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Purification and Partial Elucidation of the Structure of an Antioxidant Carbohydrate Biopolymer from the Probiotic Bacterium *Bacillus coagulans* RK-02

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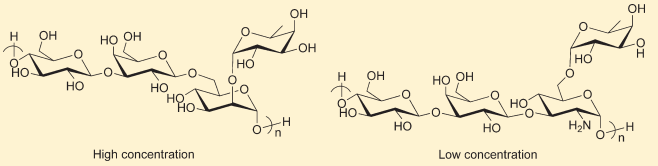
ABSTRACT: An exopolysaccharide (EPS) was isolated from *Bacillus coagulans* RK-02 and purified by size exclusion chromatography. The purified, homogeneous EPS had an average molecular weight of ~3 × 10^4 Da by comparison with FITC-labeled dextran standards. In vivo evaluations showed that, like other reported polysaccharides, this EPS displayed significant antioxidant activity. FTIR spectroscopy analysis showed the presence of hydroxy, carboxy, and α-glycosidic linkages and a mannose residue. GC analysis indicated that the EPS was a heteropolymer composed of glucose, mannose, galactose, glucosamine, and fucose as monomeric constituent units. Partial elucidation of the structure of the carbohydrate biopolymer based on GC-MS and NMR analysis showed the presence of two unique sets of tetrasaccharide repeating units that have 1→3 and 1→6 glycosidic linkages. This is also the first report of a Gram-positive bacterial polysaccharide with both fucose as a sugar monomer and 1→3 and 1→6 glycosidic linkages in the molecular backbone.



Polysaccharides are molecules with human therapeutic potential and have drawn increasing attention from researchers over the last few decades. Indeed, polysaccharides from several different origins have proven beneficial therapeutic properties. Plants synthesize polysaccharides to form exudates and slimes both in response to injury and also to serve as protective or defensive agents. Various plant polysaccharides are currently utilized in the food and nonfood industries. For instance, the plant polysaccharides starch, pectin, and guar gum significantly suppress the incidence of colon cancer.[1]

Microbial polysaccharides have also been reported to have potential therapeutic applications.[2] Recently, much attention has been devoted to the microbial exopolysaccharides (EPSs) due to their numerous health benefits.[3] EPSs from lactic acid bacteria are reported to possess antitumor effects, immunostimulatory activity, and the ability to lower blood cholesterol.[4] EPSs also offer an alternative class of biothickeners that are widely used in the food and dairy industries[5] and have been proven to provide strong emulsifying activity, which is important in many food formulations.[6]

EPSs are long-chain high molecular mass polymers secreted by microbes into the surrounding medium to protect the producer organism against phagocytosis, antibiotics, and toxic substances, to aid adhesion to solid surfaces (biofilm formation), and to survive in extreme pH and temperature conditions.[7,8] EPS-producing bacteria include a range of organisms, from animal pathogenic bacteria such as *Streptococcus pneumoniae* and *S. agalactiae*, to plant pathogenic bacteria such as *Xanthomonas compestris* and *Sphingomonas elodea*,[9] to various probiotic bacteria with the designation of generally regarded as safe (GRAS) organisms. The probiotic bacteria, e.g., lactic acid bacteria (LAB), dairy propionibacteria, bifidobacteria,[10] and various *Bacillus* strains, e.g., *B. licheniformis*[11] and *B. thermoantarcticus*,[12] produce EPSs in substantial quantities.

Depending on the monosaccharide composition, EPSs can be classified into homopolysaccharides (HoPSs) and heteropolysaccharides (HePSs). HoPSs consist of only one type of monosaccharide, mostly glucose or fructose, while HePSs comprise two or more than two types of monosaccharides, which are mostly glucose, galactose, mannose, and rhamnose. In some EPSs, there are also small quantities of fucose and hexosamines like glucosamine or galactosamine. The physicochemical and biological properties of EPSs depend mainly on the monosaccharide composition and the linkages between the monomeric units that are joined together to form the exopolymer.[10] Considering the commercial and therapeutic importance of these products, it is therefore of paramount importance to structurally characterize the EPS that was isolated and purified from a *Bacillus coagulans* RK-02 probiotic culture broth[13,14] and has shown significant antioxidant and emulsifying activities.[15,16] Herein we report that the *B. coagulans* RK-02 EPS exhibits antioxidant activity in vivo and present the first structural characterization of a microbial EPS.

