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NOTE

Initial Acidic pH Is Critical for Mycelial Cultures and Functional Exopolysaccharide Production of an Edible Mushroom, *Laetiporus sulphureus* var. *miniatus* JM 27[§]

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We conducted a time course experiment on mycelial cultures of *Laetiporus sulphureus* var. *miniatus*. The strain showed significant survival in an initial pH range of 2.0 to 7.0 for 24 days, during which time oxalic acid was accumulated. A structural analysis of purified exopolysaccharide suggested that it contained 96.1% glucose, and the mode of linkage was mainly→4-Glc*p*-(1→units, with branches at the C-6 position consisting of a Glc*p*-(1→4) linked side chain. An exopolysaccharide purified from the acidophilic strain was added to cultured U937 cells, resulting in significantly increased transcription levels of p53 and p21 genes.

***Keywords*:** mushroom, *Laetiporus sulphureus* var. *miniatus*, oxalic acid, p53, U937

Mushrooms have been widely used in nutritional and medicinal sciences (Mizuno, 1993) because they contain a variety of metabolites, such as phenolic compounds, polyketides, terpenes, and steroids (Cook and Samman, 1996). Mushroom polysaccharides have beneficial effects on the human body because they modulate the immune system and have antithrombotic, antibiotic, and anti-carcinogenic properties (Jong and Birmingham, 1993). Since the genus *Laetiporus* was first described by Murrill (1904), at least 12 species have been found (Burdsall and Banik, 2001). *L. sulphureus* is an easily cultivatable basidiomycete that grows on the living and dead trunks of hardwood tree species in the northern regions of Asian countries (Takeo and Matsuzaki, 1983). Although a structural analysis of polysaccharides generated from the fruiting bodies of the basidiomycete has been conducted (Alquini *et al.*, 2004), little is known about the structure of exopolysaccharides generated by mycelial cultures of *L. sulphureus*. In a time course experiment, we investigated the effect of the initial culture pH on the exopolysaccharide generated by submerged cultures of *L. sulphureus* var. *miniatus*. We also carried out purification and structural analyse on the exopolysaccharide produced and observed its effect on p53 and p21 genes involved in immuno-modulation (Waldman *et al*., 1995; Fridman and Lowe, 2003; Levine and Oren, 2009).

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The mycelium of *L. sulphureus* var. *miniatus* JM 27 (Hwang *et al*., 2008) was cultured in a complete mushroom medium consisting of maltose (30 g/L), bacteriological peptone (5 g/L), and $MgSO_4 \cdot 7H_2O$ (0.5 g/L). Experiments were performed in submerged cultures were performed using 250-ml Erlenmeyer flasks on an orbital shaker set at 100 rpm at 25°C. After the mycelial cultures were centrifuged at 10,000 rpm for 20 min, the resulting pellet was dissolved in deionized water and disrupted using an electric mixer, separating the exopolysaccharide extract from the microbial cells. The extract was harvested and mixed with three volumes of absolute ethanol. Protein denaturation of the strain was performed by evaporating the mixture at 95°C for 3 h. Size-exclusion chromategraphy was conducted on a Sephadex G-50 column (110 cm×1.5 cm i.d) to elute the exopolysaccharide with distilled water at a flow rate of 0.2 ml/min. Each fraction was monitored spectrophotometrically using the phenol-sulfuric acid method (Dubois *et al.*, 1956), and the eluted fractions were collected, concentrated, and lyophilized. Methylation experiments for the linkage analysis of sugars were performed with the Ciucanu and Kerek method (1984). The average molecular weight of exopolysaccharide was determined with the Adams method (2008).

