

# RAW264.7 Cell Activating Glucomannans Extracted from Rhizome of *Polygonatum sibiricum*

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**ABSTRACT:** Water-soluble polysaccharides isolated from the rhizome of *Polygonatum sibiricum* and fractionated using ion-exchange chromatography were investigated to determine their structure and immunostimulating activity. Crude and fractions (F<sub>1</sub> and F<sub>2</sub>) consisted of carbohydrates (85.1 ~ 88.3%) with proteins (4.51 ~ 11.9%) and uronic acid (1.79 ~ 7.47%), and included different levels of mannose (62.3 ~ 76.3%), glucose (15.2 ~ 20.3%), galactose (4.35 ~ 15.3%), and arabinose (4.00 ~ 7.65%). The crude contained two peaks with molecular weights (Mw) of  $151 \times 10^3$  and  $31.8 \times 10^3$ , but F<sub>1</sub> and F<sub>2</sub> exhibited one major peak with Mw of  $103 \times 10^3$  and  $628 \times 10^3$ , respectively. Little immunostimulatory activity was observed by the crude; however, F<sub>1</sub> and F<sub>2</sub> significantly activated RAW264.7 cells to release nitric oxide and various cytokines, suggesting they were potent immunostimulators. The backbone of the most immunostimulating fraction (F<sub>1</sub>) was (1→4)-manno- and (1→4)-gluco-pyranosyl residues with galactose and glucose attached to O-6 of manno-pyranoside.

**Keywords:** *Polygonatum sibiricum*, water-soluble polysaccharides, immunostimulation, molecular properties

## INTRODUCTION

The genus *Polygonatum* mostly occurs in the northern temperate zone, containing about 60 species with 39 species in China. They have been used as a functional food and as a well-known Chinese traditional medicine with health effects on removing dryness, promoting fluid secretion, and quenching thirst (1). It has been reported that a variety of chemical components of *Polygonatum* species possess different biological properties, such as antioxidant, anti-inflammatory, anti-diabetic, antitumor, and immunomodulatory activities (2). Liu et al. reported that the water extract of *Polygonatum sibiricum* was capable of reducing blood glucose and lipid levels, regulating and enhancing the immune system, and fighting aging (3). Previous phytochemical studies have shown that *Polygonatum odoratum* contains constituents such as steroidal glycosides, dipeptides, flavonoids, and polysaccharides (4-6). Among the various constituents, polysaccharides are one of the main bioactive components of *P. odoratum* with lowering blood sugar, antioxidant, and anticancer activities (7).

Polysaccharides from medicinal herbs have attracted considerable attention from biochemical and nutritional

researchers due to their potential importance as medicinal and functional food materials. Polysaccharides have therefore been isolated from various herbaceous plants and their bioactivities have been reported. In recent reports, polysaccharides from *P. odoratum* displayed a strong antioxidant activity through considerable 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity and reducing power (8). In addition, polysaccharides from *P. sibiricum* were very effective against the A $\beta$ <sub>25-35</sub>-induced neurotoxicity in PC-12 cells, revealing their plausible effect on the prevention and treatment of Alzheimer's disease (9). Moreover, *P. sibiricum* has been widely used as an ingredient or supplement in the food industry because of its sweet fragrance and taste as well as health-promoting effects (10). Despite their potential application, information on the biological activities and molecular structures of polysaccharides from *Polygonatum* species is limited.

In the current study, the water-soluble polysaccharides were extracted from the rhizome of *P. sibiricum* and purified by ion-exchange chromatography. The objective of this study was to determine the structural characteristics and ultrastructure of polysaccharides and to investigate their immunostimulating activity.

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## MATERIALS AND METHODS

### Materials

*P. sibiricum* roots were purchased from a local market on March 14, 2015 (Gangneung, Korea). The root was carefully washed with tap water, cut to small pieces, and air-dried at 60°C for 24 h. The dried sample was finely milled into a powder and stored at -20°C before the polysaccharide extraction. Cell culture media RPMI1640, penicillin/streptomycin, fetal bovine serum (FBS), and other materials required for culturing cells were purchased from Lonza Inc. (Walkersville, MD, USA). Griess reagent and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Chemicals and reagents used were of analytical grade.

### Extraction of crude polysaccharide

The powdered sample (10 g, dry basis) was treated with 95% ethanol (300 mL) under constant mechanical stirring for 2 h (twice) at room temperature. The sample was centrifuged 4,000 rpm for 15 min at 4°C, and the sediment was dried overnight at 40°C. The dried sample was extracted with hot water at 100°C with stirring for 2 h (twice), then centrifuged at 4,000 rpm for 15 min at 4°C and the supernatant was collected. The supernatant was concentrated by evaporation under reduced pressure at 50°C. Ethanol (99%) was added to obtain a final ethanol concentration of 70% to minimize structural heterogeneity due to exclusion of lower molecular weights (Mw) polysaccharides, and the solution was stirred at room temperature for 2 h. The precipitate was isolated by filtration with a nylon membrane (0.45 µm pore size, Whatman International Ltd., Maidstone, UK), washed with ethanol (99%) and acetone, and then dried at room temperature to obtain the crude polysaccharide.

### Fractionation of crude polysaccharide

The crude polysaccharide (120 mg) was dissolved in distilled water (10 mL), and the solution was injected into a DEAE-Sephacrose fast flow column (17-070-01, GE Healthcare Life Sciences, Ann Arbor, MI, USA) equilibrated with distilled water. The sample was eluted with distilled water and different concentrations of NaCl increasing from 0.5 to 1.5 M at a flow rate of 1.5 mL/min. The 2 major fractions obtained and confirmed by the phenol-sulfuric acid assay (11) were referred to as F<sub>1</sub> and F<sub>2</sub> and were extensively dialyzed against distilled water for 3 days and then lyophilized.

