

CHARACTERIZATION OF MOLECULAR MASS OF SIX WATER-SOLUBLE POLYSACCHARIDE-PROTEIN COMPLEXES FROM *GANODERMA TSUGAE* MYCELIUM*

Yan-fei Peng, Li-na Zhang** and Xiao-juan Xu

Department of Chemistry, Wuhan University, Wuhan 430072, China

Li-guo Cheng

The Laboratory of Applied Mycology, Central China Agriculture University, Wuhan 430070, China

Abstract Six water-soluble polysaccharide-protein complexes coded as GM1, GM2, GM3, GM4, GM5 and GM6 were isolated from the mycelium of *Ganoderma tsugae* by extracting with 0.2 mol/L phosphate buffer solution at 25, 40 and 80°C, water at 120°C, 0.5 mol/L aqueous NaOH solution at 25 and 65°C, consecutively. Their chemical components were analyzed by using IR, GC, HPLC and ¹³C-NMR, and some new results were obtained. The four samples GM1, GM2, GM3 and GM4 are heteropolysaccharide-protein complexes, in which, α -(1→3) linked *D*-glucose is the major monosaccharide while galactose, mannose and ribose are the secondary ones. GM5 and GM6 are β -(1→3)-*D*-glucan-protein complexes. The protein content increased from 32% to 69% with the progress of isolation. Weight-average molecular mass M_w and the intrinsic viscosity $[\eta]$ of the GM samples in 0.5 mol/L aqueous NaCl solution at 25°C were measured systematically by laser light scattering (LLS), size exclusion chromatography (SEC) combined with LLS, and viscometry. The M_w of GM1 to GM6 are 35.5, 46.8, 58.9, 41.6, 3.3 and 22.0×10^4 , respectively. The conformation and molecular mass of the two fractions of sample GM5 were characterized satisfactorily by SEC-LLS without further fractionation.

Key words *Ganoderma tsugae* mycelium, Polysaccharide-protein complex, Chemical component, Molecular mass, Light scattering, Size exclusion chromatography

INTRODUCTION

Ganoderma tsugae, namely *Lingzhi*, belongs to the family of *Heterobasidae*. It has been widely accepted as a health-maintaining food and traditional Chinese medicine especially in the Orient, e.g. in China, Japan and Korea, and been used to treat various human diseases, such as hepatitis, hypertension, hypercholesterolemia, gastric cancer and so on. Recent studies on this fungus have demonstrated that the carcinostatic substance in *Lingzhi* is polysaccharide, especially β -(1→3)-*D*-glucan^[1–3]. This polysaccharide seems to have promise as a new type of carcinostatic agent that might be useful in immunotherapy. Unlike chemicals used in chemotherapy, it has few toxic effects because it is based on immunological enhancement in the host^[4]. It has been reported^[4] that three polysaccharide-protein complexes with high antitumor activity were isolated from the mycelium of *G. tsugae*. Their protein contents were 9.3%, 29.8% and 54.4% respectively, and the molecular masses measured by gel filtration were about 8×10^4 – 10×10^4 . Sone *et al.*^[5] have obtained one kind of β -(1→3)-*D*-glucan with relatively high branching degree and obvious anti-tumor activity from the culture filtrate of *G. lucidum* mycelium cultivated in a liquid medium containing glucose and yeast extract. In our previous work, a β -(1→3)-glucan-

* This work was supported by the Research Fund for the Doctoral Program of Higher Education and the National Natural Science Foundation of China (No. 20074025).

** Corresponding author: Li-na Zhang (张俐娜); E-mail: lnzhang@public.wh.hb.cn

Received April 10, 2002, Revised July 8, 2002, Accepted August 9, 2002

protein complex with bioactivity was extracted from the *G. lucidum* mycelium by aqueous phosphate buffer solution at 80°C, and the weight-average molecular weight was 6×10^5 ^[6]; however, α -(1→3)-glucan has been isolated from the fruiting body of *G. lucidum*, and the molecular mass of several fractions was determined to be from 8×10^3 to 4.4×10^5 by light scattering^[7].

