

STRUCTURE, MOLECULAR WEIGHT AND BIOACTIVITIES OF (1→3)- β -D-GLUCANS AND ITS SULFATED DERIVATIVES FROM FOUR KINDS OF *LENTINUS EDODES**

Unursaikhan Surenjav^a, Li-na Zhang^{a,*}, Xiao-juan Xu^a, Mei Zhang^a,
Peter Chi Keung Cheung^b and Fan-bo Zeng^c

^aDepartment of Chemistry, Wuhan University, Wuhan 430072, China

^bDepartment of Biological, The Chinese University of Hong Kong, Hong Kong, China

^cTongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Abstract Lentinan samples, (1→3)- β -D-glucans containing 4.6–15.2 wt% proteins, coded as L-I₁, L-I₂, L-I₃ and L-I₄ (L-I) were isolated from four kinds of *Lentinus edodes*. These glucans were treated with acetone to remove the protein in order to obtain free protein glucans coded as LNP-I₁, LNP-I₂, LNP-I₃ and LNP-I₄ (LNP-I). The free-protein polysaccharides were sulfated to give derivatives (S-LNP-I) with degree of substitution (DS) from 0.4–0.8. The structural features and weight-average molecular weight (M_w) of the samples were investigated by using infrared spectroscopy, elemental analysis, ¹³C-NMR, size exclusion chromatography combined with laser light scattering (SEC-LLS) and viscometry. The effects of structure and conformation of the polysaccharides on antitumor activities were assayed in vivo (Sarcoma 180 solid tumors) and in vitro (Sarcoma 180, HL-60, MCF-7 and Vero tumors). The results indicated that the predominant species of the samples L-I and LNP-I in 0.2 mol/L NaCl aqueous solution existed as triple-helical chains with high rigidity and in dimethyl sulfoxide (DMSO) as single-flexible chains. Interestingly, the antitumor activities of LNP-I are lower than those of the native glucans (L-I), whereas their sulfated derivatives have higher inhibition ratio against Sarcoma 180 than LNP-I. The results reveal that the binding of protein, sulfated modification and the triple helix conformation are important factors in the enhancement of the antitumor activities of polysaccharides on the whole.

Keywords: (1→3)- β -D-glucans; *Lentinus Edodes*; Molecular weight; Protein; Sulfated derivative; Antitumor activity.

INTRODUCTION

There is growing urgency to develop renewable resource from plant, animal and fungi because of the great importance for a sustainable development. It is noted that mushroom, attracted much attention, because some β -glucan isolated from mushroom have exhibited antitumor activity^[1, 2]. Families of glucans, which contain a main chain of (1→3)-linked β -D-glucopyranosyl units, substituted at O-6 by single-unit β -D-glucopyranosyl side chains, have received considerable attention because of their antitumor activity (immunomodulators operator) and their unique solution properties^[3, 4]. Lentinan [(1→3)- β -D-glucan with β -(1→6) branches from *lentinus edodes*]^[1, 5], scleroglucan^[6, 7] and schizophyllan^[8, 9] are most well known members of this group of polysaccharides. It has been reported that the antitumor activities are closely related to the triple helical structure of the (1→3)- β -D-glucan backbone chain^[10, 11]. However, the triple helical structure of high-molecular weight (1→3)- β -D-glucans can be denatured either by increasing temperature in aqueous solutions above 135°C^[12] or dissolving in strong solvents such as dimethyl sulfoxide (DMSO)/water mixtures with water fraction (W_H) less than 0.13 at 25°C^[12, 13], or in alkali aqueous solution (0.18–0.25 mol/L NaOH/KOH)^[9, 14, 15].

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* Corresponding Author: Li-na Zhang (张利娜), E-mail: lnzhang@public.wh.hb.cn

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Lentinan has strong antitumor activity against various tumors, and prevents chemical and viral carcinogenesis^[8, 16], whereas only its hydrolyzed fractions ($M_w > 1.6 \times 10^4$) have exhibited antitumor activity^[17]. Moreover, *lentinan* sulfate and *curdlan* sulfate have showed high anti-HIV activities but with low anti-coagulant activities^[18]. Therefore, a basic understanding of the correlation between secondary structure and the antitumor activities of *lentinan* is essential for the successful application in the field of drug and food. In our laboratory, strong evidences have been given to confirm that *lentinan* exists as triple-helix in water and as single flexible chains in DMSO^[19]. Interestingly, the triple helix *lentinan* exhibits relatively high antitumor activity *in vivo*, whereas the bioactivity of its single flexible chains almost disappear^[20]. Moreover, the sulfated derivatives of (1→3)- α -D-glucan from *Canoderma lucidum* in the aqueous solution behave as expanded chains, and exhibit antitumor activity^[21]. The correlations of chemical component, molecular weight and conformation of *lentinan* to the antitumor activities have not been clarified. In this work, we isolated four water-soluble *lentinans* with different molecular weight and with different protein content from *Lentinus edodes*. The degree of substitution (DS), weight-average molecular weight, intrinsic viscosity, and chain stiffness of the native and modified *lentinan* were measured by elemental analysis (EA), size exclusion chromatography combined with laser light scattering (SEC-LLS) and viscometry, respectively. The investigation may contribute some meaningful information to the understanding the effect of structure and chain conformation on the antitumor activities.

