J. MACROMOL. SCI.—PHYSICS, B40(2), 147–156 (2001)

SOLUTION PROPERTIES OF PACHYMAN FROM *PORIA COCOS* MYCELIA IN DIMETHYL SULFOXIDE

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ABSTRACT

A linear β-(1→3)-D-glucan PCM3 (pachyman), a major polysaccharide in *Poria cocos* mycelia, formed aggregates in aqueous solution or dimethyl sulfoxide (DMSO) with absorbed moisture, thereby considerably complicating its fractionation and study of solution properties. In this work, the pachyman PCM3 having narrow polydispersity ($M_w/M_n = 1.9$) was carefully fractionated by a preparative size exclusion chromatography (SEC) using dehydrated DMSO as the mobile phase. The weight-average molecular mass M_w and intrinsic viscosity $[\eta]$ of the fractions were measured by static and dynamic laser light scattering, SEC combined with light scattering, and viscometry in DMSO at 25°C, thus eliminating the aggregation. The Mark-Houwink equation for the pachyman in DMSO is established to be: $[\eta] = 6.79 \times 10^{-4}\ M_w^{0.95}$(mL/g) in the M_w range from 1.2 to 3.5×10^5. The characteristic ratio C_∞ of the PCM3 in DMSO at 25°C, from light scattering by using Flory theory, was obtained to be 10.1, thus indicating that it behaves as a relatively expanded, flexible coil. In addition, the M_w of the pachyman from *Poria cocos* mycelia was 1.68×10^5, obviously larger than that from the sclerotium (8.9×10^4).

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Key Words: Pachyman; Weight-average molecular mass; Light scattering; Intrinsic viscosity; Charateristic ratio.

INTRODUCTION

As important natural macromolecules, polysaccharides have wide application in industry as drug, food-additive, and enhanced-oil recovery agents. Understanding of molecular mass and chain conformation of polysaccharides in solution is essential for their satisfactory application. By investigating the dilute-solution properties of polysaccharides, the conformation of polysaccharide chains can be quantitatively described from the molecular or model parameters. Many theories and models have been proposed for this purpose (1,2). β-(1→3)-D-glucans was believed to be a potential biomedical drug against bacterial or viral infection and also showed antitumor activity (3). It has been reported that pachyman, a β-(1→3)-D-glucan extracted from Chinese medicine, *Poria cocos*, has an antitumor effect (4–7). Studies on the molecular mass of *Poria cocos* polysaccharides have been already reported, but there are some discrepancies among the literature results wherein the reported values of molecular masses were from 4.1×10^4 to 5×10^6 (4,8–10). The higher values reported in the literature are believed to result from an extensive aggregation (11). The aggregation behavior seriously disturbed the fractionation and molecular mass measurements, resulting in the uncertainty of molecular mass and complicating the study of polymer solutions. Perhaps, as in the case of curdlan, the detailed behavior could not be revealed because of a large uncertainty caused by microgel aggregation in the light scattering measurements (12).

Previously, we have investigated the pachyman PC3, a linear β-(1→3)-D-glucan that is a major polysaccharide in *Poria cocos* sclerotium, and found the aggregation phenomenon in aqueous solution using dynamic and static laser light scattering (11,13). The results proved that β-D-glucan has strong intermolecular hydrogen bonding, leading to its water-insolubility; namely, it could not dissolve in water and only dissolved in strong solvents such as NaOH aqueous solution, cadoxen, a 29 wt% aqueous solution of ethylenediamine saturated with CdO, or dimethyl sulfoxide (DMSO). Moreover, the pachyman also formed aggregates in DMSO containing LiCl-absorbed moisture or DMSO containing a little water (14,15). Analytical size exclusion chromatography (SEC), viscometry, and membrane osmometry under different conditions (13,14) indicated that the aggregates can be broken in dehydrated DMSO, cadoxen, or in DMSO/LiCl at 80°C. For convenience, simple viscometry would be desirable for measurement of the viscosity-average molecular mass M_η given by the Mark-Houwink equation. However, the Mark-Houwink parameters for pachyman have not been published, probably due to the difficulty of fractionation. An attempt was made in this work to establish a Mark-Houwink equation for pachyman PCM3 isolated from *Poria cocos* mycelia (16). The solution behavior in dehydrated DMSO, to eliminate the effect of aggregation, and chain flexibility in the solution of this glucan were investigated and discussed.

