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Carbohydrate Research 341 (2006) 2261–2269
Carbohydrate RESEARCH

Physicochemical properties and antitumor activities of water-soluble native and sulfated hyperbranched mushroom polysaccharides

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Received 9 April 2006; received in revised form 19 May 2006; accepted 30 May 2006
Available online 27 June 2006

Abstract—A water-soluble hyperbranched β-glucan, coded as TM3b, extracted from sclerotia of an edible fungus (*Pleurotus tuber-regium*) was fractioned into eight fractions coded as F1–F8 by a nonsolvent addition method. Five fractions were treated with chlorosulfonic acid at 35 °C to synthesize successfully sulfated derivatives coded as S-F2, S-F3, S-F4, S-F5, and S-F8 with degree of substitution of 0.28–0.54. The ^{13}C NMR results of these sulfated β-glucans indicated that while the C-6 position was fully substituted, C-2, C-3, and C-4 were only partially substituted by the sulfate groups. The weight-average molecular weights (M_w) and intrinsic viscosities ([η]) of the native and sulfated TM3b fractions were determined using multi-angle laser light scattering and viscometry in 0.15 M aq NaCl at 25 °C, respectively. The dependences of [η] on M_w for TM3b and sulfated TM3b were found to be $[\eta] = 0.18M_w^{0.28\pm0.03}$ (M_w range from 3.30×10^4 to 3.90×10^7) and $[\eta] = 2.24\times10^{-2}M_w^{0.52\pm0.06}$ (M_w range from 3.24×10^4 to 3.15×10^5) in 0.15 M aq NaCl at 25 °C, respectively. It revealed that both the native TM3b and its sulfated derivatives exist in a spherical chain conformation in 0.15 M aq NaCl. Furthermore, the native and sulfated TM3b fractions showed potent antitumor activities in vivo and in vitro. The sulfated derivatives exhibited relatively higher in vitro antitumor activity against human hepatic cancer cell line HepG2 than the native TM3b. Water solubility and introduction of sulfate groups were the main factors in enhancing the antitumor activities.

Keywords: Hyperbranched polysaccharide; Weight-average molecular weight; Sulfation; Chain conformation; Antitumor activity

1. Introduction

Polysaccharides and their sulfated derivatives have attracted much attention in recent years because of their diverse biological activities.[1–5] Various water-soluble polysaccharides having antitumor activity have been obtained from the mycelia or sclerotia of fungi, and these substances are regarded as biological response modifiers.[6–9] Moreover, both naturally occurring and artificially synthesized sulfated polysaccharides have been shown to be inhibitors of various enveloped viruses and of the replication of HIV-1 in vitro.[10–12] Until now, there is no accepted mechanism for these biological activities, and much controversy still surrounds the relationship between structure and antitumor activity. Usually, water solubility, weight-average molecular weight (M_w), chain conformation, and introduction of suitable ionic groups with appropriate degree of substitution (DS) can change the bioactivities of polysaccharides. Interestingly, water-insoluble polysaccharides show little bioactivity, whereas their water-soluble sulfated derivatives exhibit high antitumor and/or antiviral activities.[13–15] Ionic interactions of sulfated polysaccharides with proteins are assumed to play a major role, such as the interaction between a negatively changed polysaccharide portion of heparin and three lysine residues in antithrombin-III.[16] Furthermore, the sulfated polysaccharides appear to bind strongly to the HIV virions

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doi:10.1016/j.carres.2006.05.024

and inhibit their association with the CD4 receptor of T lymphocytes.[17,18] Our previous studies showed that the relatively higher bioactivity of the sulfated polysaccharides was attributed to good water solubility and expanded chain conformation.[14,19] We are interested in chemical modification of water-soluble polysaccharide by sulfation to investigate whether its bioactivity changes concomitantly with modification, and attempt to clarify whether water-solubility and chain conformation are decisive in the enhancement of the antitumor activity.

P. tuber-regium, an edible fungus, is found growing in tropical and subtropical regions of the world.[20] It is consumed not only for its flavor and nutritive value, but also for its medicinal effects, including the treatment of asthma, smallpox, and high blood pressure.[21] In our previous work,[22] we found that a water-soluble polysaccharide, extracted from the *P. tuber-regium* sclerotia by hot water, is a hyperbranched β-glucan. It consists of (1→6)-β, (1→4)-β and (1→3)-β-linked residues with a degree of branching (DB) of 59%. In the present work, this water-soluble hyperbranched polysaccharide (coded as TM3b) was fractionated by a nonsolvent addition method. The fractions were modified by sulfation and remained water soluble. Polymer characteristics, including water solubility, M_w and chain conformation, and in vitro and in vivo antitumor activities of the sulfated derivatives and the native polysaccharide, were compared. The significance of water solubility and introduction of sulfated groups in enhancing the antitumor activity of the fungal polysaccharide was discussed.

2. Experimental

2.1. Sample preparation and sulfation

The dried sclerotia powder was defatted sequentially by using Soxhlet extraction with EtOAc and acetone for over 6 h. The resultant residue was immersed stepwise in 0.15 M aq NaCl at 20, 80, and 120 °C. In each step, the mixture was stirred overnight, and then it was centrifuged (9000 rpm, 20 min) to get a supernatant, coded as TM1, TM2, and TM3, respectively. TM3 formed two phases when cooled to room temperature, and upon centrifugation (9000 rpm, 20 min), gave two crude polysaccharide fractions: TM3a (water insoluble) and TM3b (water soluble). TM3b was purified by decolorization with 30% H_2O_2, and free protein removal by the Sevag method for over 10 times. The resulting polysaccharide was precipitated by addition of 3 vol of absolute EtOH, and recovered by centrifugation at 9000 rpm for 20 min to remove the supernatant. It was dissolved in a minimum volume of distilled water, and then dialyzed with a regenerated cellulose tube (M_w cut-off 8000, USA) against tap water for 5 days and distilled water for 4 days. The polysaccharide was finally dried with a lyophilizer (Christ Alpha 1-2, Osterode am Harz, Germany) to become the purified TM3b sample (white powder).