EXPERIMENTS

Materials

Four kinds of *Lentinus edodes* with different strains (Hua 4, FL 66, Fcro 2 and FJ 1), coded as No. 2, 36, 39, 50, which were cultivated in bags filled with log pieces, were supplied by the Laboratory of Applied Mycology in Huazhong Agricultural University. Human acute promyelocytic Leukemia HL-60 (ATCC no CCL-240) and monkey normal kidney Vero (AATCC no CCL-81) cell lines were purchased from the American type culture collection (Rockville, MD). DMSO was first treated with 0.5 nm molecular sieves to remove water, and then distilled under reduced pressure.

Preparation of Samples

Four (1→3)- β -D-glucans were isolated from four kinds of fruiting bodies of *Lentinus edodes*, by extraction with 5% NaOH / 0.05% NaBH₄ two times, and precipitation with 36% acetic acid to remove α -glucan, according to the previous method^[22]. The supernatant was subjected to the Sevag method to remove proteins, and decolorized with 30% H₂O₂. Aqueous solution of the glucan was dialyzed against distilled water for 7 d, and concentrated by using rotary evaporator at reduced pressure below 45°C, and then lyophilized to obtain the samples L-I₁ (17.4 g), L-I₂ (5.6 g), L-I₃ (15.3 g) and L-I₄ (5.6 g), respectively. The protein contents in each polysaccharide were measured to be about 5.8%, 5.5%, 4.6%, and 15.2% for L-I₁, L-I₂, L-I₃ and L-I₄, respectively, by using a KJELETC 1030 semimicro Kjeldhal self-analyzer (Switzerland). This result suggests that the *lentinans* were polysaccharides bound with proteins because the Sevag method has been repeated several times to remove free proteins.

To remove the polysaccharide bound with protein, each native β -glucan (1.5–2.5 g) was dissolved in 0.2 mol/L NaCl aqueous solution (600 mL). After standing for 12 h at 30°C, the turbid solution was centrifuged at 12,000 *r/min* to be separated into liquid and gel containing bound proteins. The gel was removed, and with an equivalent volume of acetone, was added into the supernatant to precipitate the pure polysaccharide. The purification was repeated three times. Finally, the precipitated polysaccharides were dialyzed and dried to obtain the white powder, namely free-protein polysaccharides, coded as LNP-I₁ (1.22 g), LNP-I₂ (1.94 g), LNP-I₃ (1.70 g) and LNP-I₄ (2.11 g), respectively. The nitrogen content in the samples LNP-I was measured by means of elemental analysis and was found nil.

The sulfating agent was prepared using dry pyridine and chlorosulfonic acid according to the CSA method by Yoshida *et al.*^[23]. The LNP-I free-protein β -glucans (250–400 mg) were suspended in 50 mL DMSO at room temperature with stirring for 2 h. The resulting mixture was added to chlorosulfuric acid (3.5 mL) in dry pyridine

(80 mL) with stirring for 30 min at 25°C, and then heated to 60°C, followed by stirring for 4 h. After cooling, the solution was adjusted to pH 7.5–8.0 with 10% NaOH aqueous solution, and then the mixed solution was dialyzed against slightly alkaline water (pH 9) to remove pyridine. Finally, the sulfated polysaccharide was dialyzed against distilled water for 7 d, and then the products were concentrated and lyophilized to give light yellow powder of sulfated *lentinan* coded as S-LNP-I. The yield of the samples S-LNP-I₁, S-LNP-I₂, S-LNP-I₃ and S-LNP-I₄ was 380 mg, 380 mg, 291 mg, and 360 mg, respectively.

