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Effects of molecular structure on antitumor activities of (1→3)-β-D-glucans from different *Lentinus Edodes*

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Abstract

Four (1→3)-β-D-glucans coded as L-I_1, L-I_2, L-I_3 and L-I_4 with high molecular weight (1.47×10^6–1.67×10^6) were isolated from four kinds of fruiting bodies of *Lentinus Edodes* with different strains by extracting with NaOH/$NaBH_4$, then precipitating with 1 M acetic acid. The results from element analysis indicated that the polysaccharides contained 4.6–15.2 wt% proteins. The polysaccharides were treated with acetone to remove the bound protein, coded as LNP, and by ultrasonic irradiation to degrade molecular weight, coded as LU. The weight-average molecular weight M_w, radii of gyration $<s^2>_z^{1/2}$ and intrinsic viscosity [η] of the samples in aqueous 0.2 M NaCl/0.005 M NaOH and in dimethyl sulfoxide (DMSO) were measured by size exclusion chromatography combined with laser light scattering and viscometry at 25 °C, respectively. The M_w drop by a factor of 3 from in aqueous solution to in DMSO, and M_w dependence of $<s^2>_z^{1/2}$ revealed that the β-glucan exists as triple-helical conformation in aqueous NaCl and as single flexible chains in DMSO. The single chain samples were prepared in DMSO, coded as LSC. The assay in vivo and in vitro antitumor activities against Sarcoma 180 (S-180) solid tumor for the polysaccharides showed that the native triple helical (1→3)-β-D-glucans containing protein exhibited obvious antitumor bioactivities (in vivo highest inhibition ratio ξ reached to 70.0% for L-I_3), whereas that (ξ = 2.2%) of LSC having only single flexible chains significantly decreased. Moreover, the antitumor activities of the LNP, LU and LSC samples are lower than those of the native ones, suggesting that the antitumor activities of polysaccharide were related with their conformation, molecular weight and content of the bound protein. The triple helical conformation plays an important role in enhancement of the antitumor activities.

Keywords: Lentinus Edodes; (1(3)-β-D-glucan; molecular weight; conformation light scattering; antitumor activity

1. Introduction

Mushrooms play an important role in food and medicine (Molitoris, 1994), so many researchers paid much attention on them. One (1→3)-β-D-glucan, named *Lentinan* as an antitumor polysaccharide, has been isolated from the fruiting body of *Lentinus edodes* by Chihara et al. (Maeda & Chihara, 1973; Maeda, Chihara, & Ishimura, 1974). It has been reported that the antitumor activities may be related to the triple-helical structure of the (1→3)-β-glucan backbone chain such as *Schizophyllan* (Chihara, 1992; Kashigawa, Norisuye, & Fujita, 1981; Saito, Takasuka, & Sasaki, 1977). The triple-helical structure of the (1→3)-β-D-glucan has exhibited the inhibition of growth of implanted Sarcoma 180 ascites in mice (Kulieke, Lettau, & Theilking, 1997). It is well known that the bioactivities of polysaccharides are related with the molecular weight, degree of substitution, degree of branching, chain conformation in solution, sugar component and the structures of main chain and branches (Bohn & BeMiller, 1995; Kennedy, 1989; Kennedy & White, 1983). It has been regarded that high molecular weight, triple helix and a (1→6)-β-branching are favorable structural parameters. However, some researchers have considered that single helices and (1→6)-β-glucosidic linkage are also important factors in influencing the antitumoural effect (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970). Recent studies have showed that the second structure of the polysaccharides such as conformation in dilute solution and molecular weight affects

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strongly their antitumor and immunomodulation activities (Chihara et al., 1987; Leung, Fung, & Choy, 1997; Zhang, Cheung, & Zhang, 2001; Zhang, Zhang, & Cheng, 1999). In connection with antitumoural effects, a triple-helix is believed to be important structural requirement (Adachi, Ohno, Ohsawa, & Yadomae, 1990; Kishida, Sone, & Misaki, 1992; Ohno, Asada, Adachi, & Yadomae, 1995), but Gomaa, Krauz, Franz and Roper (1991) and Saito et al. (1991) have described a single helix as a requirement for the activities. Demleitner, Kraus, and Franz (1992) have indicated that the (1→3)-β-glycosidic linkage is the essential structural feature for immunostimulatory and antitumoural effects, and no high molecular weight is required.