Polysaccharides having antitumor activity differ greatly in their chemical composition, configuration and physical properties. It has been reported that some structural features such as β -(1→3)-linkages in the main chain of glucan and further β -1,6-branch points are beneficial for some antitumor action^[5]. However, antitumor activities of polysaccharides such as α -manno-glucan^[8] and α -glucan-protein^[9] are also observed. It is worth noting that a knowledge of the solution properties of polysaccharides is also essential for predicting their anti-tumor and immunomodulatory activities^[10–12]. However, the structure, molecular mass and conformation of water-soluble polysaccharide from the *G. tsugae* mycelium have not been fully investigated. Especially in the case of highly polydisperse polysaccharides that contain two or more fractions with different composition, branching or molecular mass, complex and time-consuming isolation or fractionation is usually needed to determine the molecular mass and conformation of each fraction. In our present work, six water-soluble polysaccharide-protein complexes were isolated by successive extraction with phosphate buffer, water and alkali under hot and cold conditions from *G. tsugae* mycelium, and their chemical components, molecular weight and intrinsic viscosity were determined and discussed. In addition, we tried to characterize the fractions included in the polysaccharide-protein complex by size exclusion chromatography (SEC) combined with laser light scattering (LLS) without further fractionation.

MATERIALS AND METHODS

Cultivation of G. tsugae Mycelium

The strain of *G. tsugae*, which was isolated from the cultured fruiting body, was preserved on potato-sucrose-agar (APS) slabs. It was inoculated into a liquid medium containing 5% glucose, 15% bran extract, 1% peptone, 1% yeast extract, 0.05% KH₂PO₄, 0.05% K₂HPO₄ and 0.05% MgSO₄. The submerged cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL infusion medium. The flasks were incubated on a rotary shaker at 100 rpm at 28°C for 10 days. About 5 g beige mycelium powder per 1 liter liquid medium was obtained by filtration through cheesecloth, and lyophilized using lyophilizer (Christ Alpha 1-2, Germany) after being washed with water five times.

