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Structural analysis and molecular characterization of exopolysaccharides produced by submerged mycelial culture of *Collybia maculata* TG-1

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Abstract

Two different exopolysaccharides (EPSs) were obtained by submerged mycelial culture of an edible mushroom, *Collybia maculata* TG-1, and their chemical structures were studied by gas chromatography (GC), Fourier transform-infrared (FT-IR) spectroscopy, methylation analysis, and NMR spectroscopy. A compositional analysis result by GC indicated that both EPSs (designated as Fr-I and Fr-II) were galactomannans consisting of mainly mannose and galactose. FT-IR spectroscopy was used for obtaining vibrational spectra of the EPSs. In the anomeric region (950–700 cm^{-1}), both EPSs exhibited the characteristic absorption at 810 cm^{-1} corresponding to the existence of mannose. The obvious absorption peaks at 910 and 880 cm^{-1} in both EPSs revealed the co-existence of α and β configurations. In a ^{13}C NMR analysis, two anomeric peaks appeared at 102.0 and 99.6 ppm, which were assigned to the mannose (C-1) and galactose (C-1) residues, respectively. In a GC–MS analysis, the methlylation data confirmed the presence of a (1→3)-linked β-D-mannopyranosyl backbone mainly substituted O-6 by galactopyranosyl residues. The size exclusion chromatography/multi-angle laser light scattering (SEC/MALLS) system showed that the weight-average molecular mass of the Fr-I and Fr-II were 7.95×10^4 and 2.09×10^4 g/mol, respectively. Moreover, the SEC/MALLS revealed that the molecular conformation of the Fr-I was a random coil, with Fr-II being a rigid rod in aqueous solution.

Keywords: *Collybia maculata*; Exopolysaccharide; GC–MS; MALLS; Mushroom; NMR; Structural analysis

1. Introduction

During the past decades, much interest has been generated in extracellular polysaccharides (EPSs) produced by numerous mushrooms because of their beneficial biological activities including anti-tumor, immuno-stimulating, and hypoglycemic activities, etc. (Cheung, Cheung, & Ooi, 2003; Han et al., 1995; Shiao, Lee, Lin, & Wang, 1994; White, Hackman, Soares, Beckett, & Sun, 2002). Submerged culture of mushrooms in an attempt to produce EPSs obviously gives rise to potential advantages of higher mycelial production in a compact space and shorter time without significant contamination risk (Cheung, 1996; Wu, Cheung, Wong, & Huang, 2004; Yang, Huang, & Yang, 2003).

The difference in chemical composition, type of glycosidic linkage, and branching degree of polysaccharides, influences the secondary and tertiary structures of the single chains and their macromolecular assembly, determining the physical properties of the polysaccharide which are related to their structural or physiological functions (Gutierrez, Prieto, & Martinez, 1996). Recently, there have been extensive studies on fungal polysaccharides, especially β-glucan and galactomannan, as biological response modifiers (Bohn & BeMiller, 1995; Cheung, 1996; Duncan, Pugh, Pasco, & Ross, 2002).

Collybia maculata is an edible mushroom, which belongs to the Phylum basidiomycota, the class Basidiomycetes, and the family Tricholomataceae. Although this mushroom has been widely used as a functional food source, there is no report on culture method and elucidation of useful components that may be found in this mushroom except for our recent publication regarding optimum submerged culture conditions of this mushroom (Lim et al., 2004). In our preliminary study,

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it was found that *C. maculata* produce a galactomannan during submerged culture.

Galactomannan is a large family of natural polysaccharides extracted from endosperm of different plant seeds. They are used in many industrial domains such as food, cosmetics, paper, textile, and pharmaceuticals (Ono et al., 2003; Ramesh, Yamaki, & Tsushida, 2002). Galactomannnas are not digested in the upper gastrointestinal tract and thus are components of 'dietary fibre'. They have been reported to lower serum cholesterol and triacyglycerol levels in rats fed high-fat diets (Ide, Moriuchi, & Nihimoto, 1991; Pettolino, Hoogenraad, & Stone, 2002).

The structure and molecular mass of polysaccharides have been found to play an important role in their biological activities (Hwang, Kim, Choi, & Yun, 2003; Kulicke, Lettau, & Thielking, 1997; Zhang, Qiu, Xu, Du, & Kennedy, 1992). We therefore report here on the structural and molecular characteristics of EPS obtained by submerged culture of *C. maculata*.