In our laboratory, strong evidences showed that (1→3)-β-D-glucan, *Lentinan*, exists as triple-helix in water and as single flexible chains in dimethyl sulphoxide (DMSO) (Zhang, Li, Zhou, Zhang, & Chen, 2002; Zhang et al., 2001). In this work, the water-soluble triple-helical (1→3)-β-D-glucans were extracted from four different strains of *Lentinus edodes* to study the effect of the strain source, molecular weight, conformation and protein on the antitumor activities. The content of protein, weight-average molecular weight (M_w) and intrinsic viscosity ([η]) of the native and treated polysaccharides were measured by elemental analysis, size-exclusion chromatography combined with laser light scattering (SEC-LLS) and viscometry, respectively. The antitumor activities against the growth of S-180 solid tumor implanted in mice, of the samples were tested.

2. Materials and methods

2.1. 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. Fig. 1 shows four kinds of the fruiting bodies of *Lentinus Edodes.* DMSO was treated with 5 Å molecular sieves to remove water, and then distilled under reduced pressure. All reagents were of analytical grade, and were purchased from Tianjin Fucheng Chemical Reagent Corporation (China).

2.2. Isolation of native polysaccharides

Four (1→3)-β-D-glucans coded as L-I_1, L-I_2, L-I_3, L-I_4 were isolated from four fruiting bodies of *Lentinus edodes*, according to the method of Chihara et al. (1987) with minor modifications (Zhang et al., 1999). The homogenized materials of 154–189 g dry *Lentinus Edodes* were defatted with hot ethyl acetate and acetone by Soxlet apparatus for 5 h, respectively, and then extracted in 0.9% aqueous NaCl under high pressure five times. The resulting brown solution was centrifuged at 12,000 rpm for 20 min. The supernatants were discarded and the residues were immersed in NaOH/$NaBH_4$ (5–50 mg/100 mL) for 12 h with shaking at room temperature to obtain liquid. Solid residues were removed by centrifugation (12,000 rpm, 20 min), and the supernatants were collected, and then neutralized with 36% acetic acid to separate precipitates (α-glucan) and supernatant (β-glucan L-I). The supernatants containing β-glucan were subjected to the Sevag method to remove proteins, and treated with 30% H_2O_2 to decolorize. The glucan aqueous solution was dialyzed against running water for 3 d and then double distilled water for 4 d. Each sample was concentrated by rotary evaporator at reduced pressure below 45 °C, and finally lyophilized by using lyophilizer (CHRIST Alpha 1-2, Germany) to obtain colorless flakes. The yield of the L-I_1, L-I_2, L-I_3 and L-I_4 samples were 10.0, 3.5, 8.1 and 3.6%, respectively.

2.3. Preparation of samples

Each of 4 native β-glucans was dissolved in 0.2 M aqueous NaCl (600 mL 2%), respectively, for 12 h at 30 °C, and then the turbid solution was centrifuged at 12,000 rpm

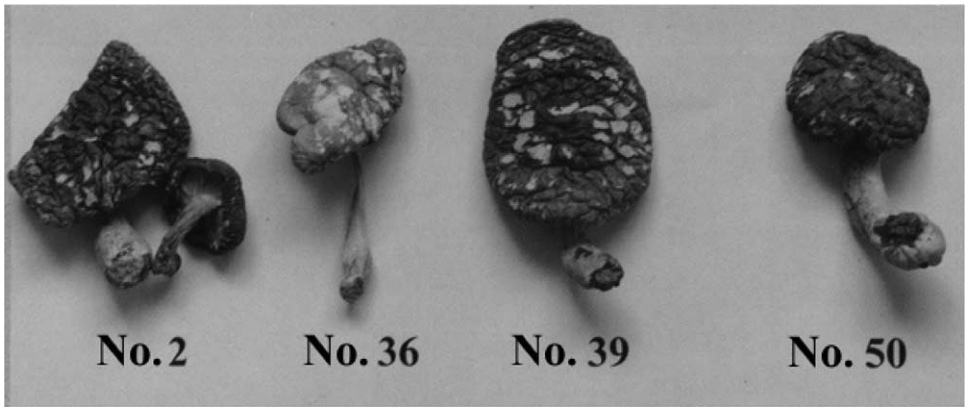

Fig. 1. Four kinds of fruiting bodies of *Lentinus Edodes* with different strains.

to separate into liquid and gel. The gel containing bound protein was removed out, and an equivalent volume of acetone was added into the liquid to obtain pure polysaccharide without bound protein. The purification was repeating three times. Finally, the precipitated polysaccharides were dialyzed and dried to obtain whitish powder, which is free of protein, coded as LNP-I_1, LNP-I_2, LNP-I_3 and LNP-I_4, respectively.