We investigated the effect of the mushroom exopolysaccharide on human leukemia U937 cells, which were purchased from the American Type Culture Collection (USA). To produce a confluent monolayer, the cells were incubated in 24-well tissue culture plates, using a Dulbecco' modified Eagle's medium

supplemented with 10% (v/v) heat-inactivated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO_2 incubator. The purified exopolysaccharide was added to the U937 monolayer (9×10^4 cells in 18-mm tissue culture plates), and the culture was incubated in a CO_2 incubator for 24 h at 37°C. The cells were harvested, washed with an ice-cold phosphate-buffered saline solution (PBS), and fixed with a 3.7% paraformaldehyde (Sigma Chemical Co., USA) solution in PBS for 10 min. The fixed cells were washed by PBS three times and stained with a 4,6-diamidino-2-phenylindile (Sigma, USA) solution for 10 min. Their apoptotic morphology was analyzed with fluorescence microscopy (Carl Zeiss, Germany).

Total RNA from the cultured U937 cells was prepared using an RNeasy Mini kit (QIAGEN, USA). For RT-PCR, the synthesis of cDNA extracted from the cells was performed using the Superscript First-Strand Synthesis System (Invitrogen, USA), according to the manufacturer's instructions. The primers of p53, p21, and GAPDH genes used in RT-PCR were as follows: p53-F (5'-GAGCGCTTCGAGATGTTCCGAGAGC-3'), p53-R (5'-GTCTGAGTCAGGCCCTTCTGTCTTG-3'), p21-F (5'-CTCAGAGGAGGCGCCATG-3'), p21-R (5'-GGG CGGATTAGGGCTTCC-3'), GAPDH-F (5'-CGGAGTCAAC GGATTTGGTCGTAT-3'), and GAPDH-R (5'-AGCCTTCTC VATGCTGGTGAAGAC-3'). Amplification of the cDNA products was carried out in 0.2 ml tubes using a PCR Thermal Cycler (TaKaRa, Japan) according to the following steps: 35 cycles each at 95°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec. The PCR products were purified using a PCR Purification kit (QIAGEN, Germany).

The optimal growth pH for mushrooms is generally considered to be pH 4-8 (Bae *et al*., 2000; van Rensburg and du Preez, 2007). The mushroom, *L. sulphureus* var. *miniatus* JM 27 actively grew in the initial culture of pH 2.0-4.0, however, the growth of the strain was dramatically reduced in cultures with initial pH values over 5.0 (Supplementary data Fig. 1). To observe the effect of initial culture pH on the growth and exopolysaccharide production of *L. sulphureus* var. *miniatus* JM 27, the mycelial cultures of the strain were analyzed at a constant time interval of 2 days for 24 days. As shown in Fig. 1A, the cultures with initial pH values of 2.0, 3.0, and 4.0 produced maximum dry weights of 7.3, 5.0, and 3.9 g/L of dry cell weight at the maximum, respectively, while the cultures with initial pH values of 5.0, 6.0, and 7.0 produced maximum dry cell weights of approximately 1.2, 0.3, and 2.1 g/L, respectively. The cultures with the lower three initial pH values produced about 2.2, 2.3, and 2.7 g/L of exopolysaccharide, respectively, while the cultures with the higher three initial pH values generated only 0.3, 0.2, and 0.4 g/L of exopolysaccharide, respectively (Fig. 1B). The mycelial cultures with initial pH values of 2.0, 3.0, and 4.0 showed a constant pH range between 2.0 and 4.0 (Fig. 1C). These results may suggest that a considerable amount of oxalic acid was accumulated in the mycelial cultures, contributing to the acidic pH conditions (Supplementary data Fig. 2). The results described above may reflect that this strain needs to grow in and maintain a certain range of acidic pH values for survival and exopolysaccharide production. In particular, an acidic environment with pH values ranging from 2.0 to 4.0, which was correlated with the accumulation of oxalic acid, may provide the most favorable conditions for the growth and survival of *L. sulphureus*, contributing to exopolysaccharide

