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# Exopolysaccharide Produced by *Pediococcus acidilactici* M76 Isolated from the Korean Traditional Rice Wine, *Makgeolli*

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**This work is aimed to increase knowledge of the functional exopolysaccharide (EPS) from lactic acid bacteria (LAB) in *makgeolli*, a Korean fermented rice wine. Among LAB strains isolated from *makgeolli*, strain M76 was selected as a functional strain producing a bioactive EPS, based on its antioxidative activity on the DPPH radical. The 16S rRNA gene sequencing analysis showed a high sequence similarity (99.0%) with *P. acidilactici*, but had different biochemical properties with the already known *P. acidilactici* type strains in the aspect of carbohydrates utilization. The obtained *P. acidilactici* M76 produced a soluble EPS above 2 g/l. One-step chromatography using gel filtration after ethanol precipitation from the supernatant of *P. acidilactici* M76 was enough to obtain purified EPS with a single peak, showing a molecular mass of approximately 67 kDa. Componential and structural analyses of EPS by TLC, HPLC, and FT-IR indicated that the EPS is a glucan, consisting of glucose units. The purified EPS had antioxidant activity on the DPPH radical of 45.8% at a concentration of 1 mg/ml. The purified EPS also showed proliferative effect on the pancreatic RIN-m5F cell line and remarkable protection activity on alloxan-induced cytotoxicity. This potent antioxidant and antidiabetic EPS by LAB in *makgeolli* may contribute to understanding the functionality of *makgeolli*.**

**Key words:** Exopolysaccharide, *Pediococcus acidilactici*, makgeolli, RIN-m5F cell, antioxidant activity

Recently, lactic acid bacteria (LAB) have received attention for their exopolysaccharides (EPSs) producing ability, indicating that a broad range of EPSs from LAB with variable functionality can be applicable for a wide range of industries [3]. EPS obtained from LAB have been known as functional materials for exhibiting sensory properties that can influence the organoleptic characteristics of various foods including beverage. In addition, EPSs play important roles in human health owing to their antitumor [4], antioxidant [1], anti-ulcer [23], antibacterial [6], immune-stimulating [16], blood glucose-regulating [5], and UV radiation-protecting activities [30]. However, owing to the lack of expansive knowledge on EPSs from the food-associated LABs, EPSs have remained largely underexploited.

*Makgeolli* is a traditional Korean rice wine, fermented with rice as the glucose source and *nuruk* as a starter culture for glycolysis and alcoholic fermentation. Unlike other alcoholic beverages, *makgeolli* has highly nutritious and functional properties, because of its bioactive compounds, various phenols, and biogenic amine produced by microorganisms, plus sugars, proteins, vitamins, and various organic acids [14]. Traditionally, *nuruk* is prepared by the natural solid fermentation of a rice or wheat source, and used as the starter culture for *makgeolli* fermentation, allowing for the complex mixed growth of fungi, yeasts, and bacteria. During the simultaneous two-step fermentation process, the microbial community secretes various amylolytic enzymes for converting starch into fermentable glucose, and follows alcoholic fermentation by yeast to produce ethanol of approximately 15–18% [13, 25]. *Makgeolli* is also kept at acidic pH 3.4–4.5 by LAB [12]. Throughout the fermentation process, LAB carry out acid fermentation to accumulate various organic acids, resulting in a sourish-sweet taste like milk punch, and significantly affect the taste and texture in the quality of the fermented *makgeolli* [13, 15]. Often, *makgeolli* contains turbid ingredients (especially abundant saccharides) by the rough filtration process of the fermented mash, creating the appropriate LAB habitat for more microbial population in the aging period than wine or beer [14]. Until now, various fermented alcoholic beverage studies have been carried out on the

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LAB diversity and their roles in grape wines or Japanese rice wines [7, 9]. However, little study has been done regarding the LAB diversity in Korean rice wines. Several strains have been introduced in the genera of LAB isolated from Korean rice wines, such as *Lactobacillus, Leuconostoc*, *Enterococcus*, *Lactococcu*s, and *Pediococcus* sp. [13, 25]. Despite the reports on the distribution of LAB, no systematic approach has been made on their role in *makgeolli*, in regard to the functional aspects.

With the aim of increasing knowledge of the functional EPS from LAB in *makgeolli*, we have isolated LAB from *makgeolli*. Among the LABs, a crude EPS was obtained and examined for its antioxidant activity to find a functionally active EPS. The selected EPS was further purified by using gel filtration chromatography and its properties characterized concerning functional and physiological properties. Moreover, we also examined the effects of the EPS on rat pancreatic β-cell proliferation and its protection activity on alloxan-induced cytotoxicity, since diabetes mellitus, a chronic metabolic disorder, continues to present major health problems worldwide.

