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Molecular Characterization of Polysaccharides in Hot-Water Extracts of *Ganoderma lucidum* Fruiting Bodies

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ABSTRACT

Polysaccharide components in hot-water extract of *Ganoderma lucidum* fruiting bodies were separated and their molecular weights and distribution profiles were measured using high-performance size-exclusion chromatography with multi-angle laser light scattering detection (HPSEC-MALLS). Three polysaccharide groups were found in the extract with a weight percentage of 31, 46 and 23%, respectively. The weight-average molecular weights of these groups were 2.08×10^6, 2.3×10^4 and 1.2×10^4 g/mol, respectively. The representative fractions of these 3 groups were obtained using differential precipitation with ethanol at 20, 60 and 80% (v/v) concentrations for further property study. The first group that had the largest molecular size was branched (1→3)-β-D-glucan with single β-D-glucosyl side chains at the *O*-6 positions, as revealed from sugar composition analysis, aniline blue assay and ^{1}H- and ^{13}C-NMR spectroscopy. (1→3, 1→6)-β-D-glucan was the major active polysaccharide and showed significant tumor necrosis factor-α (TNF-α) releasing stimulation activity from human mononuclear cells (MNC). The glucan was slightly soluble in water at ambient temperature. The second and third groups that showed slight modulating activity consisted of D-glucose, D-galactose and D-mannose at different ratios. Aggregation of polysaccharide molecules was also revealed by HPSEC-MALLS study.

Key words: *Ganoderma lucidum*, (1→3, 1→6)-β-D-glucans, polysaccharides, size-exclusion chromatography, multi-angle laser light scattering, immuno-modulating activity

INTRODUCTION

Ganoderma lucidum fruiting bodies, known as Ling-Zhi and Reishi, are a favorite herb used as an illness remedy and for promoting health in traditional Chinese medicine. This fungus has attracted considerable attention because of its antitumor[1-6] and immuno-modulating activity in many biological systems[7-9]. It has been shown that these activities are associated with its polysaccharides. Several polysaccharide and glycoprotein fractions were isolated and their immuno-modulating activities were determined. A water-insoluble branched (1→3)-*β*-D-glucan was isolated from hot-water extracts. The activity of this substance against Sarcoma 180 in mice was confirmed[4]. The D-glucan has a backbone of (1→3)-linked *β*-D-glucose units and mainly single *β*-D-glucosyl side chains attached at *O*-6 and a few short (1→4)-linked D-glucosyl units at the *O*-2 position[4,6]. The branched (1→3)-*β*-D-glucan had also been found in liquid mycelia culture[4]. Several active heteropolysaccharide fractions have also been reported, including arabinoxyloglucan[2], L-fucose and D-xylose containing mannan[3], branched D-galactoglucan mainly with α-(1→4)-linked-D-glucosyl units and various side chains[6], branched D-mannoglucan[6] and a glycoprotein contained D-glucose, D-manose, D-galactose, L-fucose and some other sugars[9].

The branched (1→3, 1→6)-*β*-D-glucans found in *G. lucidum* also occur in many other fungi, e.g. *Cordyceps*[10], *Lentinus*[11], *Schizophyllum*[12] and *Volvariella* spp[13]. Several articles reviewed the chemical, physical and immuno-modulating properties of this D-glucans[1,14,15]. (1→3, 1→6)-*β*-D-glucans from several sources have been designed as commercial carbohydrate antitumor drugs, e.g. Krestin (PSK), Lentinan and Schizophyllan (SPG)[16]. The relationships of structure and functional activity of this glucans have been intensively studied and reviewed[14,15]. Backbone linkage, side chain glucosyl units, degree of branching and molecular size of the glucan alter its biological activities, but the effects of the molecular conformation still do not have consensus among different studies.

G. lucidum fruiting bodies are widely used in medical herbs, herb supplements and functional foods (or called health foods) as active ingredients. Polysaccharide contents of *G. lucidum* extracts are often quoted as an index of the active components. The ratios of (1→3, 1→6)-*β*-D-glucans and other polysaccharide components in the extracts are often not quantified. Bao *et al.*[6] used DEAE-cellulose column and gel-filtration chromatography to characterize the polysaccharide fractions in hot-water extract of *G. lucidum* fruiting bodies. However, they provided no quantitative data. Lu *et al.*[17] attempted to use high-performance size-exclusion chromatography and molecular cut-off separation to quantify the (1→3, 1→6)-*β*-D-glucans in extracts. However, their trials were not very successful due to large molecular aggregation. To quantify (1→3, 1→6)-*β*-D-glucans, the presumed major active polysaccharides, and to investigate their molecular weights and distributions in hot-water extracts of *Ganoderma lucidum* fruiting bodies, we

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used high-performance size-exclusion chromatography coupled with multi-angle laser light scattering (HPSEC-MALLS) technique to separate polysaccharide components and to proceed the measurement. Chemical composition and specific refractive index increment [*dn/dc*] needed for measuring actual molecular weight of polysaccharide are also reported in this paper.