Structure Characterization

Structural information of the samples was obtained by using a Nicolet Fourier transform infrared (FTIR) spectrometer (Spectrum One, Elmer Co., USA). Test specimens were prepared as KBr-disk. The ¹³C-NMR spectra at 150 MHz were recorded on an INOVA-600 spectrometer (Varian Inc., USA) at ambient temperature. The samples were dissolved in DMSO-d₆. Chemical shifts were expressed relative to that of tetramethylsilane. The elemental compositions were determined by an elemental analyzer (EA, Heraeus Co., Germany). The protein contents in each polysaccharide were measured by using KJELETC 1030 semimicro Kjeldhal self-analyzer (Switzerland). Carbohydrates content were determined colorimetrically using the phenol sulfuric acid method described in the Ref. [24] with a UV-160 spectrophotometer. A calibration curve was constructed with D-glucose as the standard. Specific optical rotation at 589 nm [α]_D²⁵ was measured on a Perkin Elmer 341 polarimeter in a jacketed standard cell for 0.1% samples in 0.2 mol/L NaCl aqueous solution and pure DMSO. All the solutions were maintained at 25°C.

SEC-LLS Measurement

The weight-average molecular weight (M_w) of the samples was measured by using laser light scattering combined with size exclusion chromatography in 0.2 mol/L NaCl and DMSO, respectively. The SEC-LLS measurement was carried out with multi-angle laser light scattering instrument (DAWN DSP, Wyatt Technology Co., USA) at 633 nm combined with a size exclusion chromatography equipped with a TSK-GEL G6000 PWXL and a G4000 PWXL column (7.8 mm × 300 mm) for aqueous solution and a G4000-H8 column for DMSO at 25°C and together with a differential refractive index detector (RI-150). Optical clear solution was obtained using filtration through 0.45 μm Millipore filter (Whatman, England) before injection. The injection volume was 200 μL with the concentration of 1 mg/mL for each sample, and the flow rate was 1.0 mL/min. The specific refractive index increments (dn/dc) of the samples in 0.2 mol/L NaCl aqueous solution and DMSO at 633 nm and 25°C were determined using an interferometer refractometer (Optilab 903, Wyatt Technology, USA) to be 0.140 mL/g and 0.060 mL/g, respectively. Astra software was utilized for the data acquisition and analysis. Zimm method was used to obtain M_w and z -average radius of gyration ($\langle s^2 \rangle_z^{1/2}$) by:

$$\frac{Kc}{R_\theta} = \frac{1}{M_w} \left(1 + \frac{16\pi^2 n_0^2 \langle s^2 \rangle}{3\lambda_0^2} \sin^2 \frac{\theta}{2} + \dots \right) + 2A_2 c \quad (1)$$

where $K = 4\pi^2 n_0^2 (dn/dc)^2 / (N_A \lambda_0^4)$, with N_A , n_0 and λ_0 as Avogadro's number, refractive index of the solvent, and wavelength of incident light in a vacuum, respectively. A_2 is the second virial coefficient.

Viscosity Measurement

The intrinsic viscosities ($[\eta]$) of the samples in 0.2 mol/L NaCl aqueous solution and DMSO were determined, respectively, by using an Ubbelodhe capillary viscometer and a low-shear three-bulb capillary viscometer at 25°C. The kinetic energy correction was always negligible. Huggins and Kraemer equations were used to estimate the $[\eta]$ by extrapolating η_{sp} and $\ln \eta_r$ to the infinite dilution as follows:

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

$$(\ln \eta_r)/c = [\eta] - \beta [\eta]^2 c \quad (3)$$

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

In Vivo Antitumor Test

Sarcoma 180 cells (1×10^5 cells/mouse), provided by Tongji College of Medicine, Huazhong University of Science and Technology, were transplanted subcutaneous into the right groin of 8 week-old BALB/c mice weighting (18 ± 1) g, which were divided randomly into 10 groups with 10 mice per group. 5-Fluorouracil (5-Fu) and the test samples were dissolved or suspended in 0.9% NaCl aqueous solution, respectively, and then injected intraperitoneally once a day for 7 d, starting 24 h after tumor inoculation. The 0.4 mL 0.9% NaCl solution was injected intraperitoneally into the control mice, and 5-Fu was injected intraperitoneally into another masculine mouse at dose of 20 mg/kg. For the β -glucan samples, the doses were 20 mg/kg and 60 mg/kg, respectively. The mice were killed on the next day after the last injection, and the tumors were removed from the animal and weighted. The inhibition ratio (ξ) and enhancement ratio of body weight (f) were calculated as follows^[25]:

$$\xi = [(W_c - W_t)/W_c] \times 100\% \quad (4)$$

$$f = [(W_a - W_b)/W_b] \times 100\% \quad (5)$$

where W_c is the average tumor weight of the control group, W_t is the average tumor weight of the test group, and W_b and W_a are the body weight of mice before and after the assay. Statistical evaluations in all experiments were performed by a student's t-test. The P value of less than 0.05 was considered significant.