The ultrasonic degradation was performed in an ultrasonic cleaner (MUS-1004, Shenzhen modern Ultrasonic Industrial Co., Ltd, China) at room temperature to degrade the molecular weight of glucans. The samples (0.3 g) dispersed in 50 mL 0.2 M aqueous NaCl were exposed to 33 kHz ultrasonic irradiation by ultrasonic cleaner for 12 h. The jacket of the ultra sonication vessel was maintained below 35 °C. The samples were dried to obtain the degraded polysaccharides, coded as LU-I_2 and LU-I_3.

The L-I_2 and L-I_3 glucans (0.2 g) were dissolved in 50 mL DMSO for 3 h at room temperature, respectively. The polysaccharide solution was precipitated by the addition of acetone. The precipitates were dissolved in distilled water with stirring and then reprecipitated into acetone to give the precipitates. The resulting precipitates were redissolved in distilled water and lyophilized to obtain the products of single chain, coded as LSC-I_2 and LSC-I_3.

2.4. Characterizations

The IR spectrum was recorded with a Nicolet Fourier transform infrared (FTIR) spectrometer (Spectrum One, Elmer Co., USA). Test specimens were prepared by the KBr-disk method. The IR spectra for the β-glucans L-I_1, L-I_2, L-I_3 and L-I_4 are shown in Fig. 2. All the samples exhibit characteristic absorption at 890 cm^{-1} for the β-configuration of glucan (Mathlouthi & Koeng, 1986).

The protein contents in each polysaccharide were measured to be about 5.8, 5.5, 4.6 and 15.2% for the L-I_1, L-I_2, L-I_3 and L-I_4 samples, respectively, by using KJELETC 1030 semimicro Kjeldhal self-analyzer (Switzerland). This implies these *Lentinans* are polysaccharides bound with proteins, because the Sevag method has been repeated many times to remove free proteins.

2.5. SEC-LLS measurement

The weight-average molecular weight (M_w) of the samples, respectively, in 0.2 M aqueous NaCl and in DMSO was measured by using size-exclusion chromatography combined with laser light scattering. 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 P100 pump (Thermo Separation, Products, San Jose, USA) equipped with TSK-GEL G6000 PWXL and G4000 PWXL column (7.8 mm × 300 mm) for aqueous solution and G4000-H8 column for DMSO, at 25 °C. A differential refractive index detector