MATERIALS AND METHODS

I. *Polysaccharide Isolation*

Ganoderma lucidum fruiting bodies originating from CCRC 37033 (Culture Collection and Research Center in Taiwan) were kindly provided by the Double Crane Enterprise Co. (Tainan, Taiwan). Chemicals used in this study were purchased from Sigma Chemicals (St. Louis, MO, USA) unless specified. Dried fruiting bodies (900 g) were shredded using a grinder (RT-08, Rong-Tsong, Taichung, Taiwan) for polysaccharide extraction. The crude polysaccharides in the shredded samples were extracted 3 times with boiling water (4500 mL) for 2 hr each. The extracts were pooled and concentrated using a rotor evaporator (RE111, Buchi, Flawil, Switzerland). Four volumes of 95% (v/v) ethanol (Taiwan Tobacco and Wine Corp., Taipei, Taiwan) were added to precipitate the crude polysaccharides. The crude polysaccharides contained small amounts of proteins[18] and uronic acids[19], and reacted slightly in the iodine-stain test for starch-like α-D-glucans[20] and the Morgan-Elson assay for chitins[21,22]. The crude polysaccharides were further treated with cetylpyridinium chloride[23], amyloglucosidase (EC 3.2.1.3, A-7255, Sigma) and protease (EC 3.4.23.18, P-7026, Sigma). The treated polysaccharides, designated GLPS-T, did not contain noticeable amounts of proteins, uronic acids, starch-like α-D-glucans and hexosamines. The GLPS-T yield was 0.4% (w/w) of the fruiting body weight.

II. *Polysaccharide Fractionation*

The GLPS-T was separated further into 3 fractions, GLG, GLPS-2 and GLPS-3, using fractional precipitation with ethanol[24]. Ethanol was added by drops and stirred to the 0.3% (w/v) GLPS-T aqueous solution till to 20, 60 and 80% (v/v) ethanol concentration, determined by preliminary tests, to give GLG, GLPS-2 and GLPS-3 precipitation, respectively. After settling in a refrigerator overnight, the polysaccharide fraction precipitates were collected using a centrifuge (1500 ×g for 15 min). According to the total carbohydrate content determination using glucose as the standard[25], the GLG, GLPS-2 and GLPS-3 yields were 31, 29 and 15% (w/w) of GLPS-T, respectively. The GLG sample for NMR analysis was further purified in gel-filtration chromatography (GFC) on a Toyopearl HW-55F column (2.6 × 80 cm, Tosoh, Tokyo, Japan). The polysaccharide was eluted with NaOH-KCl buffer (pH 13.0) at a flow rate of 1.0 mL/min. The major peak was collected, dialyzed against distilled water, concentrated and collected by adding 4 volumes of 95% (v/v) ethanol, and then dehydrated using acetone and ether.

III. *Analysis Methods*

(I) *Sugar composition analysis*

A polysaccharide sample (5 mg) was hydrolyzed under vacuum with 2 mL of 2 N trifluoroacetic acid (TFA) in a sealed hydrolytic tube at 100˚C for 8 hr. TFA was removed by repeated evaporation under vacuum with water additions. The sugar compositions of polysaccharide were determined using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The HPAEC-PAD consisted of a Bioscan 817 Metrohm IC system (Metrohm, Herisau, Switzerland) including an IC pump 709, injection valve unit 812 with a 20-μL loop, and an electrochemical detector with a gold working electrode (E_1 = 0.05 V, 0.44 s; E_2 = 0.80 V, 0.18 s; E_3 = –0.30 V, 0.36 s). The column used was a CarboPac PA1 column (4 × 250 mm, Dionex, Sunnyvale, CA, USA). An isocratic elution was performed at 1 mL/min with 10 mM NaOH eluent containing 2 mM $Ba(OAc)_2$.

(II) *Aniline blue assay for (1→3)-β-D-glucans*

The selective aniline blue reaction with (1→3)-β-D-glucans (26) was employed to detect the existence of (1→3)-β-D-glucans in the crude polysaccharide. The method described by Wood and Flucher[26] and Young and Jacobs[27] was followed with some modifications. The sample was moistened with a few drops of methanol, dispersed in 0.3 N NaOH and stirred at ambient temperature for at least 30 min to completely dissolve the polysaccharides. The pH of the sample solution was then adjusted to 11.5 ± 0.1 by adding 1 N HCl and the volume was made to 10 mL using Na_2HPO_4-NaOH buffer (pH 11.5 containing 0.5 M NaCl). A 2 mL of sample aliquot (30 μg/mL) was reacted with 0.2 mL of 0.1% (w/v) aniline blue (Ferak, Berlin, Germany) for 2 hr at ambient temperature. The fluorescence was read on a Hitachi F4500 fluorescence spectrophotometer (Tokyo, Japan). The excitation and emission wavelength were set at 395 and 495 nm, respectively.