EXPERIMENTAL

Sample Preparation

The *Poria cocos* mycelia was cultured in media at 25°C for 1 week, and a polysaccharide named PCM3 was extracted from the mycelia by a 0.5 N NaOH aqueous solution. The polysaccharide was analyzed by gas chromatography and ^{13}C nuclear magnetic resonance, with the results indicating a linear β-(1→3)-D-glucan identified as pachyman (14,16). Eleven fractions were obtained by using a preparative SEC column (550 mm × 20 m) packed with cellulose gel particles prepared in our laboratory (17). Dehydrated DMSO was used as an elute at 25°C. Fractions were washed with water and ethanol five times, respectively, and dried in a vacuum in the presence of P_2O_5 to get white powders. The unfractionated sample F0 and fractions F2, F6, F8, and F9 were used in this study.

Laser Light Scattering (LLS)

In static LLS, the excess absolute scattered light intensity [also known as Rayleigh ratio, $R_{vv}(\theta)$] of a polymer solution at angle (θ) and low concentration (c) is related to the weight-average molecular mass (M_w) by

$$Kc/R_{vv}(q) = \frac{1}{M_w}\left(1 + \frac{1}{3}\langle S^2\rangle_z q^2\right) + 2A_2c \tag{1}$$

where $K = 4\pi^2 n^2 (dn/dc)^2/(N_A\lambda_0^4)$ and $q = (4\pi n/\lambda_0)\sin(\theta/2)$, with N_A, n, and λ_0 being Avogadro's number, the solvent refractive index, and the wavelength of light in a vacuum, respectively. $\langle S^2\rangle_z^{1/2}$ (or written as $\langle S\rangle_z$) is the z-average radius of gyration. A_2 is the second virial coefficient. By measuring $R_{vv}(q)$ at different c and q, we can determine M_w, $\langle S\rangle_z$ and A_2 from the Zimm plot (18).

In dynamic LLS, an intensity-intensity time correlation function $G^{(2)}(t, q)$ in the self-beating mode was measured (19,20) with

$$G^{(2)}(t, q) = A\left[1 + \beta\left|g^{(1)}(t, q)\right|^2\right] \tag{2}$$

where A is a measured baseline, β is a parameter depending on the coherence of the detection, t is the delay time, and $g^{(1)}(t, q)$ is the normalized first-order electric field time correlation function. For a polydisperse sample, $g^{(1)}(t, q)$ can be related to the line-width distribution $G(\Gamma)$ by

$$g^{(1)}(t, q) = \int_0^\infty G(\Gamma)e^{-\Gamma t}\, d\Gamma \tag{3}$$

using the analysis program CONTIN (21). $G(\Gamma)$ can be further reduced to a translational diffusion coefficient distribution *G(D)* if the relaxation is diffusive. The

average translational diffusion coefficient $\langle D \rangle$ can be obtained by

$$\langle D \rangle = \int_0^\infty G(D) D dD \tag{4}$$

DMSO (Sigma, AR), treated with a molecular sieve three times to remove water, was used as solvent. A relatively concentrated stock solution was prepared by completely dissolving a proper amount of polysaccharide in the solvent. After a complete dissolution, this stock solution was centrifuged at 15,000 rpm for 8 h to remove a trace amount of insoluble substances. A series of solutions with different desired concentrations were obtained by successive dilution of the clarified stock solution. Finally, each solution was clarified further with a 0.1-μm Whatman filter.

The light scattering (LS) measurements were performed with a modified commercial LLS spectrometer (ALV/SP-150, Langen, Hessen, Germany) equipped with an ALV-5000 multi-τ digital time correlator and a solid-state laser (ADLAS DPY 425 II, outpower = ~400 mV at $\lambda = 532$ nm) at 25.0 ± 0.1°C. The primary beam is vertically polarized. The specific refractive index increment dn/dc was determined by a novel differential refractometer that was incorporated into the LLS spectrometer (22), wherein the same laser light source is used for both the LLS spectrometer and the refractometer; thus, the wavelength correction of dn/dc was avoided. The value of dn/dc for the polysaccharide was measured to be 0.0426 mL/g in DMSO.

