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Solution properties of water-insoluble polysaccharides from the mycelium of *Ganoderma tsugae*

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Abstract

Two kinds of water-insoluble polysaccharides (GM5-1 and GM6-1) were isolated from the mycelium of *Ganoderma tsugae* with 0.5 M NaOH aqueous solution at 25 and 65 °C successively. Their chemical structures were characterized by infrared spectroscopy, gas chromatography and ^{13}C NMR. GM5-1 was composed of (1→3)-α-D-glucan and mannoxylan, while GM6-1 was mainly mannoxylan. The sample GM6-1 was fractionated into five fractions by nonsolvent addition, and their weight-average molecular mass (M_w) and intrinsic viscosity [η] were determined by laser light scattering (LLS), size-exclusion chromatography combined with multi-angle laser light scattering (SEC-LLS) and viscometer in 0.25 M LiCl/DMSO at 30 °C. The M_w dependences of [η] and the radius of gyration ($\langle S^2\rangle_z^{1/2}$) were found to be $[\eta]=8.9\times10^{-2}\,M_w^{0.57}$ (cm^3 g^{-1}) and $\langle S^2\rangle_z^{1/2}=4.1\times10^{-2}\,M_w^{0.52}$ (nm) for GM6-1 in the M_w range from 1.50×10^6 to 4.81×10^6, implying a random coil conformations in the solution. Molecular parameters of GM6-1 were calculated on the basis of the wormlike cylinder chain to be 4.2 nm for persistent length (q) and 832 nm^{-1} for molar mass per unit contour length (M_L).

Keywords: *Ganoderma tsugae*; Polysaccharides; Weight-average molecular mass; Intrinsic viscosity; Light scattering.

1. Introduction

The fruiting bodies and cultured mycelia of *Ganodema(G.) tsugae*, a traditional Chinese medicine, have been reported to be effective in the treatment of many diseases, such as chronic hepatopathy, hypertension, and neoplasia (Kimura, Okuda, & Arichi, 1988; Lin, Lin, Chiu, Yang, & Lee, 1993). Recently, this fungus has attracted much attention due to the fact that dozens of polysaccharides isolated from it have shown obvious antitumor activities (Gao et al., 2000; Wang et al., 1993; Zhang et al., 1994). However, compared with the intensive investigation of water-soluble polysaccharides, solution properties of the water -insoluble ones from the mycelium of *G. tsugae* have been scarcely reported.

In our previous work, a water-insoluble (1→3)-α-D-glucan extracted from the fruiting body of *G. lucidum* by alkali has been proved to exist as a flexible chain in 0.25 M LiCl/DMSO at 25 °C (Chen, Zhang, Nakamura, & Norisuye, 1998a; Chen, Zhou, Zhang, Nakamura, & Norisuye, 1998b). The native (1→3)-α-D-glucan hardly shows antitumor activity, while its water-soluble derivatives obtained by carboxymethylation and sulfation have significant antitumor activities, suggesting an effect of increasing chain stiffness on the enhancement of antitumor activities (Zhang, Zhang, Chen, & Zeng 2001; Zhang, Zhang, Zhou, Chen, & Zeng, 2000). Therefore, the effects of the solution properties of polysaccharides, such as chain conformation and molecular mass also could not be ignored (Zhang et al., 2001).

Recently, we have reported the isolation, structural characterization, conformational behavior and antitumor

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activities of several water-soluble polysaccharides from the mycelia of *G. tsugae* (Peng, Zhang, & Zeng, 2003a; Peng, Zhang, Zeng, & Xu, 2003b). In this work, two novel water-insoluble polysaccharides were extracted through alkali solution. Their chemical structures were characterized, and the solution properties were studied by laser light scattering and viscometry according to the theory of polymer solution.