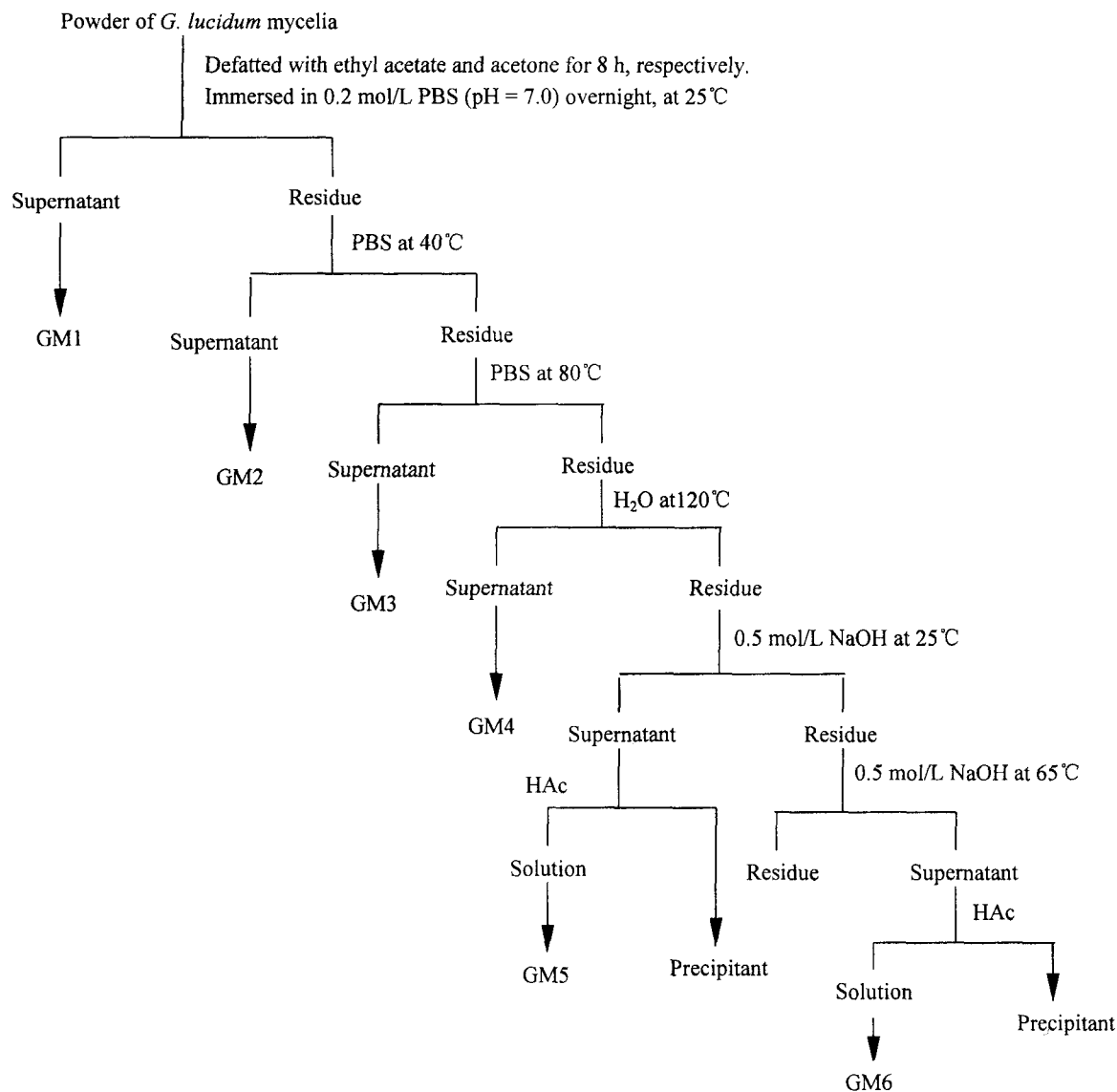
Isolation of Polysaccharides

The resulting powder was defatted with acetone and ethyl ester for 8 h, respectively. The residues were homogenized and immersed in 90% aqueous ethanol solution overnight to remove soluble compounds of low molar mass. Water-soluble polysaccharides were extracted stepwise from the mycelium with 0.2 mol/L aqueous phosphate buffer solution (PBS, pH = 7) at 25, 40 and 80°C, followed by distilled water at 120°C, 0.5 mol/L aqueous NaOH solution at 25°C and 65°C successively, and coded as GM1, GM2, GM3, GM4, GM5 and GM6. In each step, the suspension was centrifuged by ultracentrifuge at 7000 r/min for 30 min to separate the crude polysaccharide supernatant from the residue of mycelia. The concentrated supernatant was then subjected to the Sevag method to remove free proteins, and treated with 30% H₂O₂ to decolorize. The resulting aqueous solution was then dialyzed in regenerated cellulose tubing (*M_w* cut-off: 8000) against tap water for 7 days and distilled water for 3 days, and then concentrated by rotary evaporator at reduced pressure below 45°C, finally lyophilized to obtain colorless samples. This extraction procedure, similar to the one reported by Chen^[13] for the fruiting body of *G. lucidum*, is shown in Scheme 1.

Characterization

Protein content was measured on a Kjeltec1030 self-analyzer (Tecator, Switzerland) according to the semi-micro Kjeldahl principle. The N content in GM samples was measured by an elemental analyzer (Heraeus Co., Germany). Infrared spectroscopy of the samples was recorded on a Nicolet 170SX FTIR spectrometer in the range of 400–4000 cm⁻¹ with a DGTS detector and DMNIC 3.2 software. For high-performance liquid

chromatography (HPLC), the polysaccharides were hydrolyzed with 2 N trifluoroacetic acid (TFA) according to