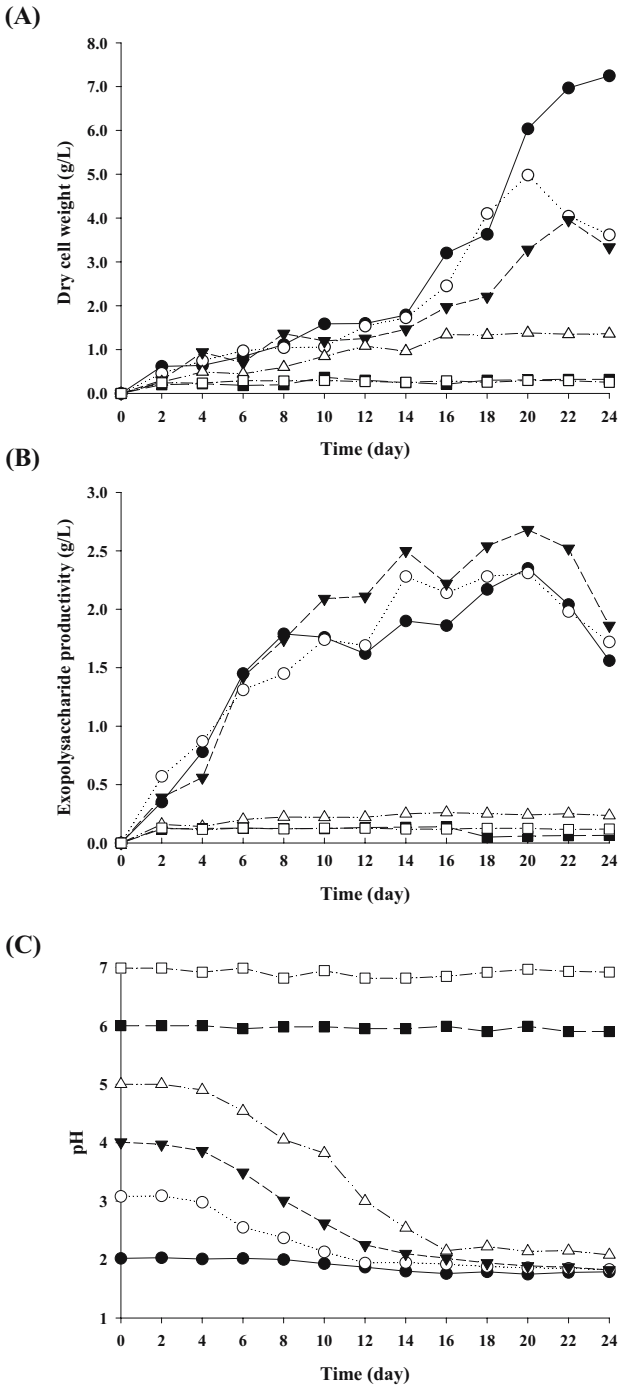

Fig. 1. Production of exopolysaccharide from submerged mycelia cultures of *L. sulphureus* var. *miniatus* JM 27. (A) pH profile, (B) Growth profile, (C) Profile for exopolysaccharide production. (●) pH 2.0, (○) pH 3.0, (▼) pH 4.0, (△) pH 5.0, (■) pH 6.0, and (□) pH 7.0. Clark and Lubs, Glycine-HCl, citric acid-Na_2HPO_4 (Mcllvaine), citric sodium Citrate, phosphate, and potassium phosphate buffers were used to adjust pH values 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0, respectively. Data are expressed as the Mean±SD of duplicate determinations in three separate experiments.