### Analytical methods

The total carbohydrate content of the crude and fractions was determined by the phenol-sulfuric acid assay using a standard solution containing glucose (11). The protein content was measured by the Lowry method using bo-

vine serum albumin (BSA) as a standard (DC protein assay kit, Bio-Rad Laboratories, Hercules, CA, USA). The uronic acid content of the polysaccharide was analyzed by a sulfamate/*m*-hydroxydiphenyl colorimetric assay using glucuronic acid as a standard (12).

### Determination of the monosaccharide composition

The monosaccharide composition was determined according to the method described by Cao et al. (13). Briefly, the polysaccharide was hydrolyzed with 4 M trifluoroacetic acid (TFA) at 100°C for 6 h followed by reduction of the hydrolysates in water using sodium borodeuteride (NaBD<sub>4</sub>) and acetylation with acetic anhydride, and analyzed by gas chromatography-mass spectrometry (GC-MS) (6890N/MSD 5973, Agilent Technologies, Santa Clara, CA, USA). The GC, equipped with a flame ionization detector using a HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm), was programmed to increase the temperature from 180 to 220°C at 4°C/min, and then hold at 220°C for 30 min. The carrier gas was nitrogen, and the flow rate was 30 mL/min. Sugar standards (arabinose, mannose, glucose, and galactose) were used to identify the sugars based on their retention times.

### Determination of Mw

The Mw of the polysaccharide was determined according to a previously reported method (13). Briefly, the polysaccharide (4 mg) was dissolved in distilled water (2 mL), heated at 75°C for 15 min prior to analysis using high-performance size exclusion chromatography (HPSEC) linked to multi-angle laser light scattering (MALLS), and a refractive index (RI) detection system. The HPSEC-MALLS-RI system was composed of a Waters 510 pump (Waters, Milford, MA, USA), Rheodyne® Model 7072 injector (Rheodyne Lp, Cotati, CA, USA) valve with a 200 µL sample loop, a guard column (7.5 × 600 mm; TSK G5000 PW, Tosoh Bioscience, Tokyo, Japan), and a MALLS detector (HELEOS, Wyatt Technology Corp., Yokohama, Japan). A solution of 0.15 M NaNO<sub>3</sub> and 0.02% NaN<sub>3</sub> was used as a mobile phase at a flow rate of 0.4 mL/min. The normalization of the MALLS detector and the determination of volume delay between the MALLS and RI detectors were carried out using BSA ( $dn/dc=0.1850$ ). The calculations of Mw and radius of gyration (R<sub>g</sub>) were carried out using ASTRA version 6 software (Wyatt Technology Corp.).

### Macrophage proliferation and nitric oxide production

Murine macrophage RAW264.7 cells in RPMI-1640 medium containing 10% FBS were plated in 96 well-plate ( $1 \times 10^5$  cells/well; obtained from American Type Culture Collection, Manassas, VA, USA) and incubated with polysaccharides at different concentrations (1, 3, 6, and 12 µg/mL). After the cells were incubated for 24 h in a hu-

modified atmosphere containing 5% CO<sub>2</sub> at 37°C, 20 µL of the water-soluble tetrazolium salts-1 solution was added to each well, and the solution was further incubated for 4 h at 37°C. The optical density was measured at 450 nm using a microplate reader (EL-800, BioTek Instruments Inc., Winooski, VT, USA). The absorbance was translated into macrophage proliferation ratio (%) = (At/Ac) × 100, where At and Ac are the absorbances of the test group and control group, respectively. The nitric oxide (NO) production in the RAW264.7 cell culture supernatant (1 × 10<sup>5</sup> cells/well), incubated in the presence of the polysaccharide at different concentrations (1, 3, 6, and 12 µg/mL) and LPS (1 µg/mL) at 37°C for 24 h, was measured using the Griess reagent (14) as previously described (15). Briefly, RAW264.7 cells (100 µL of 1 × 10<sup>6</sup> cells/mL) were plated in 96-well plates and incubated for 24 h at 37°C in the presence of 5% CO<sub>2</sub>. The cultured cells were treated with sample (100 µL) at concentrations of 1, 3, 6, and 12 µg/mL or LPS (1 µg/mL) which was used as a positive control, and incubated for 24 h at 37°C. The cultured cell supernatant (100 µL) was mixed with an equal volume of Griess reagent (1%, w/v) and incubated at room temperature for 10 min. The absorbance was measured at 540 nm using a microplate reader. The NO production from the cells was calculated with reference to a standard curve obtained with NaNO<sub>2</sub> (1 ~ 200 µM in culture medium).

#### Reverse transcription-polymerase chain reaction (RT-PCR)

To investigate the levels of inducible nitric oxide synthase (iNOS) and cytokines, RAW264.7 cells (1 mL, 1 × 10<sup>5</sup> cells/well) were treated with different concentrations of polysaccharides (1, 3, 6, and 12 µg/mL) at 37°C in the presence of 5% CO<sub>2</sub> for 18 h. The RNA was extracted from the cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and kept at -80°C until use. The concentration of the extracted RNA was measured via a spectrophotometer before constructing cDNA with an oligo(dT)20 primer and Superscript III RT (Invitrogen). The resulting cDNA was amplified by PCR using GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA). Reverse transcriptase amplification was conducted with an initial denaturation at 94°C for 3 min and 30 cycles of denaturation (94°C for 30 s), annealing (56°C for 40 s) and extension (72°C for 1 min) followed by a final extension step at 72°C for 10 min. The products of RT-PCR were separated by gel electrophoresis using 1% agarose gel stained with ethidium bromide, and the gels were viewed under UV transillumination (Kodak Digital Science, Kennesaw, GA, USA). The results were expressed by the relative intensity compared with that of β-actin. The sequences of primer used in this experiment were shown in Table 1.