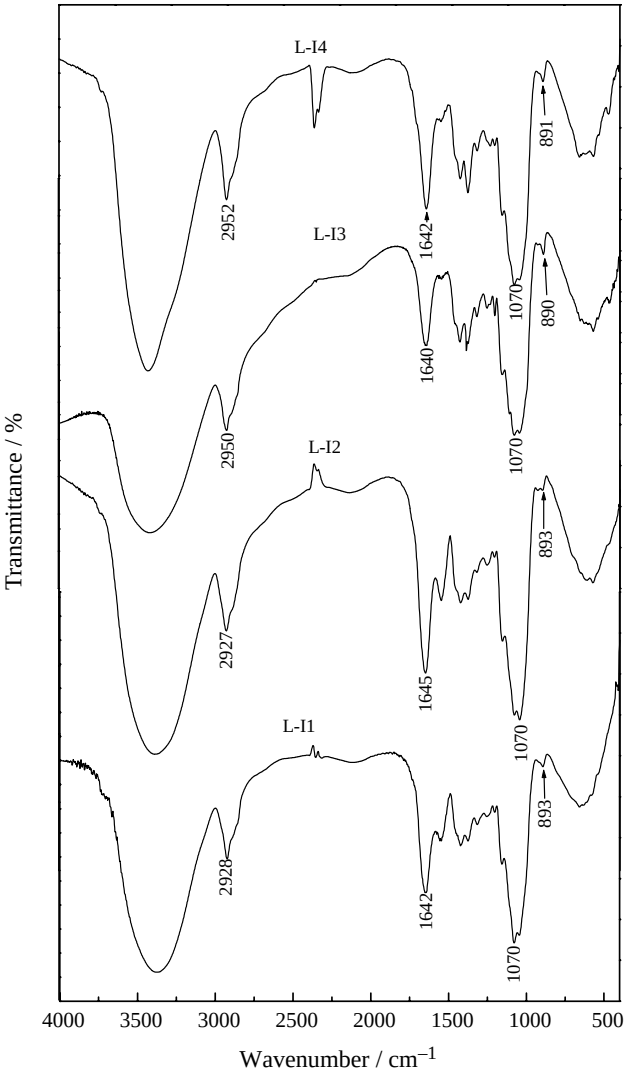

Fig. 2. Infrared spectra for β-glucans L-I_1, L-I_2, L-I_3, and L-I_4.

(RI-150) was simultaneously connected. Optical clarification of the solution was made with filtration through 0.45 μm Millipore filter (Whatman, England) before injection. The injection volume was 200 μL with the concentration of 1 mg mL^{-1} for each sample, and the flow rate was 1.0 mL min^{-1}. The specific refractive index increments (dn/dc) of the samples in 0.2 M aqueous NaCl and in DMSO at 633 nm and 25 °C were determined using an interferometer refractometer (Optilab 903, Wyatt Technology, USA) to be 0.140 and 0.060 mL g^{-1}, respectively. Astra software was utilized for the data acquisition and analysis.

2.6. Intrinsic viscosity

The intrinsic viscosities of the samples in 0.2 M aqueous NaCl and in DMSO were determined by using a capillary viscometer at 25 °C. The kinetic energy correction was always negligible. Huggins and Kraemer equations were

used to estimate the intrinsic viscosity $[\eta]$ by extrapolation to infinite dilution as follows:

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

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

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

2.7. Antitumor tests

2.7.1. In vivo antitumor test

A 0.2 mL S-180 tumor cells (5×10^6 cells mL^{-1}), provided by Tongji College of Medicine, Huazhong University of Science and Technology, were transplanted subcutaneously into the right groin of 8-week-old Kunming mice weighting 17 ± 1 g, which were divided randomly into 10 groups with 10 mice per group. 5-Fluorouracil (5-Fu) and the tested samples were dissolved or suspended in 0.9% aqueous NaCl, respectively, and injected intraperitoneally once daily for 7 d, starting 24 h after tumor inoculation. The 0.4 mL 0.9% aqueous NaCl was injected intraperitoneally into the control mice, and 5-Fu was injected intraperitoneally into another masculine mouse at dose of 20 mg kg^{-1}. For the β-glucan, the doses were 20, 40 and 60 mg kg^{-1}, respectively. The mice were killed on the next day after the last injection, and the tumors were removed from the mice and weighed. The weights of tumors were compared with those in the control mice. The inhibition ratio ξ and enhancement ratio of body weight f were calculated as follows (Ohno et al., 1995)

$$\xi = [(W_c W_t)/W_c] \times 100\% \quad (3)$$

$$f = [(W_a W_b)/W_b] \times 100\% \quad (4)$$

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.

2.7.1.1. Statistics. Data in all experiments were statistically evaluated by Student's *t*-test and difference with a *p* value of less than 0.05 were considered significant.