## Materials and Methods

### Isolation of LAB from *Makgeolli*

*Makgeolli* used in this study was manufactured using rice and *nuruk*, following the traditional method of the Jeonju Korean Traditional Wine Museum (http://urisul.net/). It was made without the use of commercially available yeasts and fungi. For LAB isolation from the *makgeolli*, 10 ml of the sample was decimally diluted in 90 ml of sterile 0.85% NaCl solution, and homogenized by vigorous vortexing (Vortex Genie 2; Scientific Industries, USA). Then 100 µl of diluted samples was respectively inoculated onto de Man, Rogosa and Sharpe (MRS) agar (Difco, Detroit, MI, USA), *Enterococcus* agar (Difco, Detroit, MI, USA), and *Lactobacillus* anaerobic MRS with vancomycin and bromocresol green agar (Lamva, MRS agar adjusted to pH 5.0 with 0.05 g cystein-HCl, 0.005 g bromocresol green, and 2 mg vancomycin per liter) plates. After incubating at 37°C for 2 days, the isolates were purified by successive streaking on MRS agar before subjecting to further experiments.

### Screening of Functional EPS-Producing Strain

To screen the functionally active EPS-producing LAB among the isolated LABs, crude EPS was collected from the broth culture of each strain by following the methods of Song *et al*. [27] with slight modifications. The cultivation was conducted in a 250 ml flask containing 50 ml of MRS broth, containing 5% (w/v) of glucose, adjusted to pH 7.0 at 37°C on a rotary shaker incubator (Model LSI-3016R; Daihan LabTech, Namyangju, Korea), at 150 rpm for 2 days. After the incubation, the culture broth was heated at 100°C for 20 min to inactivate the enzymes responsible for EPS degradation. Into the cell-free extract obtained by centrifugation (1580MGR; Gyrozen, Daejeon, Korea) at 10,000 ×*g* for 20 min, trichloroacetic acid was added to remove protein components. By re-centrifugation, the protein-free supernatant was collected and then three volumes of cold ethanol was added. The remaining solvent was removed after centrifugation, and the precipitate was collected as crude EPS produced by each strain. The residual sugar from the culture broth was determined by the dinitrosalicylic acid (DNS) method [20]. Meanwhile, the precipitated pellet was washed twice with saline and dried in a hot air oven at 90°C until a constant weight, in order to measure the cell mass. The dry weights of cell and EPS were expressed in g/l.

For functional screening, the obtained crude EPS was lyophilized with a freeze drier (PVTFD100R; Ilshin, Yangjugun, Korea) and each crude EPS was evaluated by antioxidant activity on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical spectrophotometrically, according to the method of Blois [2] with slight modifications. Briefly, 0.5 ml of the crude EPS sample at a concentration of 1.0 mg/ml was mixed with 2.0 ml of 0.2 mM DPPH solution dissolved in methanol. The mixture was shaken vigorously and left to stand for 30 min at 37°C in the dark. Next, the absorbance of the resulting solution was measured at 517 nm. The DPPH radical scavenging activity was calculated using the following formula: DPPH radical scavenging activity (%) = $[(A_0-A_1/A_0)\times100]$, where $A_0$ is the absorbance of the control, and $A_1$ is the absorbance of the test sample.

### Identification of the Isolated LAB

Identification of the selected LAB (M76) was done by 16S rDNA sequence analysis. Each colony was suspended with 60 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) and then boiled at 99°C for 10 min. After centrifugation (15,000 ×*g*, 5 min, 4°C), PCR was done on the obtained crude total genomic DNA using the *Taq* DNA polymerase (GeneAll, Seoul, Korea) with the universal primers 27F (5'-AGT TTG ATC CTG GCT CAG-3') and 1490R (5'-GTT ACC TTG TTA CGA CTT C-3'), in a PCR cycler (Primus 25; MWG-Biotech, Ebersberg, Germany) under the following conditions: 1 cycle of 2 min at 95°C; 30 cycles of 1 min at 95°C, 30 s at 54°C, and 1 min at 72°C; and 1 cycle of 5 min at 72°C. To confirm the PCR amplification, the PCR product was separated on a 1.2% agarose gel in 1× TBE buffer by gel electrophoresis. After staining with 1 mM ethidium bromide, the bands were visualized using the ChemiDoc XRS Imaging System (Bio-Rad, CA, USA).

The confirmed PCR product was directly sequenced on an ABI PRISM 3700 DNA analyzer and analyzed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). For the biochemical test, API 20E (BioMerieux, Marcy l'Etoile, France) was also used per the manufacturer's instructions to assist identification.

### Purification of EPS

For characterization of the EPS produced by the selected strain M76, the partially purified crude soluble EPS by ethanol as described above was further purified by gel-filtration chromatography. The purification was performed by using the Bio-Logic fast protein liquid chromatography system (FPLC; BioRad, CA, USA) with the Econo-column (1.5 cm × 14 cm; BioRad, CA, US) packed with a Sephadex G-200 column (GE Healthcare, Freiburg, Germany). A 0.15 M NaCl solution was used as the mobile phase at a flow rate of 1.0 ml/min. To confirm the EPS in each fraction, the fractionated sample was analyzed by the phenol-sulfuric acid method at 490 nm [18]. After collecting all EPS-containing fractions, dialysis was carried out using a dialysis membrane (3,500 MW cutoff, Thermo-Scientific, MA, USA) for 24 h at 4°C against sterile distilled water. An amount of the purified EPS sample was calculated as the dry weight after freeze drying in a lyophilizer at -18°C. The sample was used for further studies.