**Table 1.** Sequences of oligo (dT) primers used for RT-PCR

Gene		Primer sequences (5'→3')
iNOS	Forward	CTGCAGCACTTGGATCAGGAACCTG
	Reverse	GGGAGTAGC CTGTGTGCACCTGGAA
IL-1β	Forward	ATGGCAACTATTCCAGAACTCAACT
	Reverse	CAGGACAGGTATAGATTCTTCTCTTT
IL-6	Forward	TTCCTCTCTGCAAGAGACT
	Reverse	TGTATCTCTCTGAAGGACT
IL-10	Forward	TACCTGGTAGAAGTGATGCC
	Reverse	CATCATGTATGCTTCTATGC
IL-12	Forward	CCACAAAGGAGGCGAGACTC
	Reverse	CTCTACGAGGAACGCACCTT
β-Actin	Forward	ATGTGCAAAAAGCTGGCTTTG
	Reverse	ATTTGTGGTGGATGATGGAGG

#### Glycosidic linkage analysis of F<sub>1</sub> fraction

The F<sub>1</sub> fraction was per-methylated according to the method of Ciucanu and Kerek with slight modifications (16). The samples (3 mg) were dissolved in 0.5 mL of dimethylsulfoxide under nitrogen, and then methylated with 0.3 mL of methyl iodide and 20 mg of dried NaOH powder. Partially methylated alditol acetate was prepared from the fully methylated sample by acid hydrolysis with 4 M TFA at 100°C for 6 h. The hydrolysates were reduced in water with NaBD<sub>4</sub>, and acetylated with acetic anhydride. The derivative, a partially methylated alditol acetate, was analyzed by GC-MS (6890N/MSD 5973, Agilent Technologies). Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min. The oven temperature was programmed to increase from 160 to 210°C in 10 min and 240°C in 10 min at an increase of 5°C/min. The inlet temperature was kept constant at 250°C. The mass range was 35 ~ 450 (m/z), and peak assignments were based on retention time and mass spectra.

#### Nuclear magnetic resonance (NMR) spectroscopy

For structural assignment, the spectrum of the F<sub>1</sub> fraction was recorded on a solution of 10 mg in 0.5 mL D<sub>2</sub>O at 50°C. <sup>1</sup>H was recorded on a JEOL ECA-600 spectrometer (JEOL, Akishima, Japan), equipped with a 5 mm multi-nuclear auto-tuning TH tunable probe (600 MHz for <sup>1</sup>H). Chemical shifts were expressed in ppm.

#### Statistical analysis

A one-way analysis of variance was used to determine the statistical significance of the differences between the various experimental groups. Data are expressed as mean ± standard deviation (SD) values, and the results are taken from at least three independent experiments. *P*-values of ≤ 0.05 were considered to be statistically significant.

**Table 2.** Yield and chemical composition of crude polysaccharide and fractions from *Polygonatum sibiricum*

Sample	Yield (%) <sup>1)</sup>	Total carbohydrate (%)	Uronic acid	Protein (%)	Monosaccharide content (%) <sup>2)</sup>			
					Ara	Man	Glu	Gal
Crude	9.65	85.1±2.85	7.47±0.40	4.51±0.07	5.51±0.35	62.3±1.54	15.9±1.05	15.3±1.00
F <sub>1</sub>	35.6	86.4±0.75	2.94±0.28	11.9±0.10	4.00±0.28	76.3±1.10	15.2±1.10	4.50±0.02
F <sub>2</sub>	26.4	88.3±2.53	1.79±0.21	8.76±0.07	7.65±0.53	67.7±1.26	20.3±0.51	4.35±0.20

<sup>1)</sup>Crude yield, (weight of crude polysaccharide/weight of *P. sibiricum*)×100; F<sub>1</sub> and F<sub>2</sub> yield, (weight of fractions/weight of crude injected into ion-exchange chromatography)×100.

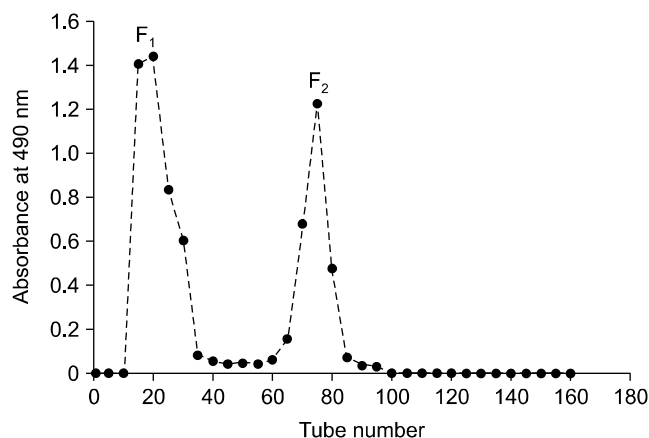
<sup>2)</sup>Ara, arabinose; Man, mannose; Glu, glucose; Gal, galactose.

## RESULTS AND DISCUSSION

### Chemical composition analysis

The yield and chemical composition of the crude and fractionated polysaccharides from the rhizome of *P. sibiricum* are shown in Table 2. The yield of the crude polysaccharide was 9.65%, this being close to the values (7.60~9.30%) reported by Jiang et al. but significantly higher than the value (2.50%) from Zhang et al. (17) exhibiting considerable variation in the yields of the *Polygonatum* polysaccharides (9). Recent studies also reported various yields (7.60~15.4%) of the *Polygonatum* polysaccharides, in which the yields were mainly dependent on extraction time, temperature, and number, and ratio of solvent to solid (8,17). The extracted polysaccharides mostly consisted of carbohydrates (85.1%) with proteins (4.51%) and uronic acids (7.47%). Monosaccharide composition analysis revealed mannose (62.3%) to be the major sugar in the crude polysaccharides with substantial amounts of glucose (15.9%), galactose (15.3%), and arabinose (5.51%), indicating their heterogeneity in the monosaccharide composition. Glucose and mannose were also the major sugars of *P. odoratum* and *P. sibiricum* polysaccharides (12,17); on the other hand, the major monosaccharides of *Polygonatum cyrtoneuma* polysaccharides were fructose and glucose, implying that the considerable variation in the monosaccharide composition might be due to the differences of the species. According to Liu et al. (8) the extraction procedures, such as extraction time, temperature, and pH as well as the use of a hydrolyzing enzyme, were critical factors affecting the monosaccharide composition of the *Polygonatum* polysaccharides.

