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Biochemical Characterization of the Exopolysaccharide Purified from *Laetiporus sulphureus* Mycelia

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The extracellular polysaccharide (EPS) was isolated from mycelial cultures of *Laetiporus sulphureus* var. *miniatus* and purified by DEAE cellulose and Sephadex G-50 column chromatography. The purified EPS (EPS-2-1) was composed of only glucose units and its molecular mass was 6.95 kDa. The chemical structure of EPS-2-1 consisted of a main chain containing (1→4)-Glc*p* units with branches at the C-6 position of the chain carrying -Glc*p*-(1→4)-linked residues. The effect of purified EPS on immunomodulatory genes and proteins of the Bcl-2 family was observed using cultured U937 human leukemia cells. Of note, the levels of Bax and Bad proteins treated with the EPS (4 mg/ml) were approximately 23- and 18-times higher than those in non-treated cells, respectively. These results may suggest that the EPS purified from the mushroom *L. sulphureus* is associated with the activation of immunomodulatory mediators, Bax and Bad proteins.

Keywords: Extracellular polysaccharide, *Laetiporus sulphureus*, immunomodulatory proteins, mycelial culture

Polysaccharides, widely existing in plants, microorganisms, algae, and animals, function as essential biomacromolecules for life activities. They play crucial roles in cell–cell communication, cell adhesion, and molecular recognition in the immune system [9]. Recently, medicinal mushroom polysaccharides have received special attention because they show potent biological and pharmacological activities, such as antitumor, immunomodulation, and anti-inflammatory properties [18].

Since the genus *Laetiporus* was first described by Murrill [21], at least 12 species have been found [3]. The basidiomycete *L. sulphureus* belongs to Polyporaceae of Aphyllophorales and is the most readily recognized among macrofungi owing to its striking yellow or orange color. Although the structural analysis of polysaccharides generated from the fruiting bodies of basidiomycetes was reported [2, 7], not many studies have studied the chemical structure of extracellular polysaccharide (EPS) purified from *L. sulphureus* mycelia and its role in U937 human leukemia cells.

Recently, the immunomodulating activities of exopolysaccharides obtained from mushroom cultures have been reported [12, 14]. The immunomodulating properties are important in maintaining tissue homeostasis and the pathogenesis of many diseases such as tumorigenesis [22]. Specifically, Bcl-2 is essential in the proliferation of kidney, melanocyte stem cells, and mature lymphoid cells [15, 20]. Bax-like proteins such as Bax and Bad are also essential in triggering programmed cell suicide [19, 23].

In this paper, we analyzed the chemical structure of mycelia-derived EPS from *L. sulphureus* and its role in U937 cells by conducting a structural analysis of the EPS and investigating its effect on immunomodulatory genes and proteins of the Bcl-2 family.

Materials and Methods

Chemicals, Fungal Strain, and Culture Condition

The mycelia of *L. sulphureus* var. *miniatus* JM 27 [11] were cultured using a 250 ml Erlenmeyer flask supplemented with 100 ml of main

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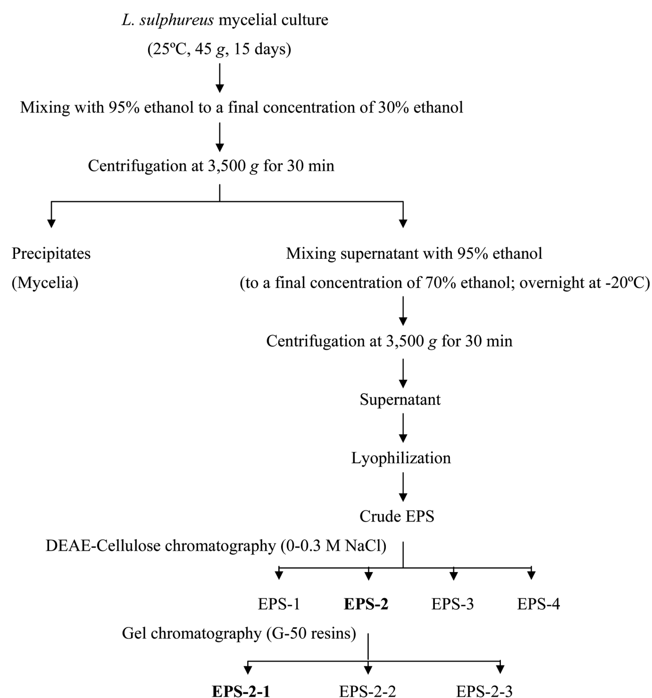