In Vitro Proliferation and Cytotoxicity Assays

The Sarcoma 180 tumor cells (1×10^5 cells/mL) and the HL-60 leukemic cells (1×10^6 cells/mL) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum containing polysaccharides at concentrations of 50, 100 and 200 $\mu\text{g/mL}$ in 0.9% NaCl aqueous solution and PBS solution, respectively, under an atmosphere of 5% carbon dioxide at 37°C for 72 h. The survival rate of the mammalian cells was assayed by a hemacytometer by counting living cells that were excluded the Trypan blue dye. According to the colorimetric MTT method for adherent cells by Department of Biology, Chinese University of Hong Kong, the Mammalian MCF-7 cells and Vero cells (1×10^6 cells/mL) were incubated separately with the polysaccharides at concentrations of 50, 100, and 200 $\mu\text{g/mL}$ and allowed to grow under the same conditions as the Sarcoma 180 cells already mentioned. The number of living MCF-7 cells and Vero cells at the end of the 72 h incubation period was determined by a colorimetric assay based on the tetrazolium salt MTT as described by Mosmann^[26]. The test samples were compared with control samples in the absence of the test samples. All in vitro results were expressed as the ratio of inhibition of tumor cell proliferation calculated as $[(A - B)/A] \times 100\%$, where A and B are the average number of viable tumor cells of the control and samples, respectively. Data in all the bioassays were statistically evaluated by Student's t-test and differences with a P value of less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Chemical Structure

The IR spectra of the all polysaccharides show characteristic absorptions at 890 cm^{-1} (Fig. 1), assigned to the β -configuration^[27]. Compared with the native samples, the —OH stretching vibration bands at 3400 cm^{-1} for the sulfated derivatives were narrowed, and two new absorption bands appeared at 820 cm^{-1} and 1240 cm^{-1} for the sulfated derivatives, as a result of the presence of the C—S—O and S=O bonds, indicating that sulfation has actually occurred. The L-I₄ polysaccharide has higher protein content (15.2%), but lower carbohydrate content (76.7%) than others.

The ¹³C-NMR spectra of the L-I₃, LNP-I₃ and S-LNP-I₃ samples are shown in Fig. 2. The strong signals around $\delta = 103.7, 85.3, 76.8, 76.5, 72.6, 70.4, 68.5$ and 61.9 for the L-I₃ are assigned as C-1, C-3, C-3', C-5, C-2, C-6's, C-4 and C-6 of (1 $\rightarrow$ 3)- β -D-glucan (see Table 1)^[28], respectively. The C-6s signal represents substituted C-6 of branched (1 $\rightarrow$ 3)- β -D-glucan^[29, 30], indicating a branched glucan. There are new peaks in ¹³C-NMR spectrum of the sulfated glucan S-LNP-I₃, namely C-1' (101.5), C-2s (78.0), C-2's (78.9), C-4's (75.0) and C-6's (68.9), resulting from sulfonation of the hydroxyl groups at positions C-6, C-4 and C-2. The peak at

61.5, which is the chemical shift of C-6 for LNP-I₃, disappears in the ¹³C-NMR spectra of S-LNP-I₃. A new peak appears at δ = 68.9 in S-LNP-I₃, which is assigned to the substituted C-6's because of the downfield shift of a carbon atom linked by a sulfate group is ~ 7 – 10 ^[31]. The new peak at 78.9 for the S-LNP-I₃ sample may be assigned to C-2s, and that at 101.5 it could be assigned to the chemical shift of adjacent C-1 as a result of an influence of partially substituted C-2^[32]. On the basis of ¹³C-NMR results, a nonselective sulfation of β -glucan has been confirmed. The data of elements, content in the sulfated derivatives are listed in Table 2. The degree of substitution (DS), which designates the average number of sulfo groups on each sugar residue, was calculated from results of EA on the basis of determined sulfur content (S%) by the following formula^[33].