The crude polysaccharide was fractionated using ion-exchange chromatography, yielding two fractions (F<sub>1</sub> and F<sub>2</sub>) with different levels of ionic strength (Fig. 1). The proximate composition of the fractions is shown in Table 2. The constituents of F<sub>1</sub> and F<sub>2</sub> were mainly carbohydrates (86.4 and 88.3%) and proteins (11.9 and 8.76%) with minor amounts of uronic acids (2.94 and 1.79%). As shown in Table 2, both fractions had similar monosaccharide patterns with major sugars of mannose (76.3 and 67.7%) and glucose (15.2 and 20.3%), containing small amounts of arabinose (4.00 and 7.65%) and galac-

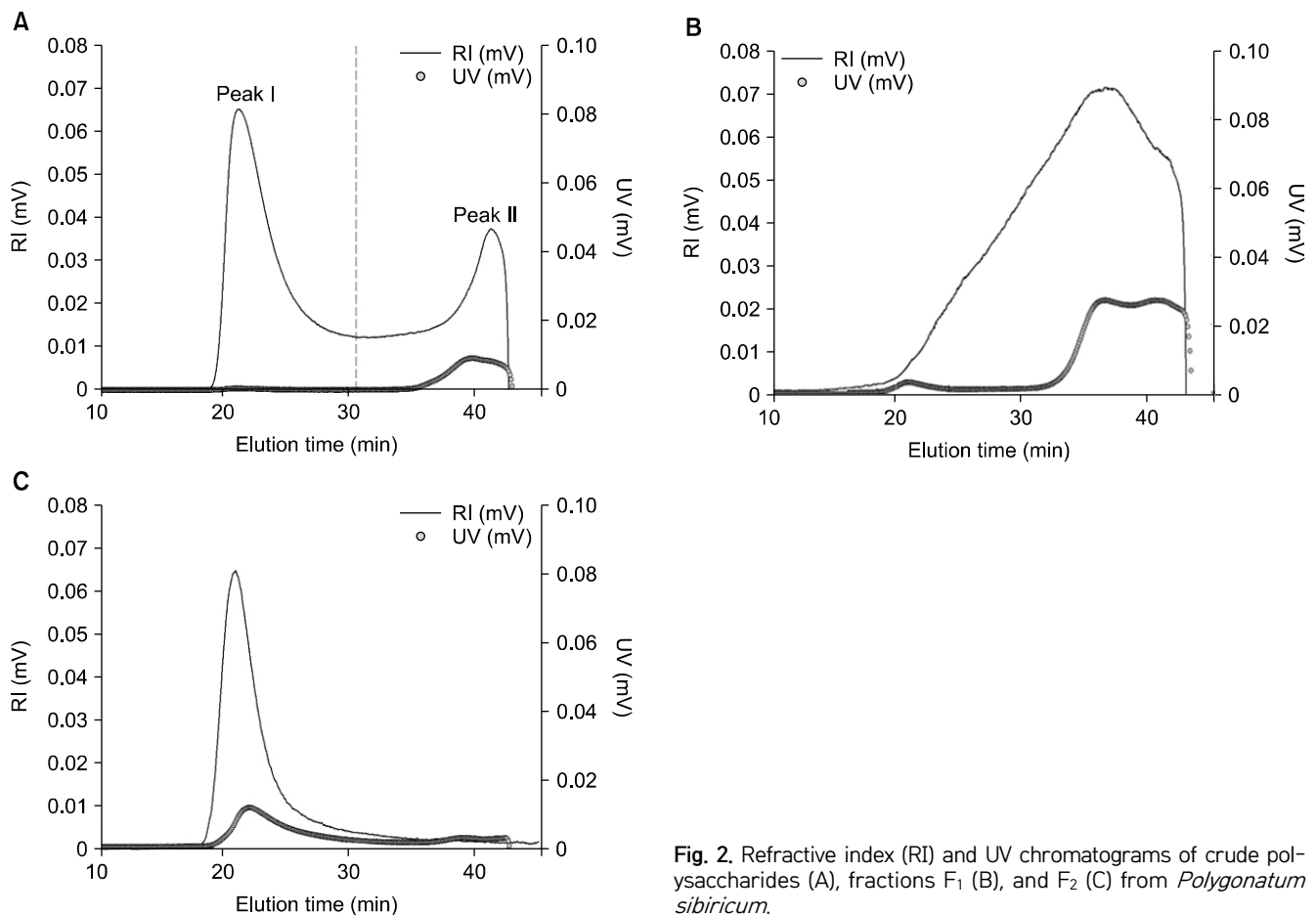


**Fig. 1.** Chromatography of eluted crude polysaccharide from *Polygonatum sibiricum* through DEAE-Sepharose fast flow column.

tose (4.50 and 4.35%), respectively.

### Molecular weights of crude and fractionated polysaccharides

The RI and UV chromatograms for the crude and fractionated polysaccharides are shown in Fig. 2. The crude polysaccharides were eluted from the SEC column between the elution times of 20~43 min with two major peaks, indicating two different polymer distributions (Fig. 2A). Proteins were mostly included in the second peak (peak II) at the elution times from 35 and 43 min as shown by the UV chromatogram. The Mw values of the peaks, obtained by the MALLS technique that provides the absolute Mw of polymers, were  $151 \times 10^3$  and  $31.8 \times 10^3$  g/mol, respectively (Table 3). The Rg of the crude was also calculated from the peaks to determine the approximate size of the polysaccharides (Table 3). The Rg values of the two peaks were 34.7 and 95.7 nm, respectively. Although peak II had significantly lower Mw, the Rg value was greater than that of peak I. This was probably due to differences in the molecular conformation of the polysaccharides, suggesting that the polysaccharides of peak II might exist in an extended conformation while those of peak I might exist in a more compact conformation. This is the first report of Mw and Rg of the water-soluble polysaccharides from *P. sibiricum*. Studies of water-



**Fig. 2.** Refractive index (RI) and UV chromatograms of crude polysaccharides (A), fractions F<sub>1</sub> (B), and F<sub>2</sub> (C) from *Polygonatum sibiricum*.

soluble polysaccharides from other medicinal herb roots, including *Rhynchosia minima*, *Actinidia Chinensis*, *Sanguisorba officinalis*, *Astragalus membranaceus*, and *Gentiana scabra* Bunge have Mw values ranging from  $20.7 \sim 2,000 \times 10^3$  g/mol (18–22), exhibiting significant variation. The variation in Mw might be due to differences not only in the species of the root polysaccharides but also in the extraction, purification, and analytical methods.

