumor activity by activating mitochondria-mediated apoptotic pathway and boosting the immune system. *J. Agric. Food Chem.* **2014**, *62*, 1581–1589.
- (22) Vinogradov, E.; Valence, F.; Maes, E.; Jebava, I.; Chuat, V.; Lortal, S.; Grard, T.; Guerardel, Y.; Sadovskaya, I. Structural studies of the cell wall polysaccharides from three strains of *Lactobacillus helveticus* with different autolytic properties: DPC4571, BROI, and LH1. *Carbohydr. Res.* **2013**, *379*, 7–12.
- (23) Wang, K.; Li, W.; Rui, X.; Chen, X.; Jiang, M.; Dong, M. Characterization of a novel exopolysaccharide with antitumor activity from *Lactobacillus plantarum* 70810. *Int. J. Biol. Macromol.* **2014**, *63*, 133–139.
- (24) Li, W.; Ji, J.; Xu, X.; Hu, B.; Wang, K.; Xu, D.; Dong, M. Extraction and antioxidant activity in vitro of capsular polysaccharide from *Lactobacillus helveticus* MB2-1 in Sayram yogurt from Xinjiang. *Food Sci.* **2012**, *33*, 34–38.
- (25) Li, W.; Ji, J.; Chen, X.; Jiang, M.; Rui, X.; Dong, M. Structural elucidation and antioxidant activities of exopolysaccharides from *Lactobacillus helveticus* MB2-1. *Carbohydr. Polym.* **2014**, *102*, 351–359.
- (26) Li, W.; Ji, J.; Tang, W.; Rui, X.; Chen, X.; Jiang, M.; Dong, M. Characterization of an antiproliferative exopolysaccharide (LHEPS-2) from *Lactobacillus helveticus* MB2-1. *Carbohydr. Polym.* **2014**, *105*, 334–340.
- (27) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356.
- (28) Li, W.; Xiang, X.; Tang, S.; Hu, B.; Tian, L.; Sun, Y.; Ye, H.; Zeng, X. Effective enzymatic synthesis of lactosucrose and its analogues by β -D-galactosidase from *Bacillus circulans*. *J. Agric. Food Chem.* **2009**, *57*, 3927–3933.
- (29) Villares, A.; García-Lafuente, A.; Guillamón, E.; Mateo-Vivaracho, L. Separation and characterization of the structural features of macromolecular carbohydrates from wild edible mushrooms. *Bioact. Carbohydr. Diet. Fibre* **2013**, *2*, 15–21.
- (30) Rodríguez-Carvajala, M. A.; Ignacio Sánchez, J.; Campelo, A. B.; Martínez, B.; Rodríguez, A.; Gil-Serrano, A. M. Structure of the high-molecular weight exopolysaccharide isolated from *Lactobacillus pentosus* LPS26. *Carbohydr. Res.* **2008**, *343*, 3066–3070.
- (31) Tallon, R.; Bressollier, P.; Urdaci, M. C. Isolation and characterization of two exopolysaccharides produced by *Lactobacillus plantarum* EP56. *Res. Microbiol.* **2003**, *154*, 705–712.
- (32) Svensson, M. V.; Zhang, X.; Huttunen, E.; Widmalm, G. Structural studies of the capsular polysaccharide produced by *Leuconostoc mesenteroides* ssp. *cremoris* PIA2. *Biomacromolecules* **2011**, *12*, 2496–250.
- (33) Cui, J.; Qiu, J. Production of extracellular water-insoluble polysaccharide from *Pseudomonas* sp. *J. Agric. Food Chem.* **2012**, *60*, 4865–4871.
- (34) Niu, Y.; Yan, W.; Lv, J.; Yao, W.; Yu, L. Characterization of a novel polysaccharide from tetraploid *Gynostemma pentaphyllum* makino. *J. Agric. Food Chem.* **2013**, *61*, 4882–4889.
- (35) Bubb, W. A. NMR spectroscopy in the study of carbohydrates: Characterizing the structural complexity. *Concepts Magn. Reson. A* **2003**, *19*, 1–19.
- (36) Francius, G.; Alsteens, D.; Dupres, V.; Lebeer, S.; Keersmaecker, S. D.; Vanderleyden, J.; Gruber, H. J.; Dufrene, Y. F. Stretching polysaccharides on live cells using single molecule force spectroscopy. *Nat. Protoc.* **2009**, *4*, 939–946.
- (37) Marszalek, P. E.; Dufrêne, Y. F. Stretching single polysaccharides and proteins using atomic force microscopy. *Chem. Soc. Rev.* **2012**, *41*, 3523–3534.
- (38) Wang, Y.; Li, C.; Liu, P.; Ahmed, Z.; Xiao, P.; Bai, X. Physical characterization of exopolysaccharide produced by *Lactobacillus plantarum* KFS isolated from Tibet Kefir. *Carbohydr. Polym.* **2010**, *82*, 895–903.
- (39) Costa, N. E.; Wang, L.; Auty, M. E.; Hannon, J. A.; McSweeney, P. L. H.; Beresford, T. P. Rheological, microscopic and primary chemical characterisation of the exopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* DPC6532. *Dairy Sci. Technol.* **2012**, *92*, 219–235.
- (40) Yadav, V.; Prappulla, S. G.; Jha, A.; Poonia, A. A novel exopolysaccharide from probiotic *Lactobacillus fermentum* CFR2195: Production, purification and characterization. *Biotechnol. Bioinform. Bioeng.* **2011**, *1*, 415–421.
- (41) Xu, S.; Xu, X.; Zhang, L. Effect of heating on chain conformation of branched β -glucan in water. *J. Phys. Chem. B* **2013**, *117*, 8370–8377.
- (42) Ma, L.; Qin, C.; Wang, M.; Gan, D.; Cao, L.; Ye, H.; Sun, Y.; Zeng, X. Preparation, preliminary characterization and inhibitory effect on human colon cancer HT-29 cells of an acidic polysaccharide fraction from *Stachys floridana* Schuttl. ex Benth. *Food Chem. Toxicol.* **2013**, *60*, 269–276.
- (43) Wang, Y.; Fang, J.; Ni, X.; Li, J.; Liu, Q.; Dong, Q.; Duan, J.; Ding, K. Inducement of cytokine release by GFPBW2, a novel polysaccharide from fruit bodies of *Grifola frondosa*, through dectin-1 in macrophages. *J. Agric. Food Chem.* **2013**, *61*, 11400–11409.