SEC-LS Measurements

SEC combined with static LS is convenient for the determination of true molecular mass and its distribution without the aid of standard samples. SEC-LS measurements were performed using a multiangle laser photometer (DAWN-DSP, Wyatt Technology Co., Santa Barbara, CA) combined with a pump P100 (Thermo Separation Products) equipped with a TSK-GMH6 column (300 × 7.5 mm, Tosoh Corporation, Japan) packed with cross-linked polystyrene-divinylbenzene particles of 8–10 μm and a differential refractive index detector RI-150 (Thermo Separation Products) at 25°C. The elute was dehydrated DMSO, and the flow rate was 1.0 mL/min. All solutions used were first filtered with a sand filter and then injected into the SEC column by a 0.2-μm filter (Whatman, Maidstone, UK). The injected volume was 200 μL. Astra software was used for data acquisition and analysis.

Viscometry

Viscosities of the unfractionated pachyman and fractions in dehydrated DMSO were measured at 25 ± 0.1°C by using a modified capillary viscometer that was a gift from the Institute of Industrial Science, Tokyo University. The kinetic energy

correction was always negligible. Huggins and Kraemer plots were used to estimate the intrinsic viscosity $[\eta]$ and the Huggins constant k'.

RESULTS AND DISCUSSION

The analytical SEC is more sensitive and direct for detection of aggregates than other methods (14). Figure 1 shows SEC chromatograms of the pachyman PCM3, namely F0, detected by LS and differential refractometer. The two chromatograms both gave only a single peak in DMSO at 25°C, which is similar to the differential weight distribution of molecular mass $F_w(M)$ of pachyman PC3 for the same conditions by dynamic LS, indicating no aggregates in this case. The values of M_w and M_n were calculated to be 1.7×10^5 and 9×10^4, respectively, for F0. Therefore, the polydispersity index (M_w/M_n) was 1.9, corresponding with the result from dynamic LS (15).

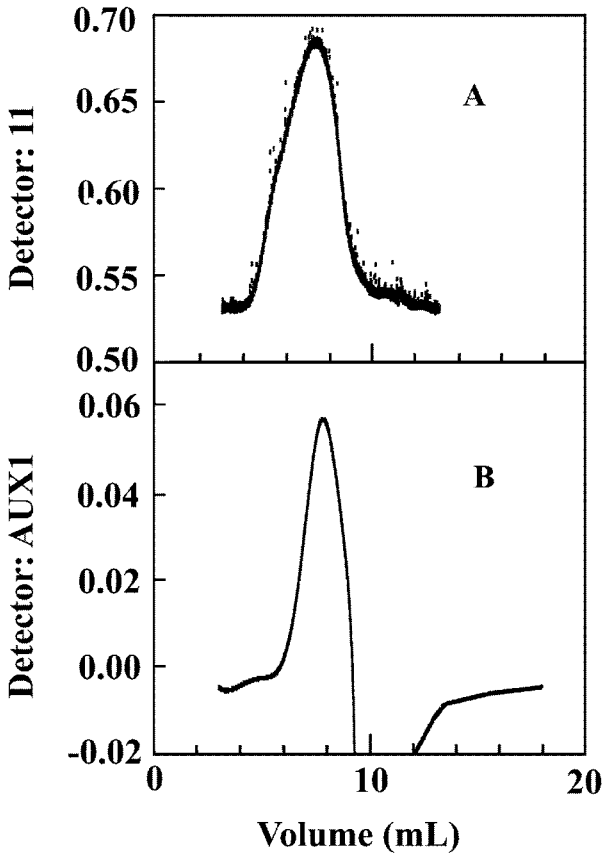

Figure 1. SEC chromatograms of pachyman PCM3 in DMSO at 25°C detected by LS (A) and differential refractometer (B). The Detector.11 and Detector.AUX1 values are signals from the LS at 90° and the refractive index detectors.