2. Materials and methods

2.1. Microorganism and culture conditions

The *C. maculata* in our culture collection was isolated from a mountainous district in Korea. The strain was maintained on potato dextrose agar (PDA) slants at 4 °C. The seed culture was grown in 250 ml flasks containing 50 ml mushroom complete medium (MCM) (2% glucose, 0.2% yeast extract, 0.2% peptone, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4, 0.046% KH_2PO_4) at 25 °C on a rotary shaker incubator at 150 rpm for 3 days. The fermentation medium was inoculated with 4% (v/v) of the seed culture and them cultivated at 20 °C in a 5-l stirred-tank reactor (Ko Biotech Co., Seoul, Korea). Fermentations were performed under the following conditions: temperature, 20 °C; aeration rate, 2 vvm; agitation speed, 150 rpm; initial pH 5.5; working volume, 3-l; media (3% glucose, 2% Martone A-1, 0.1% $CaCl_2$, and 0.1% K_2HPO_4).

2.2. Preparation of EPS

The fermentation broths from the 5-l fermenter were centrifuged at 10,000*g* for 20 min, and the supernatant was mixed with four volumes of absolute ethanol, stirred vigorously and kept overnight at 4 °C. The precipitated EPS was centrifuged at 10,000*g* for 20 min, the supernatant was discarded, (Kim, Hwang, Xu, Choi, & Yun, 2003) and the remaining EPS was used throughout the experiments.

2.3. Fractionation of EPS

The crude EPSs were dissolved in 0.2 M NaCl buffer, and loaded onto a Sepharose CL-6B column (2.4 cm × 100 cm, Sigma Chemical Co., St Louis, MO). The column was eluted with the same buffer at a flow rate of 0.6 ml/min. Protein concentration was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. The total sugar content in the EPS was determined by phenol sulfuric acid method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein moiety in the EPS was monitored by measuring the absorbance at 280 nm, whilst the carbohydrate moiety was monitored at 480 nm.

2.4. Compositional analysis

The EPSs were hydrolyzed by treatment with 2 M trifluoro-acetic acid (TFA) at 121 °C for 3 h, and then the resulting monosaccharides were analyzed by gas chromatography (Varian Co. Model: Star 3600CX, Lexington, MA) with a fused silica capillary column (Na form, 15 m × 0.25 mm, Supelco, Inc., Bellefonte, PA) and a flame ionization detector.

2.5. FT-IR spectroscopy

FT-IR (Genesis II, Mattson, Inc., Fremont, CA) was analyzed using the KBr disc for detecting functional groups. The spectra were obtained from 1 mg of freeze–dried EPS and 300 mg of KBr. FT-IR spectra were recorded on a Mattson Instrument from 400 to 4000 cm^{-1}, with a resolution 4 cm^{-1} and 20 scans. Mattson spectra of the samples were recorded on a near-infrared Mattson microspectrometer, built in-house, using 100 mW of laser light of 850 nm on the sample. A signal integration time of 30–120 s per sample was used. Spectra were corrected for wavenumber-dependent signal-detection efficiency of the set up using the white light spectrum of a temperature calibrated tungsten band lamp (Grosev, Bozac, & Puppels, 2001; Römer et al., 1998).

2.6. ^1H and ^{13}C NMR spectroscopy

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance 400 MHz spectrometer (Bruker Co., Billerica, MA) with a 5 mm inverse probe. Proton spectra were run at a probe temperature of 25 °C, while carbon spectra were obtained at 25 °C. The samples were dissolved in D_2O (99.96%) at concentrations of 5 mg/ml (for ^1H NMR) and 20 mg/ml (for ^{13}C NMR). ^1H chemical shifts were referenced to internal D_2O (4.7 ppm at all temperatures).