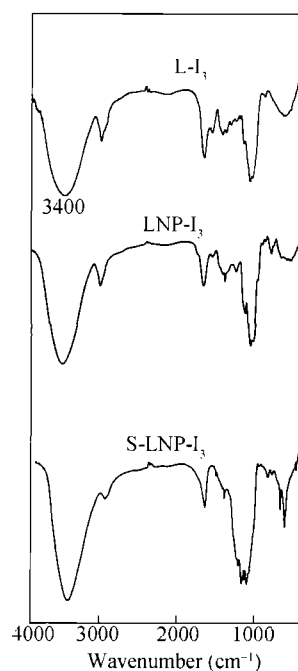


Fig. 1 FT-IR spectra of the samples L-I₃, LNP-I₃, and S-LNP-I₃

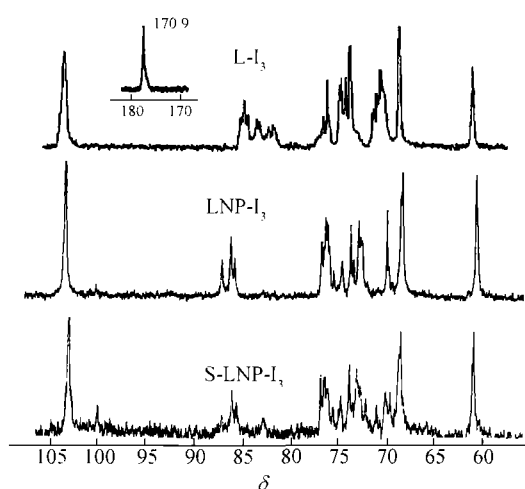


Fig. 2 ¹³C-NMR spectra of the samples L-I₃, LNP-I₃ and S-LNP-I₃ in DMSO-*d*₆

Table 1. ¹³C-NMR chemical shifts of the polysaccharides in DMSO-*d*₆

Sample	Chemical shifts													Source
	C-1	C-2	C-3	C-3'	C-4	C-5	C-6	C-6s	C-1'	C-2s	C-2's	C-4's	C-6's	
L-I ₃	103.7	72.6	85.3	76.8	68.5	76.5	61.9	70.4						This work
LNP-I ₃	103.5	72.4	86.7	76.9	68.5	76.7	61.5	70.5						This work
S-LNP-I ₃	103.5	72.6	86.6	76.7	68.6	76.4	61.5	70.0	101.5	78.0	78.9	75.0	68.9	This work
Glucan L-FV-1A	103.1	72.9	86.3	76.8	68.5	76.2	61.0	70.1						Ref. 35
Sulfated β -glucan	103.1	72.4	86.1		68.4	76.2			101.5	79.4	80.3	77.4	67.7	Ref. 28

Table 2. Results of elemental analyses and yield on sulfated derivatives

Sample	C (%)	H (%)	N (%)	S (%)	DS	Yield (%)
S-LNP-I ₁	33.7	5.3	n.d. ^a	7.8	0.49	~100
S-LNP-I ₂	33.8	5.2	n.d.	6.0	0.36	94.7
S-LNP-I ₃	34.9	5.1	n.d.	7.5	0.49	~100
S-LNP-I ₄	31.7	5.0	n.d.	11.5	0.82	81.8

^aNot detected

$$DS = (162 \times S\%) / (32 - 80 \times S\%) \quad (6)$$

The estimation errors for DS were calculated to be around 0.6% from the experimental error of the instrument. The DS values of sulfated β -glucans S-LNP-I were found to be from 0.36–0.82, and they are well soluble in aqueous solution.

Molecular Parameters

The SEC chromatograms of L-I, LNP-I and S-LNP-I samples are shown in Fig. 3. The experimental results from SEC-LLS and viscometry for the native L-I, free-protein LNP-I and S-LNP-I sulfated glucans, in 0.2 mol/L NaCl aqueous solution and DMSO at 25°C are summarized in Table 3. Obviously, the values of M_w , $\langle s^2 \rangle_z^{1/2}$ and $[\eta]$ for the native samples in two solvents are quite different, implying a conformation transition. The ratios of the apparent mean $M_{w, app}$ in the aqueous solution to those in DMSO were roughly estimated to be in the range of 2.9–3.2 for L-I glucans, and of 2.7–3.3 for LNP-I glucans, confirming the existence of triple stranded chains in aqueous solution. This can be explained that DMSO is a strong polar solvent, and can break the intra- and intermolecular hydrogen bonds, which sustain the helical structure and triple strands of the polysaccharide, leading to the change from triple helix to single flexible chains.

The $[\eta]$ values of L-I and LNP-I samples in 0.2 mol/L NaCl aqueous solution are much higher than those in DMSO, and the ratios of $[\eta]$ in water to that in DMSO are close to 4.0–5.6 for L-I glucans, and 4.1–5.3 for LNP-I glucans. The high $[\eta]$ also reflects the characteristic of stiff chain such as helix. More information about the conformation could be obtained from the SEC-LLS chromatogram. The SEC-LLS is an absolute method, since the power law $\langle s^2 \rangle_z^{1/2} = KM_w^\alpha$ can be estimated from a number of experimental points of M_w and $\langle s^2 \rangle_z^{1/2}$ in the SEC. The exponent (α) values for the L-I glucans in NaCl aqueous solution are in the range of 0.81–0.89, and of 0.47–0.54 in DMSO. The exponent is related to the chain conformation, and the high values are corresponding to the stiff chains for the L-I glucans in aqueous solution, and the values of about 0.5 suggest random coil chains in DMSO.