RESULTS AND DISCUSSION

EPS Purification and Molecular Weight Determination. The crude EPS was purified by gel filtration chromatography

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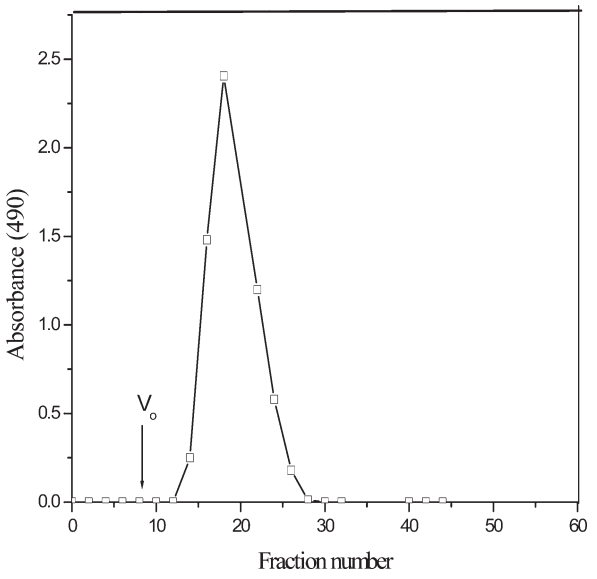

Figure 1. Gel filtration chromatography of the EPS on a Sephadex G-100 column. Flow rate was 0.5 mL/min. Fraction size was 1 mL/min. Void volume was 9 mL.

Table 1. Carbohydrate and Protein Contents of Crude and Pure EPS

sample no.	fraction	total carbohydrate (%)	protein (%)	phenolic content (%)[a]
1	crude EPS[b]	89 ± 2	1.8 ± 0.4	0.0
2	purified EPS[c]	96 ± 3	0	0.0

[a] The contents were measured by phenol/sulfuric acid, Lowry, and Folin−Ciocalteu methods, respectively. [b] Alcohol precipitated EPS. [c] Gel filtration purified EPS.

using a Seralose CL-4B column and then repurified using a Sephadex G-100 column. A gel permeation chromatogram (Figure 1) showed that the elution of EPS started at the 12th fraction and ended at the 28th fraction. It also indicated that there were no other proteins associated with EPS. The average EPS molecular mass was 3×10^4 Da based on comparison with a standard plot of FITC-labeled dextrans. The carbohydrate and protein contents of the crude and purified EPS are listed in Table 1. While the total sugar content (%, w/w) of the EPS was found to increase by 7% following purification, the percentage content of contaminating proteins decreased from 1.8% before purification to zero after purification. These observations suggested that gel filtration had produced a highly purified EPS. To evaluate EPS homogeneity, the purified EPS was resolved on a 1% agarose gel. These results indicated that the purified EPS was homogeneous (Figure 2). On the basis of the results of a Folin−Ciocalteu test performed to check for the presence of phenolic compounds, the purified EPS was completely free of these compounds.

Analysis of EPS in Vivo Antioxidant Activity. *DPPH Radical Scavenging Assay.* In this assay, which assesses the DPPH radical scavenging ability of a serum sample, the percentage of DPPH radical scavenging activity of serum obtained from mice treated with EPS (100 mg/kg body weight) was higher than that of serum from mice treated with vitamin C (100 mg/kg body

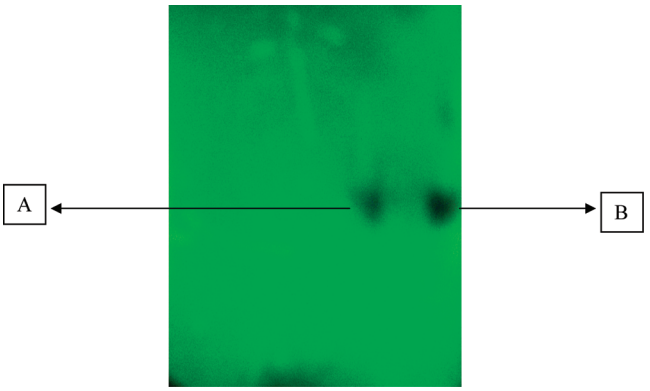

Figure 2. Agarose gel electrophoresis of the purified EPS. 1% agarose in Tris HCl buffer (pH 9.1) and stained with toluidine blue in Ac_2O/EtOH/H_2O (0.1:5:5, v/v/v): (A) rat liver glycogen; (B) purified EPS.