2.7. GC–MS analysis

Methylation analysis was carried out according to the Hakomori method (Hakomori, 1964). The EPS (5 mg) was dissolved in dimethyl sulfoxide (0.1 ml) by ultrasonication in a nitrogen atmosphere. Sodium dimethylsulfinyl-anion solution was added to the Hypovial, and the mixture

was stirred for 2–4 h at room temperature. Methyl iodide (0.1 ml) was slowly added for 10–15 s to the stirred mixture, which was maintained at 0~–20 °C by cooling the Hypovial in water. Each methylated EPS was purified using a Sep-pek C_{18} cartridge (Waters Co., Milford, MA). A purification technique was developed to utilize reversed-phase chromatography on Sep-pek C_{18} cartridge. A Sep-pek C_{18} cartridge (preflushed with 40 ml of 100% ethanol to remove contaminants from the cartridge and to increase the recovery of the per-*O*-methylated carbohydrate) was preconditioned by passing through the cartridge with 2 ml of 100% acetonitrile, followed by 4 ml of water. The methylated reaction mixture containing the per-*O*-methylated carbohydrate was diluted with an equal volume of water to produce a 50% (v/v) dimethyl sulfoxide–water solution. This solution was slowly (1–2 drops per second) pushed, using the syringe plunger, through the cartridge bed until the liquid level was just above the resin bed. The Hypovial was rinsed with 1:1 (v/v) dimethyl sulfoxide–water. The permethylated EPS was hydrolyzed with 2 M TFA (1.5 ml) for 3 h at 121 °C, and then the hydrolyzates were evaporated to dryness, followed by successive reduction with $NaBH_4$ and acetylation. The resulting methylated alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS). The GC–MS (70 eV) was performed on a Shimadzu QP5050 instrument (Shimadzu Co., Nakagyo-ku, Kyoto, Japan) equipped with the same capillary column. Peaks were identified on the basis of relative retention time and fragmentation patterns. The molecular percentage for each sugar was calibrated using the peak areas.

2.8. SEC/MALLS analysis

The molecular weights of the EPS were estimated by SEC coupled with MALLS system (Wyatt Technology, Santa Babara, CA). The EPS samples were dissolved in a phosphate/chloride buffer (ionic strength = 0.1, pH 6.8) containing 0.04% diaminotetraacetic acid–disodium salt (Na_2EDTA) and 0.01% sodium azide and filtered through 0.025 μm filter membranes (Millex HV type, Millipore Co., Bedford, MA) prior to injection into the SEC/MALLS system. The chromatographic system consisted of a degasser (Degasys, DG-1200, uniflow, HPLC Technology, Macclesfield, UK), a high performance pump (Model 590 Programmable Solvent Delivery Module, Waters Co., Milford, MA), an injection valve (Rheodyne, Inc., Cotati, CA) fitted with a 100 μl loop, and two SEC columns (Shodex PROTEIN KW-803, 804, Showa Denko K.K., Tokyo, Japan) connected in series. The flow rate was 0.5 ml/min and the injection volume and concentration was 100 μl and 3 mg/ml, respectively. During the calculation of molecular weights of each EPS, the value of d*n*/d*c* (specific refractive index increment) was used from the data in literature (Jumel, Fiebrig, & Harding, 1996), in which the estimated d*n*/d*c* was 0.14 ml/g. Calculations of molecular weight and root mean square (RMS) radius of gyration for each EPS were performed by the Astra 4.72 software (Wyatt Technology). The RMS radii of each polysaccharide were determined from the slope by extrapolation of the first-order Debye plot (Astafieva, Eberlein, & Wang, 1996; Wyatt, 1993). The gross conformation of EPS in aqueous solution could be identified from the double logarithmic plot of RMS radius vs. molecular mass of EPS according to the following equations:

$$\text{Spheres}: \; r_i^3 \propto M_i \rightarrow \log r_i = k + 1/3 \log M_i$$

$$\text{Random coils}: \; r_i^2 \propto M_i \rightarrow \log r_i = k + 1/2 \log M_i$$

$$\text{Rigid rods}: \; r_i^1 \propto M_i \rightarrow \log r_i = k + \log M_i$$

where r_i is the RMS radius of an EPS molecule, M_i is the molar mass of EPS, k is the intercept at the *Y*-axis (RMS radius), and 1/3, 1/2, and 1 are the critical slope values for determining the molecular conformation of each EPS (Majdoub et al., 2001; Peng, Zhang, Zeng, & Xu, 2003; Wyatt, 1993; Zhang, Zhang, & Cheng, 2002).