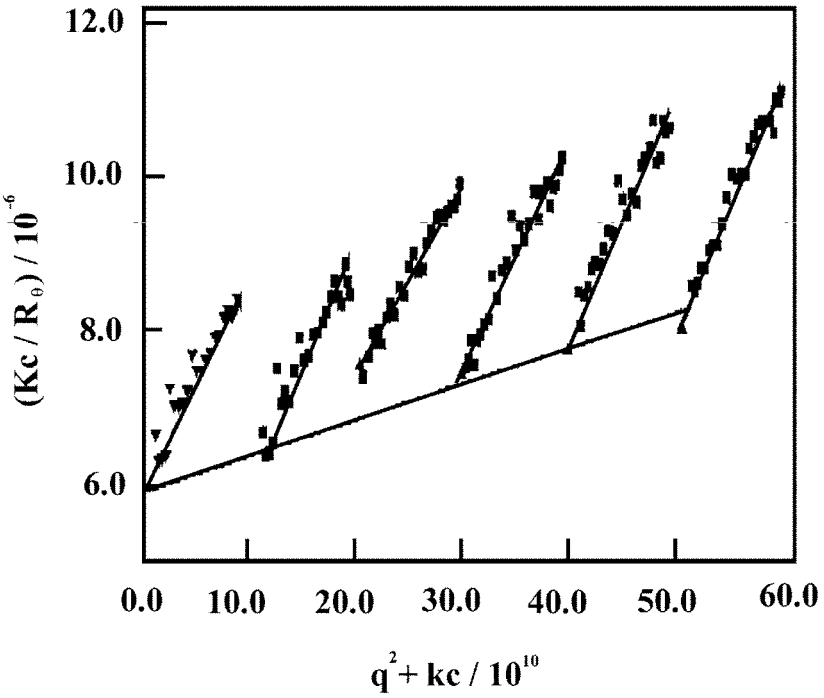

Figure 2. Zimm plot for pachyman F0 in DMSO at 25°C.

Figure 2 shows a typical Zimm plot of F0 in DMSO at 25°C, where c ranges from 1×10^{-3} to 5×10^{-3} g/mL. Based on Eq. 1, M_w, A_2, and $\langle S^2 \rangle_z$ calculated from the static LLS are summarized in Table 1. The difference of molecular mass of the fractions is small, because the unfractionated sample F0 has a narrow distribution (polydispersity index, $M_w/M_n = 1.9$); thus, it is difficult to fractionate. The M_w value of F0 from *Poria cocos* mycelia is much larger than that from the sclerotium (8.9×10^4) (11).

Figure 3 shows the translational diffusion coefficient distributions $G(D)$ of three fractions in DMSO at 25°C by using dynamic LLS. The $\langle D \rangle$ values from dynamic LLS are also summarized in Table 1. Using a previously established

Table 1. Results of LLS and Intrinsic Viscosity for the Pachyman Unfractionated and Fractioned Fractions in DMSO at 25°C

Sample	$10^{-5}M_w$ (g/moL)	$10^4 A_2$ (moL mL/g^2)	$10^{12}\langle S^2 \rangle_z$ (cm^2)	$10^8\langle D \rangle$ (cm^2/s)	$[\eta]$ (mL/g)	k'	M_w/M_n	C_∞
F0	1.68	1.82	7.02	7.12	65.3	0.50	1.9	10.1
F2	3.48	0.76	19.39	6.73	124.0	0.43	1.3	13.4
F6	2.20	2.35	10.74	8.92	87.0	0.45	1.5	11.8
F8	1.83	2.30	7.59	—	67.5	0.38	—	10.0
F9	1.28	—	—	—	48.1	0.42	—	—

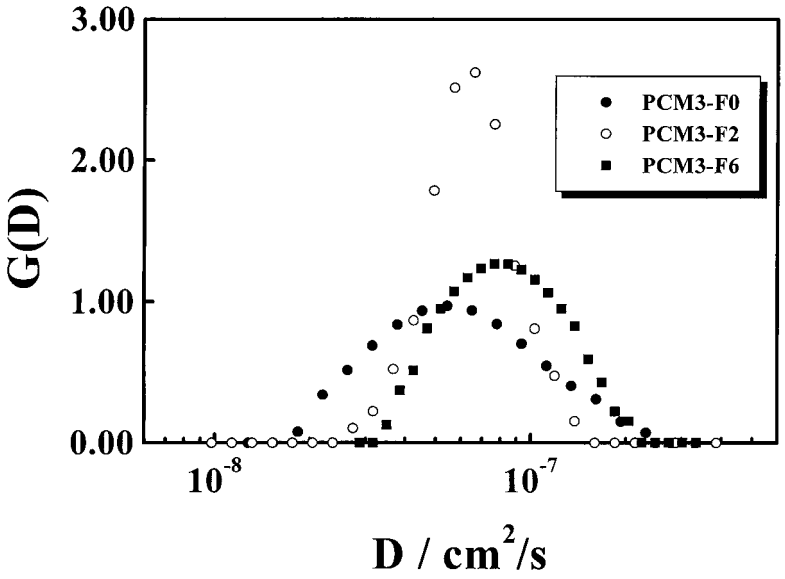

Figure 3. Translational diffusion coefficient distributions *G(D)* of three pachyman fractions in DMSO at 25°C.

method of combining static and dynamic LLS results [M_w and *G(D)*] of two or more broadly distributed samples (23), a calibration between D and M, namely D (cm^2/s) $= k_D M^{-\alpha D}$ with $k_D = 6.32 \times 10^{-4}$ cm^2/s and $\alpha_D = 0.65$, from three pachyman samples was obtained. It should be noted that this calibration is only an approximation. The high value of $\alpha_D \sim 0.65$ suggests the chain of the pachyman PCM3 has an extended conformation. The values of polydispersity ($d = M_w/M_n$) of the fractions were lower (1.3–1.5) than that of the unfractionated sample, suggesting that fractionation was satisfactory.