### Characterization of EPS

**Molecular weight of EPS.** The molecular weight of the EPS was estimated by gel-filtration chromatography with a Sephadex G-200 and 0.15 M NaCl solution as the mobile phase at a flow rate of 1.0 ml/min. To confirm the EPS in each fraction, the fraction was analyzed by the phenol-sulfuric acid method. The molecular weight of the EPS was calculated with blue dextran and a gel filtration standard (GE Healthcare, NJ, USA) to calibrate the relationship between retention time and molecular weight.

**Monosaccharide composition.** The purified EPS (10 mg) was hydrolyzed with 1 ml of 2 N trifluoroacetic acid by heating at 100°C for 6 h. The resulting hydrolysate was diluted with the same volume of distilled water and neutralized with 1 N NaOH solution after cooling at room temperature. The remaining solution was filtered by a membrane filter (0.45 μm; Advantec, Toyo, Japan) before analysis. To determine the monosaccharide component of the hydrolysate, TLC was performed on silica gel 60 F254 plates (Merck, Darmstadt, Germany) with *n*-butanol/2-propanol/water/acetic acid [7:5:4:2 (v/v/v/v)] as a developing solvent system. After the development process was performed twice, the developed spots were visualized by spraying the plates with ethanol/sulfuric acid/*p*-anisaldehyde [18:1:1 (v/v/v)] solution following heating of the plates at 120°C.

The hydrolysate was also analyzed using the Agilent 1100 Series HPLC system (Agilent, CA, USA) with a Supelcosil LC-$NH_2$ 4.6 mm × 25 cm column (Supelco, Bellefonte, PA, USA) at room temperature and an Alltech 500 ELSD detector (Alltech, IL, USA) at 55°C. HPLC-grade water/acetonitril [25:75 (v/v)] was used as the mobile phase for isocratic elution at a flow rate of 1.0 ml/min.

**FT-IR analysis.** The structure of the EPS sample was determined by Fourier transform infrared (FT-IR) spectrometry. The sample was ground with spectroscopic-grade potassium bromide (KBr) powder and then pressed into 1 mm pellets for FT-IR measurement. The FT-IR spectrum of the composite was recorded using a Bruker IFS 66v FT-IR spectrophotometer (Bruker, Karlsruhe, Germany) at the absorbance mode from 4,000 to 400 $cm^1$ with 32 accumulated scans.

### Antidiabetes Effects of EPS on RIN-m5F Cells

**Cell culture condition.** The insulin-secreting rat insulinoma cell line (RIN-m5F) was obtained from the American Type Culture Collection (ATCC, MD, USA). The obtained RIN-m5F cells were cultured in RPMI 1640 medium (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, New York, USA) and 1% penicillin-streptomycin solution 10,000 units/ml (Hyclone, Utah, USA) as the antibiotic in the presence or absence of glucose. Before screening, RIN-m5F cells were maintained at 37°C in a humidified $CO_2$ incubator (Model MCO-19AIC; Tokyo, Japan), supplied with 5% $CO_2$. Cells were passaged at regular intervals of 3–4 days when they reached a confluency of 70–80%. For the assay, the EZ-Cytox Enhanced Cell Viability Assay kit (Daeil, Seoul, Korea) was used.

**Proliferative activity of EPS on RIN-m5F cell.** The proliferative activity of the EPS produced by strain M76 was evaluated according to the assay with RIN-m5F cells. Cells were seeded in the 96-wells plate at a density of $1 \times 10^4$ cells/well in 100 μl of RPMI-1640 medium containing 10% FBS and 1% antibiotic. After incubating overnight, the prepared samples at different concentrations (0.1, 1, 2, 5, 10, and 20 μg/ml of purified EPS) were added and incubated for 48 h to investigate its proliferative effect on RIN-m5F cell viability. After refreshing the medium at every 24 h for 48 h, RIN-m5F cell viability was analyzed by measuring the absorbance at 420–480 nm, using the EZ-Cytox Enhanced Cell Viability Assay kit.

**Cytoprotection activity of EPS on alloxan induced cytotoxicity.** RIN-m5F cells were cultured in the 96-wells plate at a density of $2 \times 10^5$ cells, using 100 μl of RPMI-1640 medium containing 10% FBS and 1% antibiotic, overnight. After discarding the medium by aspiration, the grown cells were replenished with fresh culture medium containing different doses of the purified EPS (1, 2, 5, 10, and 20 μg/ml) and incubated further for 24 h. After 24 h, the culture medium was replaced again with the identical procedure above, and further incubated for 1 h. Then, the cells were treated with 10 or 15 mM of alloxan and followed further incubation for 1 h. After washing twice with fresh culture medium, a cell viability assay was done per the protocol provided by the manufacturer.