3. Results and discussion

3.1. Purification of EPS

The crude EPS from submerged culture of *C. maculata* was purified by gel filtration in Sepharose CL-6B column. Two fractions of EPSs (Fr-I and Fr-II) were coeluted as shown in Fig. 1. Fr-I and Fr-II peaks appeared between 34–51 and 53–65 tubes, respectively. It was revealed that Fr-I and Fr-II were obviously polysaccharides in the absence of protein moiety. The sugar compositions in Fr-I and Fr-II are shown in Table 1. The compositional analysis revealed that both of the two fractions consisted of mainly mannose and galactose and their compositions were slightly different.

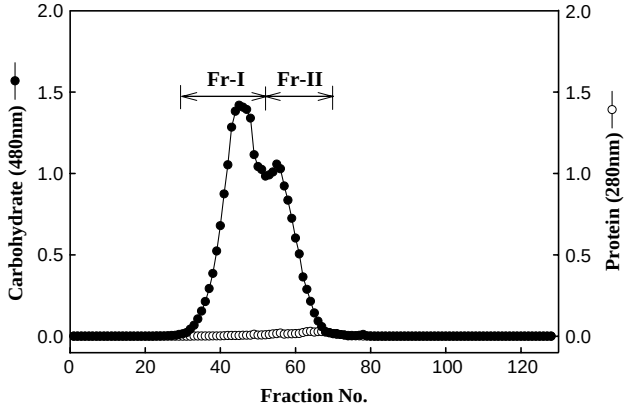

Fig. 1. Elution profiles of the exopolysaccharides produced by submerged mycelial culture of *Collybia maculata* TG-1 in Sepharose CL-6B chromatography.

Table 1
Compositional analysis of the exopolysaccharides (Fr-I and Fr-II) produced by submerged mycelial culture of *Collybia maculata* TG-1

Fraction	Sugar composition[a] (%)		
	Mannose	Galactose	Ribose
Fr-I	81.4	17.1	1.5
Fr-II	78.9	18.1	3.0

[a] Determined by GC.

3.2. Chemical characterization of EPS

In order to investigate the functional groups of the purified EPS, the FT-IR spectra were measured in KBr pellets. Typical IR spectra for the two fractions are presented in Fig. 2. In the anomeric region (950–700 cm^{-1}), both Fr-I and Fr-II exhibited the obvious characteristic absorption at 810 cm^{-1}, suggesting the existence of mannose (Peng et al., 2003; Zhang, Yang, Ding, & Chen, 1995). In comparison with the IR spectra of polysaccharides documented in literature, a characteristic absorption band appeared at 1650 cm^{-1} and was assigned to the stretching vibration of the carboxyl group (C=O) of the Fr-I and Fr-II, while another absorption band at 2935 cm^{-1} was intensified and assigned to the stretching vibration of the methylene group (C–H). Furthermore, a continuous absorption beginning at approximately the region of 3400 cm^{-1} is characteristic of a carbohydrate ring. The obvious absorption peaks at 910 and 880 cm^{-1} in Fr-I and Fr-II revealed

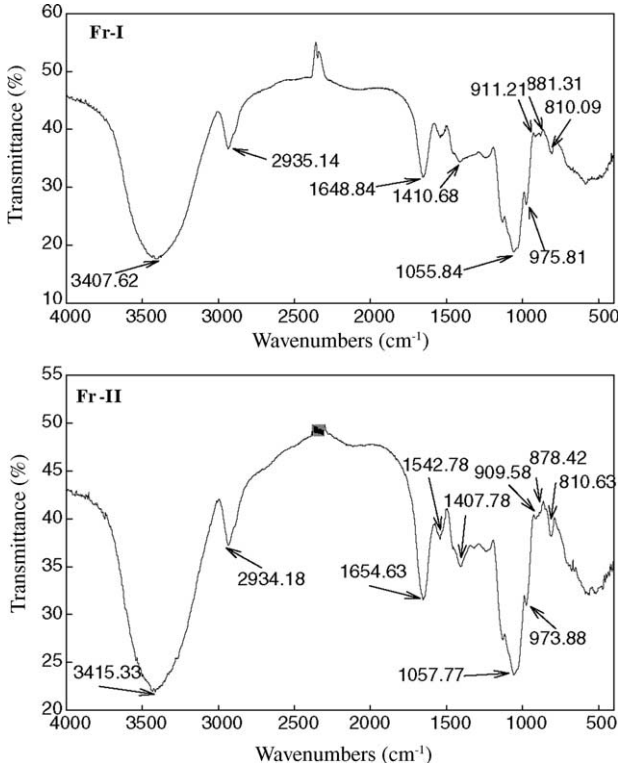

Fig. 2. The FT-IR spectra of the exopolysaccharides (Fr-I and Fr-II) produced by submerged mycelial culture of *Collybia maculata* TG-1.

the coexistence of α and β glycosidic bonds (Kim, Park, Nam, Lee, & Lee, 2003; Peng et al., 2003).