The TM3b was redissolved in distilled water and fractionated according to a nonsolvent addition method. A mixture of 4:1 acetone–water was slowly added to an aq solution of TM3b at 25 °C until the solution turned slightly milky. The turbid liquid was warmed at 50 °C to become transparent again. After standing for 12 h at 25 °C, the turbid solution was centrifuged to separate it into liquid and gel phases. The gel was removed, and the supernatant was subjected to the next fractionation. In this way, the TM3b sample was divided into eight fractions and coded as F1, F2, F3, ..., and F8. Each TM3b fraction was reprecipitated by the addition of acetone, then washed with anhyd acetone three times, and finally vacuum dried to form a white powder.

Individual TM3b fractions (300 mg) dissolved in 25 mL DMSO were kept at room temperature overnight with stirring, followed by a slow addition of 4.5 mL of pyridine. The mixture was stirred for 30 min, and then 1.85 mL of chlorosulfonic acid (mole ratio of pyridine to chlorosulfonic acid was 2:1) was added dropwise to the solution in an ice bath with stirring. The resulting solution was heated to 35 °C, and the reaction was continued for 2 h. After the solution was cooled to room temperature, 5% NaOH solution was added to adjust the pH to 7. The solution was dialyzed in slightly alkaline water (pH 9) to remove pyridine. Finally, the sulfated polysaccharide was dialyzed against distilled water and freeze-dried to afford samples coded as S-TM3b fractions.

2.2. Physicochemical characterization

Infrared spectra (IR) of the TM3b and S-TM3b samples were recorded with a Nicolet 170SX FTIR spectrometer (Spectrum One, Perkin–Elmer Corp., USA) in the range 4000–400 cm^{-1} using the KBr-disk method. ^{13}C NMR spectra of the TM3b, F4, and S-F4 samples were recorded on a Mercury 600 NMR spectrometer (Varian Inc., USA) at 30 °C. The samples were dissolved in D_2O to obtain a concentration of 50 mg/mL. The elemental compositions for C, H, O, and S in the S-TM3b fractions were determined by using an elemental analyzer (EA) (Heraeus Co., Germany).

Intrinsic viscosity ($[\eta]$) of the TM3b fractions and their sulfated derivates in 0.15 M aq NaCl solutions were measured at 25 ± 0.1 °C by using an Ubbelohde capillary viscometer. The kinetic energy correction was assumed to be negligible. The Huggins equation[23] was used to estimate the $[\eta]$ value by extrapolation to infinite dilution as

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

where k' is constant for a given polymer at a given temperature in a given solvent, and η_{sp}/c is the reduced specific viscosity.

M_w and radius of gyration ($\langle S^2\rangle^{1/2}$) of the samples solution in 0.15 M aq NaCl were measured with a multi-angle laser light-scattering instrument equipped with a He–Ne laser (MALLS, $\lambda = 633$ nm; DAWN®DSP, Wyatt Technology Co., Santa Barbara, CA, USA) at angles of 43°, 60°, 69°, 80°, 90°, 100°, 111°, 121°, and 132° at 25 °C. The polysaccharide solution with desired concentrations was prepared, and optical clarification of the solution was achieved by filtration through a 0.2-μm pore size filter (PTFE, Puradisc 13-mm Syringe Filters, Whatman, England) into the scattering cell. The refractive index increment (dn/dc) was determined by using an Optilab refractometer (Dawn-DSP, Wyatt Technology Co., Santa Barbara, CA, USA) at 633 nm and 25 °C. Figure 1 shows the plot of dn against c for the samples in 0.15 M aq NaCl. The dn/dc values for TM3b and S-F4 were determined to be 0.133 $cm^3\ g^{-1}$ and 0.120 $cm^3\ g^{-1}$ in 0.15 M aq NaCl, respectively. Astra software (v. 4.90.07) was utilized for data acquisition and analysis.

2.3. Antitumor test

2.3.1. In vivo antitumor test. Sarcoma 180 (S-180) tumor cells (1×10^5 cells/mouse) were subcutaneously inoculated into 8-week-old male BALB/c mice weighting 18 ± 2 g. 5-Fluorouracil (5-Fu) and the tested samples were dissolved in 0.9% aq NaCl and injected intraperitoneally once a day for 8 days, starting 24 h after tumor inoculation. The same volume of 0.9% aq NaCl was injected intraperitoneally into the control mice. The mice were killed on the next day after the last injection, and the tumors were excised. The tumor weights were compared with those in the control mice.

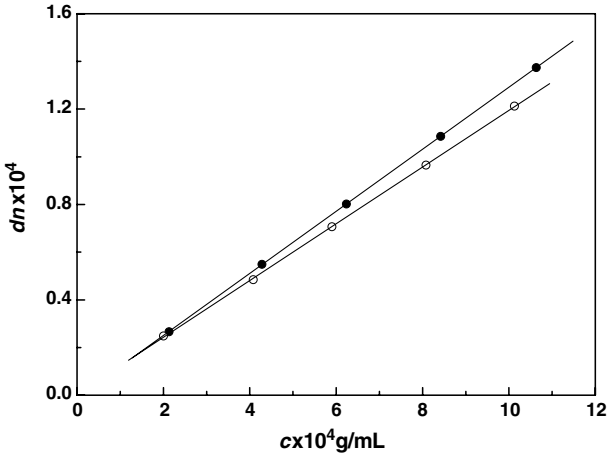

Figure 1. Plots of dn versus of c for TM3b (●) and S-F4 (○) in 0.15 M aq NaCl, respectively.

The inhibition ratio (ζ) and enhancement ratio of body weight (ϕ) were calculated as follows:

$$\xi = [(W_c - W_t)/W_c] \times 100\% \quad (2)$$

$$f = [(W_a - W_b)/W_b] \times 100\% \quad (3)$$

where W_c is the average tumor weight of the control group, W_t is the average tumor weight of the tested group; and W_b and W_a are the body weight of mice before and after the assay. Complete regression is indicated as the ratio of the number of tumor-free mice to the number of mice tested.