weight) (58 ± 3% vs 50 ± 2.7%, respectively, Figure 3). The DPPH free radical scavenging activity of EPS was significantly different from mice dosed with the control vitamin C at all doses tested, although EPS radical scavenging activity did increase with increasing EPS concentrations. Indeed, EPS administered at the doses of 50, 100, 200, and 400 mg/kg significantly scavenged DPPH free radicals in all tested animals. In general, EPS dosed at 50 mg/kg exhibited stronger effects than vitamin C at a dose of 100 mg/kg. These data suggested that the EPS administration induced effective DPPH free radical scavenging activities in the serum of all mice tested.

Lipid Peroxidation Inhibition Assay (TBA Assay). In biological systems, lipid peroxidation generates a number of lipid degradation products, such as malondialdehyde (MDA), that have been found to be important causes of cell membrane destruction and cell damage. In the MDA assay, the potential of EPS to inhibit lipid peroxidation was measured in liver homogenates from mice that had been treated with different doses of EPS. The assay results indicated that EPS inhibition of lipid peroxidation increased with escalating EPS doses (Figure 4). The percentage of peroxidation inhibition for mice dosed with EPS at 100 mg/kg body weight was much higher (62 ± 3.3%) than for mice (39 ± 2.9%) dosed with 100 mg/kg body weight vitamin C (62 ± 3.3% vs 39 ± 2.9%, respectively, Figure 4).

MDA is a primary marker of endogenous lipid peroxidation and is a widely used research tool. In the present study, MDA levels in liver microsomes significantly increased with aging (Figure 4). This increase in MDA production indicates that peroxidative damage increases during the aging process.[17] While EPS treatment at a lower dose (50 mg/kg) considerably decreased liver MDA levels, EPS at the doses of 100, 200, and 400 mg/kg significantly inhibited liver MDA formation in all mice tested. In general, EPS at the 100 mg/kg dose exhibited stronger effects than the same dose of vitamin C. These data suggest that EPS administration effectively inhibited endogenous lipid peroxidation in aging mice.

There is a vast amount of evidence indicating that aging is associated with a decrease in endogenous antioxidant status and that age-dependent increases in lipid peroxidation are a consequence of diminished antioxidant protection. Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from undergoing oxidative damage. Superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) are two important endogenous enzymatic antioxidant defense

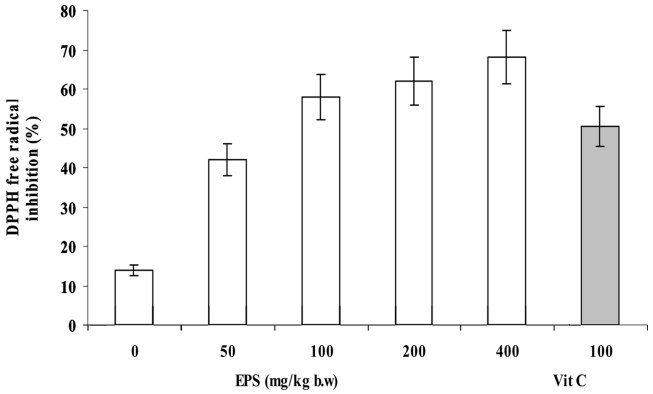

Figure 3. Scavenging effects of EPS against DPPH radical: EPS (---□---), vitamin C (---■---). Vitamin C used as positive control. The values are means ± SD (n = 3).

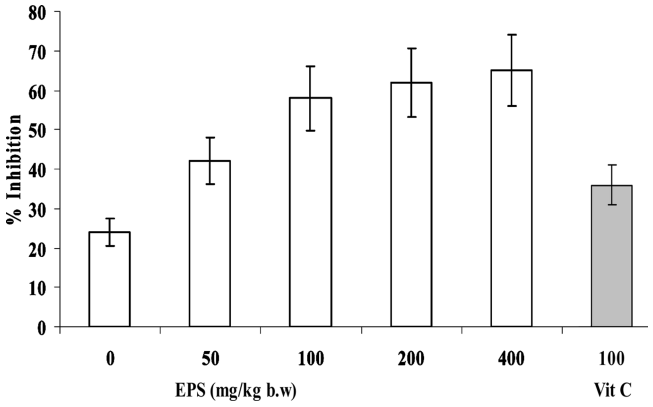

Figure 4. Lipid peroxidation inhibition of EPS: EPS (---□---), vitamin C (---■---). Vitamin C used as positive control. The values are means ± SD (n = 3).