2. Experimental

2.1. Isolation and purification of polysaccharides

Mycelia of *G. tsugae* were obtained through submerged cultivation and extracted as described elsewhere (Peng, et al., 2003b). After being defatted, 100 g mycelia were extracted with 10L 0.2 M phosphate buffer (pH, 7.0, PBS), which was made of potassium dihydrogen phosphate and dipotassium hydrogen phosphate, and then 3.0L hot water, the residue of *G. tsugae* mycelium was extracted by 2%NaOH/0.02%$NaBH_4$ at 25 and 65 °C, successively. The supernatants were neutralized with acetic acid. Both the precipitates after neutralization were re-purified as follows: the precipitates were dissolved in dimethylsulfoxide containing 0.25 M LiCl (0.25 M LiCl/DMSO), and then centrifuged to remove the insoluble fractions. The resulting supernatants were re-precipitated by adding 80% acetone aqueous solution, then washed with 50% acetone aqueous solution and pure acetone five times, finally vacuum-dried for 7 days to obtain white powders coded as GM5-1 for the water-insoluble polysaccharide extracted at 25 °C and GM6-1 for the one extracted at 65 °C, respectively.

2.2. Fractionation of polysaccharides

The non-solvent addition method is a method whereby successive precipitation of polymer species from solution occurs by addition of a miscible non-solvent. The sample GM6-1 was fractionated with 0.25 M LiCl/DMSO as the solvent and a mixture of acetone and 0.25 M LiCl/DMSO (4:1 by volume) as precipitant. Precipitant was gradually added to the 0.25 M LiCl/DMSO solution of the sample (400 mL, 0.50%) at 30 °C until the solution turned milky. The solution was warmed at 60 °C for 3 h, and then kept at 30 °C for 12 h, resulting in a separation into a liquid phase and a gel phase. The gel, namely the precipitated polymer fraction, was removed by centrifugation, and the liquid was subjected to the next fractionation. Each fraction was re-purified through dissolving in 0.25 M LiCl/DMSO, and precipitated by adding 80% aqueous acetone. Then the precipitate was washed with 50% aqueous acetone and pure acetone five times each, and finally vacuum-dried for 7 days to obtain white powders. From the fractions thus prepared, 5 fractions in middle ones were chosen for studying the solution properties, and coded as GM6-1-F1, GM6-1-F2, GM6-1-F3, GM6-1-F4 and GM6-1-F5.

2.3. General methods

The ^{13}C NMR spectra for the samples GM5-1 and GM6-1 were recorded on an INOVA-500 spectrometer (Varian Inc., America) with 125 MHz at 60 °C, using 0.25 M LiCl/DMSO-d_6 as solvent. The spectral width was 200 ppm. Chemical shifts were expressed in δ (ppm) relative to the resonance of 2,2′-dimethyl-2-silapentane-5- sulphonate (DSS, $\delta = 0$). The N element content was measured on an elemental analyzer (Heraeus Co., Germany). Infrared spectroscopy of the samples was recorded with Nicolet 170SX FT-IR (Perkin Elmer Co. USA) spectrometer equipped with DGTS detector and DMNIC 3.2 software in the range of 400–4000 cm^{-1} using the KBr-disk method. Gas chromatography (GC) of the alditol acetates derivatives of monosaccharides according to the literature (Englyst, Quigley, & Hudson, 1994) was performed with a HP 6890 instrument (Hewlett Packard, USA) using an Alltech DB-225 capillary column (15 m × 0.25 mm) programmed from 180 to 220 °C at 4 °C/min and held at 220 °C for 30 min.

2.4. Size exclusion chromatography combined with laser light scattering (SEC-LLS) measurements

SEC-LLS measurements were carried out on a DAWN-DSP multi-angle laser photometer ($\lambda = 633$ nm, Wyatt Technology Co., USA) combined with a P100 pump (Thermo Separation Products, San Jose, USA) equipped with TSK-GEL G4000H6 column (7.8 mm × 300 mm) at 30 °C. A differential refractive index detector (RI-150) was simultaneously connected. The eluent was 0.25 M LiCl/DMSO at a flow rate of 1.0 mL/min. All the solutions used were first filtered with a sand filter and then with a 0.20 μm filter (Whattman, England). Astra software was utilized for data acquisition and analysis. The refractive index increments (d*n*/d*c*) were determined by using an optilab refractometer (OPTILAB-DSP, Wyatt Technology Co., USA) at 30 °C. The values of d*n*/d*c* at 633 nm obtained were 0.058 mL g^{-1} for GM6-1 in 0.25 M LiCl/DMSO.