2.3.2. In vitro antitumor test. The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method was used for measuring the proliferation of an adherent human hepatoma cell HepG2, provided by Union Hospital of Tongji Medical College in Huazhong University of Science and Technology. HepG2 cells (1×10^6 cells/mL) were incubated with 180 μL RPMI 1640 medium supplemented with 10% fetal bovine serum solution and 20-μL sample solutions (at concentrations of 0.02, 0.2, and 2 mg/mL in 0.9% NaCl, respectively) under an atmosphere of 5% CO_2 at 37 °C for 24 h. The tumor cells were continuously incubated for another 4 h after 20 μL of MTT (5 mg/mL) had been added. The supernatant was removed by centrifuging, and then 150 μL of Me_2SO was added to terminate the reaction. The survival rate of tumor cells was assayed by measuring the optical intensity by an auto enzyme-labeled meter (Bio-Tek EX-800, USA) at 570 nm. The sample groups were compared with control groups in the absence of the tested samples. All in vitro results were expressed as the inhibition ratio (ϕ) of tumor cell proliferation as follows:

$$\phi = [(A - B)/A] \times 100\% \quad (4)$$

where A and B are the average number of viable tumor cells of the control group and test group, respectively. All assays were done in triplicate.

Student's t-test was used to evaluate the differences between the control and tested group. Significant differences between the two groups were defined as $p < 0.05$, and no difference was defined as $p > 0.05$.

3. Results and discussion

3.1. Chemical structure

The FTIR spectra of native TM3b and the sulfated derivatives are shown in Figure 2. The absorption peak at 890 cm^{-1} for TM3b is characteristic of the β-glucan.[24] Compared with the FTIR spectrum of the native TM3b, two new absorption peaks appeared at 817 and 1260 cm^{-1} for the sulfated derivatives, due to the presence of the C–S–O and S═O bonds, respectively,

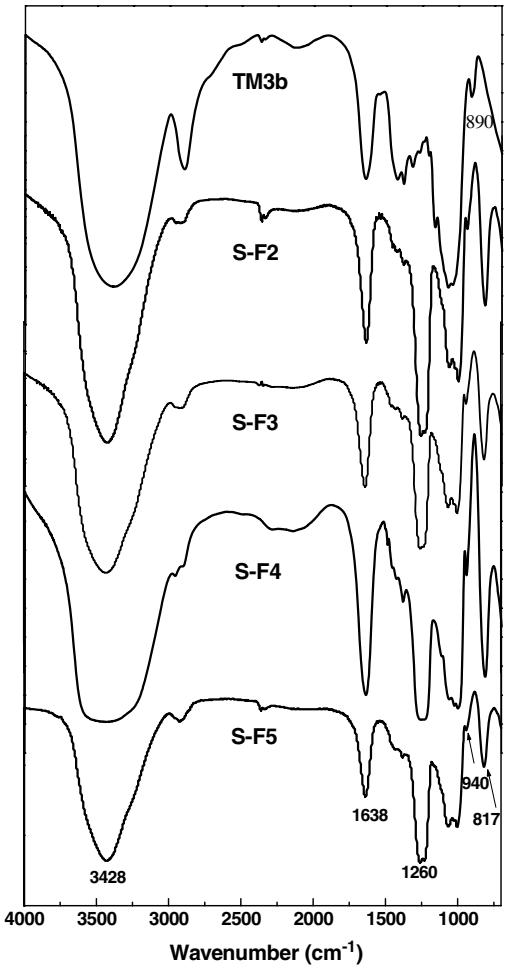

Figure 2. FTIR spectra of the native TM3b and sulfated derivatives.

indicating that sulfated reaction had taken place.[25] The absorption at 890 cm^{-1}, corresponding to the native β configuration, had shifted to 940 cm^{-1} in the sulfated derivatives. In a previous work,[22] GC–MS has been carried out and there are five components, namely 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, 2,3,4-tri-*O*-methylglucose, and 2,3-di-*O*-methylglucose. This indicates that TM3b consists mainly of terminal glucose, 1,3-linked glucose, 1,4-linked glucose, 1,6-linked glucose, and 1,4,6-linked glucose in a molar ratio of 6:1:4:2:4. The proportion of terminal glucose in methylation of TM3b is much higher than others, suggesting a hyperbranched polysaccharide. Hawker et al.'s equation[26] can be used to calculate the degree of branching (DB) by

$$\mathrm{DB} = \frac{N_T + N_B}{N_T + N_B + N_L} \quad (5)$$

where N_T, N_B, and N_L are the total numbers of the terminal residues, branched residues, and linear residues, respectively. According to the analogous method as described in the reference,[27] all of the parameters can be obtained from the GC–MS, that is $N_T{:}N_B{:}N_L = 6{:}4{:}(1+4+2)$. So we calculated the value of the DB to be 59%.

The ^{13}C NMR spectra of the native TM3b, F4, and the sulfated derivative (S-F4) in D_2O are shown in Figure 3. The chemical shifts for the samples and literature data are summarized in Table 1. The major signals of TM3b are assigned to 102.6 ppm for C-1, 76.0 ppm for C-3 and C-5, 73.2 ppm for C-2, 69.8 ppm for C-4, and 60.8 ppm for C-6, similar to that of the carbons for β-D-glucopyranose.[28] Furthermore, a bigger signal at 69.8 ppm is assigned to some substituted residues at C-6, and the smaller signals at 79.3 and 84.3 ppm represent substituted residues at C-4 and C-3, respectively. These results further indicate that TM3b is a polysaccharide having numerous terminal units, which is the characteristic of a hyperbranched polymer.[29] There is only one