Table 2. Effect of EPS on the Activity of SOD in Liver Homogenate in Aging Mice

group	SOD, NU/mg
control (normal mice)	6.2 ± 0.8[a]
EPS (50 mg/kg b.w.) treated	7.2 ± 0.4
EPS (100 mg/kg b.w.) treated	9.6 ± 0.3
EPS (200 mg/kg b.w.) treated	10.9 ± 0.6
EPS(400 mg/kg b.w.) traeted	11.3 ± 0.4
Vit C(100 mg/kg b.w.) treated	7.1 ± 1.1

[a] The values are means ± SD (n = 3).

mechanisms. SOD is an intracellular antioxidant enzyme that protects against oxidative processes initiated by the superoxide anion, while GSH-Px reduces lipid hydroperoxides to their corresponding alcohols and free hydrogen peroxide to water. EPS successfully inhibited lipid peroxidation, as demonstrated by the reduction in MDA production following treatment of aging mice with EPS. These results are consistent with the in vitro inhibitory effects of EPS on liver microsomal lipid peroxidation.

Polysaccharides have been reported to stimulate the production of both SOD and GSHPx.[17−19] Enhanced SOD and GSH-Px activity along with increased total antioxidant capacity can be effective in scavenging the various types of oxygen free radicals

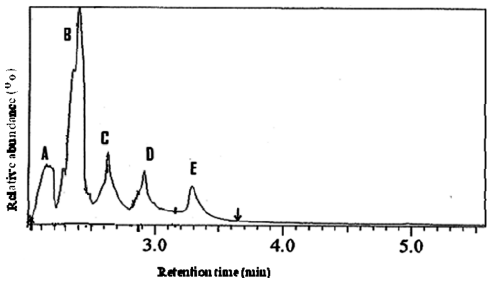

Figure 5. GC analysis of alditol acetates of monosaccharides of the EPS. The alditol acetates of monosaccharides were analyzed by a Shimadzu GS-MS QP 2010 using a ZB-1 column. Helium was used as carrier gas. (A: fucitol; B: galactitol; C: mannitol; D: aminoglucitol; and E: glucitol).

and their byproducts in aging animals. Thus, the inhibitory effect of EPS on lipid peroxidation might be attributed to its influence on the expression or activity of antioxidant enzymes. As shown in Table 2, there was no significant difference in SOD activity between young and aging mice. Importantly, treating aging mice with the EPS significantly increased SOD activity in liver homogenates in a dose-dependent fashion. These SOD assay results clearly indicate that EPS considerably stimulated the production of the antioxidant enzyme SOD.

EPS Compositional Analysis. The purified EPS was hydrolyzed, derivatized, and then analyzed for its sugar composition by gas chromatography (GC). The GC chromatogram of the alditol acetates of monosaccharides shown in Figure 5 showed that the EPS from *B. coagulans* RK-02 was composed of galactose, mannose, fucose, and glucose. In terms of peak area, galactose and mannose were the two major monosaccharides, whereas glucose, fucose, and glucosamine were present as minor peaks. The proportions of these monosaccharides were calculated on the basis of chromatograms using each of the five monosaccharides as the standard and are presented in Table 3. A literature search did not find any previously reported bacterial exopolysaccharides with this typical monosaccharide composition, and in particular one containing fucose.