Compared to the crude polysaccharides, RI and UV chromatograms of the F<sub>1</sub> and F<sub>2</sub> fractions were different, both showing one major peak at the elution times of 20 min and 43 min for F<sub>1</sub> and 20 min and 30 min for F<sub>2</sub> fraction, respectively (Fig. 2B and 2C). These results indicated that the fractionation of the crude polysaccharides by ion-exchange chromatography successfully produced the polysaccharides with relatively homogeneous distribution. The UV response observed in fraction F<sub>1</sub> and F<sub>2</sub> indicated that a considerable amount of proteins were included in both fractions. This is in agreement with the chemical composition of F<sub>1</sub> and F<sub>2</sub> in which the protein contents were 11.9 and 8.76%, respectively (Table 2). Regarding the Mw values for the two fractions revealed by the elution times in Fig. 2B and 2C, the Mw value of F<sub>1</sub> was significantly lower than that of F<sub>2</sub>, and both are dramatically different from those of the crude polysaccharides (Table 3). In fact, it was unclear why the

proximate composition of F<sub>1</sub> and F<sub>2</sub> were relatively similar after the fractionation of the crude polysaccharides. It is plausible that significant difference in the Mw of the fractions may result in the separation of the fractions. The Rg values of the fractions F<sub>1</sub> and F<sub>2</sub> were 68.6 and 66.2 nm, respectively.

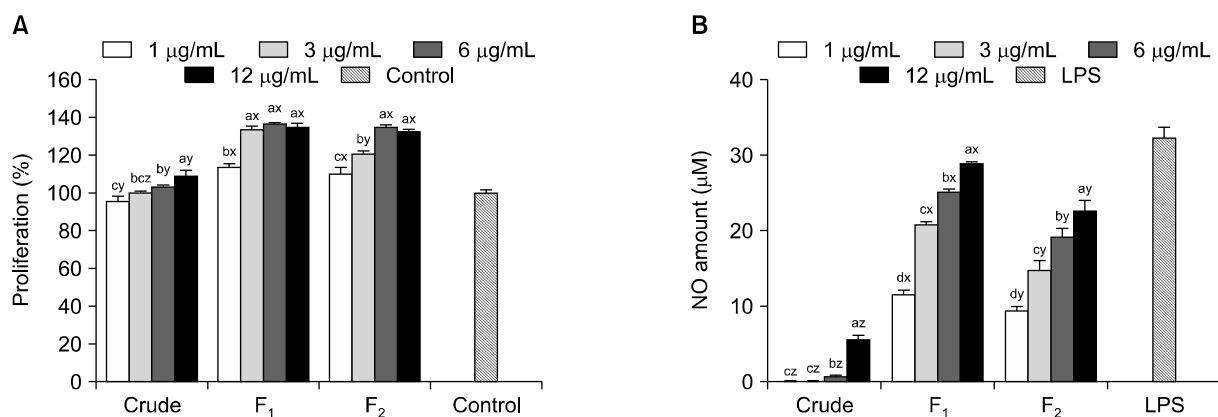
#### Immunomodulatory activity of crude polysaccharide and fractions

Murine macrophage RAW264.7 cells have been known to release various cytokines and chemokines in response to the stimulation of LPS commonly found in the outer membrane of Gram-negative bacteria. This system has been used to determine the immunomodulating activities of compounds (23). In this study, the immunomod-

**Table 3.** Average molecular weight (Mw) and radius of gyration (Rg) of crude and fraction polysaccharide from *Polygonatum sibiricum*

<i>P. sibiricum</i>	Mw $\times 10^3$ (g/mol)		Rg (nm)	
	Peak I	Peak II	Peak I	Peak II
Crude	151 $\pm$ 12.1	31.8 $\pm$ 1.80	34.7 $\pm$ 2.10	95.7 $\pm$ 7.00
F <sub>1</sub>	ND	103 $\pm$ 4.90	ND	68.6 $\pm$ 4.40
F <sub>2</sub>	628 $\pm$ 35.9	ND	66.2 $\pm$ 4.31	ND

ND, not detected.