### Statistical Evaluation

Data were analyzed by one-way ANOVA using SPSS version 16.0. All analyses were performed in triplicates, and the results were expressed as the mean ± standard deviation (SD). The differences among groups were assessed using Duncan's multiple range tests. Statistical significance was defined as $p < 0.05$. Different letters (*e.g.*, a–e) were assigned to significantly different groups, and the same letters were assigned to similar groups.

## RESULTS AND DISCUSSION

### Isolation of Functional EPS-Producing LAB from *Makgeolli*

The number of LAB strains isolated from *makgeolli* was log 4.63 CFU/ml on MRS agar medium, log 4.73 CFU/ml on Lamva agar medium, and log 5.26 CFU/ml on *Enterococcus* agar medium, respectively. Among them, 54 isolates were screened as EPS-producing LAB forming a mucoidal colony. To find the functionally active EPS-producing strain, we obtained the crude EPS extracts and examined their antioxidant activity as a first screening for functional EPS, due to its significant relationship with other activities.

Among the examined 54 strains, strain M76 showed the highest DPPH radical scavenging activity of 48.1% at a concentration of 1 mg/ml. The scavenging activity of the EPS produced by strain M76 increased proportionally with the sample concentration ranging from 0.5 to 5.0 mg/ml (Fig. 1A). Five milligram per milliliter of the EPS showed significantly higher scavenging activity against the DPPH radical at 54.2%.

### Identification of Strain M76

Since the crude EPS from *P. acidilactici* M76 at the first step of screening showed higher antioxidative activity compared with those from other strains, we selected this strain for further experiments. The 16S rRNA gene sequencing analysis showed a similarity of 99%, 97%, 98%, and 97% with *Pediococcus pentosaceus, P. claussenii, P. stilesii,* and *P. argentinicus*, respectively. The highest

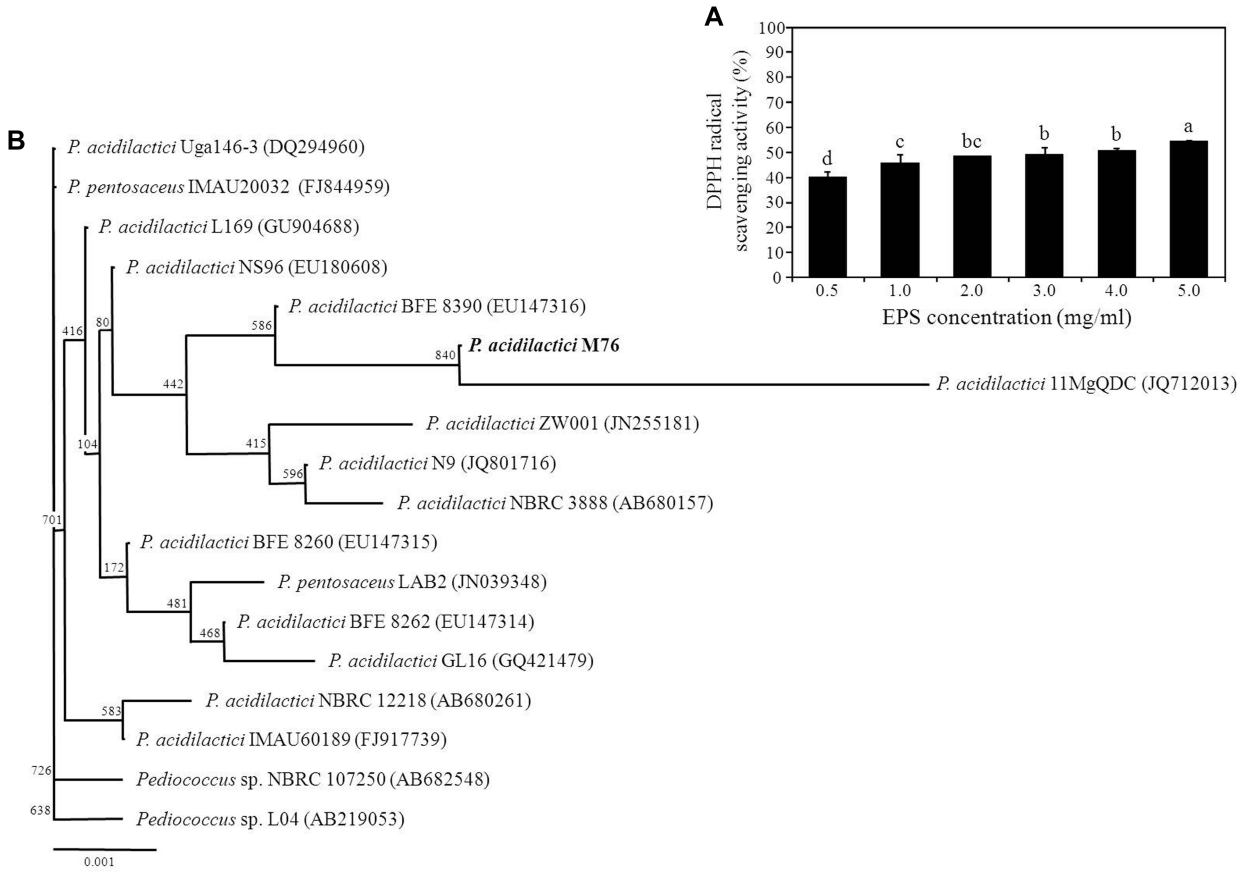


**Fig. 1.** Scavenging activity against DPPH radical of crude EPS produced by the strain M76, $p < 0.001$ (**A**) and a phylogenetic tree, based on 16S rDNA sequence analysis, showing the taxonomic position of isolate M76 with members of *Pediococcus* (**B**).

sequence similarity (99.0%) was shown with *P. acidilactici*, as shown in Fig. 1B, indicating the isolated strain M76 might be closely related with *P. acidilactici*.