Scheme 1 Isolation procedure of polysaccharides from *G. tusgae* mycelia

the following procedure^[14]. Each sample in a sealed tube was kept at 100°C for 18 h, the TFA left in the product was removed by repeated rotary evaporation under reduced pressure at 50°C. The monosaccharides were identified by a HPLC instrument (Shimadzu LC-6A) with a refractive index detector and a Shodex Sugar SC1011 column at 80°C. Distilled water was used as the mobile phase with flow rate of 0.7 mL/min. Gas chromatography (GC) of the alditol acetate derivatives of the saccharides according to the literature^[15] was done on a HP 6890 instrument (Hewlett Packard, USA) with an DB-225 column (15 m × 0.25 mm), and at temperatures programmed from 180°C to 220°C at 4 K/min. ¹³C-NMR spectrometry was performed on a DRX-400BB spectrometer (Bruker Co. Germany) at 25°C, DSS and TMS as the internal standard. D₂O was used as the solvent for GM1 and GM3, and DMSO-*d*₆ for GM5, respectively.

Viscosity Measurement

Viscosities of the water-soluble polysaccharides in 0.5 mol/L aqueous NaCl solution were determined at 25°C

using an Ubbelohde viscometer. The kinetic energy correction was always negligible. Huggins and Kraemer plots were used to estimate the intrinsic viscosity $[\eta]$.

Laser Light Scattering (LLS)

The light-scattering intensities were measured with a multi-angle laser light scattering instrument equipped with a He-Ne laser ($\lambda = 632.8$ nm) (Dawn-DSP, Wyatt Technology Co., USA) at 25°C. The samples were dissolved in 0.5 mol/L aqueous NaCl solution. Optical clarification of the solution was done by filtration through a 0.2 μm filter (Whatman). The refractive index increments (dn/dc) were measured with an optilab refractometer (OPTILAB-DSP, Wyatt Technology Co., USA) at 633 nm and 25°C. The values of dn/dc of samples in 0.5 mol/L NaCl aqueous solution were determined to be 0.141 mL g⁻¹ for GM1, GM2, GM3, GM4, and 0.152 mL g⁻¹ for GM5, GM6, accordingly. Astra software was utilized for data acquisition and analysis.

SEC-LLS Measurements

Size-exclusion chromatography combined with LLS (SEC-LLS) were performed on the laser photometer mentioned above combined with a pump P100 equipped with TSK-GEL a G6000PWXL and a G4000PWXL column (7.8 mm $\times$ 300 mm) and differential refractive index detector (RI-150) at 25°C. The eluent was 0.5 mol/L NaCl aqueous solution at a flow rate 1.0 mL/min. All the solutions used were filtered through a sand filter, and then a 0.45 μm filter (Wattman). Astra software was utilized for data acquisition and analysis.

RESULTS AND DISCUSSION

The six GM samples were all water-soluble. Their IR spectra are shown in Fig. 1. GM1, GM2, GM3 and GM4 all have absorption peaks at 925, 850 and 822 cm⁻¹, namely, the characteristic bands of α -D-glucan^[16]. Interestingly, GM5 and GM6, extracted with 0.5 mol/L aqueous NaOH solutions, have absorption peaks at 890 cm⁻¹, indicating that they contain β -D-glucan. The strong absorption at 1650 and 1547 cm⁻¹, corresponding to the secondary —CONH— group^[17], showed the existence of protein. Besides, no absorption band over 1700 cm⁻¹ indicates the absence or trace of uronic acids in the samples^[18].

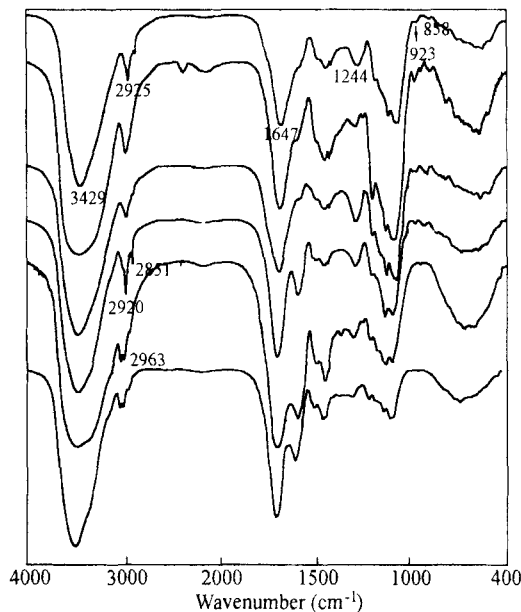


Fig. 1 FT-IR spectra of water soluble polysaccharides from *G. tsugae* mycelia

The HPLC results indicate that the GM polysaccharides mainly consist of D-glucose, and GM1, GM2, GM3 and GM4 also contain other sugars. Usually, GC analysis could give the accurate content of sugars in the