Data of intrinsic viscosity [η] are summarized in Table 1. Figure 4 illustrates the molecular mass dependence of [η] for the pachyman PCM3 in DMSO at 25°C. The fitting curve is represented by the relationship:

$$[\eta] = 6.79 \times 10^{-4} M_w^{0.95} \text{ (mL/g)} \quad (5)$$

The high exponent 0.95 of the Mark-Houwink equation can be explained by third-power type theories, and reflects the polysaccharide chains to possess small unperturbed dimensions and undergo large excluded-volume effects. Data of [η] and M_w lay in the region of flexible polysaccharides, such as pullulan, curdlan, and amylose (24–26), and was much lower than that of semiflexible β-(1→3)-D-glucan from *Auricularia auriculae judae* (27). This implies that the pachyman exists as a flexible chain in DMSO. Interestingly, data of PC3, from the sclerotium, by using SEC (14), is also close to the data line, but its exponent ($\alpha = 0.50$) was much lower than that of PCM3. The origin of this is not understood at present. It is worth noting that the molecular dimension changes of the polysaccharide in the M_w range from 10^4 to 10^5 sometimes occurred, leading to the up-bending or down-bending of

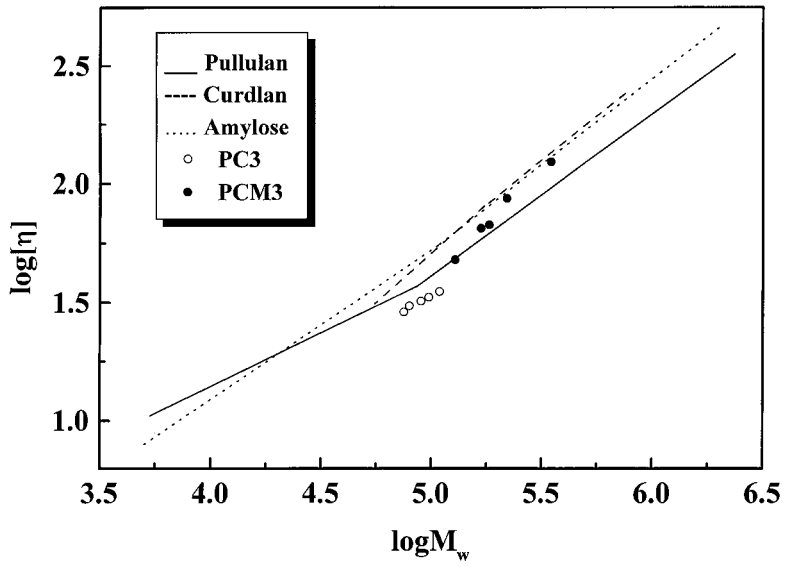

Figure 4. Molecular mass dependence of $[\eta]$ for PCM3 (●) in DMSO, PC3 (○) in DMSO by SEC (14), pullulan in water (—) (24), curdlan in 1:1 water-diluted cadoxen (- - - - -) (25), and amylose in DMSO (26) (· · · · · ·) at 25°C.

$[\eta]$-M curves. For instance, the turning points of pullulan, curdlan, and amylose in Figure 4 are 8.6×10^4, 1.2×10^5, and 1×10^4, respectively. A double-logarithmic plot of $\langle S^2 \rangle^{1/2}$ is shown in Figure 5; the equation representing the line is:

$$\langle S^2 \rangle^{1/2} = 5.26 \times 10^{-3} M_w^{0.71} \text{ (nm)} \tag{6}$$