(III) *Molecular weight determination using high-performance size-exclusion chromatography coupled with multi-angle laser light scattering detection (HPSEC-MALLS)*

The molecular weight and distribution of polysaccharide were determined using HPSEC-MALLS. The system included a SSI single pump (Scientific Systems, Inc., State College, PA, USA), a column oven (Super Co-150, Enshine, Tainan, Taiwan) equipped with a Rheodyne injector (Cotati, PA. USA) and a 100-μL sample loop, a mulit-angle laser light scattering photometer DAWN EOS

(30 mW GaAs linearly polarized laser at 685 nm, Wyatt Technology Co., Santa Barbara, CA) and an OPTILAB DSP interferometric refractometer (P10 cell, 690 nm, Wyatt) with the temperature controlled at 35˚C. The data and chromatograms were recorded and were processed using the ASTRA software (Wyatt) on a personal computer. The DWAN EOS photometer was calibrated using puriss p.a. grade toluene (Fluka, St. Gallen, Switzerland) and normalized with a pullulan standard (Shodex Standard P-82, Showa Denko, Kawasaki, Japan) of 50,000 g/mol molar mass. Two column and elution systems were used to compare the molecular properties of polysaccharide samples prepared in 0.3 N NaOH (denatured) and hot water (native), respectively. The samples, dissolved in 0.3 N NaOH at ambient temperature and neutralized with 0.3 N HCl prior to injection, were analyzed using 3 TSK-Gel columns (7.8 × 300 mm), PW_{XL} 4000, PW_{XL} 3000 and PW_{XL} 2500, connected in series along with the TSK-Gel PW_{XL} Guard column, and eluted with 0.3 N $NaNO_3$ at a flow rate of 0.4 mL/min at 70˚C. The samples, directly dissolved in 80˚C hot water, were analyzed using a TSK-Gel GMPW column (7.5 × 300 mm, Tosoh, Tokyo, Japan) along with a TSK-Gel PWH Guard column (7.5 × 75 mm, Tosoh, Tokyo, Japan) and eluted with 0.02% NaN_3 aqueous solution at a flow rate of 0.5 mL/min at 80˚C. All sample solution was filtered through a 1.2 μm nylon syringe filter before injection. The weight-average molecular weight of GLG fraction was also determined in 0.3 N NaOH using batch-type analyses. The sample solution containing 2.5 mg/mL glucans, determined by phenol-sulfuric acid method[25], was prepared as previously described with the exception of the neutralizing step, and introduced into the flow cell on the DAWN EOS detector using a syringe pump. The measurement was made as solution was static. The specific refractive index increment [*dn/dc*] of polysaccharides, a parameter needed for actual molecular weight determination, was determined on an Optilab DSP interferometric refractometer at 35˚C using 0.02% NaN_3 aqueous solution as solvent. The [*dn/dc*] values for GLG, GLPS-2 and GLPS-3 were 0.133, 0.145 and 0.145 mL/g, respectively; the value for GLG in 0.3 N NaOH was 0.154 mL/g.

(IV) *NMR spectroscopy*

High-resolution nuclear magnetic resonance (NMR) spectra of GLG and other references were recorded using a Bruker DMX-600 NMR spectrometer (Bruher Biospin GmbH, Germany) operated at 600 MHz for ^1H and 150 MHz for ^{13}C. The samples were dissolved in DMSO-d_6 : D_2O = 6 : 1 (v/v) (Cambridge Isotope Lab., Andover, MA, USA) at 80˚C. The sample concentration was 20 mg/mL. Chemical shifts were referenced to tetramethylsilane (TMS, 0 ppm) for ^1H and to DMSO-d_6 (39.5 ppm) for ^{13}C. The degree of branching (DB) of glucan was determined using the method described by Kim *et al.*[28]. Curdlan (Megazyme, Cork, Ireland), laminarin (Sigma, St. Louis, MO, USA) and Lentinan (a generous gift from Ajinomoto, Tokyo, Japan) were used as the references.

(V) *TNF-α release activity*

The effect of *G. lucidum* polysaccharides on stimulating tumor necrosis factor-α (TNF-α) production by human blood mononuclear cells (MNC)[8] was investigated. MNC was obtained from the peripheral blood of normal adult volunteers with informed consent. MNC was separated from the blood preparation using density centrifugation in a Ficoll-Hypaque solution (1.077 g/mL, Amersham Bioscience, Amersham, UK) at 400 × g for 30 min. MNC was recovered at the interface, washed with Hank's balanced salt solution/HBSS (Gibco, Carlsbad, CA, USA) solution and re-suspended in a RPMI 1640 medium (Gibco, Carlsbad, CA, USA) containing 10% (w/v) fetal bovine serum/FBS (Hyclone, Logan, Utah, USA). The polysaccharide fractions were incubated with MNC (5×10^4 cell/mL) at 37˚C in a humidified 5% CO_2 incubator for 1 and 5 days. TNF-α levels in the cultured medium were assayed using an ELISA kit (Cistron, Pine Brook, NJ, USA). Lentinan, a commercial antitumor drug from Ajinomoto, was used as a positive control.

RESULTS AND DISCUSSIONS

I. *Polysaccharide Components in the Hot-water Extracts*

The water-soluble polysaccharide portion of *G. lucidum* fruiting bodies, designated as GLPS-T, was prepared using hot-water extraction, the most common method for preparing traditional Chinese medical herb servings. The HPSEC chromatogram of GLPS-T (Figure 1A) showed a complicated heterodiperse profile in which 5 polydispersed populations of molecules were observed (the peak of each populations was indicated by arrow sign). The first 3 peaks (according to the eluting order) in the chromatogram were grouped into the same category (Gr. I), because they could precipitate with 20% (v/v) ethanol and were all glucans (Table 1 and 2). The partially overlapped 2 peaks before the solvent peaks were designated as second (Gr. II) and third (Gr. III) groups (Figure 1A). The first polysaccharide group in the chromatogram had a significantly larger molecular size than the other 2 groups. Representative fractions of these polysaccharide groups could be obtained using fractional precipitation with ethanol at 20, 60 and 80% (v/v) concentrations and designated as GLG, GLPS-2 and GLPS-3 fraction, respectively. In each fraction, the major component was composed of about 80% weight or higher of the fraction and had the same distribution coefficient as the corresponding peak in HPSEC chromatogram of GLPS-T (Figure 1 and 2). Therefore, GLG, GLPS-2 and GLPS-3 fractions were used to explore the physiochemical properties of the polysaccharide groups observed in the HPSEC chromatogram (Figure 1) in this study.