polysaccharides. The experimental results from GC and protein analysis are summarized in Table 1. The predominant monosaccharide in GM samples is *D*-glucose, which is consistent with the results of HPLC. At the same time galactose, mannose and ribose co-exist in GM1, GM2, GM3 and GM4. Clearly, the protein content is relatively high, and increases from 32% to 69% with the progress of isolation. Because the procedure of Sevag methods was repeated for more than 10 times, it could be concluded that the protein remains in the samples are mainly bound with polysaccharides.

Table 1. Components of monosaccharides and protein content of GM samples from the mycelium of *G. tsugae*

Sample	Sugar component (mole ratio)									Protein (%)	
	Rha	Fuc	Rib	Ara	Xyl	Man	Gal	Glc	GlcN	A	B
GM1	–	–	0.2	–	–	0.4	0.6	1.0	–	31.8	33.9
GM2	0.1	–	–	0.1	0.2	0.2	0.4	1.0	0.3		34.5
GM3	–	–	0.4	–	–	0.2	0.3	1.0	–	38.1	40.2
GM4	–	–	0.2	–	–	0.4	0.8	1.0	–		52.2
GM5	–	–	–	–	–	–	–	1.0	–	68.1	69.4
GM6	–	–	–	–	–	–	–	1.0	–	64.1	67.3

Abbreviation: Rha means Rhamnose; Fuc, Fucose; Rib, Ribose; Ara, Arabinose; Xyl, Xylose; Man, Mannose; Gal, Galactose; Glc, Glucose; GlcN, Glucosamine; “–” means that not detected. A means data obtained from KJELETC1030 self-analyzer. B, data obtained from elemental analysis.

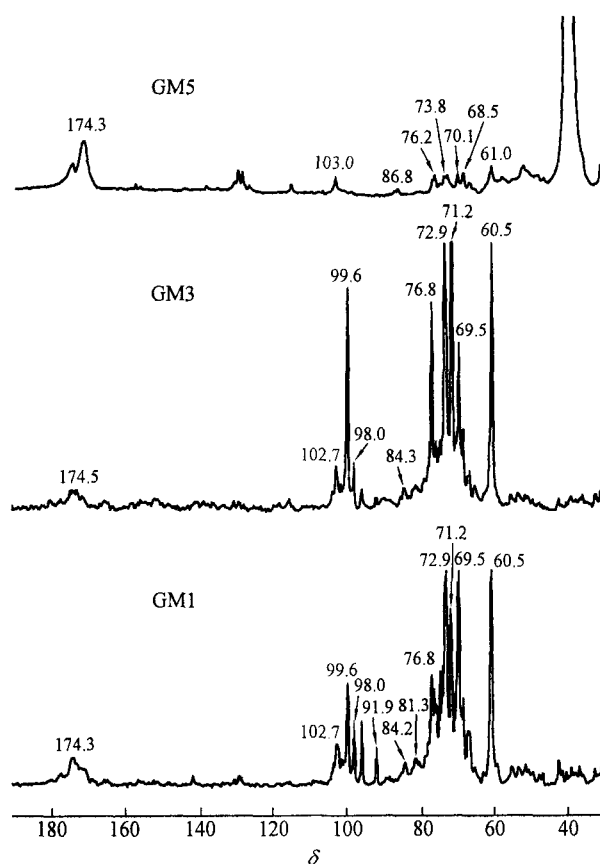


Fig. 2 ^{13}C -NMR spectra of GM1, GM3 in D_2O and GM5 in $\text{DMSO}-d_6$ at 25°C