3.3. Structural analysis of EPS

The Fr-I and Fr-II were individually methylated and hydrolyzed, and the products were then converted into alditol acetates for GC–MS analysis. As summarized in Table 2, hydrolysis of the permethylated Fr-I EPS yielded six components, viz. 2,3,4,6-tetra-*O*-methyl (0.36%), 1,3,4-tri-*O*-methyl (39.04%), 2,4,6-tri-*O*-methyl (6.81%), 2,3,4-tri-*O*-methyl (5.39%), 2,3,6-tri-*O*-methyl (5.29%), and 2,4-di-*O*-methyl-D-manitol (43.11%). Meanwhile, the predominant peaks of Fr-II were characterized as 2,3,4,6-tetra-*O*-methyl (0.51%), 1,3,4-tri-*O*-methyl (28.48%), 2,4,6-tri-*O*-methyl (17.24%), 2,3,4-tri-*O*-methyl (12.64%), 2,3,6-tri-*O*-methyl (4.72%), and 2,4-di-*O*-methyl-D-manitol (36.41%). These results imply that Fr-I and Fr-II contain non-reducing (2→6), (1→3), (1→4), and (1→6) linked mannopyranosyl residues with 3,6-substituted mannopyranosyl residues. The methlylation data is consistent with the presence of a (1→3)-linked β-D-mannopyranosyl backbone mainly substituted O-6 by galactopyranosyl residues.

Resonances of the anomeric protons in ^{1}H NMR spectra are well separated and identified. The ^{1}H NMR spectra of the Fr-I and Fr-II (data not shown) exhibited signals in the high field region attributed to the β-mannopyranosyl residues (H-1, 4.80 ppm) and α-galactopyranosyl residues (H-1, 4.92 ppm) (Ganter, Heyraud, Petkowicz, Rinaudo, & Reicher, 1995). The ^{13}C NMR spectra of both EPSs are shown in Fig. 3. The high resolution ^{13}C NMR spectra of the Fr-I and Fr-II contained two main signals in the anomeric region at 101–102.6 ppm and 99.6–98.7 ppm, which were assigned to the β-D-mannopyranosyl and α-galactopyranosyl residues, respectively (Gübitza, Laussamauer, Zsilavccz, & Steiner, 2000; Petkowicz, Sierakowski, Ganter, & Reicher, 1998; Whitney, Brigham, Darke, Reid, & Gidley, 1998). The position of the C-6 of the β-D-mannopyranosyl unit was of special interest because of its potential information regarding the substitution patterns. This resonance was observed at 67.1 and 68.1 ppm for both Fr-I and Fr-II, which is for β-D-mannopyranosyl branched at O-6. This is clear evidence for β-D-mannosyl substitutions. Further, the assignment given for the carbon resonances of the D-galactose residues agrees well with the reported spectrum of monomeric α-D-galactose pyranose. (Chaubey & Kapoor, 2001; Joshi & Kapoor, 2003; Kapoor et al., 1998; Ramesh, Yamaki, Ono, & Tsushida, 2001). A low field shift of +5.9 ppm was observed in the δ value of the C-6 of the O-6 substituted β-D-mannose (δ 69.2 ppm) over that of the unsubstituted one (δ 63.3 ppm). In this study, the observed δ value for the C-6 of the β-D-mannosyl residue was 67.1 ppm (Fr-I) and 68.1 ppm (Fr-II) indicating its O-6 substitution (Ramesh et al., 2002). The broad C-3 signal at 78.7 ppm (Fr-I) and 77.8 ppm (Fr-II) could be ascribed to the presence

Table 2
Identification of partially methylated alditol acetates of the exopolysaccharides (Fr-I and Fr-II) produced by submerged mycelial culture of *Collybia maculata* TG-1