Fig. 1. A scheme for the extraction and purification of an EPS from mycelia cultures of *L. sulphureus* mycelia.

media (3% maltose, 0.2% soy peptone, and 2 mM $MnSO_4 \cdot H_2O$) inoculated with a cutter square (0.7 cm × 0.7 cm) of solid cultivation mycelia. The mycelial cultures were conducted using a rotary shaker incubator (Jeio Tech Co., Seoul, Korea) at 45 ×*g* at 25°C for 24 days. DEAE cellulose, Sephadex G-50, and monosaccharides were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All other reagents and chemicals were of analytical grade.

Extraction and Purification of the EPS

The crude EPS was produced by the sequential extraction of the mycelial culture of *L. sulphureus* using 30% and 70% ethanol solutions, respectively (Fig. 1). *L. sulphureus* mycelial cultures were mixed with 95% ethanol, resulting in a 30% ethanol solution at a final concentration. The solution was placed overnight at 4°C and centrifuged at 3,500 ×*g* for 30 min. The resulting supernatant was treated with 95% ethanol to provide a 75% solution at final concentration. After the mixture was centrifuged at 3,500 ×*g* for 30 min, the precipitates were lyophilized. The precipitates carrying the crude EPS were dissolved in 80 ml of distilled water and applied to a DEAE cellulose column (300 mm × 26 mm i.d.) equilibrated with distilled water. Polysaccharides were fractionated, eluted with distilled water, and subjected to different concentrations of stepwise NaCl solutions (0, 0.1, 0.2, and 0.3 M NaCl). To estimate the total carbohydrate content, all fractions were analyzed spectrophotometrically using a phenol-sulfuric acid method [8]. The main homogeneous fraction EPS-2 (2.46 g) was collected and lyophilized. Then, it was dissolved in 40 ml of distilled water and fractionated by size-exclusion chromatography on a Sephadex G-50 column (110 cm × 1.5 cm i.d.) at a flow rate of 0.2 ml/min. All fractions were monitored spectrophotometrically using the phenol-sulfuric acid method. The main fraction EPS-2-1 (1.67 g) was collected, concentrated, and lyophilized. Then, the EPS was dissolved and diluted using 0.1% phosphate-buffered saline for the additional experiments.

Polysaccharide Characterization

Monosaccharide composition. EPS-2-1 was determined by HPLC determination of monosaccharides labeled by 1-phenyl-3-methyl-5-pyrazolone (PMP) [10].

High-performance liquid chromatography (HPLC) analysis of a PMP-derivatized monosaccharide. HPLC analysis was performed by the HPLC system (Perkin-Elmer, Waltham, MA, USA) with a Perkin-Elmer Brownlee Choice ODS column (150 mm × 4.6 mm i.d.). The PMP-derivatized monosaccharide was monitored by an UV detector (Perkin-Elmer) at 245 nm and the flow rate was maintained at 1 ml/min. The sample was eluted with a gradient mobile phase consisting of (A) 0.1 M phosphate buffer (pH 7.0) containing 10% CH_3CN and (B) 0.1 M phosphate buffer (pH 7.0) containing 25% CH_3CN. The gradient was programmed as follows: 0−20 min, 100−80% A; 20−35 min, 80−40% A; 35−40 min, 40−0% A; 40−60 min, 0% A; and hold at 100% A.

Molecular mass determination. The average molecular mass of EPS-2-1 was determined by the Waters HPLC system (GPC Breeze System; Milford, MA, USA) coupled with the Ultrahydrogel Linear (Ultrahydrogel 500, 250, and 120) column (300 mm × 7.8 mm i.d.) and differential refractive index detector (Waters, Milford, MA, USA). Fifty µl of 0.1% sample supplemented with a 0.02 N $NaNO_3$ solution was injected into the system in the mobile phase at 0.8 ml/min (column temperature: 30°C). Pullulans of different molecular masses (788, 404, 212, 120, 47.3, 22.8, 11.8, and 5.9 kDa) were used as standard markers.