However, when the biochemical test using the API kit was done, it showed different biochemical properties with already known *P. acidilactici* type strains in the aspect of carbohydrates utilization, such as galactose (+), ribose (+), glycerol (+), trehalose (+), gluconate (+), maltose (-), sorbose (+), erythritol (+), and xylose (+). This result indicates that the isolated *P. acidilactici* M76 from *makgeolli* must be a different strain to the already known *P. acidilactici* strains. *P. acidilactici* M76 is deposited in the Korean Agricultural Culture Collection (KACC, Suwon, Korea) under the accession number KACC 91683P as a novel strain.

Previously, *L. paracasei* and *L. arixonensis* were identified as the major populations existing in *makgeolli*, whereas minor populations included *L. plantarum*, *L. harbinensis*, *L. parabuchneri*, and *L. brevis*, among others [13]. Seo *et al*. [25] reported the major population in *makgeolli* as *L. plantarum*. It is difficult to identify the major dominant LAB strain of *makgeolli* fermentation, owing to the varying conditions of the manufacturing process and regional differences of the prepared microbial source of *nuruk*. However, interestingly, one recent study has found that the major LAB population in freshly stored *makgeolli* at 4°C is *Pediococcus* sp., whereas the major population in *makgeolli* stored at 20°C, which may lead to spoilage, is *Lactobacillus* sp. [13]. This result indicates that *Pediococcus* sp. may contribute a positive effect to *makgeolli* fermentation. Thus, we selected 40 isolates randomly from the obtained LAB to determine the distribution of the LAB, identified by 16S rRNA gene sequencing analysis; the distribution of the strains were as follows: *Pediococcus* sp. (53%) > *Lactobacillus* sp. (40%) > others (7%) including *Staphylococcus* sp. and *Enterococcus* sp. This result is in good agreement that *Pediococcus* sp. might be a dominant strain in traditional *makgeolli* fermentation and may contribute its functionality.

## Recovery of EPS from Strain M76

To examine the EPS productivity of the selected M76 strain, fermentation was conducted. As shown in Table 1, the *P. acidilactici* M76 strain can produce above 2,048 mg/l

**Table 1.** Fermentation[a] kinetics of *P. acidilactici* M76.

| Kinetic parameters | Values |
|---|---|
| Maximum biomass concentration, X (g/l) | 6.21 |
| Maximum EPS concentration , P (mg/l) | 2,048.3 |
| Specific growth rate, m ($h^{-1}$) | 0.13 |
| Productivity for EPS, $Q_P$ (mg $l^{-1}$ $h^{-1}$) | 42.7 |
| Productivity for biomass, $Q_X$ (g $l^{-1}$ $h^{-1}$) | 129.4 |
| Specific product yield, $Y_{P/X}$ (mg EPS per g biomass) | 329.7 |
| Product yield, $Y_{P/S}$ (mg EPS per g glucose) | 146.8 |
| Cell yield, $Y_{X/S}$ (g biomass per g glucose) | 0.45 |
| Specific production rate of EPS, $Y_{P/S}$ (mg EPS per g glucose $h^{-1}$) | 30.6 |

[a]Fermentation condition: 37°C at 150 rpm for 48 h.

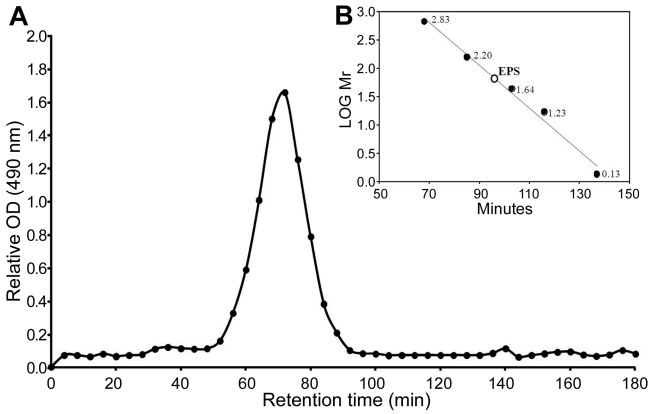


**Fig. 2.** GFC chromatogram (**A**) and molecular mass estimation (**B**) of EPS using Sephadex G-200.
The apparent profile of EPS was detection by the phenol-sulfuric acid method at 490 nm. The molecular mass of the EPS was calculated with thyroglobulin (670 kDa), bovine gamma-globulin (158 kDa), chicken ovalbumin (44 kDa), equine myogolbin (17 kDa), and vitamin B12 (1.35 kDa) as standards.