Methylated sugar	Mol%[a]		Major mass spectral fragments (*m/z*)	Linkage
	Fr-I	Fr-II		
2,3,4,6-tetra-*O*-Me-Man[b]	0.36	0.51	43,45,71,87,101,117,129,145,161,205	$Man^1\rightarrow$
1,3,4-tri-*O*-Me-Man	39.04	28.48	43, 45, 71, 87, 101, 117, 129, 145, 161, 205	$\rightarrow{}^6Man^2\rightarrow$
2,4,6-tri-*O*-Me-Man	6.81	17.24	43, 45, 87, 101, 117, 129, 161, 233	$\rightarrow{}^3Man^1\rightarrow$
2,3,4-tri-*O*-Me-Man	5.39	12.64	43, 87, 99, 101, 117, 129, 161, 189, 233	$\rightarrow{}^6Man^1\rightarrow$
2,3,6-tri-*O*-Me-Man	5.29	4.72	43, 45, 87, 99, 101, 113, 117, 233	$\rightarrow{}^4Man^1\rightarrow$
2,4-di-*O*-Me-Man	43.11	36.41	43, 87, 101, 117, 129, 189	$\rightarrow{}^{3,6}Man^1\rightarrow$

[a] Calculated from peak areas and response factors of hydrogen flame ionization detector on GC.
[b] 2,3,6-tri-*O*-Me-Man = 2,3,6-tri-*O*-methyl-Manitol.