FTIR Analysis. An FTIR spectrum of the *B. coagulans* RK-02 EPS revealed the purified product's major functional groups and the chemical bonds (Figure 6). The broad peak at 3406 cm^{-1} was an OH stretching peak, the carbonyl (C═0) stretching was at 1670 cm^{-1}, and the peak between 2800 and 3000 cm^{-1} was a C−H stretching peak. Absorption at 1110 cm^{-1} was typical for D-glucose in pyranose form, and that at 847 cm^{-1} was indicative of the presence of an α-glycosidic linkage between individual glycosyl residues in EPS. In the anomeric region (950−700 cm^{-1}), polysaccharides are reported to exhibit an obvious characteristic absorption at 812 cm^{-1} that corresponds with the presence of mannose.[20]

Linkage Analysis. *Methylation Analysis.* The sugar linkages in alditol acetates of the EPS methylated sugars were elucidated by GC-MS analysis (Table 3). The linkages between the monomers were analyzed according to the description by Bjorndal et al.[21] GC-MS data showed the presence of 1,2,6-tri-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol, 1,4-di-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol, 1,3-di-*O*-acetyl-2,4-di-*O*-methyl-L-fucitol, 1,4,6-tri-*O*-acetyl-3-mono-*O*-methyl-2-amino-D-glucitol, and 1-mono-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

NMR Analysis. ^{1}H NMR analysis confirmed the presence of five different monosaccharides in the purified EPS (data not shown). Subsequent ^{13}C NMR analysis of the purified polysaccharide showed

Table 3. Partially Methylated Alditol Acetate Derivatives of the EPS

sugar derivative	relative proportions (%)[a]	mass spectral fragments (m/Z)	mode of linkage
1,2,6-tri-*O*-acetyl-3,4-di-*O*-methyl-D-galactitol	55.7	43, 87, 99, 129, 189	→1, 2)-Gal-(6→
1,4-di-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol	18.0	43, 45, 87, 102, 118, 129, 143, 161, 173, 203, 233	→1)-Man-(4→
1,3-di-*O*-acetyl-2,4-di-*O*-methyl-L-fucitol	12.5	43, 87, 101, 117, 139, 189, 203	→1)-Fuc-(3→
1,4,6-tri-*O*-acetyl-3-mono-*O*-methyl-2-amino-D-glucitol	9 0.8	43, 102, 189, 261	→1, 4)-GlcN-(→6
1-mono-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol	7 0.3	43, 45, 101, 102, 118, 129, 161, 162, 205	→1)-Glc→

[a] Calculated from peak areas and response factors obtained using a flame ionization detector.[33]

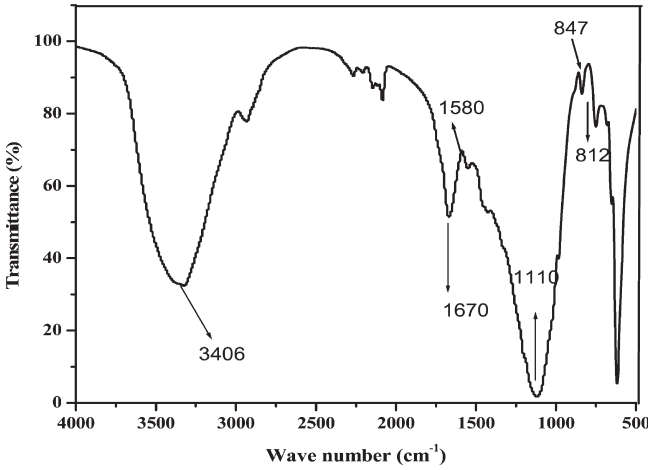

Figure 6. FTIR spectrum of the purified EPS recorded by using a Shimadzu FTIR-8300/8700 in the frequency range 4000−500 cm^{-1}.

three major peaks at δ_C 102.2, 102.1, and 100.5 and three minor peaks at δ_C 99.37, 103.0, and 98.2. This pattern clearly indicated the presence of two sets of tetrasaccharide repeating units. The ^{13}C NMR spectrum (Figure 7) also indicated that two of the sugar moieties in the polysaccharide possessed a β-configuration (δ_C 102.2 and 102.1) and that two sugar moieties have an α-configuration (δ_C 100.5 and 98.2). Observation of a ^{13}C NMR signal at δ_C 20.2 indicated the presence of α-fucose in the polysaccharide, while the C-6 signals observed at δ_C 65 and 64, which were further downfield from where they are usually observed (δ_C∼60 ppm), might indicate the presence of (1→6)-linked glycosides in the EPS. However, the three C-3 signals at δ_C 79.0, 78.7, and 78.6 clearly indicated the presence of (1→3)-linked glycosides.[22,23] Figure 8 presents a possible EPS molecular structure based on the results of its partial characterization by GC-MS and ^{13}C NMR studies. No other reported polysaccharide of bacterial origin has (1→3) and (1→6) glycosidic linkages in its backbone. Therefore the heteropolysaccharide purified from *B. coagulans* RK-02 is truly unique based on the presence of fucose as a monosaccharide unit and of (1→3) and (1→6) glycosidic linkages within the molecule.