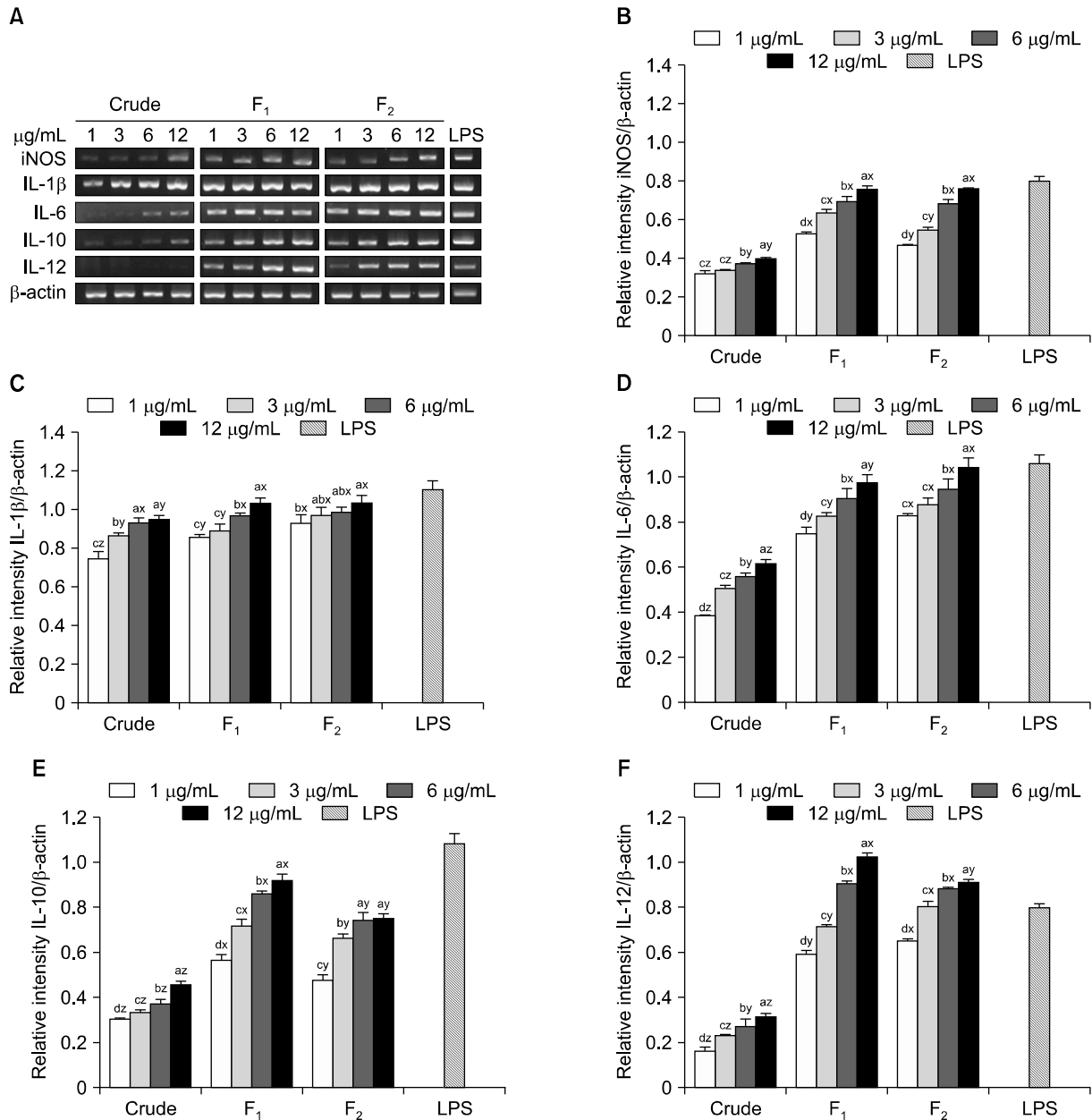
**Fig. 3.** Effect of various polysaccharides from *Polygonatum sibiricum* on proliferation and nitric oxide (NO) of RAW264.7 cells. RAW264.7 cells ( $1 \times 10^5$  cells/well) were incubated for 24 h with different concentrations of polysaccharides (1–12  $\mu\text{g/mL}$ ) (A). Amounts of NO released from RAW264.7 cells by stimulation with crude polysaccharides and fractions or 1  $\mu\text{g/mL}$  of lipopolysaccharide (LPS) (positive control) (B). The data are presented as the means  $\pm$  SD ( $n=3$ ). Means with different letters (a–c) above bars are significantly different among samples (a–c) and between same concentrations (x–z) at  $P < 0.05$ .

ulatory activities of the crude and fractions (F<sub>1</sub> and F<sub>2</sub>) were investigated using RAW264.7 cells by determining the released levels of NO and a group of cytokines, including interleukin (IL)-1 $\beta$ , IL-6, IL-10, and IL-12. Fig. 3A shows the cytotoxic effect of crude, F<sub>1</sub>, and F<sub>2</sub> fractions at concentrations of 1–12  $\mu\text{g/mL}$  on the proliferation of RAW264.7 cells. The presence of the samples considerably improved cell proliferation. Compared to the crude polysaccharide, fractions F<sub>1</sub> and F<sub>2</sub> exhibited slightly higher cell proliferation, suggesting that the crude and fractions of polysaccharides were not toxic to the RAW264.7 cells over the concentration range tested. Fig. 3B shows the released levels of NO from RAW264.7 cells by the crude and fractions with LPS as a positive control. The amount of NO produced from RAW264.7 cells by the crude polysaccharide was found to be very low, even at a polysaccharide concentration of 12  $\mu\text{g/mL}$ , which indicated that the crude had relatively low capacity to stimulate RAW264.7 cells. Conversely, the fractions (F<sub>1</sub> and F<sub>2</sub>) significantly increased the levels of NO production from RAW264.7 cells. The levels of NO by F<sub>1</sub> and F<sub>2</sub> were found, in a dose-dependent manner ( $P < 0.05$ ), to be 30.1 and 22.8  $\mu\text{M}$  at the highest concentration (12  $\mu\text{g/mL}$ ), respectively (Fig. 3B). The amount of NO production was comparable to the amount of NO produced by the positive control, LPS (1  $\mu\text{g/mL}$ ), indicating that F<sub>1</sub> and F<sub>2</sub> fractions were strong stimulants of RAW264.7 cells. It was not clear why fractions F<sub>1</sub> and F<sub>2</sub> exhibited more NO production than crude polysaccharides. This result therefore implied that when the F<sub>1</sub> and F<sub>2</sub> fractions co-exist, the respective fraction seems to lose its capacity to stimulate RAW264.7 cells. In the study of sulfated polysaccharides from *Laminaria japonica*, the fractionated polysaccharides exhibited higher antioxidant activity than their crude polysaccharides in which polymeric aggregation of the polysaccharides before fractionation was a main reason not to exert their bioactivity (24). It was al-

so reported by Schepetkin et al. (25) that higher-Mw polysaccharides induced a greater production of NO from RAW264.7 cells, observing a relationship between the Mw and NO production from macrophages. In this study, an opposite trend was shown because F<sub>1</sub> had lower Mw than those of the crude and F<sub>2</sub> fraction (Table 3). Currently, it was too complex to obtain a strong correlation between the structural and biological activities of the polysaccharides because of their considerable compositional and structural heterogeneity. Further research is necessary to obtain a better understanding of their fine structures and biological activities.