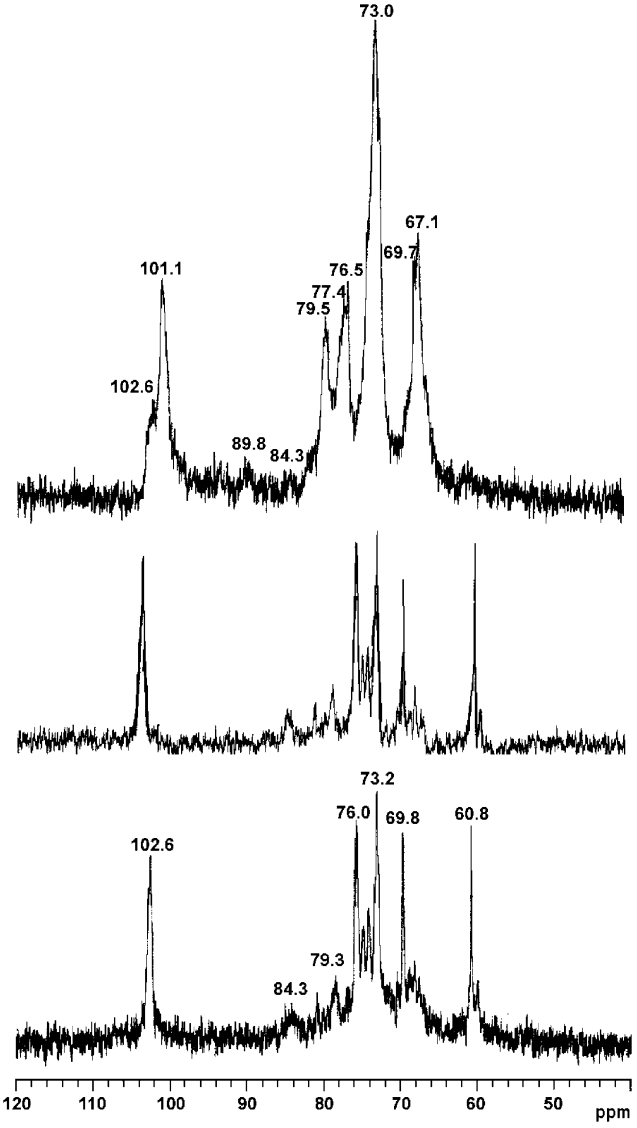

Figure 3. ^{13}C NMR spectra of TM3b (bottom), F4 (middle), and S-F4 (top) in D_2O.

Table 1. ^{13}C NMR chemical shifts (δ/ppm) of native TM3b, F4, S-F4, and monosaccharide in D_2O solution

Chemical shifts	C-1	C-2	C-3	C-4	C-5	C-6	C-1′	C-3′	C-4′	C-6′	C-2s	C-3s	C-4s	C-6s
α-Glucose[a]	92.9	72.5	73.8	70.6	72.3	61.6								
β-Glucose[a]	96.7	75.1	76.7	70.6	76.8	61.7								
TM3b	102.6	73.2	76.0	69.8	76.0	60.8		84.3	79.3	69.8				
F4	102.6	73.1	76.0	69.8	76.0	60.8		84.3	79.1	69.8				
S-F4	102.6	73.0	76.5	69.7	76.5		101.1	84.3	79.5	69.7	79.5	89.8	77.4	67.1

[a] Data from Ref. 28.

anomeric peak at 102.6 ppm in the ^{13}C NMR spectrum of TM3b; this suggests that the polysaccharide contains one kind of configuration for the glucose residue, namely the β configuration. In view of the results from GC–MS and ^{13}C NMR spectroscopy, TM3b is a hyperbranched β-D-glucan mainly containing β-(1→6), β-(1→4), and β-(1→3)-linked residues. Compared with the ^{13}C NMR spectrum of the F4 sample, there are four new peaks appearing in the S-F4 spectrum, namely C-1′ (101.1), C-3s (89.8), C-4s (77.4), and C-6s (67.1 ppm). The peak at 60.8 ppm, which is the chemical shift of C-6 for F4, disappeared in the ^{13}C NMR spectrum for S-F4 (Table 1). The new peak that appeared at 67.1 ppm in S-F4, is assigned to the substituted C-6 because the downfield shift of a carbon atom linked by a sulfated group is ~7–10 ppm.[30] This indicates that C-6 has been fully substituted by sulfo groups, and the C-6 signal is shifted downfield to 67.1 ppm. Similarly, the new peaks at 89.8 and 77.4 ppm for S-F4 are assigned to C-3 and C-4, which have been partially substituted by sulfo groups. The peak at 79.5 ppm for the S-F4 is higher than that for F4, resulting from substitution of the hydroxyl group at position 2. Furthermore, a new peak at 101.0 ppm is assigned to C-1, because C-2 and C-6 have been substituted to influence the chemical shift of the adjacent C-1, leading to the splitting of the C-1 carbon signal. The upfield shift of C-1 to C-1′ is 1.6 ppm, which is in line with the description found in Gorin.[30] In view of the intensity of the signals of the O-substituted carbons, we may conclude that nonselective sulfation of β-glucan has occurred, that is, C-6 is fully substituted and C-2, C-3, and C-4 are partially substituted. The substitution at O-6 is the major reaction compared with other positions in a glucosyl residue due to steric hindrance.[25] The degree of substitution (DS), which designates the average number of sulfo groups on each sugar residue, was calculated from the sulfur content on the basis of Schoniger's formula:[31]

$$DS = (162 \times S\%)/(32 - 102 \times S\%) \quad (6)$$

The sulfur content and the DS of the sulfated TM3b fractions are summarized in Table 2.

3.2. Mark–Houwink equation

Figure 4 shows the Berry plot for F3 (a) and S-F8 (b) in 0.15 M aq NaCl at 25 °C, respectively. The Berry plot differs from the more familiar Zimm plot by the fact that root of Kc/R_θ is now plotted, and the Berry plot has the advantage that it also takes into account the influence of the third viral coefficient.[32] From the intercept of two straight lines and the slope of the angular dependence, the M_w and $\langle S^2\rangle^{1/2}$ values were calculated, respectively. The angular dependences of $(Kc/R_\theta)^{1/2}_{c=0}$ for the TM3b and S-TM3b in 0.15 M aq NaCl at 25 °C are shown in Figure 5. Usually, 0.15 M aq NaCl (0.9% NaCl) as a medium is widely used in various bioactivity assays, so the investigation of M_w and $[\eta]$ for the TM3b and S-TM3b fractions in such a solvent is essential to clarify the correlation of the secondary structure to bioactivities of biopolymers in the same aq solution. In our previous work, 0.15 M aq NaCl was used as a solvent for the sulfated Pi-PCM3-I fractions with a DS range from 0.86 to 1.38, and the viscosity measurements indicated that electrostatic repulsion had been eliminated.[33] The experimental results of the TM3b and S-TM3b fractions in 0.15 M aq NaCl are listed in Table 2. The results indicate that the M_w values of TM3b decrease as the fractionation proceeds. Therefore, we have successfully fractionated TM3b to obtain a series of fractions having different M_w values. It is noted that the M_w values of the S-TM3b fractions were much lower than those of the native one due to the extensive degradation of the polysaccharides during the sulfation. The effect of DS of the sulfated polysaccharides on the $[\eta]$–M_w relationship

Table 2. The experimental results of M_w, $\langle S^2\rangle^{1/2}$, $[\eta]$, yield (%), sulfur content and DS for the TM3b and S-TM3b fractions in 0.15 M NaCl at 25 °C