EXPERIMENTAL SECTION

EPS Isolation. The probiotic bacterium *B. coagulans* RK-2 was isolated from a soil sample and grown at 37 °C, pH 6.7, and 180 rpm in glucose mineral salt medium. EPS isolation and quantification have been described previously.[13−15]

EPS Purification. The crude EPS was dissolved in a 0.2 M NaCl solution to a concentration of 10 g/L and initially loaded onto a Seralose CL-4B column (50 × 1.2 cm, SRL, India). The EPS fractions were pooled, dialyzed, and dissolved in a fresh 0.2 M NaCl solution. This EPS solution was loaded on a Sephadex G-100 column (50 × 1.2 cms, SRL, India), the column was eluted with the same solution (0.2 M NaCl) at a flow rate of 0.5 mL/min, and 1 mL fractions were collected. The EPS protein content was monitored at 280 nm, and its carbohydrate content was estimated at 490 nm using the phenol sulfuric acid method with glucose as a standard.[24] The total phenolic content was measured using the Folin−Ciocalteu method as described previously.[25]

EPS Molecular Weight Determination. The average EPS molecular mass was determined by gel filtration chromatography on a Sepharose-6B column (75 × 2.2 cm; Amersham Pharmacia Biotech, Uppsala, Sweden), calibrated with FITC-labeled dextran standards (M_w 1000, 70, 30, 10 kDa, Fluka Chemie GmbH, Buchs, Switzerland), and using 50 mM phosphate buffer, pH 6.0, containing 150 mM NaCl as the eluent. The flow rate was 0.2 mL/min, and 2 mL fractions were collected. The carbohydrate content of each fraction was determined using the phenol sulfuric acid assay as described previously.[24] The average molecular weight of EPS was calculated from a standard plot of molecular weight versus elution volume for FITC-labeled dextrans (data not shown). To determine EPS homogeneity, the purified EPS was resolved on a 1% agarose gel by electrophoresis in Tris-HCl buffer (pH 9.1) and stained with 0.1% toluidine blue (Sigma Aldrich Co., St. Louis, MO, USA) in HOAc/EtOH/H_2O (0.1:5:5, v/v/v).[26]

In Vivo Testing of EPS Antioxidant Activity. Male Swiss albino mice (weight 20 ± 2 g) were used to study EPS antioxidant activity. Mice were housed under standardized conditions (temperature 21−25 °C and a light/dark cycle of 12 h/12 h) and fed a normal laboratory diet. After one week of acclimatization, the mice were divided into a control group and six experimental groups with six animals in each group. All mice received daily intraperitoneal (ip) injections of D-galactose (150 mg/kg body weight) for 4 weeks to produce oxidative stress in the mice.[18] From day 3, mice in groups 1 to 4 received ip injections of EPS at 50, 100, 200, and 400 mg/kg body weight per day for 14 days. Group 5 served as a positive control group and was given ip injections of ascorbic acid (100 mg/kg body weight per day). Group 6 served as a negative control and received only saline. At the end of the 14th day, blood was collected from all mice and was used to evaluate in vivo antioxidant activity. This protocol followed the methods described by Wang et al., 2008.[27]

Blood Collection. Approximately 1 mL of blood was collected from each mouse through the sinus orbital. Each blood sample was centrifuged at 2000*g* for 10 min to separate the serum, and individual serum samples were stored at −20 °C until their use in evaluating antioxidant activities.