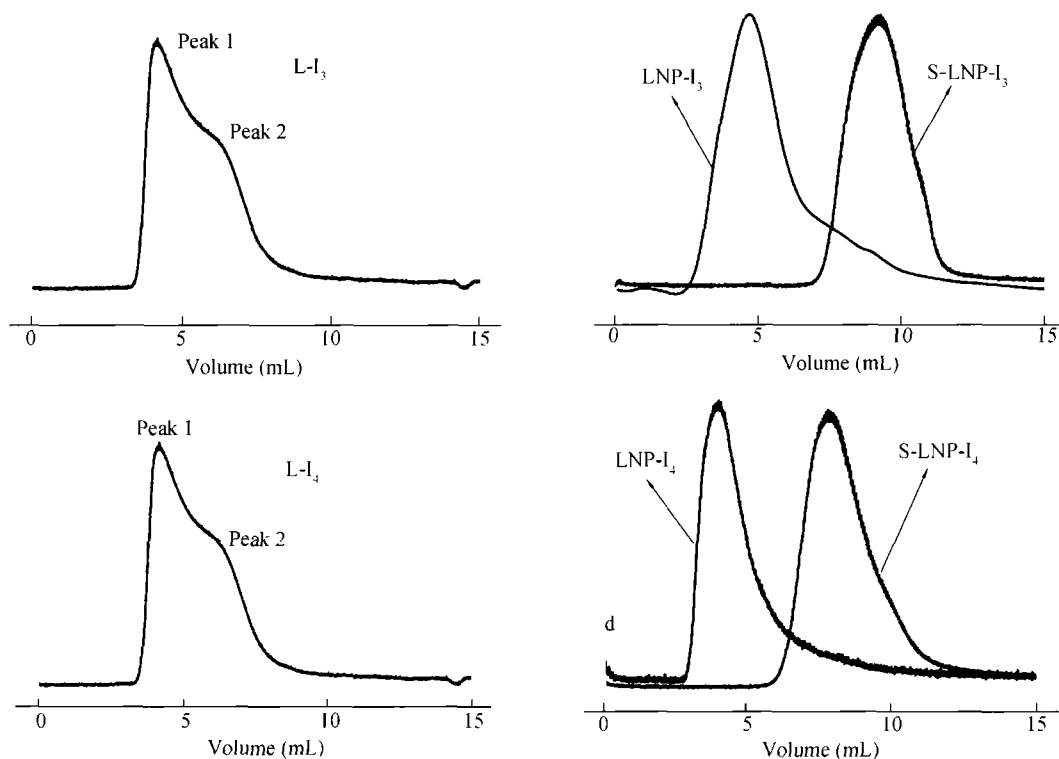


Fig. 3 SEC chromatograms of the samples L-I₃, LNP-I₃, S-LNP-I₃ and L-I₄, LNP-I₄, S-LNP-I₄ in 0.2 mol/L NaCl aqueous solution at 25°C detected by LLS

Table 3. Results from light scattering measurements and viscosity in water and DMSO at 25°C and the protein content in the polysaccharides

Sample	In 0.2 mol/L NaCl		In DMSO		$M_{w, app}$	$[\eta]_{in aq.}$	Content of protein (%)
	$M_{w, app} \times 10^{-4}$	$[\eta] (cm^3 g^{-1})$	$M_w \times 10^{-4}$	$[\eta] (cm^3 g^{-1})$	$M_{w, DMSO}$	$[\eta]_{in DMSO}$	
L-I ₁	143.5*	1050	49.0	224	2.9	4.6	5.8
L-I ₂	164.0*	1123	53.7	288	3.1	4.0	5.5
L-I ₃	165.0*	1162	56.8	275	2.9	4.1	4.6
L-I ₄	177.0*	1250	56.0	222	3.2	5.6	15.2
LNP-I ₁	57.0	568	21.4	135	2.7	4.2	<0.01
LNP-I ₂	51.8	377	16.2	84	3.2	4.5	<0.01
LNP-I ₃	53.7	307	16.1	75	3.3	4.1	<0.01
LNP-I ₄	111.6	1035	35.3	195	3.2	5.3	<0.01
S-LNP-I ₁	26.9	122	18.4	98	1.2	1.0	<0.01
S-LNP-I ₂	15.4	82	14.4	78	1.1	1.1	<0.01
S-LNP-I ₃	20.1	97	17.1	87	1.2	1.1	<0.01
S-LNP-I ₄	36.9	149	32.4	136	1.1	1.1	<0.01

*Apparent weight-average molecular weight

In addition, the L-I₃ and LNP-I₃ in aqueous solution have a low positive specific rotation ($[\alpha]_D^{25}$) of +19.2° and +17.5° cm²/g in 0.2 mol/L NaCl aqueous solution, and -20.1° and -18.9° cm²/g in DMSO, suggesting triple helix conformation in aqueous solution, and the conformation transition has occurred in DMSO^[34]. On the basis of the results mentioned above and previous evidence^[35], the L-I and LNP-I glucans mainly exist as triple-helical chains in aqueous solution and as single chain in DMSO. The exponents of $\langle s^2 \rangle_z^{1/2} - M_w$ for sulfated glucans S-LNP-I were measured to be 0.49–0.52, indicating a flexible chain conformation.