Table 1. Sugar compositions and specific interaction with aniline blue of polysaccharide fractions from *G. lucidum* hot-water extracts

Sample	Molar ratio (%)			Relative fluorescence intensity (%)*
	Glucose	Galactose	Mannose	
GLPS-T	76.13 ± 1.45	14.65 ± 0.10	9.22 ± 0.53	16.88 ± 0.10
GLG	94.37 ± 1.23	5.60 ± 1.03	Trace	57.45 ± 0.32
GLPS-2	62.08 ± 2.45	28.90 ± 0.31	9.02 ± 0.64	0.42 ± 0.01
GLPS-3	65.08 ± 1.83	22.76 ± 1.09	12.16 ± 1.19	0.41 ± 0.02

* Fluorescent excitation and emission wavelength were set at 395 nm and 495 nm, respectively.

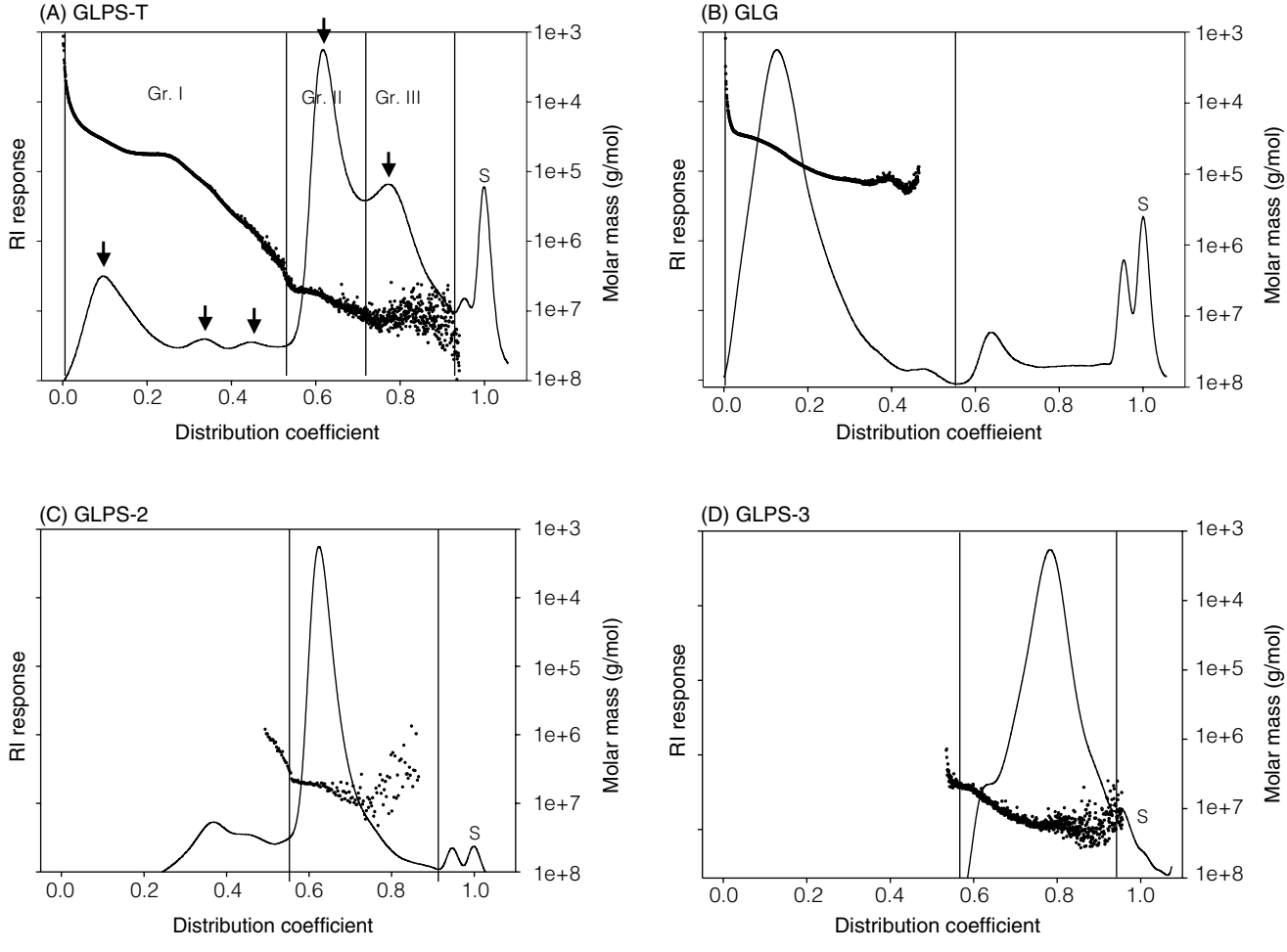

Figure 1. High-performance size-exclusion chromatograms of polysaccharides from hot-water extracts of *G. lucidum* fruiting bodies (GLPS-T, A) and their fractions (GLG, B; GLPS-2, C; GLPS-3, D). S: solvent peak; ——— : RI response; ·····: molar mass.
Analytical conditions: 3 TSK-Gel columns (7.8 × 300 mm), PW_{XL} 4000, PW_{XL} 3000 and PW_{XL} 2500, connected in series along with the TSK-Gel PW_{XL} guard column and eluted with 0.3 N $NaNO_3$ at a flow rate of 0.4 mL/min at 70°C. The polysaccharide samples were denatured with 0.3 N NaOH, neutralized and passed through 1.2 μm filter membrane before analysis.

GLPS-T was a mixture of polysaccharides and contained 76 molar % of glucose. The existence of (1→3)-β-D-glucan was confirmed by the selective fluorescence reaction with aniline blue. GLG fraction was the largest molecular size polysaccharide group of *G. lucidum* and was composed of ~ 94 molar % of glucose and small amount (~6 molar %) of galactose (Table 1). A strong specific fluorescence with aniline blue indicated that it had a (1→3)-β-D-glucan backbone. The small amount of galactose found in the sample was believed from contaminant of the second polysaccharide group (Figure 1B). GLPS-2 and GLPS-3 were composed of 62~65% of glucose, 23~29% of galactose and 9~12% of mannose in molar ratio. They showed very weak interaction with aniline blue and might be mixtures of heteropolysaccharides as reported[3,4,6,9,29].

II. *Molecular Structure of (1→3, 1→6)-β-D-glucans*