of soluble EPS for 48 h in the given fermentation condition, showing 42.7 mg/l/h of EPS productivity. According to the several reports on *Pediococcus* strains, some strains such as *P. pentosaceus* and *P. parvulus* from beverages can produce EPS (Table 2) [11, 24, 26, 29, 31]. Velasco *et al*. [29] reported that *P. parvulus* 2.6 strain isolated from Basque natural ciders produced 140 mg/l of EPS for 96 h with 50 g/l of glucose source. Walling *et al*. [31] reported on *P. damnosus* IOEB8801, isolated from spoiled wine, producing 102 mg/l of EPS with 20 g/l of glucose for 15 days. Semjonovs and Zikmanis [24] also reported that *P. pentosaceus* P773 isolated from a spoiled beer produced 3,900 mg/l of EPS with 100 g/l of sucrose source for 48 h. Compared with the above results, our *P. acidilactici* M76 produced a relatively significant amount of EPS, indicating the EPS production level is enough for industrial application. Moreover, when we examined the EPS concentration in *makgeolli*, we found that approximately 8 mg/l of EPS in the *makgeolli* sample, indicating that the EPS can be successfully produced by *P. acidilactici* strain M76 during the *makgeolli* fermentation period.

## Characterization of EPS Produced by *P. acidilactici* M76

**Molecular mass of EPS.** The molecular mass estimated by gel filtration chromatography was around 67.2 kDa, as shown in Fig. 2. It has been reported that the EPS derived from various LAB is usually from 10 to 2,700 kDa [28]. Moreover, EPSs from *Pediococcus* strains until now have a molecular mass ranging from 2,000 to 10,000 kDa, showing 10,240 kDa of EPS by *P. damnosus* IOEB8801 [31] or 2,000 kDa of EPS by *P. pentosaceus* P773 [24]. Thus, the EPS from *P. acidilactici* M76 has a relatively lower molecular mass than EPSs of other LAB strains including *Pediococcus* sp.

**Monosaccharide composition of EPS.** The monosaccharide composition of the EPS produced by *P. acidilactici* M76 was determined by TLC and HPLC-ELSD analysis. Based on the results of TLC analysis (Fig. 3A), the sulfuric acid

**Table 2.** Exopolysaccharide production by different strains of *Pediococcus* sp. from foods.

| IS[a] | Strain | CS (g/l) | CT | EP (mg/l) | MW (kDa) | References |
|---|---|---|---|---|---|---|
| Rice wine | *P. acidilactici* M76 | Glu(20)[b] | 2 days | 2,048 | 67.2 | This study |
| Ropy and malted cider | *P. parvulus* CUPV1 | Glu(50) | 4 days | 233 | 10,200 | [11] |
| | CUPV22 | Glu(50) | 4 days | 228 | 10,500 | |
| | CUPV23 | Glu(50) | 4 days | 243 | 9,900 | |
| Ropy cider | *P. parvulus* 2.6 | Glu(50) | 4 days | 140 | - | [29] |
| Spoiled wine | *P. damnosus* IOEB8801 | Glu(20) | 15 days | 102 | - | [31] |
| Spoiled beer | *P. pentosaceus* P773 | Suc(100)[c] | 2 days | 3,900 | 2,000 | [24] |
| Pork sausage | *P. pentosaceus* AP-1 | Suc(20) | - | 6,000 | 14 | [26] |
| | AP-3 | Suc(20) | - | 2,500 | 6,000 | |

[a]IS, Isolation source; CS, Carbon source; CT, cultural time; EP, exopolysaccharide production; MW, molecular mass.
[b]Glu, glucose.
[c]Suc, sucrose.

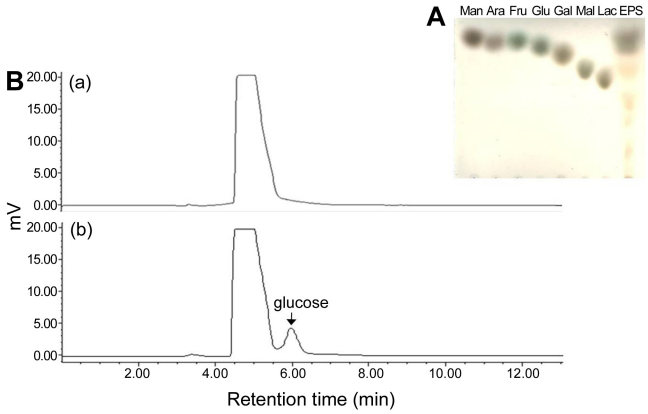


**Fig. 3.** Chromatograms of TLC (**A**) and HPLC-ELSD (**B**) analysis of monosaccharide composition of EPS produced by *P. acidilactici* M76.
Each chromatogram of the hydrolysis product with water (**a**) and the hydrolysis product with purified EPS (**b**) was presented by HPLC-ELSD: arabinose, 5.2 min; fructose, 5.7 min; glucose, 6.0 min; galactose, 6.8 min; maltose, 9.1 min; and lactose, 9.8 min, as retention times of standards.