2.5. Laser light scattering

The light-scattering intensities were measured with a multi-angle laser light scattering instrument equipped with a He–Ne laser ($\lambda = 633$ nm) (DAWN-DSP, Wyatt Technology Co., USA) at 30 °C. The samples were dissolved in 0.25 M LiCl/DMSO. Optical clarification of the solution was done by filtration through a 0.2 μm filter (Whattman, England). Astra software was utilized for data acquisition and analysis.

Table 1
The yield and sugar composition of water-insoluble polysaccharides from *G. tsugae* mycelium

Samples	Yielding (%)	Sugar component (%)					
		Fuc	Xyl	Man	Gal	Glc	Nac
GM5-1	2.1	0.81	21.78	41.44	nd[a]	31.29	4.68
GM6-1	1.9	0.93	32.26	57.58	nd	4.74	4.09

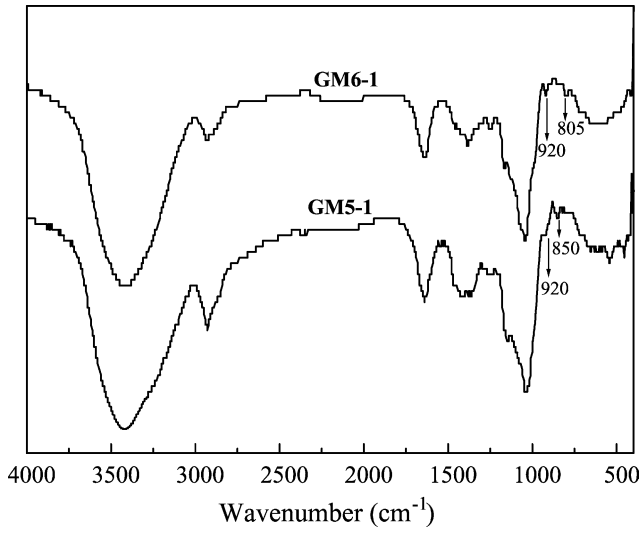

Fig. 1. FT-IR spectra of water-insoluble polysaccharides GM5-1 and GM6-1 from *G. tsugae* mycelium.

2.6. Viscosity

The viscosity of GM6-1 fractions in 0.25 M LiCl/DMSO was determined by the Ubbelohde type viscometer at 30±0.1 °C. The kinetic energy correction was negligible. Huggins and Kraemer equations were used to estimate the intrinsic viscosity [η] by extrapolation to infinite dilution as follows

$$\eta_{sp}/c = [\eta] + k'[\eta]^2 c \quad (1)$$

$$(\ln \eta_r)/c = [\eta] - k''[\eta]^2 c \quad (2)$$

where k' and k'' are constants for a given polymer under given conditions in a given solvent; η_{sp}/c, the reduced specific viscosity; $(\ln \eta_r)/c$, inherent viscosity.