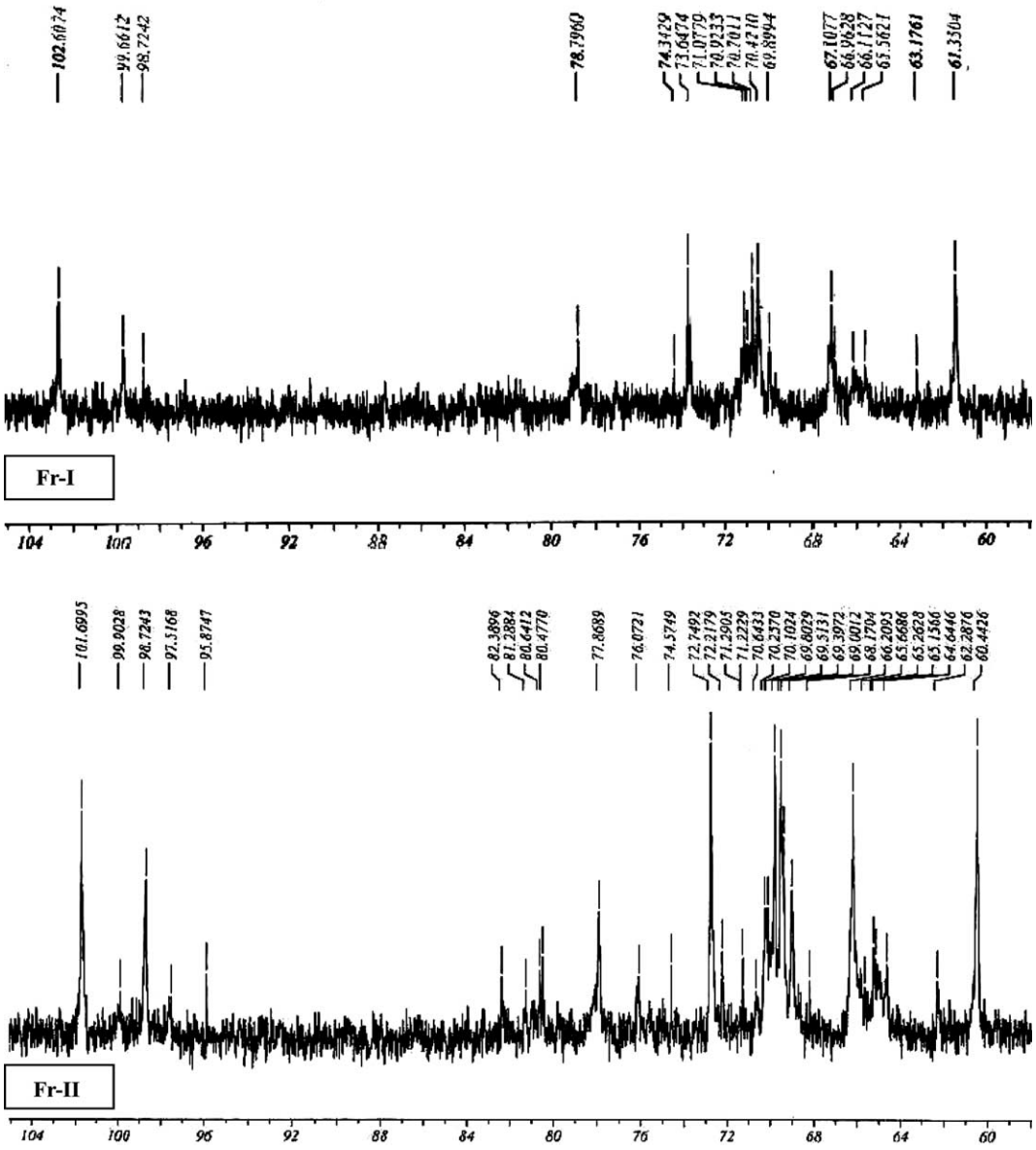

Fig. 3. ^{13}C NMR spectra of the exopolysaccharides (Fr-I and Fr-II) produced by submerged mycelial culture of *Collybia maculata* TG-1.

of liner D-(1→3), branched D-(1→6), and terminal D-residues. The peaks of C-2, C-4, and C-5 were 70.9, 66.9, and 73.6 ppm, respectively. It has been reported that a splitting of the C-3 of the β-D-mannopyranosyl residue in galactomannan arises from nearest neighbor probabilities (Kapoor et al., 1998). The methylation analysis and ^{13}C NMR spectra evidenced similarities between the galactomannan which constitutes the Fr-I and Fr-II of the extracellular polysaccharides produced by *C. maculata*.

Since these results still provide limited information concerning the branching pattern in the EPS, a more detailed characterization and interpretation would require additional enzymatic and biosynthetic studies (Joshi & Kapoor, 2003; McCleary, Clark, Dea, & Rees, 1985).

3.4. Characterization of EPS with SEC/MALLS system

The incorporation of a MALLS into a SEC system is, a powerful new development in characterization of biopolymers (Carceller & Aussenac, 2001; Wyatt, 1993). SEC coupled with MALLS and RI detectors was performed, and eventually the absolute molecular weight peaks were determined for each Fr-I and Fr-II (Fig. 4). Fr-I and Fr-II peaks appeared between the elution volume of 14–21 and 19–24 ml, respectively. The molecular mass values for two eluted fractions were calculated for the portions of peaks, which lie within the peak ranges. The relevant molecular parameters of each EPS are summarized in Table 3. The weight average molar mass (M_w) of Fr-I and Fr-II were determined to be 7.95×10^4 and 2.09×10^4 g/mol, respectively. The low values of polydispersity ratio for both EPSs mean that these EPS molecules exist much less dispersed in aqueous solution without forming large aggregates (Hwang et al., 2003). This information is important because the functional properties of polysaccharides can be greatly influenced by the molecular weight distribution. For each of these moments of the distribution, the root mean square (RMS) radii of the EPSs were calculated (Table 3). These data provide a measure of the EPS molecular size in terms of

Table 3
Relevant molecular parameters of exopolysaccharides (Fr-I and Fr-II) produced by submerged mycelial culture of *Collybia maculata* TG-1 in MALLS analysis

Parameters[a]	Fr-I (error %)	Fr-II (error %)
M_n (g mol^{-1})	7.583×10^4 (0.7)	2.047×10^4 (0.7)
M_w (g mol^{-1})	7.950×10^4 (0.7)	2.086×10^4 (0.7)
M_z (g mol^{-1})	8.352×10^4 (1.5)	2.127×10^4 (1.6)
M_w/M_n	1.048	1.017
R_n (nm)	18.8 (1.6)	23.2 (1.6)
R_w (nm)	19.8 (1.5)	23.3 (1.6)
R_z (nm)	20.9 (1.4)	23.4 (1.7)

[a] M_n, M_w, and M_z refer number-, weight-, *z*-average molecular weight, respectively. M_w/M_n means polydispersity ratio. R_n, R_w, and R_z refer number-, weight-, *z*-average square mean radius of gyration, respectively.