The ^{13}C -NMR spectra for GM1 and GM3 in D_2O and GM5 in $\text{DMSO}-d_6$ are shown in Fig. 2. The strong signal at $\delta = 99.6, 71.2, 84.2, 69.5, 72.9$ and 60.5 for samples GM1 and GM3 is assigned to C1, C2, C3, C4, C5 and C6 of branched α -(1→3)-*D*-glucan^[19, 20]. The relatively small peaks at $\delta = 102.7, 76.8, 67.3, 98.0$ and 81.3

may be attributed to chemical shifts of C1, C4 and substituted C6' for α -linked-*D*-galactose residues, as well as C1 and C3 for β -linked *D*-mannose. This indicates that the polysaccharide in GM1 and GM3 are mainly composed of α -linked *D*-glucose, α -linked *D*-galactose and β -linked *D*-mannose, and the predominant saccharide of the heteropolysaccharide is α -(1→3)-linked *D*-glucose. The resonance peaks at $\delta = 172\text{--}178$ can be assigned to the --CONH-- ^[21] group of protein. Therefore, GM1 and GM3 are heteropolysaccharide-protein complexes. In ¹³C-NMR spectrum of GM5, the strong signals at $\delta = 171\text{--}74$ are assigned to protein, and the relatively small peaks at $\delta = 103.0, 73.8, 86.8, 68.5, 76.2, 61.0$ and 70.1 are assigned to C1, C2, C3, C4, C5, C6 and substituted C6' for β -(1→3)-linked *D*-glucan^[22–24]. Thus GM5 is a β -(1→3)-*D*-glucan-protein complex, and protein is the predominant species. The results are in good agreement with the data obtained from GC, IR, HPLC and protein analysis. In view of these results and their isolation procedure, GM2 and GM4 can be referred to as heteropolysaccharide-protein and GM6 as β -(1→3)-*D*-glucan-protein complex.

The Zimm plot of GM1 in 0.5 mol/L aqueous NaCl solution at 25 °C is shown in Fig. 3. The values of M_w and $[\eta]$ for the GM samples are listed in Table 2. Similar to the arabinogalactan – protein complexes^[25, 26], they all have relatively low $[\eta]$ as compared with normal polysaccharides having the same molecular mass. This is understandable because of the relatively high content of protein, which usually has low $[\eta]$, and adopts a global conformation in aqueous solution^[27]. β -(1→3)-*D*-glucan as the main chain of GM5 and GM6 often adopt a relatively expanded conformation in aqueous solution, resulting in higher values of $[\eta]$ as compared with the complex containing α -*D*-glucose. M_w of the samples determined by SEC-MALLS are also collected in Table 2 and are consistent with the results measured by MALLS.

Table 2. Experimental results from LLS and viscosity for the polysaccharides from the mycelium of *G. tsugae* in 0.5 mol/L aqueous NaCl solutions at 25 °C

Sample	$[\eta]$ (g ⁻¹ cm ³)	$M_w \times 10^{-4}$		
		LLS	SEC-LLS	Mean-value
GM1	10.7	33.8	37.2	35.5
GM2	23.1		46.8	46.8
GM3	29.4	54.8	62.9	58.9
GM4	27.0	42.8	40.3	41.6
GM5	14.7	3.4	3.1	3.3
GM6	23.7	23.4	20.5	22.0