hydrolysate of the EPS showed a single apparent spot on TLC ($R_f$ = 0.545), indicating the identical $R_f$ value to glucose ($R_f$ = 0.548). To reconfirm the monosaccharide composition of the hydrolysate of EPS, HPLC-ELSD analysis was carried out with a $NH_2$ column, resulting in the glucose peak shown in Fig. 3B. All together, it seems that the EPS from *P. acidilactici* M76 may be a glucan comprising the glucose monomer only. Previously, it was reported that the EPS synthesized by a *P. pentosaceus* P 773 strain was mainly composed of glucose and fructose residues at a ratio 3:1, respectively [24], whereas the EPSs synthesized by *P. parvulus* 2.6 and *P. damnosus* IOEB8801 strains were reported as glucan having only a single constituent glucose molecule [29, 31].

## FT-IR Analysis

In order to examine the structure of the EPS produced by *P. acidilactici* M76, FT-IR analysis was carried out (Fig. 4). As expected, FT-IR analysis showed a carbohydrate structure characterized by a broad absorption band at the 3,492 $cm^{-1}$ region, and it assigned the hydroxyl stretching vibration of the polysaccharide, indicating a strong O-H band. The two bands at 2,932 $cm^{-1}$ and 2,892 $cm^{-1}$ indicate a C-H stretching band, assigned to the C-H asymmetric stretch and C-H symmetric stretch of $CH_2$ and $CH_3$ groups, respectively. The absorption at 1,383 $cm^{-1}$ also indicates C-H band variation. In addition to the characteristic carbon-hydrogen absorptions, the attributions are also related to vibrations of the O=C-O structure (1,670 and 1,230 $cm^{-1}$) and strong C-O bending bands (at 1,230 and 1,028 $cm^{-1}$) by glycosidic bond vibration of the EPS (attributed to asymmetrical and symmetrical stretching vibrations of the carboxylic group). Conclusively, the EPS obtained in this

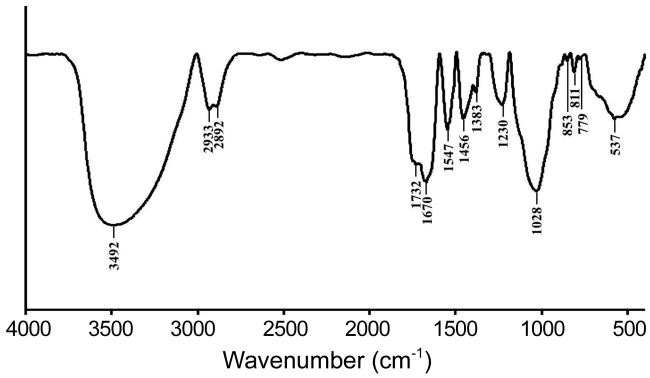


**Fig. 4**. Structural analysis of the EPS produced by *P. acidilactici* M76 by FT-IR.

study from *P. acidilactici* M76 is assigned to glucose residues [10, 17, 18, 21, 22].

## Activity of EPS from *P. acidilactici* M76

For the functional activity of the purified EPS, we first examined its effects on β-cell proliferation by an MTT assay. As shown in Fig. 5, the EPS produced by *P. acidilactici* M76 showed a positive activity on RIN-m5F cell proliferation in a dose-dependent manner, ranged from 0.1 to 20 μg/ml. A significantly high percentage viability of 124% ($p < 0.001$) was observed at a dose of 20 μg/ml, indicating that pancreatic function improved in the insulin secretion with the EPS produced by *P. acidilactici* strain M76.

We also examine the cytoprotection activity on the alloxan-induced cytotoxicity of the purified EPS from *P. acidilactici* M76. Since alloxan is a prompt and potent inducer of diabetes in experimental animals due to its damaging effects on insulin-producing β-cells by inducing

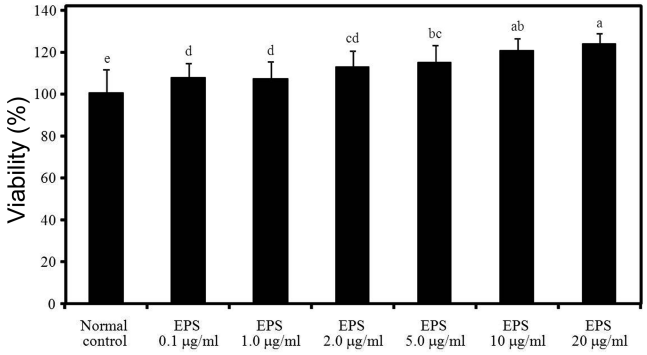


**Fig. 5.** Effects of proliferation on insulin secretion rat insulinoma cell line (RIN-m5F) of EPS produced by *P. acidilactici* M76.
The cells ($1\times10^4$ cells/well) were treated with purified EPS sample (0.1, 1, 2, 5, 10, or 20 μg/ml) and incubated for 48 h at 37°C and 5% $CO_2$. The RIN-m5F viability was detected by an EZ-Cytox Enhanced Cell Viability Assay kit. The quantitative data were calculated as means ± SD; control versus treatment groups, $p < 0.001$.