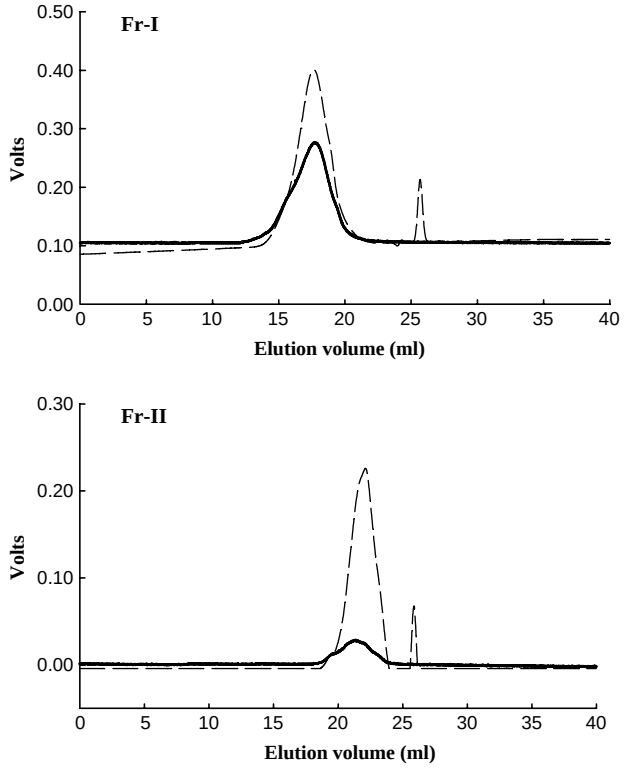

Fig. 4. Elution profiles of the polysaccharides (Fr-I and Fr-II) for the determination of molecular mass in SEC/MALLS system. For detailed analysis conditions, see Materials and methods. (–·–) MALLS detector, (—) refractive index detector.

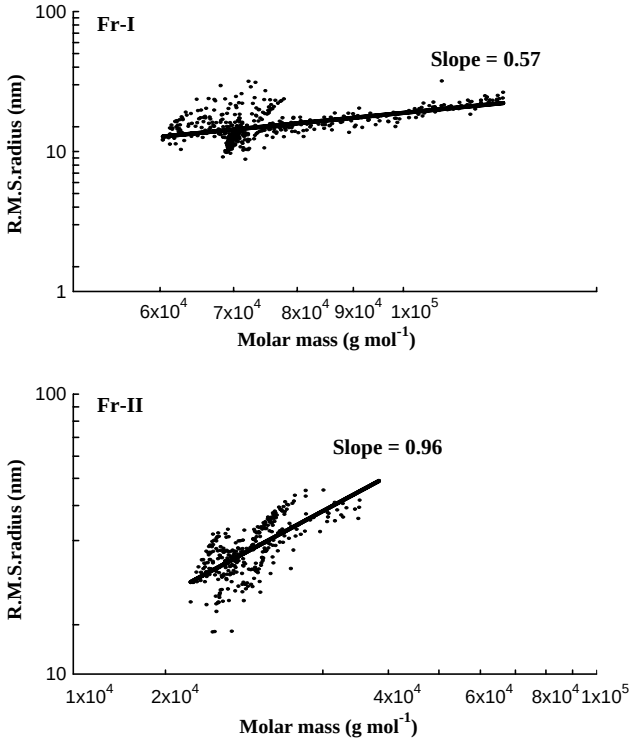

Fig. 5. The double logarithmic plots of root mean square radius (RMS) vs. molecular mass of the exopolysaccharides (Fr-I and Fr-II) produced by submerged mycelial culture of *Collybia maculata* TG-1.

the RMS distance from the molecular center of gravity to its edge (Hwang et al., 2003). The RMS radii for both peaks ranged from 18.8 to 23.4 nm with no clear trends. It appeared that the RMS radii (R_n, R_w, and R_z) of Fr-II were larger than those of Fr-I, in spite of the smaller molecular weight (Table 3).

The overall slopes for each EPS in the double logarithmic plots of RMS radius versus molecular mass were shown in Fig. 5. The values of slope indicated 0.57 and 0.96 for Fr-I and Fr-II, respectively. This implies that the Fr-I molecule exists nearly as random coils, whereas Fr-II exists as a rigid rod form in aqueous solution (see Materials and Methods). It should be mentioned here that most real coils of biopolymers are frequently slightly more extended, having the slopes between 0.55 and 0.6 in a good solvent (Wyatt, 1993; Zhang, Zhang, & Cheung, 2003; Zhang, Zhang, Zhou, Chen, & Zeng, 2000). This result gives us the useful information that Fr-I of higher molecular weight and coiled conformation are more branched than Fr-II.

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