Effects of Structure on Bioactivities

The results from *in vivo* assay of the antitumor activities of the samples are listed in Table 4. The LNP-I₁, LNP-I₂ and LNP-I₃ samples show lower inhibition ratio than the native L-I₁, L-I₂ and L-I₃ glucans. The results suggest that antitumor activities depend on the binding of protein on the polysaccharides. The L-I₂ and L-I₃ samples at high dose have a higher inhibition ratio than 5-Fu, indicating an important role of both triple helix and bound protein. It is worth noting that the enhancement ratios of body weight of the mice injected with the polysaccharide are higher than that with 5-Fu, implying that β -glucans do not have the same toxicity as 5-Fu, which kills normal cells as well as cancer cells. Interestingly, all sulfated β -D-glucans have significantly higher antitumor activity against Sarcoma 180 than the LNP-I original glucans. The effect of expanded chains of the sulfated glucan in aqueous solution on the improvement of antitumor activity could not be neglected. The M_w value of S-LNP-I₄ (36.9×10^4) is higher than those of other S-LNP-I, and shows higher antitumor activity. The results reveal that antitumor activities of the polysaccharides are related with their bound protein, molecular weight, chain conformation, and origin. The chemically modified samples having sulfur content from 5.0% to 11.5% are soluble in 0.2 mol/L NaCl aqueous solutions, and show antitumor activity. In view of the results obtained from *in vivo*, the antitumor activities of the samples on the whole are in the order of L-I > S-LNP-I > LNP-I. The water solubility of the samples is different, but their effects on bioactivities are not obvious. In addition, it has been reported that^[36, 37] sulfated schizophyllan with a sulfur content of 5% can be useful as an anti-HIV agent for treatment of HIV-infected hemophiliacs. We will continually investigate the anti-HIV activity of the sulfated β -glucans.

Table 5 shows the inhibitory effects of L-I₃ and LNP-I₃ against HL-60, MCF-7 and Vero cells line at three concentrations. The sample L-I₃ shows relatively strong inhibitions against tumor cell growth at three concentrations, and has dose dependent relationship. However, LNP-I₃ exhibits lower activities than native L-I₃. No antiproliferation effect of these polysaccharides on Vero cells is observed, suggesting that the bioactivities of the polysaccharide have selectivity. The inhibition ratios to the proliferation of Sarcoma 180 cell with different concentrations (5000, 500, 50 and 5 μ g/mL) of the samples are shown in Fig. 4. The samples also show a dose-response relationship in inhibiting the Sarcoma 180 cells proliferation. Interestingly, S-LNP-I₄ also exhibits the

highest inhibition ratio (57%) to the proliferation of S-180 cell line at the high concentrations. From these results, the antitumor activities *in vitro* are in the order of L-I > S-LNP-I > LNP-I. These results support the conclusion from *in vivo*, namely that the antitumor activities of the polysaccharides are related with their molecular weight, bound protein and chain conformation. The relatively high molecular weight, certain content of bound protein and chain stiffness may be helpful to improve the antitumor activities.

Table 4. In vitro antitumor activities against Sarcoma 180 Solid tumor of the samples

Sample	Structural feature	Water-solubility ^a	Dose (mg/kg × 7days)	Inhibition ratio (%)	Enhancement ratio of body weight (%)	Complete regression
Control					44.2	0/10
FU-5			20	48.6	19.1	0/10
L-I ₁	Triple helix with protein	+	20	12.1	55.0	0/10
			60	47.8*	29.1	0/10
L-I ₂		+	20	25.9	41.3	0/10
			60	54.5*	31.4	0/10
L-I ₃		+	20	70.0	27.4	0/10
			60	63.0	37.1	0/10
L-I ₄		+	20	20.3*	33.3	0/10
			60	37.1	45.0	0/10
Control					45.4	0/10
FU-5			20	51.7	25.6	0/10
LNP-I ₁	Triple helix without protein	++	20	7.5	57.8	0/10
			60	17.1	48.6	0/10
LNP-I ₂		++	20	0.6	52.2	0/10
			60	30.4*	45.3	0/10
LNP-I ₃		++	20	12.1	50.9	0/10
			60	41.4	25.0	0/10
LNP-I ₄		++	20	37.6*	45.4	0/10
			60	9.8	49.6	0/10
Control					48.6	0/10
FU-5			20	54.24	25.1	0/10
S-LNP-I ₁	Semi flexible chain with sulfate group	+++	20	19.5	39.3	0/10
			60	26.5	30.9	0/10
S-LNP-I ₂		+++	20	14.5*	33.0	0/10
			60	47.6	29.3	0/10
S-LNP-I ₃		+++	20	21.5*	55.3	0/10
			60	51.5	21.7	0/10
S-LNP-I ₄		+++	20	35.5*	42.4	0/10
			60	53.0	24.9	0/10