3. Results and discussion

3.1. Chemical structure

The yield and sugar composition of the samples GM5-1 and GM6-1 are listed in Table 1. No protein was found in both samples. The sugar composition of the polysaccharide GM5-1 was mainly mannose, glucose and xylose. For GM6-1, mannose was the major sugar followed by xylose. The IR spectra of the polysaccharides GM5-1 and GM6-1 are shown in Fig. 1. The main peaks were 920 and 850 cm^{-1} for GM5-1, characteristic for α-glucan, while 920 and 800 cm^{-1} for GM6-1, indicating an existence of mannose. Fig. 2 shows the ^{13}C-NMR spectra of GM5-1 and GM6-1 in 0.25 M LiCl/DMSO-*d6* at 60 °C. The six strong signals at 99.4, 82.49, 71.88, 70.9, 69.5 and 60.4 ppm in the spectrum of GM5-1 were assigned to the C-1, C-3, C-5, C-2, C-4 and C-6 of (1→3)-α-D-glucan (Chen et al., 1998a). Interestingly, the other signals with

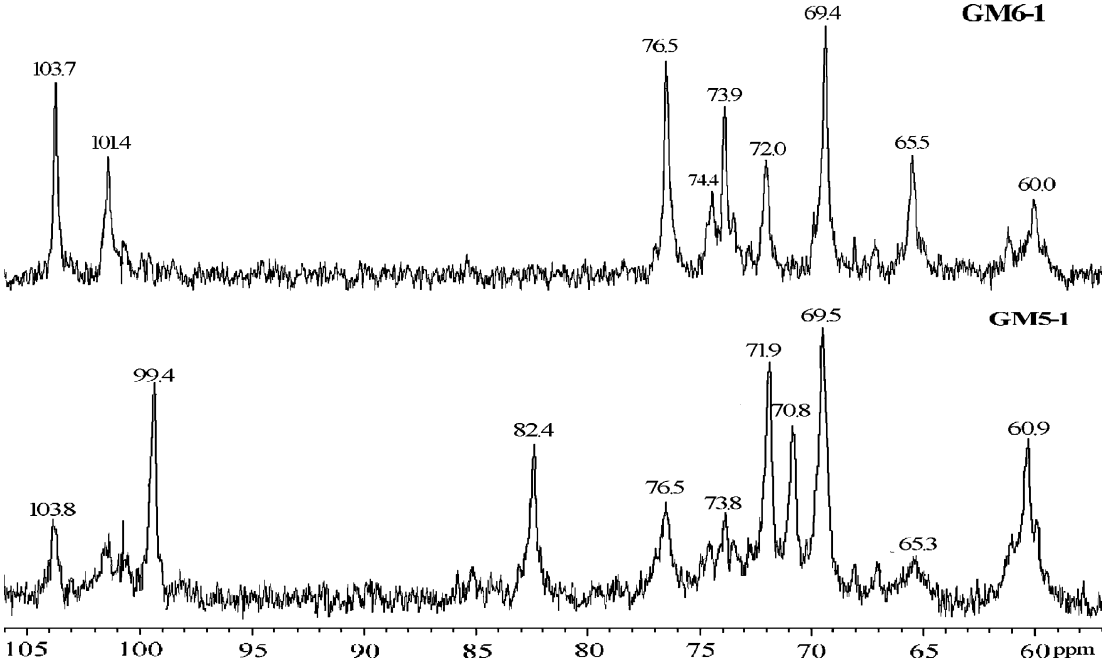

Fig. 2. The ^{13}C NMR spectra of GM5-1 and GM6-1 in 0.25 M LiCl/DMSO-*d6* at 60 °C.

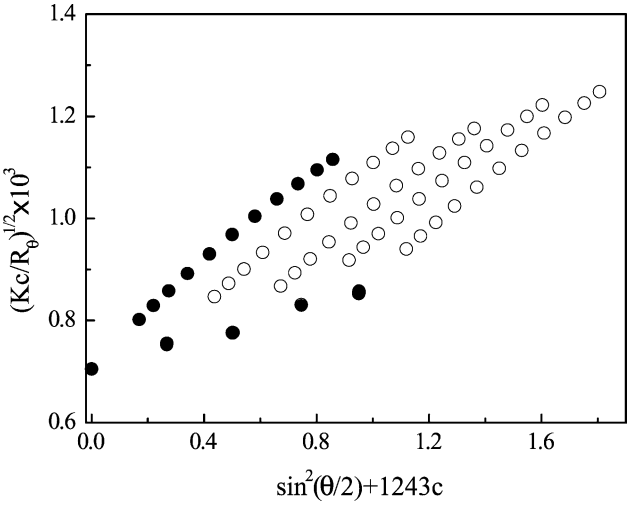

Fig. 3. Berry plot of GM6-1-F2 in 0.25 M LiCl/DMSO at 30 °C.