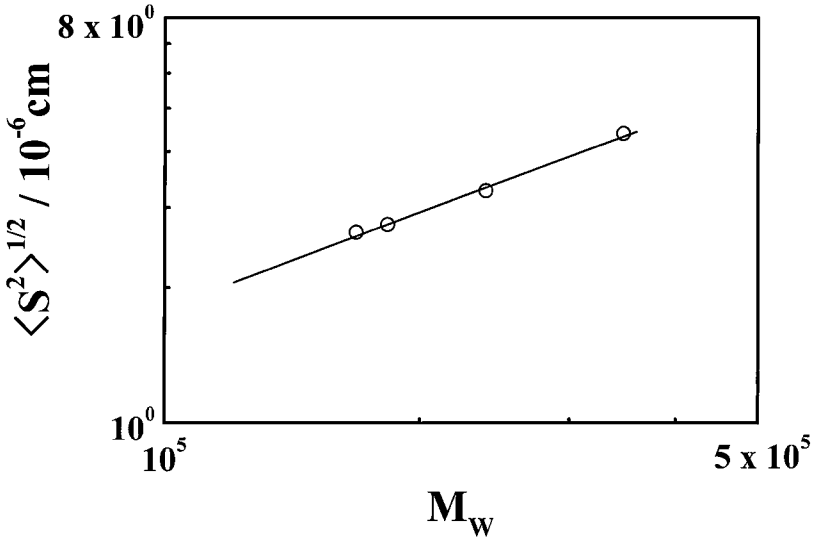

Figure 5. Molecular mass dependence of radius of gyration $\langle S^2 \rangle^{1/2}$ for pachyman in DMSO at 25°C.

The exponent 0.71 is larger than that of normal coil-chain (0.5–0.6), supporting the interpretation previously described.

Polymer chain dimensions can be obtained from LS or viscosity by using the Flory theory (28). The characteristic ratio C_∞ reflects the flexibility and conformation of polymers, and was evaluated from LS as follows (28,29):

$$C_\infty = 6(\langle S^2\rangle_z/M_w)(M_0/\alpha^2 l^2) \tag{7}$$

$$\alpha^2 = 1 + 0.223[\exp(5.73\Psi) - 1] \tag{8}$$

$$\Psi \equiv (4\pi^{3/2}N_A)^{-1}A_2 M^{1/2}(\langle S^2\rangle/M)^{-3/2} \tag{9}$$

where M_0 is the molecular mass of a glucose residue, namely 162; α is the expansion factor; l is the length of the virtual bond [for a linear dextran chain, it is 5.7 Å (30)]; N_A is Avogadro's number, and A_2 is the second virial coefficient. The values of Ψ and α^2 for the F0 sample, obtained as described from Eqs. 8 and 7, were 20.6 and 1.236, respectively. Data of C_∞ for samples F0–F8 are given in Table 1. It shows that the C_∞ decreased with a decrease of M_w, suggesting a possible effect of M_w on C_∞. In this case, the C_∞ of the whole glucan PCM3 was 10.1, similar to values of flexible D-glucan in DMSO containing 0.2 M LiCl ($C_\infty = 7.2$) (31), curdlan ($C_\infty = 10$), and schizopyllan ($C_\infty = 7$ for single coils in DMSO), and in accord with those predicted from the modeling studies for linear β-(1→3)-D-glucan (1). These results indicate that the pachyman PCM3 behaves as an expanded flexible coil in DMSO.

CONCLUSIONS

The fractionation by preparative SEC for pachyman PCM3 in dehydrated DMSO at 25°C was satisfactorily performed, eliminating the effect of aggregation. Molecular mass dependences of $[\eta]$ and $\langle S^2\rangle^{1/2}$ for pachyman in DMSO were established as: $[\eta] = 6.79 \times, 10^{-4}M_w^{0/95}$ (mL/g) and $\langle S^2\rangle^{1/2} = 5.26 \times 10^{-3}M_w^{0.71}$ (nm) in the range of weight-average molecular mass studied (1.2–3.5 $\times$ 10^5). The combination of static and dynamic LLS results gave D (cm^2/s) $= k_D M^{-\alpha D}$, with $k_D = 6.32 \times 10^{-4}$ cm^2/s and $\alpha_D = 0.65$. The value of characteristic ratio C_∞ obtained from LS is 10.1, which agrees with that of β-(1→3)-D-glucan predicted from a model. The pachyman behaves as an expanded flexible coil in DMSO. In addition, the β-D-glucan PCM3 produced from *Poria cocos* mycelia has almost identical chain flexibility, but different M_w than PC3 from the *Poria cocos* sclerotium.

ACKNOWLEDGMENTS

We acknowledge the support of the National Nature Science Foundation of China (29374170) and the Research Grants Council of the Hong Kong Government Earmarked Grants (CUHK 4161/99M).

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Received June 28, 2000
Revised August 16, 2000
Accepted August 23, 2000