The GLG fraction was known to have (1→3)-β-D-glucan backbone as the results of sugar composition (Table 1) and selective interaction with aniline blue. To further elucidate the detailed structure of this (1→3)-β-D-glucan, the GLG fraction was further purified using GFC to remove the small molecular size portion and subjected to NMR

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Table 2. ^{13}C-NMR chemical shifts (ppm) of (1→3, 1→6)-β-D-glucans from *G. lucidum* (GLG) and other standards

	Chemical shifts δ (ppm)					
Glucans*	C-1	C-2	C-3	C-4	C-5	C-6
Curdlan						
BC	103.5	73.1	86.1	68.6	76.4	61.1
Laminarin						
BC	103.2	73.2	86.1	68.6	76.5	61.1
TSC	—**	—	—	—	76.3	61.3
Lentinan						
BC	103.1	73.1	86.0 (86.5)	68.6	76.4	61.2
BP	103.0	72.9	85.8	68.6	75.0	68.6
TSC	103.0	73.7	76.6	70.3	76.2	61.0
GLG						
BC	103.1	73.2	86.1 (86.7)	68.7	76.5	61.2
BP	103.0	73.0	85.8	68.7	75.0	68.7
TSC	103.1	73.8	76.5	70.3	76.3	61.0

* BC: backbone; BP: branching point; TSC: terminal unit in the side chain.

** Not detectable.

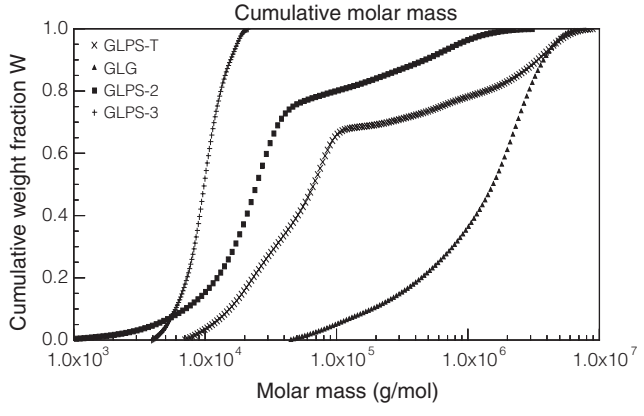

Figure 2. Cumulative molar mass distribution plot of polysaccharides (GLPS-T) from hot-water extracts of *G. lucidum* fruiting bodies and their fractions, GLG, GLPS-2 and GLPS-3.

analysis. The chemical shift assignments for ^{1}H and ^{13}C described by Kim *et al.*[28] were followed. The ^{1}H- and ^{13}C-NMR spectra of GLG were referred as linear (1→3)-β-D-glucan, curdlan, a slightly branched laminarin, and a highly branched Lentinan. The ^{13}C-NMR chemical shifts of GLG and three commercial (1→3)-β-D-glucan references are listed in Table 2. The ^{13}C-NMR spectrum (Figure 3) of GLG indicated that the glucan was a (1→3, 1→6) mix-linked β-D-glucan. It showed that C-3 and C-1 resonance peaks located at 86.1 and 103.1 ppm respectively, indicating that the (1→3)-β-D-glucan backbone (BC) referred to the linear curdlan and branched Lentinan. C-4 peak located at 70.3 ppm away from 68.6 ppm. The 3 separated C-3 peaks located around 85.8~86.7 ppm and the 3 separated C-2 peaks with slightly different chemical shifts (73.0~73.8 ppm) indicated the presence of β-(1→6) branches in the GLG. The high intensity of C-4 peak located at 70.3 ppm and the separated C-2 and C-5 peaks located at 73.8 and 75.0 ppm indicated a large amount of terminal anhydro D-glucose units (AGU) in a highly branched molecule.