lower intensity for GM5-1 became the main ones in the spectrum of GM6-1, which were assigned to mannoxylan. Therefore, the water-insoluble polysaccharides from the mycelium have obviously different structure with the corresponding water-insoluble linear (1→3)-α-D-glucan from the fruiting body and spores of *G. lucidum* (Bao, Duan, & Fang, 2001).

3.2. Mark–Houwink equation

Fig. 3 is the typical Berry plot of the fraction 2 of GM6-1 in 0.25 M LiCl/DMSO at 30 °C. The values of weight-average molecular mass (M_w), radii of gyration ($\langle S^2\rangle^{1/2}$) and polydispersity index (M_w/M_n) for the fractions are summarized in Table 2, along with [η]. It is noteworthy that the M_w of GM6-1 fractions were above 1×10^6, and much more than that of the polysaccharides from the mycelium of *Poria cocos* (Jin et al., 2003). The molecular mass dependence of [η] is shown in Fig. 4. The Mark–Houwink equation was established to be: $[\eta]=8.9\times10^{-2}\ M_w^{0.57}$. For flexible polymer in good solvent, the exponent α-value is in the range from 0.5 to 0.8. The α-value of 0.57 indicated that the macromolecules of GM6-1 in the M_w range from 1.5×10^6 to 4.8×10^6 exist as random coil chains in 0.25 M LiCl/DMSO at 30 °C.

Table 2
Results from SEC-LLS, LLS and viscosity measurement on fractions of GM6-1 in 0.25 M LiCl/DMSO at 30 °C

Fractions	M_w/M_n	[η] (mLg^{-1})	$M_w\times10^4$	$\langle S^2\rangle_z^{1/2}$ (nm)
GM6-1-F1	1.4	585	481	120
GM6-1-F2	1.3	519	390	105.1
GM6-1-F3	1.4	440	295	93.9
GM6-1-F4	1.3	360	200	76.6
GM6-1-F5	2.0	298	150	64.6

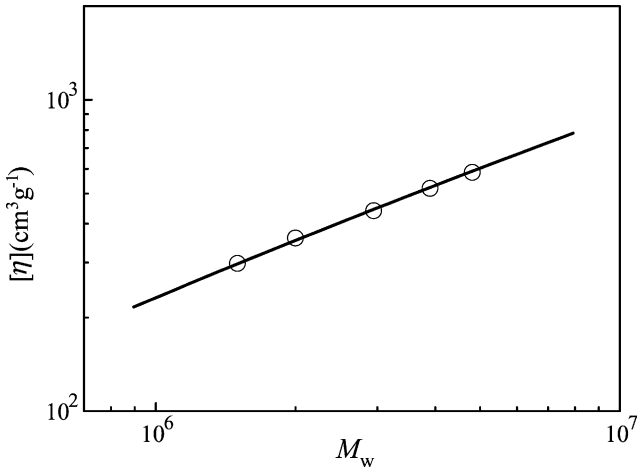

Fig. 4. Molecular weight dependence of [η] for GM6-1 in 0.25 M LiCl/DMSO at 30 °C, compared with the theoretical curves (the solid line) for the unperturbed wormlike chain with $d=0.9$ nm, $q=4.4$ nm. $M_L=830\ \text{nm}^{-1}$, $B=0.0$ nm.