Methylation analysis. A methylation analysis was performed by the method described by Ciucanu and Kerek [6]. The methylation of EPS-2-1 (5.0 mg) was repeated using powered NaOH dissolved in DMSO-CH_3I three times until the O-H stretching vibration (3,500 to 3,200/cm) of the IR spectrum had disappeared. The methylated EPS-2-1 was acid-hydrolyzed with 2 M TFA, followed by successive reduction with $NaBH_4$ and acetylation with acetic anhydride−pyridine (1:1) at room temperature for 16 h [26, 27], yielding their corresponding methylated alditol acetates for a GC/MS analysis. The resulting methylated alditol acetates were identified by their typical electron impact breakdown profiles and retention times [13]. The gas chromatography (GC)−mass spectrum (MS) used in the analysis was the Hewlett-Packard model 6890 (Palo Alto, CA, USA) and Agilent 5973N MSD (Santa Clara, CA,USA) system coupled with a fused silica capillary column (30 m × 0.25 mm i.d.). Helium was used as the carrier gas. The temperature was programmed from 50°C to 220°C at 40°C intervals/min [2].

Nuclear magnetic resonance (NMR) analysis. The ^1H and ^{13}C NMR spectra, including two-dimensional (2D) NMR, were recorded on a DRX-500 spectrophotometer (Bruker, Madison, WI, USA). EPS-2-1 (50 mg) was dissolved in D_2O (0.4 ml) and analyzed at 60°C. The chemical shifts are expressed in δ (ppm) values relative to HOD (δ_H 4.816) as an internal reference for ^1H NMR and to acetone chemical shifts (δ_C 31.08) as an external reference for ^{13}C NMR.

Cell Cultures

The effect of purified EPS on immunomodulatory mediators of a Bcl-2 family member was analyzed using U937 human leukemia cells, which were purchased from the American Type Culture

Table 1. Primers for RT-PCR used in this study.

Primers		Sequences
Bax	Forward	5'-ATGGACGGGTCCGGGGAG-3'
	Reverse	5'-TCAGCCCATCTTCTTCCA-3'
Bcl-2	Forward	5'-CAGCTGCACCTGACG-3'
	Reverse	5'-ATGCACCTACCCAGC-3'
Bcl-xL	Forward	5'-CGGGCATTCAGTGACCTGAC-3'
	Reverse	5'-TCAGGAACCAGCGGTTGAAG-3'
GAPDH	Forward	5'-CGGAGTCAACGGATTTGGTCGTAT-3'
	Reverse	5'-AGCCTTCTCVATGCTGGTGAAGAC-3'

Collection (Rockville, USA). The cells were incubated in 24-well tissue culture plates at 37°C in a 5% CO_2 incubator to form a confluent monolayer using a Dulbecco' modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, and supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. EPS-2-1 was added to the U937 monolayer (9×10^4 cells in 18 mm tissue culture plates) and incubated for 24 h at 37°C in a CO_2 incubator. The cells were harvested, washed using an ice-cold phosphate-buffered saline (PBS) solution, and fixed by 3.7% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) dissolved in PBS for 10 min. The fixed cells were washed with PBS and stained by a 4,6-diamidino-2-phenylindile (Sigma Chemical Co.) solution for 10 min.

Reverse Transcription-Polymerase Chain Reaction (RT−PCR)

Total RNA was prepared from the U937 cells using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The synthesis of cDNA from the cells was performed by using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The primer sequences of Bax, Bcl-2, Bcl-xL, and GAPDH genes are listed in Table 1. The cDNA products from the genes were amplified by repeating 35 cycles, each cycle consisting of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, respectively.

Western Blot Analysis

Total cellular proteins were isolated by the lysis of the cells for 20 min in an ice-cold lysis buffer solution (20 mM sucrose, 1 mM EDTA, 20 μM Tris-Cl, pH 7.2, 1 mM DTT, 10 mM KCl, 1.5 mM $MgCl_2$, 5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). The protein concentrations were estimated by a Bio-Rad protein assay (Bio-Rad Lab., Hercules, CA, USA), according to the supplier's instructions. For the Western blot analysis, an equal amount of the proteins were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred by electroblotting to a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). The blots were probed with the desired antibodies, incubated with the diluted enzyme-linked secondary antibody, and visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL, USA). The antibodies that were used to detect Bax and Bcl-xL were rabbit monoclonal. The antibodies for the detection of Bcl-2 and Bad proteins were mouse monoclonal. Goat monoclonal antibody was used to detect β-actin protein. All the antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The dilution rate of the antibodies was 1:500. The peroxidase-labeled donkey anti-rabbit immunoglobulin (dilution rate 1:4,000) and peroxidase-labeled sheep anti-mouse immunoglobulin (dilution rate 1:4,500) were purchased from Amersham Corp. (Arlington Heights, IL, USA).