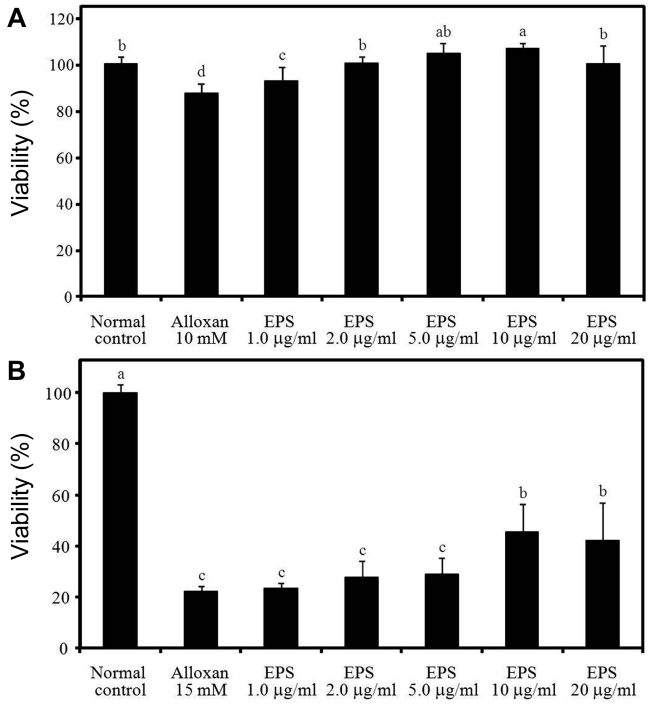


**Fig. 6.** Protective effect of EPS on damage of the RIN-m5F cell by alloxan.

RIN-m5F cells were preincubated with different concentrations (1, 2, 5, 10, or 20 μg/ml) of EPS for 24 h, and then alloxan of 10 mM (**A**) or 15 mM (**B**) was added to the culture medium for 1 h except the control group. The RIN-m5F viability was detected by an EZ-Cytox Enhanced Cell Viability Assay kit. Control group vs. alloxan group vs. alloxan groups after treating EPS, $p < 0.001$.

oxygen free-radical and inhibiting the enzyme activity of glucokinase and hexokinase *etc*., its inhibition activity usually showed potent antidiabetic effects [32]. As expected, alloxan administration to the isolated insulinoma cell line for 1 h caused dose-dependent toxicity, showing viabilities of 87.7% at 10 mM and 21.9% at 15 mM. However, when we supplied our EPS purified from the culture of *P. acidilactici* M76, the damage by alloxan on the prepared RIN-m5F cells was decreased in a dose-dependent manner (Fig. 6). With higher alloxan treatment of 15 mM, the supplied EPS showed more cytoprotective effect significantly ($p < 0.001$). Interestingly, the supplied EPS (10 μg/ml) showed more proliferative effect on RIN-m5F cells compared with the control untreated alloxan, significantly ($p < 0.001$), when RIN-m5F cells were treated with 10 mM alloxan. The supply of EPS at the concentration of 1 to 20 μg/ml increased the cell viability up to 107% and 45.4% from 87.7% and 21.9% in the absence of EPS, respectively. *Makgeolli* must be functionally active for cell viability that is effective for diabetes.

The functional effects of EPS for the diabetes-related protective and hypoglycemic activity have been studied with fungi, particularly EPS extracted from mushroom, whereas there are few studies on EPS produced by bacterial species. The EPS from *Sorangium cellulosum* showed 30% decrease of serum glucose levels on the experimental animals during the trial period, when 200 mg $kg^{-1}$ $day^{-1}$ of EPS was administered orally for 7 days [8]. Meanwhile, the polysaccharide of *Ganoderma lucidum*, Lingzhi mushroom, had protective effects on alloxan-induced pancreatic islets damage, and increased the cell viability from 47.9% to 80.15% in doses of 100 μg/ml concentration [32]. Compared with those results, the EPS from *P. acidilactici* M76 showed similar effects at 10 μg/ml concentration, indicating that the EPS produced by the M76 strain exhibited excellent protective activity on the damaging effects on the insulin-producing cells. The current study reveals that the EPS produced by *P. acidilactici* M76 is non-toxic, biocompatible, and safe for use as a biomaterial for biomedical applications.

Consequently, a *P. acidilactici* M76 strain was isolated from Korean traditional rice wine *makgeolli*, which was able to produce a functionally active EPS in this study. The EPS with the molecular mass of approximately 67 kDa, consisting of a single glucose unit, was able to stimulate the growth of insulin-secreting β-cell and protect the damage of pancreatic RIN-m5F cell lines. It can be inferred, therefore, that the EPS from *P. acidilactici* M76 isolated from *makgeolli* may contribute as a functional ingredient for a variety of functional foods, especially in the diabetes-related field.

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