Sample	$M_w \times 10^{-5}$	$\langle S^2\rangle_z^{1/2}$ (nm)	$[\eta]$ ($cm^3\ g^{-1}$)	Yield (%)	Sulfur content (%)	DS
F2	389.6	113.0	23.0	23.3		
F3	290.5	97.7	18.9	18.3		
F4	83.30	64.7	14.6	20.6		
F5	37.10	67.2	12.1	9.19		
F6	3.28	45.9	5.8	8.45		
F7	1.83	47.6	5.5	1.25		
F8	0.33	42.5	3.0	10.5		
S-F2	3.15	30.9	14.9		5.05	0.30
S-F3	2.02	25.3	13.0		6.48	0.41
S-F4	1.30	22.0	10.9		10.29	0.54
S-F5	0.91	17.8	10.4		4.74	0.28
S-F8	0.32		4.5		5.82	0.36

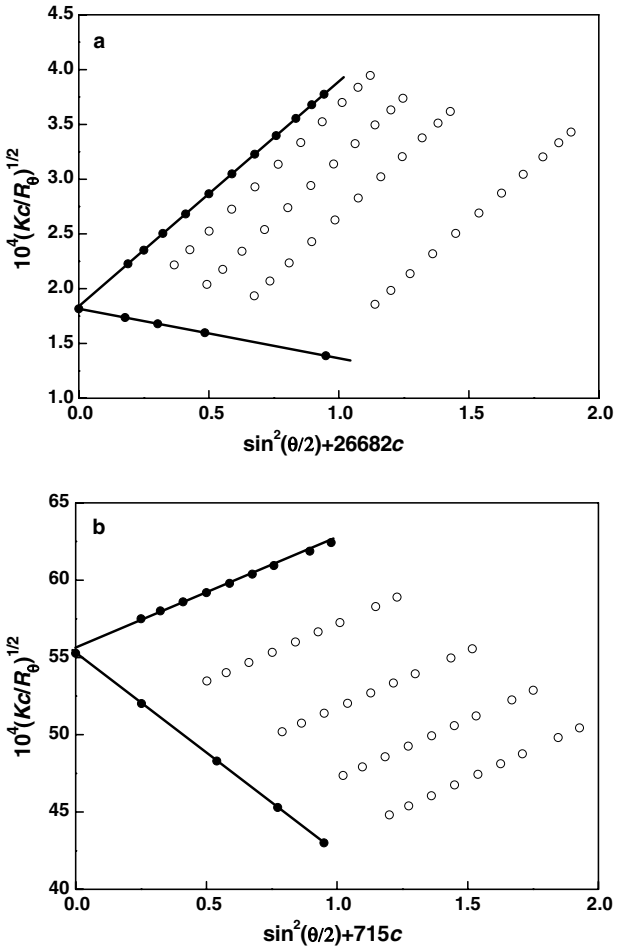

Figure 4. Berry plots for F3 (a) and S-F8 (b) in 0.15 M aq NaCl at 25 °C, respectively.

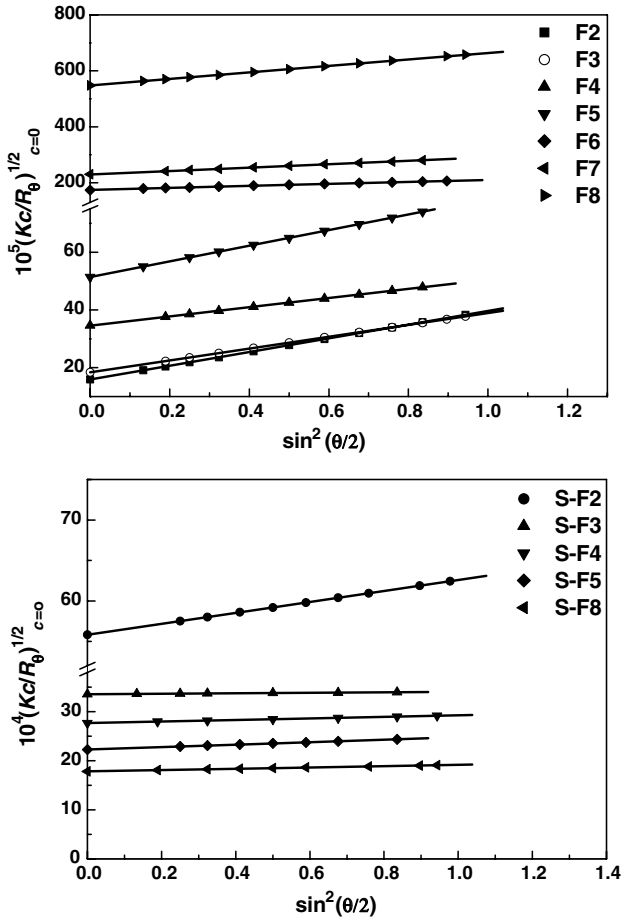

Figure 5. The angular dependences of $(Kc/R_\theta)^{1/2}_{c=0}$ for the TM3b and S-TM3b fractions in 0.15 M aq NaCl at 25 °C, respectively.

could be neglected because the changes of DS were relatively small (Table 2).

The M_w dependence of $[\eta]$ for TM3b and S-TM3b in 0.15 M aq NaCl at 25 °C is shown in Figure 6. The Mark–Houwink equations for TM3b in the M_w range from 3.30×10^4 to 3.90×10^7, and for S-TM3b in the M_w range from 3.24×10^4 to 3.15×10^5 are, respectively, established as

$$[\eta] = 0.18M_w^{0.28\pm0.03} \quad (7)$$

$$[\eta] = 2.24\times10^{-2}M_w^{0.52\pm0.06} \quad (8)$$

The exponent (α) is related to the shape of the macromolecule and the nature of the solvent. In general, $\alpha\sim0.5$ suggests that the polymer molecules behave as dense spheres, $\alpha\sim0.6$–0.8 for a flexible chain, and α is greater than one for an elongated rod. Our experimental value of 0.28 for TM3b was noticeably low, which is ascribed to the compact spherical structure. If the structure of the polymer chain is a perfect hard sphere, the exponent will be 0.[34] Furthermore, the α value depends on the degree of branching (DB). The Mark–Houwink exponents typically vary between 0.34 and 0.20 for