Additionally, the question of whether the NO production from RAW264.7 cells by the polysaccharides was due to the increased mRNA expression of NO synthase, called iNOS, was also examined. The mRNA expression of iNOS in RAW264.7 cells by the crude and F<sub>1</sub> and F<sub>2</sub> fractions were determined by an agarose gel analysis of the RT-PCR products using primers for iNOS mRNA. As shown in Fig. 4A and 4B, relatively more distinct and stronger bands were observed in the agarose gel with F<sub>1</sub> and F<sub>2</sub> than the crude polysaccharide, indicating that the mRNA expression of iNOS was considerably enhanced by the treatment of F<sub>1</sub> and F<sub>2</sub>. Therefore, the results suggest that the increased NO production may be because of the augmented mRNA expression of iNOS that is up-regulated in RAW264.7 cells through the activation by F<sub>1</sub> and F<sub>2</sub> fractions. These higher levels of NO production were also observed in those of root polysaccharides from *Actinidia eriantha*, *S. officinalis*, and *Actinidia Chinensis* (26, 20, 19).

Similar trends were observed for mRNA expression of other cytokines such as IL-1 $\beta$ , IL-6, IL-10, and IL-12 from RAW264.7 cells by the crude and fractions at the concentrations of 1–12  $\mu\text{g/mL}$  (Fig. 4A and 4C–4F). The crude extract and fractions F<sub>1</sub> and F<sub>2</sub> significantly increased mRNA expressions of these cytokines in a dose-



**Fig. 4.** Effects of polysaccharides on cytokines mRNA expression in RAW264.7 cells. The iNOS and cytokines mRNA expression was determined by RT-PCR (A). Graphic analysis of the polymerase chain reaction products for RNA levels of different cytokines (B~F). β-Actin was used as a control. Data are presented as the mean±SD (n=3). Means with different letters (a-c) above bars are significantly different among samples (a-c) and between same concentrations (x-z) at  $P<0.05$ .

dependent manner, which was comparable to the levels by LPS. It has been reported that the release of pro-inflammatory cytokines such as IL-1β and IL-6 is essential for host survival from the injured and/or abnormal cells and tissues through the stimulation of the immune system (27,25). However, excessive secretion of pro-inflammatory cytokines by immoderate immune cell activation could produce harmful effects on the repair process of damaged cells and tissues by aggravating sites with severe inflammation. Therefore, anti-inflammatory cytokines such as IL-10 and IL-12 also need to be released to prevent potential detrimental effects from excessive macrophage activation (28). Our current data demonstrate

that the considerable expression of IL-10 and IL-12 mRNA also occurred with the F<sub>1</sub> and F<sub>2</sub> fractions (Fig. 4). This suggests that released IL-10 might suppress the excessive activation of RAW264.7 cells, thus avoiding the potential aggravation of the recovering sites. Therefore, the present data suggest that the polysaccharides from *P. sibiricum* might be immunostimulating polysaccharides that can activate macrophages via the release of pro-inflammatory mediators while suppressing their over-activation by producing anti-inflammatory cytokines. Strong macrophage activation was also observed with polysaccharides from *A. eriantha*, which considerably enhanced the secretion of NO and both pro- and anti-in-

**Table 4.** Glycosidic linkage analysis of constituent sugar F<sub>1</sub> of polysaccharides from *Polygonatum sibiricum*

Retention time (min)	Methylation products	Glycosidic linkage	Peak ratio (%)
10.7	1,5-di- <i>o</i> -acetyl-2,3,4,6-tetra- <i>o</i> -methyl-Glu	Glu-(1→	3.50
11.8	1,5-di- <i>o</i> -acetyl-2,3,4,6-tetra- <i>o</i> -methyl-Gal	Gal-(1→	4.50
11.2	1,4,5-tri- <i>o</i> -acetyl-2,3-di- <i>o</i> -methyl-Ara	→5)-Ara-(1→	4.00
12.5	1,4,5-tri- <i>o</i> -acetyl-2,3,6-tri- <i>o</i> -methyl-Man	→4)-Man-(1→	68.2
12.6	1,4,5-tetra- <i>o</i> -acetyl-2,3,6-tri- <i>o</i> -methyl-Glu	→4)-Glu-(1→	11.7
14.7	1,4,5,6-tetra- <i>o</i> -acetyl-2,3-di- <i>o</i> -methyl-Man	→4,6)-Man-(1→	8.10

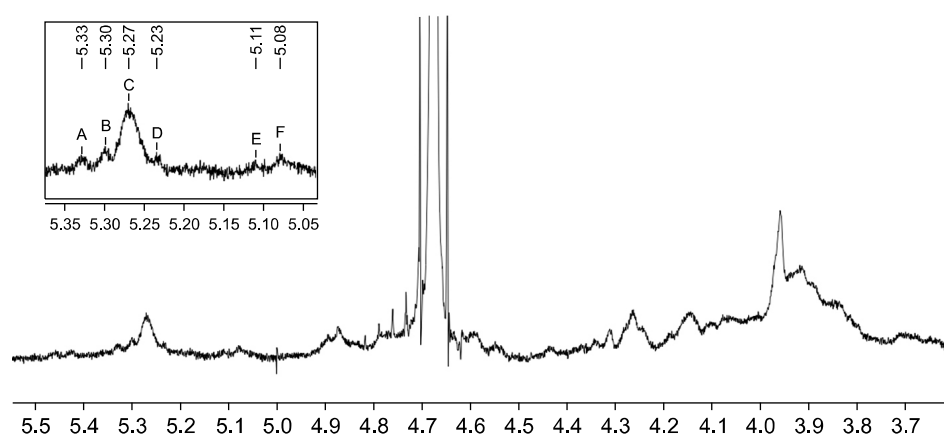
flammatory cytokines (TNF- $\alpha$ , IL-10, IL-1 $\beta$ , and IL-6) from RAW264.7 cells (26). Moreover, it was reported by Xu et al. (29) that the water-soluble polysaccharides from *A. eriantha* could enhance acquired immunity by activating natural killer and cytotoxic T splenocyte cells. A linear gluco-mannan extracted from the rhizome of *Anemarrhena asphodeloides* Bunge also enhanced the productions of IL-10, interferon- $\gamma$ , and IL-6 from Peyer's patch immunocompetent cells, exhibiting a potent immunomodulating activity (30).