DPPH Radical Scavenging Assay. This assay measured the ability of serum to inhibit DPPH radical formation. For this assay, 20 μL of mouse serum was added to 3 mL of DPPH solution (0.1 mM/L in EtOH), and the reaction mixture was shaken vigorously. After incubation at room temperature for 10 min, the absorbance was measured at 517 nm. The DPPH solution without serum was used as a blank, and DPPH solution incubated with serum from mice treated with ascorbic acid was used as a positive control.[28]

Lipid Peroxidation Assay. For the MDA assay, 10 μM $FeSO_4$ and 0.1 mM L-ascorbic acid in 1 mL of K_3PO_4 buffer solution (0.2 M, pH 7.4)

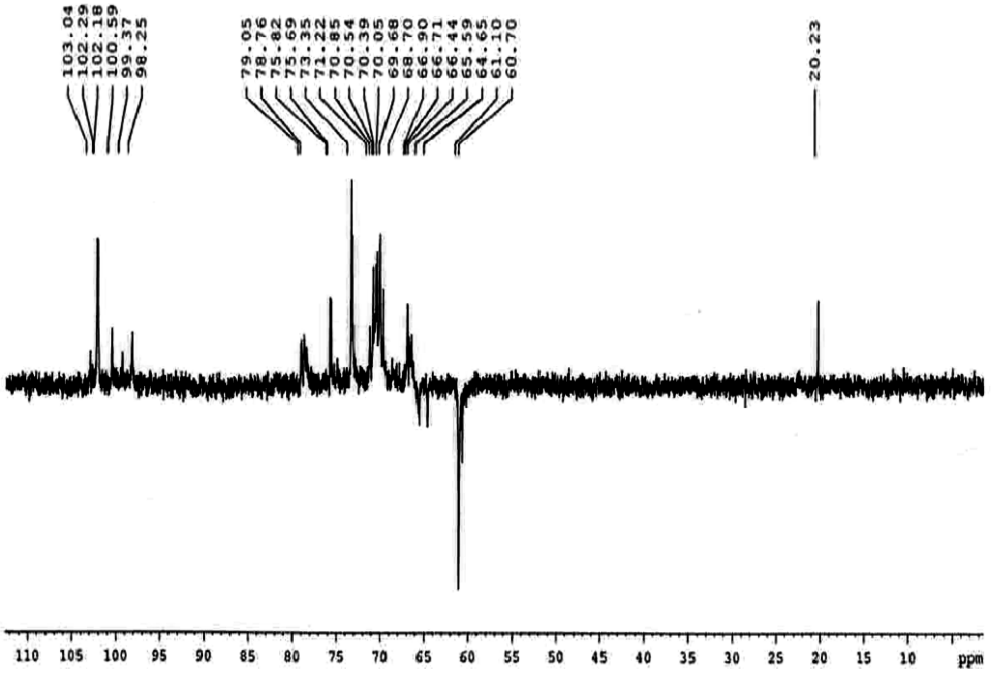

Figure 7. ^{13}C NMR (DEPT) spectrum of purified EPS recorded in D_2O at 70 °C using a Bruker AMX-400 MHz instrument.

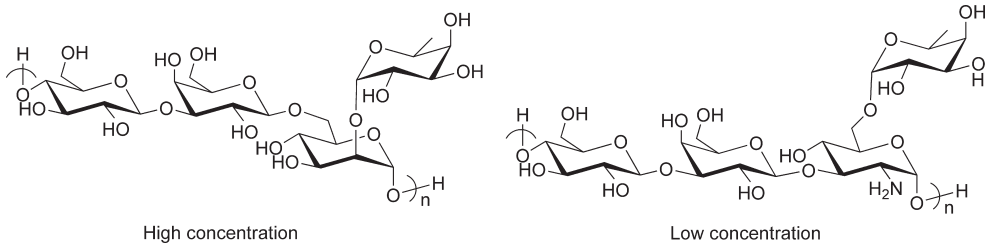

Figure 8. The possible structure of the EPS isolated from *Bacillus coagulans* RK-02.

were added to 100 μL of serum, and the mixture was incubated at 37 °C for 60 min. The reaction was stopped by adding 28% (w/v) TCA (1 mL) and 1% (w/v) TBA (1.5 mL) in succession, and the solution was then heated at 80 °C for 30 min. The samples were cooled and centrifuged at 3000*g* for 15 min, and the absorbance of the supernatant was measured at 532 nm using a spectrophotometer. The percentage inhibition of lipid peroxidation was calculated by the equation:

$$\text{Inhibitionoflipidperoxidation}\ (\%) = \left[1 - \frac{A_{\text{Sample}}}{A_{\text{Blank}}}\right] \times 100$$

where A_{Blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{Sample} is the absorbance of the test compound.[29]

Superoxide Dismutase Assay. Individual liver homogenates from both normal and oxidative stress mice were centrifuged, and the SOD content of the supernatant was assessed using a SOD kit (Sigma Aldrich Co., St. Louis, MO, USA).