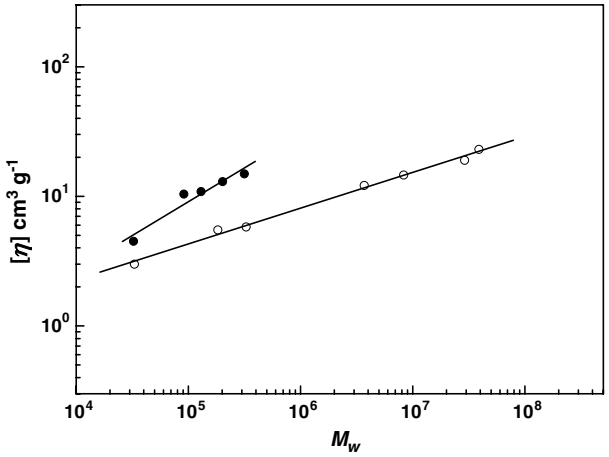

Figure 6. The $[\eta]$ dependences on the M_w for the TM3b (○) and S-TM3b fractions (●) in 0.15 M aq NaCl at 25 °C.

hyperbranched glycopolymers with different DB values.[35] The exponent for TM3b was much smaller than that for linear polymers, but within the range for hyperbranched polymers. It has been confirmed that the

TM3b polysaccharide has a hyperbranched spherical conformation,[22] which is indicated by the relatively low value of the exponent. The α value of 0.52 for S-TM3b was larger than that of TM3b. It was probable that the sulfo groups in the derivatives enhance the steric hindrance between the polymer chains, leading to the relatively expanded conformation of the sulfated derivatives.

3.3. Fractal dimension

The relevant structural quantities and further insight into the nature of the derivative of the hyperbranched polysaccharide can be obtained by investigating the fractal dimension. The fractal dimension (d_f) of monodisperse polymers can be extracted directly from the angular dependence of the scattered light or neutron intensity.[36] Alternatively, the fractal dimension can also be determined from the M_w dependence of $\langle S^2\rangle_z^{1/2}$, and is defined as the inverse of the exponent v:

$$\langle S^2\rangle_z^{1/2} \sim M^v \tag{9}$$

$$d_f = 1/v \tag{10}$$

The plot of $\langle S^2\rangle_z^{1/2}$ versus M_w for the S-TM3b sample in 0.15 M aq NaCl at 25 °C is shown in Figure 7. The resulting relation is expressed as

$$\langle S^2\rangle_z^{1/2} = 0.10M^{0.45\pm0.02} \tag{11}$$

On the basis of the theory of polymer solutions, the values of the exponent (v) of 0.33, 0.50–0.6, and 1.0 reflect the chain shape in adapting for a sphere, a random coil, or a rigid rod, respectively. The v value of 0.45 suggests that S-TM3b molecules are in the state between hard sphere and random coil. Furthermore, the value of d_f was calculated to be 2.22 for S-TM3b according to Eq. 10. The d_f value of 2.22 is characteristic for a particle

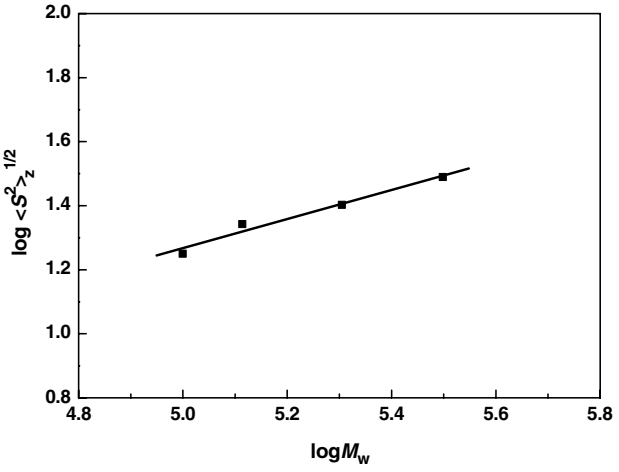

Figure 7. Plot of $\log\langle S^2\rangle_z^{1/2}$ versus $\log M_w$ for the S-TM3b fractions in 0.15 M aq NaCl at 25 °C.

that has an internal structure between a hard sphere ($d_f = 3.0$) and a fully swollen branched macromolecule in a thermodynamically good solvent ($d_f = 2.0$).[37] Scherrenberg and co-workers[38] have found that d_f for some dendrimers is 3, suggesting a uniform distribution of segments in the space pervaded by the polymer.

From the Mark–Houwink equation, the d_f value of the polymers can also be derived by using the following relation:[39]

$$d_f = 3/(1+\alpha) \tag{12}$$

where α is the exponent of the Mark–Houwink equation. Thus, the value of the d_f was calculated to be 1.97 for S-TM3b, which is similar to that from Eq. 10. Since the fractal dimension is a measure of the compactness of a polymer chain, the larger the fractal dimension, the more compact the structure. In view of the result from the d_f value for S-TM3b, we may further confirm that the sulfated derivative of the water-soluble hyperbranched β-glucan has a globular chain conformation.

3.4. Antitumor activity

The results of the in vivo assay of the TM3b fractions against Sarcoma 180 tumor cells in mice are summarized in Table 3. 5-Fu, a well-known anticancer agent, was included for comparison. More potent antitumor activities were observed in the TM3b fractions at high dose except for F8. The F3 sample having M_w of 2.91×10^7 at high dose exhibits higher inhibition ratio than the other TM3b samples, indicating the effect of M_w on the bioactivity. It is worth noting that the antitumor activities of the two fractions at high dose (F3 and F5) are slightly higher than that of 5-Fu. Moreover, the enhancement ratios of body weight for the four TM3b samples are higher than that for 5-Fu. This implies that the fungal polysaccharides might be less toxic than 5-Fu that kills the normal cells as well as tumor cells. Mushroom polysaccharides are regarded as biological response modifiers (BRMs) that cause no harm and place no additional stress on the body, but help the body adapt to various environmental and biological stresses.[40] The polysaccharides from mushrooms do not attack cancer cells directly, but produce their antitumor effects by activating different immune responses in the host.