3.3. Chain conformation

Based on above analysis, a wormlike cylinder model can be used for conformational characteristic of GM6-1. Yamakawa et al (Yamakawa & Fujii, 1974) independently showed that Yomakawa–Fujii–Yoshizaki (YFY) theory for [η] of unperturbed wormlike cylinder can be represented approximately by

$$(M_w^2/[\eta])^{1/3} = A_\eta + B_\eta M_w^{1/2} \quad (3)$$

$$A_\eta = A_0 M_L \Phi_{0,\infty}^{-1/3}\ \ (\text{g}^{1/3}\ \text{cm}^{-1}) \quad (4)$$

$$B_\eta = B_0 \Phi_{0,\infty}^{-1/3}(2q/M_L)^{-1/2}\ \ (\text{g}^{1/3}\ \text{cm}^{-1}) \quad (5)$$

where q and M_L are the persistence length and the molar mass per unit contour length, respectively. A_0 and B_0 are

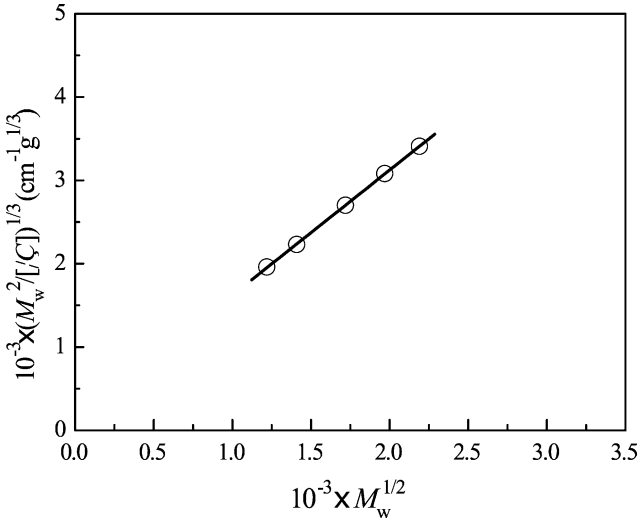

Fig. 5. Plot of $(M_w^2/[\eta])^{1/3}$ vs $M_w^{1/2}$ for GM6-1 in 0.25 M LiCl/DMSO at 30 °C.

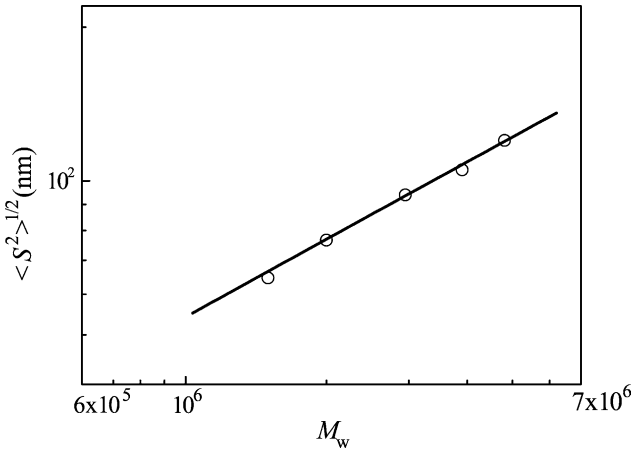

Fig. 6. Radii of gyration for the GM6-1 fractions in 0.25 M LiCl/DMSO at 30 °C, compared with the theoretical curves (the solid line) for the unperturbed wormlike chain (a) with $q=6.8$ nm. $M_L=760$ nm^{-1}, $B=0.0$ nm.

tabulated in Bohdanecky's paper (Bohdanecky, 1983), and $\Phi_{0,\infty}$ is 2.87×10^{23}. The $(M_w^2/[\eta])^{1/3}$ vs $M_w^{1/2}$ is plotted in Fig. 5. Substitution of the intercept and slope of this plot into Eqs. (3)–(5) yielded 824 nm^{-1} for M_L and 4.2 nm for q, indicating a flexible chain conformation.