#### Glycosidic linkage analysis of F<sub>1</sub>

The strong immunostimulating fraction (F<sub>1</sub>) with highest yield was per-methylated by the method of Ciucanu and Kerek (16), followed by hydrolysis, reduction, and acetylation of partially methylated alditols for investigation of glycosidic linkage patterns. Table 4 shows the types and ratios of glycosidic linkages of F<sub>1</sub> polysaccharides determined by GC-MS. The most abundant component of the per-methylated and acetylated derivatives from F<sub>1</sub> was 2,3,6-Me<sub>3</sub>-Manp, which suggested that the backbone of F<sub>1</sub> was mainly manno-pyranosyl residues connected by (1→4)-linkages. In addition, fraction F<sub>1</sub> included small amounts of 2,3,6-Me<sub>3</sub>-Glu<sub>p</sub> and 2,3-Me<sub>2</sub>-Araf, implying that the backbone was also connected through (1→4)-linked gluco-pyranosyl and (1→5)-linked arabinofuranosyl residues with non-reducing terminals of gluco- and galacto-pyranosides. Furthermore, the presence of (1→4,6)-linked manno-pyranosyl residues indicates that some branches may exist along the backbone. The total percentage of terminal glucose and gal-

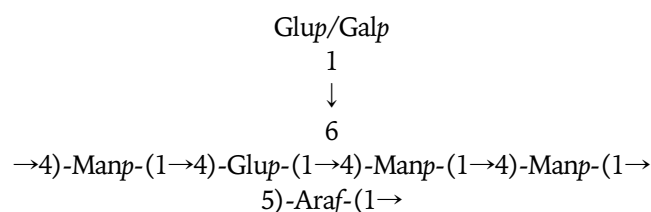
actose was 8.00%, which was in good agreement with the branched portions of (1→4,6)-linked manno-pyranoside. The glycosidic linkage pattern of F<sub>1</sub> was also partially inferred from the <sup>1</sup>H-NMR spectrum (Fig. 5). Six anomeric proton signals at 5.33, 5.30, 5.27, 5.23, 5.11, and 5.08 ppm were observed in the spectrum designated as A, B, C, D, E, and F with a molar ratio of 3.13:3.00:58.5:1.00:1.00, respectively. The chemical shift of the resonance at 5.33 ppm (residue A) was assigned to the  $\alpha$  anomeric proton of (1→4)-linked-glucopyranosyl residues (31). Residue B was assigned as an (1→4,6)-linked-manno-pyranosyl moiety with  $\alpha$  configuration at 5.30 ppm (32). The stronger signal at 5.27 ppm (residue C) appeared to be  $\alpha$ -(1→4)-linked-manno-pyranoside (32, 33). The <sup>1</sup>H resonance at 5.23 ppm for residue D was assigned as  $\alpha$ -linked terminal gluco-pyranosyl moiety (34). The chemical shifts of the anomeric proton at 5.11 ppm (residue E) corresponded to the anomeric proton of  $\alpha$ -linked terminal galacto-pyranoside (35). In case of residue F, anomeric proton at 5.08 ppm indicated  $\alpha$ -(1→5)-linked-arabino-furanoside (36). These assignments are consistent with the reports in the methylation analysis and monosaccharide composition of F<sub>1</sub> fraction.

According to Lui et al. (3), the backbone of water-soluble polysaccharides (PSW-1a) from *P. sibiricum* was also (1→4)-linked manno-pyranoside with a galacto-pyranosyl residues attached to O-6 of mannose residues, whereas (1→4)-linked galacto-pyranoside was a main backbone of PSW-1b-2 with galactose substituted at O-6 of the backbone. The structures of glucomannans from *Polygonatum* species have been widely studied, revealing their

**Fig. 5.** <sup>1</sup>H-NMR spectrum of F<sub>1</sub> polysaccharides recorded at 600 MHz in D<sub>2</sub>O at 50°C.



various structural features with different mannose and glucose ratios (37-39). Overall, the above data indicate that fraction F<sub>1</sub> may be a branched glucomannans, consisting mainly of (1→4)-linked-mannose and (1→4)-linked-glucose backbone with galactose and glucose residues attached to O-6 of the mannose residues. Based on the above results, the schematic structure of F<sub>1</sub> could be proposed as below:



## CONCLUSION

In the present study, the polysaccharides extracted from the rhizome *P. sibiricum* and fractionated by ion-exchange chromatography were used to investigate their molecular features and RAW264.7 cell activation. The extracted polysaccharides and their fractions mostly consisted of carbohydrates (85.1~88.3%), proteins (4.51~11.9%) and uronic acid (1.79~7.47%) with different levels of arabinose (4.00~7.65%), mannose (62.3~76.3%), glucose (15.2~20.3%), and galactose (4.35~15.3%). Ion-exchange chromatography yielded two fractions having relatively similar chemical profiles and monosaccharide composition; however, they had significantly different Mw values. Although the crude exhibited little stimulatory action on RAW264.7 cells, F<sub>1</sub> and F<sub>2</sub> were strong immunostimulators, inducing NO and various cytokines from RAW264.7 cells. The results of current study, therefore, demonstrate the possible application of these fractions as medicinal, pharmacological, and functional food ingredients. Further study on molecular structure-activity relationship of the individual fraction is in progress to determine the relationship between structural features and immune-enhancing activity of these fractions.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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