The in vitro inhibition ratios to the proliferation of HepG2 by the TM3b fractions at different concentrations (0.02, 0.2, and 2 mg/mL) are shown in Figure 8. All the samples show certain inhibition ratios against tumor-cell growth at all concentrations. The four samples (F2, F3, F4, and F5) with relatively higher M_w have relatively higher inhibition ratios than those having lower M_w, suggesting that relatively higher M_w enhances antitumor activity.[41]

Studies on various chemical modifications for water-insoluble glucans have been carried out to elucidate

Table 3. Antitumor activity of the TM3b and S-TM3b fractions from the sclerotia of *P. tuber-regium* against Sarcoma 180 solid tumor grown in BALB/c mice

Sample	$M_w \times 10^{-5}$	Dose (mg/kg × days)	Inhibition ratio (%)	Enhancement ratio of body weight (%)	Complete regression
5-Fu		60 × 8	48.6[a]	16.7	0/10
F2	389.6	20 × 8	17.9	22.6	0/10
		60 × 8	39.7	45.3	0/10
F3	290.5	20 × 8	30.5	29.2	0/10
		60 × 8	67.9[a]	39	0/10
F5	37.10	20 × 8	32.1	32.5	0/10
		60 × 8	51.3[a]	40.5	0/10
F8	0.33	20 × 8	42.3	37.2	0/10
		60 × 8	38.5	26.0	0/10
S-F2	3.15	20 × 8	21.6	35.1	0/10
		60 × 8	72.1[a]	10.3	0/10
S-F3	2.02	20 × 8	17.0	30.9	0/10
		60 × 8	24.3	29.1	0/10
S-F4	1.30	20 × 8	39.6[a]	22.9	0/10
		60 × 8	36.9[a]	30.5	0/10
S-F8	0.32	20 × 8	25.2	25	0/10
		60 × 8	44.0[a]	26.9	0/10

[a] $P < 0.05$, significant difference when compared to the control.

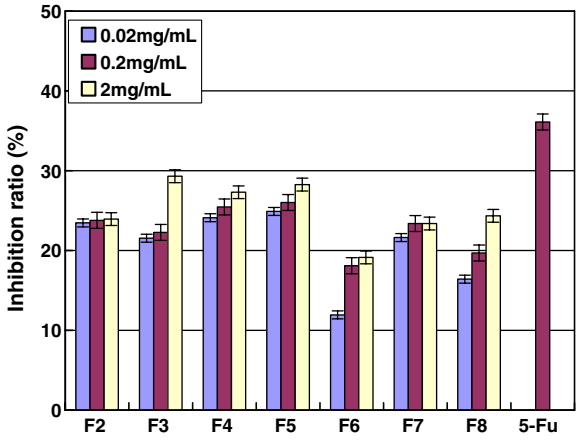

Figure 8. Inhibition ratio of proliferation of HepG2 liver cancer cells in vitro by different concentrations of the TM3b fractions.

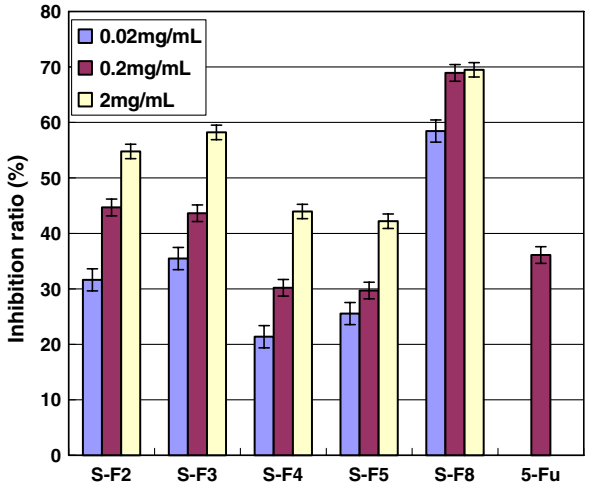

Figure 9. Inhibition ratio of proliferation of HepG2 liver cancer cells in vitro by different concentrations of the S-TM3b fractions.

the correlation of structure to antitumor effects.[15,19,42,43] To further clarify the main factor relating to bioactivity, we examined the antitumor activity of the sulfated derivatives of the water-soluble hyperbranched glucan. The in vivo antitumor activities of the four sulfated derivatives against Sarcoma 180 tumor cells are summarized in Table 3. The S-F2 sample having M_w of 3.15×10^5 had the highest inhibition ratio at high dose than the other three derivatives, and the in vivo inhibition ratios of the other three derivatives (S-F3, S-F4, and S-F8) were relatively lower than those of the native samples, respectively. In comparison with the TM3b fractions, the in vivo antitumor activities of the sulfated derivatives did not change significantly. Figure 9 shows the in vitro inhibition ratios to the proliferation of HepG2 by the S-TM3b fractions at different concentrations (0.02, 0.2, and 2 mg/mL). It is worth noting that all the sulfated derivatives exhibited much higher antitumor activities in vitro than the original samples.

On the basis of the assay of the in vivo inhibition ratio against Sarcoma 180 tumor cells, and the in vitro proliferation of HepG2 tumor cell lines, it is likely that good water solubility, relatively higher M_w, chain stiffness, and introduction of sulfated groups are crucial to the enhancement of the antitumor activities. In view of the results mentioned above, the contributions of the factors related to the antitumor activity of the polysaccharide are of the order of water solubility > introduction of sulfo groups > chain conformation > M_w. In our previous work,[14,19] we have found that derivatives of water-insol-

uble polysaccharides had good water solubility and relatively expanded chain conformation, leading to enhancement in antitumor activity. According to the present results, water solubility is the main factor in the improvement of bioactivity.

4. Conclusions

Eight fractions of the TM3b hyperbranched water-soluble polysaccharide isolated from the sclerotia of *P. tuber-regium* were reacted, respectively, with the chlorosulfonic acid–pyridine complex to obtain successfully sulfated derivatives having different M_w values with DS from 0.28 to 0.54. The Mark–Houwink equations for TM3b and S-TM3b in 0.15 M aq NaCl at 25 °C were established. It indicated that both of them possessed a spherical chain conformation in aqueous solution. The spherical chains of the sulfated derivatives appeared to be slightly more expanded than those of the native samples. In view of the fractal dimension, S-TM3b had an internal structure between a hard sphere and a swollen branched macromolecule in 0.15 M aq NaCl. All of the native TM3b samples and the sulfated derivatives exhibited potent in vivo and in vitro antitumor activities, owing to their good water solubility. Moreover, the sulfated derivatives had a higher in vitro inhibition ratio on HepG2 cells than the native ones. The water solubility and chain conformation of mushroom polysaccharides seemed to be tightly related to their antitumor effects.