RESULTS AND DISCUSSION

Isolation, Purification, and Properties of EPS-2-1

According to the phenol-sulfuric acid method, the EPS purified from *L. sulphureus* contained 98.9% of carbohydrates and no absorbance was observed under the UV_{280nm} spectrophotometer, indicating that it was not contaminated with other substances including proteins [16]. The EPS was purified by a DEAE cellulose column with gradient elution using 0−0.3 M NaCl solutions. As a result, the yields of four fractions, designated as EPS-1, EPS-2, EPS-3, and EPS-4, were 0.10, 2.46, 0.61, and 0.24 g, respectively. The main fraction EPS-2 was further purified by size-exclusion chromatography on a Sephadex G-50 column, yielding EPS-2-1 (1.67 g), EPS-2-2 (0.21 g), and EPS-2-3 (0.54 g). The molecular mass of the main fraction, EPS-2-1, was approximately 6.95 kDa (data not shown).

Monosaccharide Composition and Linkage Analysis of EPS-2-1

The monosaccharide composition of EPS-2-1 was determined by a PMP-derivatization method of sugar using a HPLC analysis technique [29]. EPS-2-1 was hydrolyzed with TFA and the hydrolysate was PMP-derivatized. HPLC

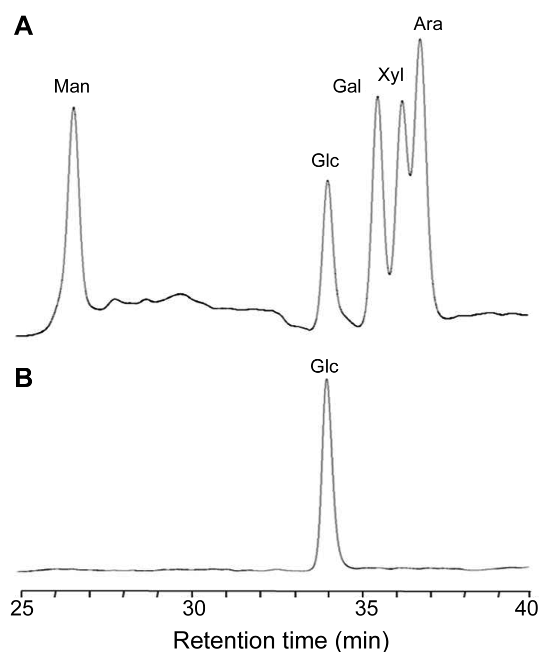

Fig. 2. HPLC profiles of a PMP-derivatized monosaccharide.
(**A**) Standard sugar. (**B**) Acidic hydrolysate of EPS-2-1.

Table 2. GC and GC−MS data for alditol acetates of the methylated EPS-2-1 purified from *L. sulphureus* mycelia.

Retention time (min)	Molar ratios[a]	Methylated sugar[b]	Mass fragments (*m/z*)	Linkage type
19.77	0.68	2,3,4,6-Me$_4$-Glc*p*	43, 71, 87, 101, 117, 129, 145, 161, 205	1→
21.28	2.01	2, 3,6-Me$_3$-Glc*p*	43, 45, 71, 87, 99, 101, 117, 129, 161, 173, 233	1, 4→
22.84	0.35	2,3-Me$_2$-Glc*p*	43, 58, 85, 87, 101, 117, 127, 142, 159, 161, 201, 261	1, 4, 6→

[a]Molar ratio was calculated with the peak area. [b]2,3,4,6-Me$_4$-Glc*p* means 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol.

analysis suggested that EPS-2-1 consisted of only glucose as a monosaccharide unit (Fig. 2).