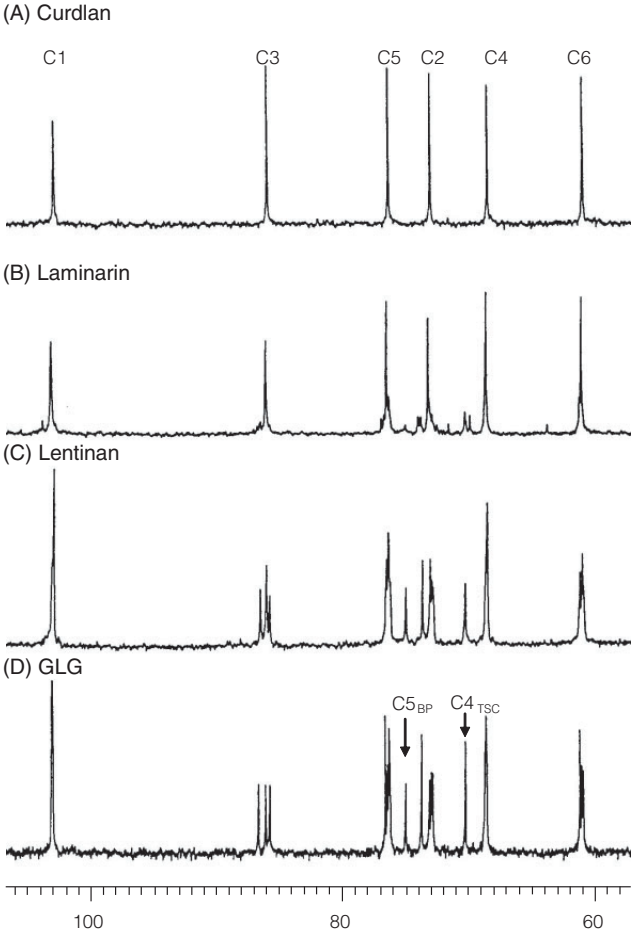

Figure 3. ^{13}C-NMR spectra of (A) curdlan, (B) laminarin, (C) Lentinan and (D) GLG, (1→3)-β-D-glucan from *G. lucidum*. BP: branching point; TSC: terminal unit in the side chain.

The degree of branching (DB) of GLG was determined using the integrated peak areas of assigned H-1s for backbone (BC) and for the branched single or terminal AGU in the side chain (TSC)[28] referred to the (1→3)-β-D-glucan references. The H-1 peaks of BC and TSC AGUs were located at 4.54 ppm (d, *J* 7.3 Hz) and 4.24 ppm (d, *J* 7.3 Hz), respectively (Figure 4). The DB, which equals to the ratio of TSC H-1 peak area to all H-1 peak areas in the backbone, was 0.35 for GLG. This value agreed with the value (0.33) reported by Sone *et al.* [4] using methylation analysis. For comparison, the DB value for Lentinan was 0.42, in agreement with the value (0.4) provided by the manufacturer and report of Sasaki and Takasuka[11].

III. *Molecular Weight and Distribution of the Polysaccharides*

The actual molecular weight and distribution of the polysaccharide components of GLPS-T were determined by multi-angle laser light scattering detection coupled with the HPSEC. The polysaccharide samples were prepared in 0.3 N NaOH to dissociate (denature) the molecular aggregation. The sample solution was neutralized and filtered through

Table 3. Molecular weights and weight percentages of polysaccharides in hot-water extract of *G. lucidum* fruiting bodies

Sample*	Weight-average molecular weight $\times 10^{-4}$ *(Mw)*	Number-average molecular weight $\times 10^{-4}$ *(Mn)*	Polydispersity *(Mw/Mn)*	Weight percentage (%)
GLPS-T				
Group I	207.8 ± 17.4	161.1 ± 13.2	1.79 ± 0.69	30.9 ± 2.6
Peak 1	306.6 ± 12.0*			
Peak 2	81.1 ± 4.0*			
Peak 3	26.7 ± 3.9*			
Group II	2.3 ± 0.2	1.9 ± 0.1	1.12 ± 0.05	45.8 ± 0.8
Peak 4	2.1 ± 0.2*			
Group III	1.2 ± 0.1	1.1 ± 0.1	1.00 ± 0.00	23.4 ± 0.5
Peak 5	1.1 ± 0.2*			

* Sample and chromatographic peaks are indicated in Figure 1.

1.2 μm nylon membrane instead of 0.45 μm (commonly used for HPLC sample preparation) right before the HPSEC analysis. This preparation method allowed us to achieve the molecular weight measurement within a reasonable deviation, although it also caused a higher level of noise on both RI and MALLS signals. The sample solution of GLPS-T and GLG were difficult to pass through 0.45 μm filtration membrane and obvious viscosity was observed. The sample preparation of GLG was difficult because the solubility of this fraction was poor due to the molecular aggregation. An attempt to prepare a stable GLG aqueous solution with a concentration higher than 0.25% was not successful. The specific refractive index increment [*dn/dc*] values of GLG, GLPS-2 and GLPS-3 fractions were applied for molecular weight determination of the polysaccharides group I, II and III, respectively.