The double logarithmic plot of $\langle S^2\rangle^{1/2}\sim M_w$ is shown in Fig. 6. The equations was established to be: $\langle S^2\rangle^{1/2}=4.1\times10^{-2}\,M_w^{0.52}$. Usually, the exponent α-value of 0.5–0.6 in $\langle S^2\rangle^{1/2}=KM_w^{\alpha}$ reflects a flexible chain of polymers like to random coil. The α-value of 0.52 further indicated that macromolecules of GM6-1 exist as random coil chains in 0.25 LiCl/DMSO at 30 °C.

The unperturbed mean-square radius of gyration $\langle S^2\rangle_0$ of the wormlike chain is expressed by (Xu, Zhang, Nakamura, & Norisuye, 2002).

$$\langle S^2\rangle_0=\frac{qL}{3}-q^2+\frac{2q^3}{L}-\frac{2q^4}{L^2}[1-\exp(-L/q)] \tag{6}$$

Where L denoted the contour length. Since L equals the ratio of M (the molecular weight) to M_L, the molecular weight dependence of $\langle S^2\rangle_0$ is determined by q and M_L. The theory (Yamakawa & Yoshizaki, 1980) for $[\eta]_0$ (the unperturbed intrinsic viscosity) of the wormlike chain contains one additional parameter, the chain diameter d. On the basis of the values obtained through YFY theory, a set of q and M_L were tried to consistently explain the experimental data in Figs. 4 and 6. The solid line in Fig. 4 represents the $[\eta]_0$ theoretical values (the cylinder model) computed with the resulting parameters, $d=0.9$ nm, $q=4.4$ nm, $M_L=830$ nm^{-1}, and $B=0.0$ nm, with B being the excluded-volume strength (the binary cluster integral divided by the square of the bead spacing). It fits the data points well. To check this, the excluded-volume effects on $\langle S^2\rangle_z$ was also taken into account in the quasi-two-parameter (QTP) scheme (Hayashi, Tstsumi, Nakajima, Norisuye, & Teramoto, 1995), in which the expansion factors $\alpha_s^2(=\langle S^2\rangle/\langle S^2\rangle_0)$ and α_η^3 $(=[\eta]/[\eta]_0)$ are universal functions of the scaled excluded-volume parameter $\tilde{z}$ defined (for the wormlike chain) by

$$\tilde{z}=\frac{3}{4}K(L/2q)z \tag{7}$$

and the functions are expressed by

$$\alpha_s^2=\left[1+10\tilde{z}+\left(\frac{70\pi}{9}+\frac{10}{3}\right)\tilde{z}^2+8\pi^{3/2}\right]^{2/15}\times[0.933+0.067\exp(-0.85\tilde{z}-1.39\tilde{z}^2)] \tag{8}$$

$$\alpha_\eta^3=(1+3.8\tilde{z}+1.9\tilde{z}^2)^{0.3} \tag{9}$$

The solid line in Fig. 6 shows the theoretical values calculated as an unperturbed wormlike chain with $q=6.9$ nm, $M_L=750$ nm^{-1}, and $B=0.0$ nm. Good fit to the experimental data was also obtained. The difference between the q values obtained from $[\eta]_0$ and $\langle S^2\rangle_0$ may be related to the scatter of the points obtained from $\langle S^2\rangle_0$. Thus both the resulting parameters and the values calculated above indicated that the GM6-1 exist as a flexible chain without intramolecular excluded-volume effect in 0.25 M LiCl/DMSO at 30 °C.

4. Conclusions

Water-insoluble heteropolysaccharides GM6-1 isolated from the mycelium of *G. tsuage* by extracting with 0.5 M NaOH aqueous solution was fractionated successively to five fractions by non-solvent addition method. The $[\eta]$–M_w relationship of GM6-1 in 0.25 M LiCl/DMSO at 30 °C was established. Analysis of experimental data in terms of the theories for wormlike chain, without excluded volume effect, gave the conformational parameters of GM6-1 to be 4.2 nm for q, 832 nm^{-1} for M_L, indicating that the macromolecules exist as a flexible chain like random coil in the solution.

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