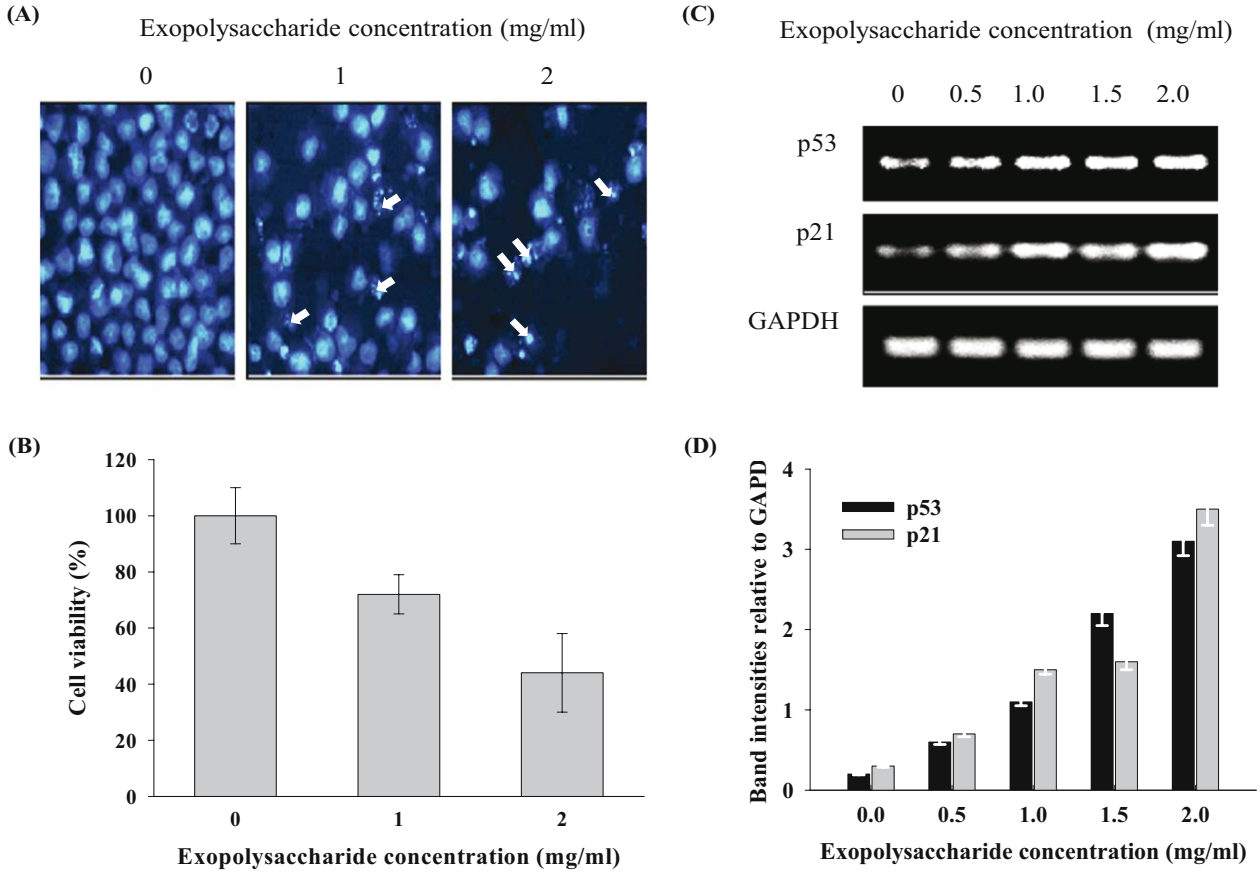

Fig. 2. Effect of purified exopolysaccharide on cultured U937 cells (A, B) and RT-PCR of immunomodulation-conferring p53 and p21 genes (C, D). (A) treatment of purified exopolysaccharide on U937 cells. After being treated with the exopolysaccharide for 48 h, the cells were fixed and stained with DAPI. Nuclei stained with DAPI were then photographed with a fluorescent microscope using a blue filter (original magnification 400×), (B) quantification of the viable cells, (C) RT-PCR of p53, p21, and GAPDH genes, (D) quantification of the gene expression levels. The values represent the polysaccharide concentration added to the cells. In Figs. C-D, p53 and p21 genes are represented using symbols (■) and (■), respectively. Measurement of the band intensity was performed using PD-Quest 2-D software, version 7.3.1.07 (Bio-Rad). The levels of gene transcription were normalized to GAPDH. Data are expressed as the Mean+SD for three independent experiments.

production in the mycelial culture.