Acknowledgements

This work was supported by major Grant from the National Natural Science Foundation of China (30350850), and UGC-AoE Plant and Fungal Biotechnology Project of the Chinese University of Hong Kong.

References

1. Liu, G. G.; Borjihan, G.; Baigude, H.; Nakashima, H.; Uryu, T. *Polym. Adv. Technol.* **2003**, *14*, 471–476.
2. Kolender, A. A.; Matulewicz, M. C. *Carbohydr. Res.* **2002**, *337*, 57–68.
3. Zierer, M. S.; Mourão, P. A. S. *Carbohydr. Res.* **2000**, *328*, 209–216.
4. Ahmad, A. S.; Matsuda, M.; Shigeta, S.; Okutani, K. *Mar. Biotechnol.* **1999**, *1*, 102–106.
5. Hussein, M. M.; Helmy, W. A.; Salem, H. M. *Phytochemistry* **1998**, *48*, 479–484.
6. Riccio, R.; Kinnel, R. B.; Bifulco, G.; Scheuer, P. J. *Tetrahedron Lett.* **1996**, *37*, 1979–1982.
7. Mizuno, T. *Int. J. Med. Mushrooms* **1999**, *1*, 9–29.
8. Falch, B. H.; Espevik, T.; Ryan, L.; Stokke, B. T. *Carbohydr. Res.* **2000**, *329*, 587–596.
9. Suzuki, I.; Hashimoto, K.; Oikawa, S.; Sato, K.; Osawa, M.; Yadomae, T. *Chem. Pharm. Bull.* **1989**, *37*, 410–413.
10. Hasui, M.; Matsuda, M.; Okutani, K.; Schigeta, S. *Int. J. Biol. Macromol.* **1995**, *17*, 282–293.
11. Uryu, T. *Prog. Polym. Sci.* **1993**, *18*, 717–761.
12. Tockikura, T. S.; Nakashima, H.; Yamamoto, N. *Antiviral. Immunodofic. Syndr.* **1989**, *2*, 441–447.
13. Zhang, M.; Cheung, P. C. K.; Zhang, L. *J. Agric. Food Chem.* **2001**, *49*, 5059–5062.
14. Zhang, M.; Cheung, P. C. K.; Ooi, V. E. C.; Zhang, L. *Carbohydr. Res.* **2004**, *339*, 2297–2301.
15. Bao, X.; Duan, J.; Fang, X.; Fang, J. *Carbohydr. Res.* **2001**, *336*, 127–140.
16. Uryu, T.; Ikushima, N.; Katsuraya, K.; Shoji, T.; Takahashi, N.; Yoshida, T.; Kanno, K.; Murakami, T.; Nakashima, H.; Yamamoto, N. *Biochem. Pharmacol.* **1992**, *43*, 2385–2392.
17. Lederman, S.; Gulick, R.; Chess, L. *J. Immunol.* **1989**, *143*, 1149–1154.
18. Jeon, K. J.; Katsuraya, K.; Kaneko, Y.; Mimura, T.; Uryu, T. *Macromolecules* **1997**, *30*, 1997–2001.
19. Wang, Y.; Zhang, L.; Li, Y.; Hou, X.; Zeng, F. *Carbohydr. Res.* **2004**, *339*, 2567–2574.
20. Zoberi, M. H. *Nigerian Field* **1973**, *38*, 81–90.
21. Oso, B. *Mycologia* **1977**, *69*, 271–279.
22. Tao, Y.; Zhang, L. *Biopolymers*, submitted for publication.
23. Huggins, M. L. *J. Am. Chem. Soc.* **1942**, *64*, 2716–2718.
24. Kiho, T.; Sakushima, M.; Wang, S.; Nagai, K.; Ukai, S. *Chem. Pharm. Bull.* **1991**, *39*, 798–800.
25. Katsuraya, K.; Nakashima, H.; Yamamoto, N.; Uryu, T. *Carbohydr. Res.* **1999**, *315*, 234–242.
26. Hawker, C. J.; Lee, R.; Frechet, J. M. J. *J. Am. Chem. Soc.* **1991**, *113*, 4583–4588.
27. Bolton, D. H.; Wooley, K. *Macromolecules* **1997**, *30*, 1890–1896.
28. Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27–66.
29. Satoh, T.; Imai, T.; Ishihara, H.; Maeda, T.; Kitajyo, Y.; Sakai, Y.; Kaga, H.; Kaneko, N.; Ishii, F.; Kakuchi, T. *Macromolecules* **2005**, *38*, 4202–4210.
30. Gorin, P. A. J. *Adv. Carbohydr. Chem. Biochem.* **1981**, *38*, 13–104.
31. Schoniger, W. *Mikrochem. Acta* **1956**, 869–876.
32. Ioan, C. E.; Aberle, T.; Burchard, W. *Macromolecules* **1999**, *32*, 7444–7453.
33. Huang, Q.; Zhang, L. *Biopolymers* **2005**, *79*, 28–38.
34. Park, I. H.; Choi, E. J. *Polymer* **1996**, *37*, 313–319.
35. Muthukrishnan, S.; Mori, H.; Müller, A. H. E. *Macromolecules* **2005**, *38*, 3108–3119.
36. Geladé, E.; Goderis, B.; Koster, C.; Meijerink, N.; Benthem, R.; Fokkens, R.; Nibbering, N. *Macromolecules* **2001**, *34, 3552–3558.*
37. Hanselmann, R.; Burchard, W.; Ehrat, M.; Widmer, H. M. *Macromolecules* **1996**, *29*, 3277–3282.
38. Scherrenberg, R.; Coussens, B.; Van, V. P.; Edouard, G.; Brockman, J.; De Brabander, E. *Macromolecules* **1998**, *31*, 456–461.
39. Burchard, W. *Adv. Polym. Sci.* **1999**, *143*, 113–194.
40. Wasser, S. P. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 258–274.
41. Adachi, Y.; Ohno, N.; Ohsawa, M.; Oikawa, S.; Yadomae, T. *Chem. Pharm. Bull.* **1990**, *38*, 477–481.
42. Kulicke, W. M.; Lettau, A. I.; Thielking, H. *Carbohydr. Res.* **1997**, *297*, 135–143.
43. Zhang, L.; Zhang, M.; Zhou, Q.; Chen, J.; Zeng, F. *Biosci. Biotechnol. Biochem.* **2000**, *64*, 2172–2178.