A linkage analysis was performed by full methylation, reduction, and acetylation of EPS-2-1, and GC/MS analysis yielded three types of glucitol derivatives with a relative molar ratio of 0.68:2.01:0.35 corresponding to 2,3,4,6-Me$_4$-Glc*p*, 2,3,6-Me$_4$-Glc*p*, and 2,3-Me$_4$-Glc*p* (Table 2). This indicated that the monosaccharide residues of the EPS-2-1 moiety consisted of nonreducing Glc*p*-(1→) residues, →4-Glc*p*-1→ linked residues (main chain or side chain), and small amounts of →4,6-Glc*p*-1→ linked residues (branch point). These results suggest that EPS-2-1 is a polysaccharide in which the main chain consisted of (1→4)-Glc*p* units, with branches at the C-6 position of a main chain consisting of a Glc*p*-(1→4)-linked side chain.

The chemical structure of EPS-2-1 resembles that of amylopectin, which is composed of linearly linked α-1,4-linked glucose units with occasional α-1,6 glycosidic bonds in branching points [17]. Each amylopectin molecule may contain 100,000−200,000 glucose units and each branch is about 20 or 30 glucose units in length. Amylopectin molecules may contain up to two million glucose units. The chemical structure of EPS-2-1 also resembles that of glycogen, which is the main storage carbohydrate of mammals [17]. Glycogen is mainly composed of α-1,4 glycosidic linkages and forms an α-1,6 glycosidic linkage with another glucose at every 12−14 glucose subunits. Although EPS-2-1 is a polymer of α-D-glucose, it is distinguished from amylopectin and glycogen in that, as shown in Table 2, branches in EPS-2-1 are much shorter (about 6 glucose units) and more frequent.

NMR Analysis of EPS-2-1

As shown in Fig. 3, ^{1}H NMR data of EPS-2-1 suggested that two main peaks at δ_H 5.843 ppm and δ_H 5.466 ppm corresponded to an anomeric proton of the →4-Glc*p*-1 linked residue (main chain or side chain) and →6-Glc*p*-1 linked residue (side chain), respectively. The chemical shifts that ranged from δ_H 4.0 ppm to δ_H 4.5 ppm are designated as H2 to H6 on a glucopyranosidic ring [5]. In addition, the anomeric configuration of →4-Glc*p*-1→ and →6-Glc*p*-1→ linked residues were present in the α-conformation by a coupling constant at $J_{1,2}$ 2.5 Hz and $J_{1,2}$ 3.0 Hz, respectively [24].

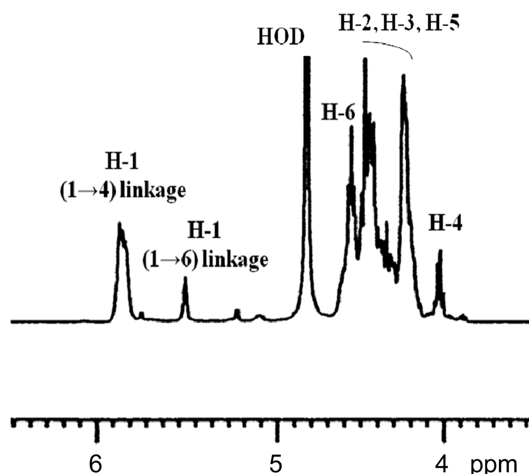

Fig. 3. The 500 MHz ^{1}H NMR spectrum of EPS-2-1.
The spectrum was recorded in D_2O, and values of chemical shifts were recorded with respect to the HOD signal fixed at 4.816 ppm at 80°C.