* $P < 0.05$, significant difference when compared to the control

^a+++ good water-soluble; ++ water-soluble; + water-soluble with a few of gels

Table 5. The inhibition ratios of the samples L-I₃ and LNP-I₃ against HL-60, MCF-7 and Vero cells

Name of cell	Concentration (μg/mL)	Inhibition ratio (%)	
		L-I ₃	LNP-I ₃
HL-60 cell	50	37.0 [*]	17.1
	100	31.9	31.4
	200	27.2 [*]	-12.9
MCF-7 cell	50	22.7	16.9
	100	32.8	-7.9
	200	33.2	-18.6
Vero cell	50	3.0 [*]	1.1 [*]
	100	2.3 [*]	1.2 [*]
	200	0.9 [*]	0.2 [*]

* $P < 0.05$, significant difference when compared to the control

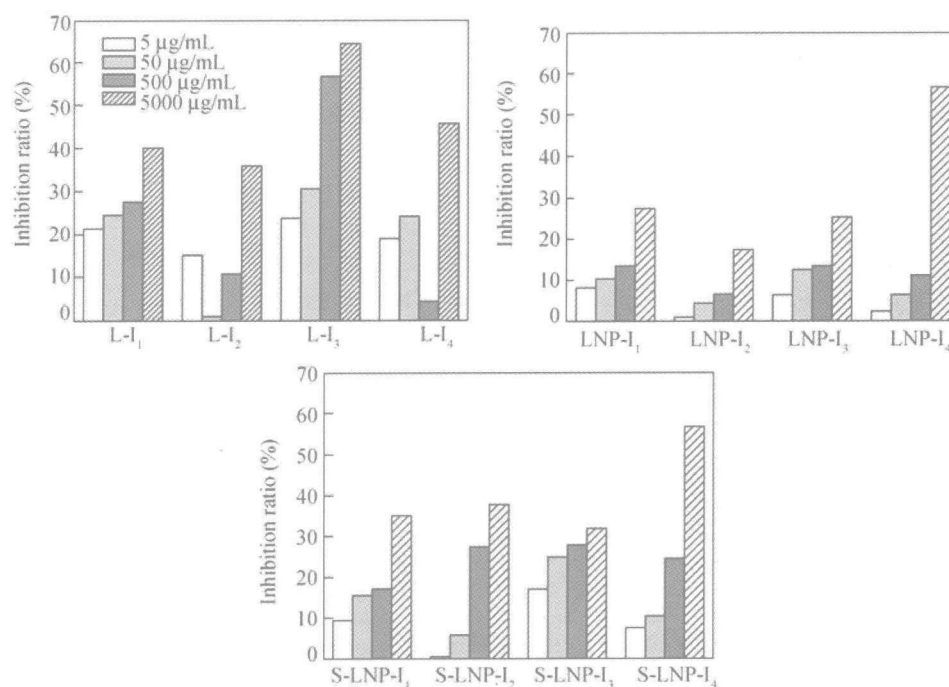


Fig. 4 Inhibition ratios in vitro against Sarcoma 180 cells with different concentrations of the samples

CONCLUSION

The results from SEC-LLS, viscometry and optical rotation measurements indicate that L-I native (1→3)- β -D-glucans, and the LNP-I protein free glucans exist as triple-helical chains with high rigidity in 0.2 mol/L NaCl aqueous solution, and as single-flexible chains in DMSO. The antitumor activities of the LNP-I glucans are lower than those of the L-I native glucans. Interestingly, water-soluble sulfated derivatives synthesized from LNP-I exhibit higher antitumor activities against Sarcoma 180 than the original LNP-I. The samples have different antitumor activities, depending on their bound protein content, molecular weight, chain conformation, and modified derivatives. On the whole, the inhibition ratio against tumor in vivo and in vitro is in the order of L-I > S-LNP-I > LNP-I. Therefore, the triple helix conformation, certain bound protein and sulfated group are important factors in the enhancement of the antitumoral activities of samples from *Lentinus edodes*.

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