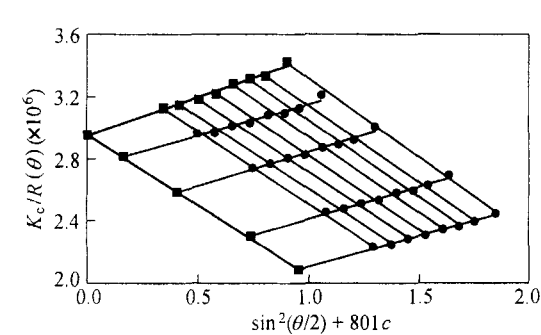


Fig. 3 Zimm Plot for GM1 in 0.5 mol/L NaCl aqueous solution at 25 °C

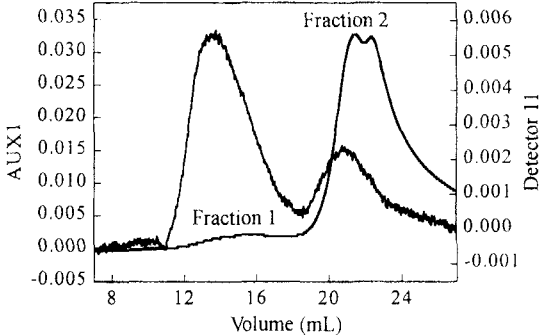


Fig. 4 The SEC chromatogram of GM5 in 0.5 mol/L NaCl aqueous solution at 25 °C detected by LLS (- - -) and differential refractometer (—)
The detector 11 and detector AUX1 represent signals from the LLS at 90° and the refractive index detectors

Figure 4 shows the SEC chromatograms of GM5 in 0.5 mol/L aqueous NaCl solution at 25 °C. There are two peaks in the chromatograms, indicating that two fractions exist in GM5. The M_w , $\langle r^2 \rangle_z^{1/2}$ and relative content of each fraction are summarized in Table 3. Since each SEC-LLS chromatogram can produce two functions: $M = f$

(v) and $\langle s^2 \rangle_z^{1/2} = f(M)$, the power law of the latter can be estimated from many experimental points detected by LLS^[28]. Figure 5 shows the $\langle s^2 \rangle_z^{1/2} = f(M)$ power law for the two fractions of GM5 as shown in Fig. 4. The exponents are approximately 0.3 for the fraction 1 and 0.6 for the fraction 2. Usually, the exponent of flexible polymer in good solvent is 0.6–0.8, and that of protein is 0.3–0.5. Therefore, it is possible that fraction 1 may be mainly composed of protein while fraction 2 mainly consists of polysaccharide. These imply that SEC-LLS is really efficient for determining the molecular mass and conformation of the fractions in the complex polysaccharide.

Table 3. The experimental values of molecular mass and parameters of the two fractions included in GM5

GM5	Content (%)	$M_w \times 10^{-4}$	M_w/M_n	$\langle s^2 \rangle_z^{1/2}$ (nm)	α
Fraction 1	6.3	32.0	1.7	42.9	0.3
Fraction 2	93.7	1.3	1.2	47.8	0.6

α means the exponent in the formula $\langle s^2 \rangle_z^{1/2} = kM^\alpha$

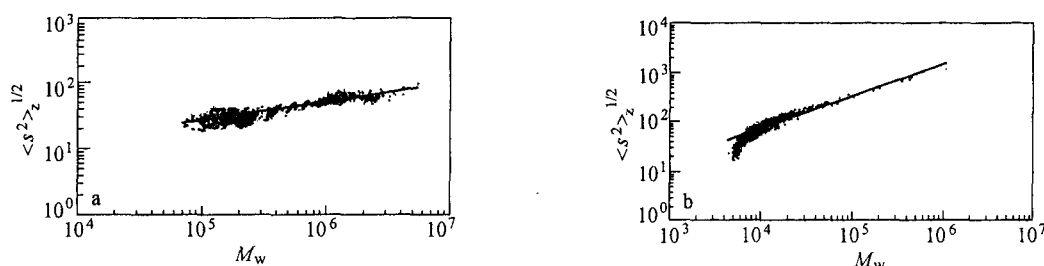


Fig. 5 M_w dependence of $\langle s^2 \rangle_z^{1/2}$ of the sample GM5 in 0.5 mol/L NaCl aqueous solution at 25 °C (a) for fraction 1 and (b) for fraction 2 as shown in Fig. 4

CONCLUSIONS

The chemical component and molecular mass of six water-soluble polysaccharide-protein complexes by extraction with different solvent from *G. tusgae* mycelium were clarified. Their molecular mass was determined to be in the range of 3.3×10^4 to 58.9×10^4 . The SEC-LLS was successfully used to measure the molecular mass and conformation of GM5 as an example. This work provides a simple procedure for the determination of complex polymer without further fractionation.