A HPLC chromatogram showed that the exopolysaccharide purified from the mycelial culture contained 96.1% glucose, 2.0% mannose, 1.0% galactose, and 0.8% arabinose (Supplementary data Fig. 3). No absorbance was estimated at 280 nm in the UV spectrophotometer. This indicated that the exopolysaccharide was not contaminated with other substances, including proteins (Kobayashi and Kim, 2003). A linkage analysis was performed with full methylation, reduction, and acetylation of purified polysaccharide. The mode of linkage was mainly →4)-Glcp-(1→ units, with branches at the C-6 position consisting of a Glcp-(1→4) linked side chain. A GC/MS analysis showed that the relative molar ratio of purified polysaccharide corresponding to 2,3,4,6-Me_4-Glcp, 2,3,6-Me_3-Glcp, and 2,3-Me_2-Glcp was 28.5:57.1:10.6 (Table 1). An analysis of GPC data suggests that the molecular mass of purified exopolysaccharide is 0.5-0.8×10^4 Da (data not shown). The chemical structure of polysaccharide purified from the *L. sulphureus* mycelia was distinguished from that of the fruiting bodies of *L. sulphureus* in that the major linkage type of polysaccharide occurring in the mycelia includes→4)-Glcp-(1→units. This linkage feature is similar to the chemical structure of polysaccharide from the mycelia of the basidiomycete *Flammulia velutipes* (Pang *et al*., 2007).

The effect of purified exopolysaccharide on the viability of cultured human leukemia U937 cells was observed using

Table 1. GC-MS data for alditol acetates of the methylated exopolysaccharide purified from *L. sulphureus* var. *miniatus* JM 27

Methylated sugar (as alditol acetate)	Ratio (%)	Mode of linkage
2,3,4,6-Me_4-Glc*p*	28.5	Glc*p*-(1→
2,3,6-Me_3-Glc*p*	57.1	→4)-Glc*p*-(1→
2,3-Me_2-Glc*p*	10.6	→4,6)-Glc*p*-(1→
2,3,4-Me_3-Gal*p*	1.0	→6)-Gal*p*-(1→
2,3,4-Me_3-Ara*p*	0.8	Ara*f*-(1→
2,4,6-Me_3-Man*p*	2.0	→3)-Man*p*-(1→

[a] Molar ratio was calculated with a 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

fluorescence microscopy. Untreated cells showed a fairly typical round morphology (Panel a in Fig. 2A, whereas the cells exposed to 1 and 2 mg/ml of exopolysaccharide displayed the appearance of apoptotic bodies and condensation of chromatin (Panels b-c in Fig. 2A). In comparison with the untreated cells, the viability of U937 cells exposed to 2 mg/ml of exopolysaccharide was reduced to 58%, indicating that it may have an inhibitory effect on cell viability (Fig. 2B). RT-PCR analysis showed that the expression levels of the immuno-modulation genes p53 and p21 in the U937 cells exposed to 2 mg/ml of exopolysaccharide were significantly higher than those in non-treated cells (Figs. 2C-D). This finding may suggest that the exopolysaccharide purified from the mycelial cultures of a mushroom, *L. sulphureus* var. *miniatus* performs a functional activity in the cell, since it increased the transcription of p53, a gene involved in tumor suppression, and p21, a gene that encodes a potent cyclin-dependent inhibitor (CKI) and regulates cell cycle progression (Waldman *et al*., 1995; Seoane *et al*., 2002).

In conclusion, we conducted a time course experiment of *L. sulphureus* var. *miniatus* using submarine cultures. Mycelial growth and exopolysaccharide production were maximized in cultures with a pH range of 2.0 to 4.0. We suggest that a considerable amount of oxalic acid accumulated in the mycelial cultures contributes to the acidic environment. The chemical structure of purified polysaccharide consisted of a main chain containing Glc*p*-(1→4) units with branches at the C-6 position of the chain carrying Glc*p*-(1→4) linked residues. The treatment of the cells with the mushroom exopoly-saccharide significantly increased the transcription levels of the p53 and p21 genes. This study, which found that a mushroom exopolysaccharide contributed to the the immuno-modulation of p51 and p21 genes, may contribute to the growing sciences studying therapeutic potential mushrooms.

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