The first polysaccharide group (Gr. I), the (1→3, 1→6)-β-D-glucans, in the GLPS-T chromatogram (Figure 1 A) was widely dispersed molecules (polydispersity = 1.79) and had an overall weight-average molecular weight (M_w) of 2.08×10^6 g/mol and number-average molecular weight (M_n) of 1.61×10^6 g/mol (Table 3). The molecular weight values at peaks of these 3 molecules were 3.07×10^6, 8.11×10^5, 2.67×10^5 g/mol. The first polysaccharide group was composed 30.9% of the GLPS-T mass (Figure 2). The M_w and M_n of the second (Gr. II) and third (Gr. III) polysaccharide groups in the GLPS-T chromatogram were 2.3×10^4, 1.9×10^4 and 1.2×10^4, 1.1×10^4 g/mol, respectively. The second and third polysaccharide groups had much smaller molecular sizes and narrower dispersed molecules (polydispersity close to 1) than those of the first group (Table 3). The second and third polysaccharides were composed of 45.8% and 23.4% of the GLPS-T mass (Figure 2). The molecular size distribution of the first group was significantly larger than the size of second and third groups and so they could be separated from the others using only fractional precipitation with ethanol.

To confirm the molecular weight and distribution similarity between the GLG, GLPS-2 and GLPS-3 fractions and those polysaccharide groups, all polysaccharide fractions were subjected to the HPSEC-MALLS. The chromatogram of GLG consisted of one major asymmetric peak with shoulder and small peak corresponding to the first 3 peaks of the first group (Gr. I, Figure 1B). The M_w value of GLG

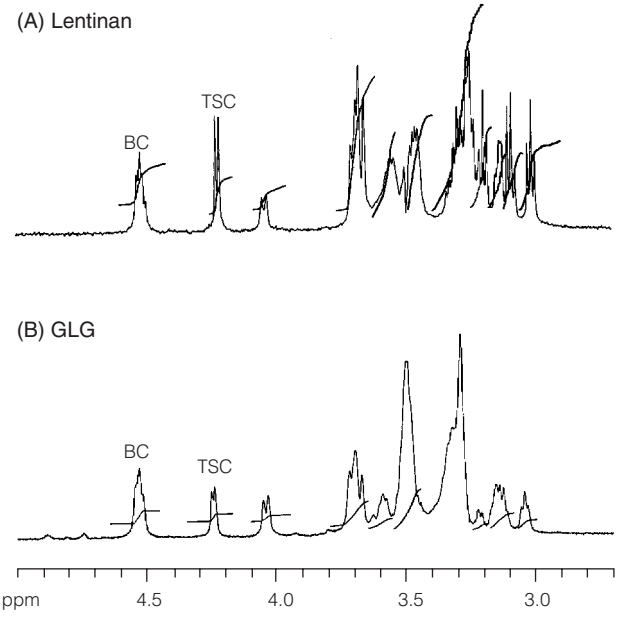

Figure 4. ^{1}H-NMR spectra of Lentinan (A) and (1→3)-β-D-glucan from *G. lucidum* (B, GLG). BC: backbone; TSC: terminal unit in the side chain.

was 1.80×10^6 g/mol. Contamination of second and third groups was excluded upon the molecular weight calculation (Figure 1B). The M_w value of GLG was smaller than the value of corresponding peaks in GLPS-T chromatogram. The decrease in the determining value might be the result of the loss of large molecules during the fractionation process. According to the sugar composition, NMR and HPSEC results of GLG, the first polysaccharide group (Gr. I) in the GLPS-T was (1→3, 1→6)-β-D-glucans. The 3 components in the group were polymer homologs differing in molecular size. The Mw values of major peak of GLPS-2 and GLPS-3 were equivalent to that of the second and third polysaccharide groups, respectively (Figure 1). GLPS-2 included about 15% larger molecular contamination from GLG (Gr. I).

To evaluate the possibility of molecular re-aggregation in the neutral eluent of HPSEC-MALLS, the molecular weight of GLG was re-measured in 0.3 N NaOH aqueous solution ([*dn/dc*] = 0.154 mL/g) using batch-type analyses on the MALLS photometer. The value was 1.5×10^6 g/mol

in agreement with the value from HPSEC-MALLS. The results indicated that using of 0.3 N NaOH followed by neutralization during sample preparation was sufficient to dissociate the aggregation of (1→3, 1→6)-β-D-glucans of *G. lucidum* for molecular weight measurement.

The samples prepared and eluted in 0.02% NaN_3 aqueous solution with the method reported in many of aqueous SEC studies[30-32] showed abnormal SEC elution behaviors (Figure 5). According to refractive index signals, 3 normal separated peaks were observed on the HPSEC chromatogram of GLPS-T, accompanied with some abnormal elution behaviors deviating from the normal relationship of molar mass and distribution coefficient (elution time or retention time). The relationship plot of the molar mass and distribution coefficient showed a concave curve. The curve-up indicated that some large molecules aggregated and retained in the SEC column longer than smaller molecules, because the aggregated molecules showed a small root mean square radius (RMS). This abnormal elution behavior did not occur in the alkali-denatured samples in Figure 1. The chromatogram in Figure 5 showed a significantly low ratio of large molecules because parts of (1→3, 1→6)-β-D-glucans were aggregated and lost in the filtration. The loss was predictable because the (1→3, 1→6)-β-D-glucans in GLPS-T had high tendency to aggregate and the solubility was poor[4,12,14,27].