REFERENCES

- 1 Mizuno, T., Wang, G., Zhang, J., Kawagishi H., Nishitoba T. and Li, J., Food Rev. Intern., 1995, 11: 151
- 2 Won, S., Lin, M. and Wu, W., Japan J. Pharm., 1992, 59: 171
- 3 Lin, B. and Chen, M., FASEB J., 2000, 14: 1122
- 4 Zhang, J., Wang, G. and Li, H., Biosci. Biotech. Biochem., 1994, 58: 1202
- 5 Sone, Y., Okuda, R., Wada, N., Kishida, E. and Misaki, A., Agric. Biol. Chem., 1985, 49: 2641
- 6 Chen, J., Zhang, L., Yu, D. and Zhu, R., Chem. J. Chinese Universities., 2000, 21: 961
- 7 Chen, J., Zhang, L., Nakamura, Y. and Norisuge, T., Polym. Bull., 1998, 41: 471
- 8 Mizuno, T., Saito, H., Nishitoba, T. and Kawagishi, H., Food Rev. Intern., 1995, 11: 23
- 9 Kawagishi, H., Kanao, T., Inagaki, R., Mizuno, T., Shimura, K., Ito, H. and Hugiawara, T., Carbohydr. Res., 1990, 12: 393
- 10 Tanigami, Y., Kuaumoto, S., Nagao, S., Kokeyuchi, S., Kato, K., Kotani, S. and Shiba, T., Chem. Pharm. Bull., 1991, 39: 1782
- 11 Ogawa, K. and Tsurugi, J., Carbohydr. Res., 1973, 29: 397

- 12 Mischnick, P., *Macromol. Symp.*, 1995, 99: 3
- 13 Chen, J., Zhou, J., Zhang, L., Nakamura, Y. and Norisuye, T., *Polym. J.*, 1998, 30: 838
- 14 Adilma, S., Daniela, M. and Ronaldo, J., *Intern. J. Biomacromolecules*, 1997, 21: 115
- 15 Hans, N., Michael, E. and Geoffrey, J., *Analyst*, 1994, 119:1497
- 16 Jozef, S., Grigorij, K., Marta, K. and Eva, M., *Carbohydr. Polym.*, 1999, 38: 247
- 17 Dudley H. and Lan F., *Spectroscopic methods in organic chemistry*. McGraw-Hill Book Company Limited (NK), 1980, p.58
- 18 Krcmar, P., Novotny, C., Marais, M. and Joseleau, J., *Intern. J. Bio. Macromolecules*, 1999, 24: 61
- 19 Zhang, P., Zhang, L. and Cheng, S., *Biosci. Biotechnol. Biochem.*, 1999, 63: 1197
- 20 Zhang, L., Zhang, M., Zhou, Q., Chen, J. and Zeng, F., *Biosci. Biotechnol. Biochem.*, 2000, 64: 2172
- 21 Andrzej, T., Romana, A. and Iwona, W., *Carbohydr. Res.*, 2001, 334: 71
- 22 Satio, H., Tabeta, R., Sasaki, T. and Yoshioka, Y., *Bull. Chem. Soc. Jpn.*, 1986, 59: 2093
- 23 Kogan, G., Alfoldi, J. and Masler, L., *Biopolymers*, 1988, 27: 1055
- 24 Deng, C., Yang, X., Gu, X., Wang, Y., Zhou, J. and Xu, H., *Carbohydr. Res.*, 2000, 328: 629
- 25 Picton, J., Bataille, I. and Muller, G., *Carbohydr. Polym.*, 2000, 42: 23
- 26 Connolly, S., Fenyó, J. and Vandeveld, M., *Carbohydr. Polym.*, 1988, 8: 23
- 27 Wu, Q. and Zhang, L., *J. Appl. Polym. Sci.*, 2001, 82: 3373
- 28 Mendichi, R., Giammona, G., Cavallaro, G. and Giacometti, S., *Polymer*, 2000, 41: 8649