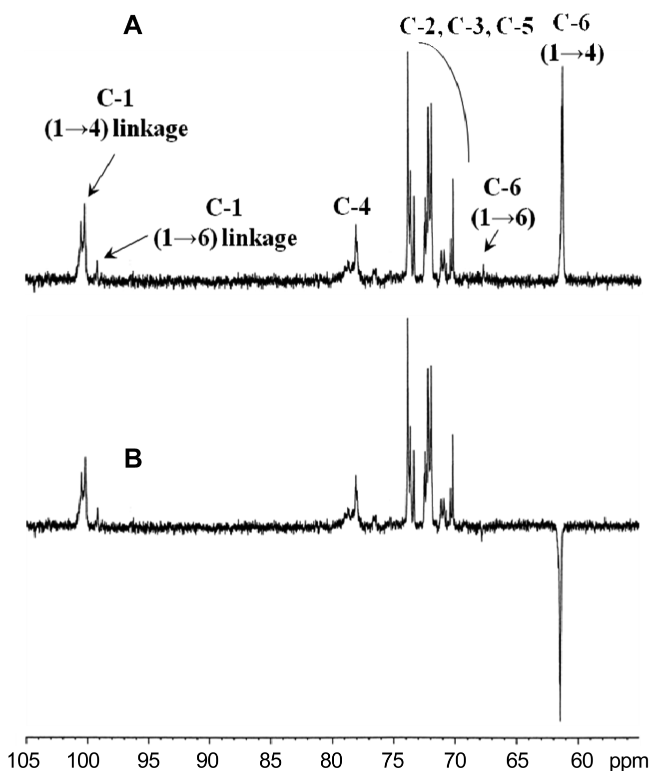

Fig. 4. The 125 MHz ^{13}C NMR (**A**) and DEPT spectrum (**B**) of EPS-2-1.
The spectra were recorded in D_2O and values of chemical shifts were recorded with respect to the acetone signal fixed at 30.08 ppm at 80°C.

Table 3. ^{1}H and ^{13}C NMR chemical shifts of EPS-2-1.

Residues	Chemical shift (δ, ppm)						
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6a/C-6	H-6b
α-D-Glc*p*-(1→	5.82	4.09	4.22	4.24	4.07	4.34	4.37
	100.28	72.12	73.48	73.97	72.27	61.40	-
→4)-α-D-Glc*p*-(1→	5.84	4.14	4.24	4.14	4.45	4.28	4.34
	100.61	72.31	72.06	78.02	73.97	61.47	-
→4)-α-D-Glc*p*-(1→	5.47	4.14	4.26	4.12	4.32	4.39	4.50
	99.48	72.27	72.06	78.07	73.75	67.85	-

The ^{13}C NMR and DEPT data of EPS-2-1 were further analyzed (Fig. 4). The ^{13}C NMR spectrum of EPS-2-1 was compared with that of α-D-glucans [28]. Two main peaks at δ_C 100.28 ppm and δ_C 99.48 ppm corresponded to the anomeric proton of the →4-Glc*p*-1 linked residue (main chain or side chain) and →6-Glc*p*-1 linked residue (side chain), respectively. In addition, the chemical shifts in the high field, 61−68 ppm, were also investigated by distortionless enhancement by polarization transfer pulse sequence (DEPT). As a result, the methylene carbon of Glc*p* showed opposite amplitudes to the methylene carbon [4]. The carbon peaks at 61.47 and 67.85 ppm were present in the C-6 of 1,4-linked and 1,6-linked Glc*p*, respectively. The C-6 of 1,6-linked Glc*p* was downfield at 5−9 ppm, compared