IV. *TNF-α Release Activity*

An activity assay for stimulating human blood mononuclear cells to release TNF-α was carried out *in vitro* to evaluate the immunuo-modulating activities[8] of *G. lucidium* polysaccharides. Lentinan, a commercial antitumor drug, was used as a positive control. All polysaccharide fractions demonstrated significant activities in stimulating TNF-α production (Table 4). On the first day, the TNF-α concentration in all polysaccharides treated media was significantly higher than the control at 20 and 40 μg/mL dosage. On the fifth day, the TNF-α values decreased and only the GLG treated group was still significantly at 20 μg/mL dosage. GLG demonstrated the strongest activity among fractions with 4~10 fold activity higher than the unfractionated polysaccharide GLPS-T, but lower than Lentinan possibly due to the limited solubility of GLG. The dose-dependent increases in TNF-α production were found in all fractions except GLG and Lentinan. That might reach a plateau for TNF-α production of the MNC at the concentrations used. The results indicated that (1→3, 1→6)-β-D-glucans (GLG) was the major component contributing to TNF-α stimulating activity.

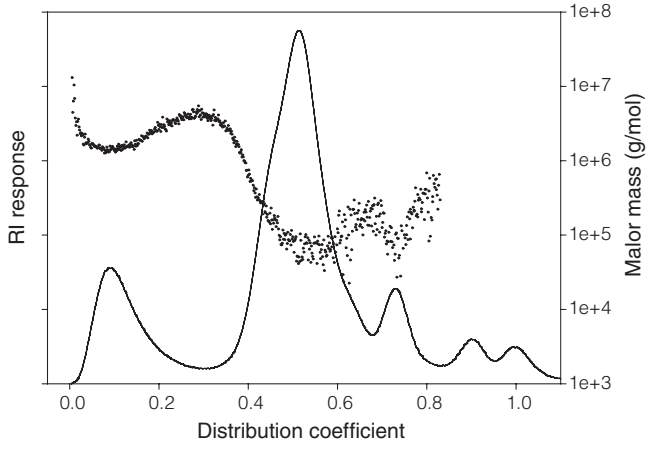

Figure 5. High-performance size-exclusion chromatogram of aggregated polysaccharides from hot-water extracts of *G. lucidum* fruiting bodies (GLPS-T). S: solvent peak; ———: RI response; ·····: molar mass.
Analytical conditions: TSK-Gel GMPW column (7.5 × 300 mm) along with a TSK-Gel PWH Guard column (7.5 × 75 mm) and eluted with 0.02% NaN_3 aqueous solution at a flow rate of 0.5 mL/min at 80°C. The polysaccharide sample was prepared in 80°C hot-water, and passed through 1.2 μm filter membrane.

CONCLUSION

The molecular weights and distributions of *G. lucidum* (1→3, 1→6)-β-D-glucans were directly determined in a hot-water extractable polysaccharide mixture using HPSEC-MALLS. The weight-average molecular weight of the glucans was 2.08×10^6 g/mol. Population of these molecules was widely polydispersed, without noticeable amount of molecules having molecular weight smaller than 2.0×10^5 g/mol. The isolated (1→3, 1→6)-β-D-glucans became sparingly soluble in water and its aqueous solution was unstable. (1→3, 1→6)-β-D-glucan was the major active polysaccharide for stimulating MNC to release TNF-α in a hot-water extract of *G. lucidum* fruiting bodies. The active glucans comprised about one third of water-extractable polysaccharides of *G. lucium* fruiting bodies. To produce a carbohydrate-enriched *G. lucidum* health food product, it is important to monitor the content and molecular weight of the (1→3, 1→6)-β-D-glucans in the raw materials and the end products, since they might vary with the strain, cultivating method (mycelium or fruiting body), cultivating condition and processing. Developing accessible methods such as fluorometric and enzymatic

Table 4. Induction of TNF-α release into the cultured medium using isolated human mononuclear cells (MNC) treated with Lentinan and the polysaccharide fractions from *G. lucidum* hot-water extracts

Sample	Conc. (μg/mL)	TNF-α (pg/mL) Day 1	Day 5
Control	0	3.2 ± 6.7	15.8 ± 6.0
GLPS-T	20	73.6 ± 7.1	20.2 ± 11.6
	40	174.7 ± 3.4	108.2 ± 47.1
GLG	20	754.1 ± 101.6	325.3 ± 34.4
	40	785.3 ± 134.4	400.8 ± 10.5
GLPS-2	20	41.9 ± 11.6	9.7 ± 1.1
	40	461.2 ± 61.6	150.7 ± 25.0
GLPS-3	20	16.3 ± 17.2	17.1 ± 4.1
	40	138.6 ± 45.9	70.4 ± 16.1
Lentinan	20	1350.1 ± 78.1	748.8 ± 5.2
	40	1343.0 ± 32.1	768.1 ± 13.1

methods for determining (1→3, 1→6)-β-D-glucan content are demanded for routine quality control.

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