EPS Compositional Analysis. *EPS Acid Hydrolysis.* EPS isolated from a 36 h culture was dissolved in deionized H_2O and dialyzed against deionized H_2O at 4 °C for 24 h and then lyophilized. Lyophilized EPS (10 mg) was hydrolyzed with 2 M TFA for 6 h at 100 °C. The TFA was removed using a rotary vacuum evaporator, the hydrolyzed solution was neutralized with 15 M ammonia solution (0.32 mL), and the resultant product was reduced and acetylated as described previously.[30]

Reduction of Hydrolyzed Monosaccharides. Monosaccharides were reduced with a solution of $NaBH_4$ in DMSO prepared by dissolving $NaBH_4$ (2 g) in anhydrous DMSO (100 mL) at 100 °C. Monosaccharides were reduced for 90 min at 40 °C by adding 1 mL of the $NaBH_4$ solution to 0.1 mL of the monosaccharide mixture in 15 M ammonia. After reduction, the excess $NaBH_4$ was decomposed by the addition of 18 M acetic acid (0.1 mL).

Acetylation. For acetylation, 0.2 mL of 1-methylimidazole and 2 mL of Ac_2O were added sequentially to the reduced monosaccharides, and the components were mixed. After 10 min at room temperature, 5 mL of H_2O was added to decompose the excess Ac_2O. When cooled, 1 mL of DCM was added and the mixture was agitated vigorously on a vortex mixer. After the phases had separated, the lower phase was removed with a pipet and stored in a 1 mL septum-cap vial at −20 °C.[31]

GC Analysis of Alditol Acetates. Alditol acetates were separated and quantified on a ZB-1-packed column fitted to a Shimadzu GC-MS QP 2010 gas chromatograph equipped with a flame-ionization detector.[23] Ultra-high-purity He was used as a carrier gas at a flow rate of 5 mL/min. The initial 38 °C oven temperature was maintained for 30 s following injection, raised to 190 °C at a rate of 70 °C/min and then from 190 to 230 °C at 3 °C/min, and finally maintained at 230 °C for 10 min. The detector temperature was held at 250 °C.

FTIR Spectroscopic Analysis of Purified EPS. In order to detect the functional groups present in EPS, FTIR spectra of EPS were recorded using a Shimadzu FTIR-8300/8700 with a resolution of 4 cm^{-1} auto

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gain and an average of 30 scans in the frequency range 4000−500 cm^{-1}. For the analysis, purified, lyophilized EPS (20 mg) was ground with KBr to form pellets at room temperature.

Linkage Analysis. *Methylation Analysis (GC-MS Analysis).* A solution of 5 mg of EPS in 0.5 mL of DMSO was permethylated by adding finely powdered NaOH (20 mg) and MeI (0.1 mL) prior to sonication for 15 min. The permethylated EPS was extracted with $CHCl_3$ (1 mL) and H_2O (3 mL). The $CHCl_3$ phase was separated and dried under N_2 and hydrolyzed in 2 M TFA at 100 °C for 6 h. The hydrolyzed EPS was reduced with 50 mM $NaBH_4$ at room temperature for 4 h, evaporated three times from a mixture of HoAc/MeOH (1:1), and acetylated in 50:50 acetic Ac_2O/pyridine at 100 °C for 90 min. Alditol acetates of the methylated sugars were analyzed using a Shimadzu GCMS-QP2010. Analysis conditions including the temperature program and other column conditions have been described previously.[32]

NMR Spectroscopic Analysis. NMR spectra were obtained using a Bruker Avance 400 MHz spectrometer (Bruker Co., Billerica, MA) with a 5 mm inverse probe. Proton spectra were run at a probe temperature of 25 °C, while carbon spectra were obtained at 70 °C. The purified sample was dried in a vacuum over P_2O_5 and then exchanged with deuterium by lyophilizing several times with D_2O. The EPS was dissolved in 0.7 mL of D_2O (99.96%) at concentrations of 10 mg/mL (for ^1H NMR) and 30 mg/mL (for ^{13}C NMR).[20]

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