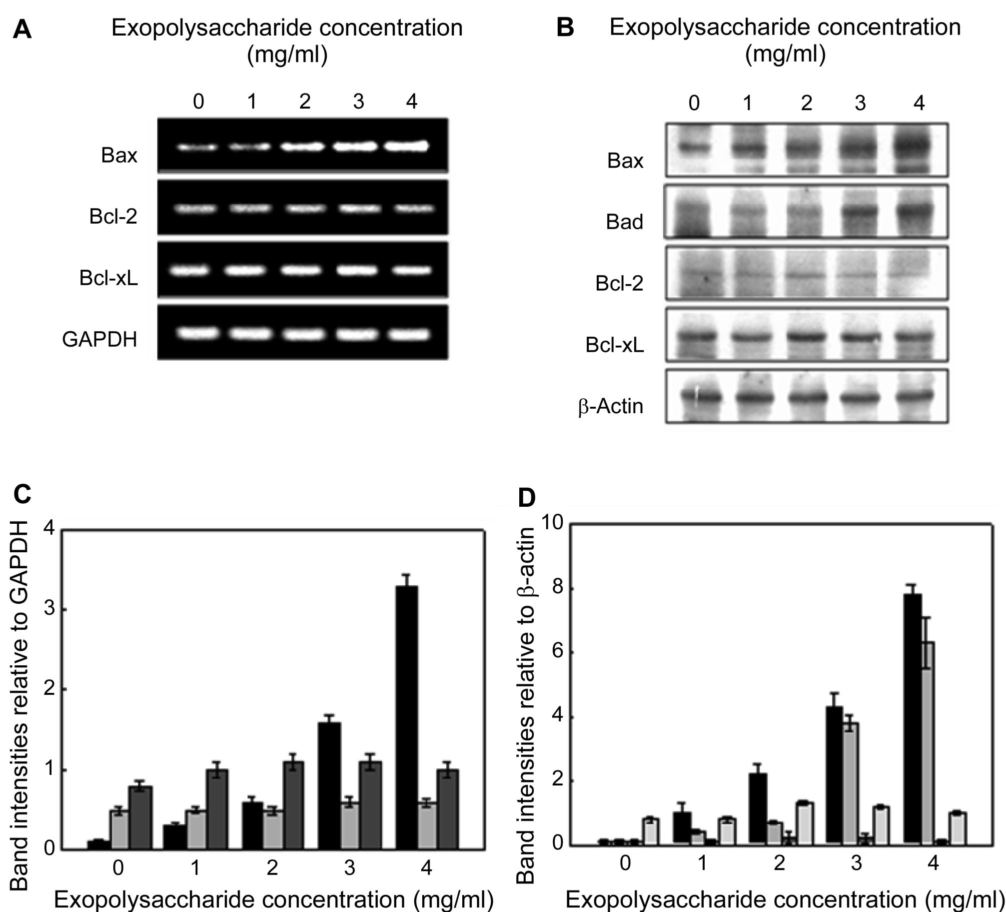

Fig. 5. RT-PCR and Western blotting of the Bcl-2 family members.

(**A**) RT-PCR of Bax, Bcl-2, Bcl-xL, and GAPDH genes. (**B**) Western blotting of Bax, Bad, Bcl-2, Bcl-xL, and β-actin proteins. (**C**) Quantification of Bax (■), Bcl-2 (■), and Bcl-xL (■) genes. (**D**) Quantification of Bax (■), Bad (■), Bcl-2 (■), and Bcl-xL (■) proteins. The values represent the polysaccharide concentration added to the cells. Measurement of the band intensity was performed using PD-Quest 2D software, version 7.3.1.07 (Bio-Rad, CA, USA). The gene expression and protein levels were normalized to GAPDH and β-actin, respectively. Data are expressed as the mean±SD of three independent experiments.

with that of 1,4-linked Glc*p*. Other signals in the ^{1}H and ^{13}C NMR spectra were compared with other known data [1] and were assigned on the basis of the 2D NMR correlation [25]. The resulting ^{1}H and ^{13}C NMR chemical shifts are summarized in Table 3.

Effect of the EPS on Immunomodulating Genes and Proteins in Cultured U937 Cells

To identify the effect of mediators on the immunomodulatiing process, we conducted a RT-PCR analysis on cDNA derived from human leukemia U937 cells. The RT-PCR analysis, as shown in Fig. 5A and 5C, indicated that no significant change was observed in the expression of cell proliferation factors, Bcl-2 and Bcl-xL genes, on the treatment of 4 mg/ml EPS, whereas the expression level of the Bax gene was significantly increased. Of note, the expression level of the Bax gene treated with 4 mg/ml EPS was about 12-times higher compared with that of non-treated cells.

Western blotting was conducted to confirm the specific proteins of the Bcl-2 family associated with immunomodulation. The levels of Bax and Bad proteins were increased in a concentration-dependent manner (Fig. 5B and 5D). Notably, the protein levels of Bax and Bad treated with the 4 mg/ml EPS were approximately 23- and 18-times higher than those in non-treated cells. Therefore, this study supports that the EPS produced by the *L. sulphureus* mycelia may have a significant effect on the genes, contributing to the immunomodulation of the Bcl-2 family members.

In conclusion, the EPS was purified from mycelial cultures of *Laetiporus sulphureus* by DEAE cellulose and Sephadex G-50 column chromatography. The purified EPS was composed of only glucose residues of 6.95 kDa molecular mass. Structural analysis suggests that EPS-2-1 contained a main chain consisting of (1→4)-Glc*p* units, with branches at the C-6 position consisting of a -Glc*p*-(1→4)-linked side chain. Specifically, the levels of Bax and Bad proteins treated with the EPS (4 mg/ml) in cultured human leukemia U937 cells were approximately 23- and 18-times higher than those in non-treated cells, respectively. Therefore, these studies may support that EPS purified from *L. sulphureus* mycelia has a crucial effect on the apoptosis mediators Bax and Bad, thereby contributing to the immunomodulation of Bcl-2 family members.

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