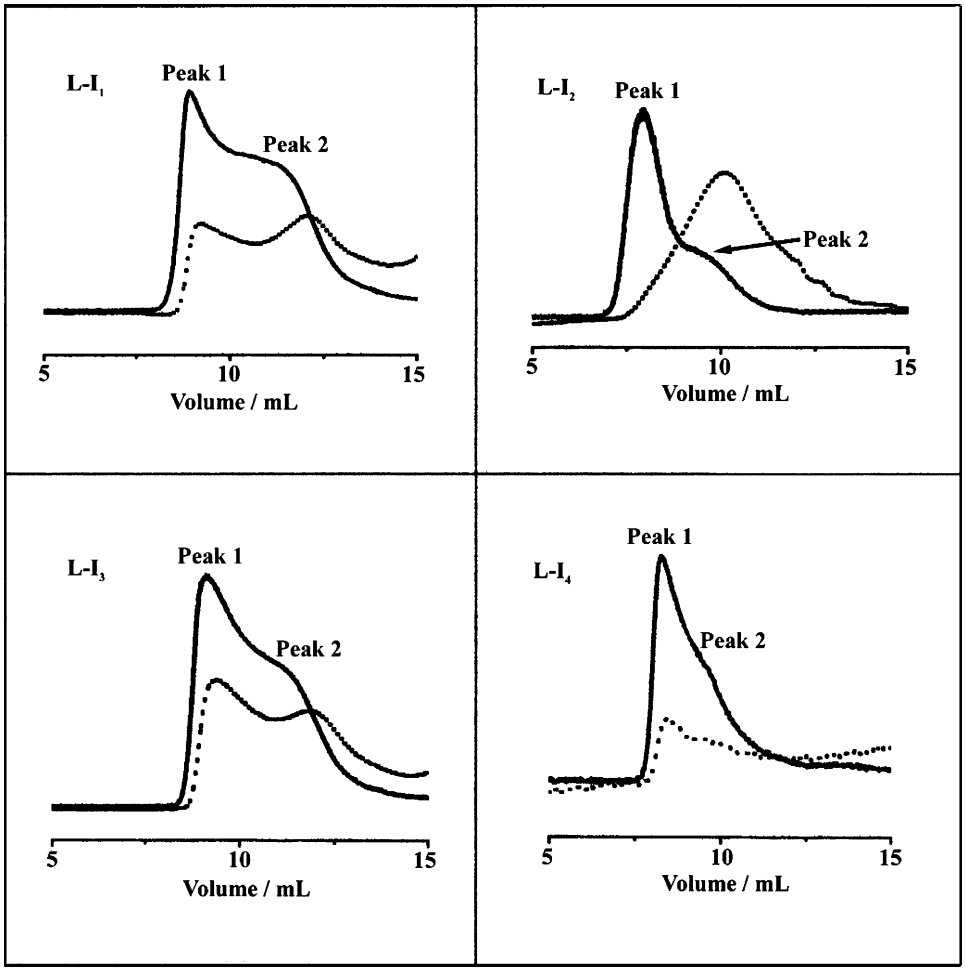

Fig. 3. SEC chromatograms of the samples L-I_1, L-I_2, L-I_3 and L-I_4 in 0.2 M aqueous NaCl at 25 °C detected by using on-line LLS (—) and differential refractometer (…).

2.7.2. In vitro antitumor test

S-180 tumor cells (5×10^6 cells mL^{-1}) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum under an atmosphere of 5% carbon dioxide at 37 °C for 72 h containing polysaccharides at concentrations of 0.005, 0.05, 0.5 and 5 μg mL^{-1} in 0.9% aqueous NaCl. The survival rate of the mammalian cells was assayed by counting the living cells that excluded the Trypan blue dye by a hemacytometer. The number of living S-180 tumor 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 (Mosmann, 1983). All in vitro results were expressed as the ratio of inhibition of tumor cell proliferation calculated as $[(A-B)/A]\times100\%$, where A and B are the average number of viable tumor cells of the control and samples, respectively. All samples were done in triplicates.

3. Results and discussion

3.1. Chain conformation

The SEC chromatograms of the β-(1→3)-D-glucans in 0.2 M aqueous NaCl and in DMSO are shown in Figs. 3 and 4, respectively. There are double peaks in the SEC chromatograms in aqueous solution, corresponding to the triple helix chains bounded with protein having high molecular weight and the fragments of triple-helix and single chains having low molecular weight. It has been proved that DMSO is a strong solvent, and can break the intra- and intermolecular hydrogen bonds. As shown in Fig. 4, the triple-helix was completely disrupted into single chains corresponding to the main peak. The ratios of the apparent mean M_w in the aqueous solution to those in DMSO have been roughly estimated to be in the range from 3.0 to 3.3, confirming the existence of the triple stranded chains in aqueous solution.

In view of the data in Table 1, the values of M_w, $<s^2>_z^{1/2}$ and [η] for the four samples in two solvents are much different, implying an effect of different *Lentinan* strains on the molecular parameters. The [η] values of β-glucans in 0.2 M aqueous NaCl are much higher than those in DMSO, and the ratios of [η] in water to those in DMSO are close to 4.0–5.6. The high [η] also reflects the characteristic of stiff chain such as triple helix. More information about the conformation could be obtained from the SEC-LLS chromatogram. The power law $<s^2>_z^{1/2}=f(M_w)$ can be estimated from many experimental points in the SEC chromatogram. Fig. 5 shows the relationship between

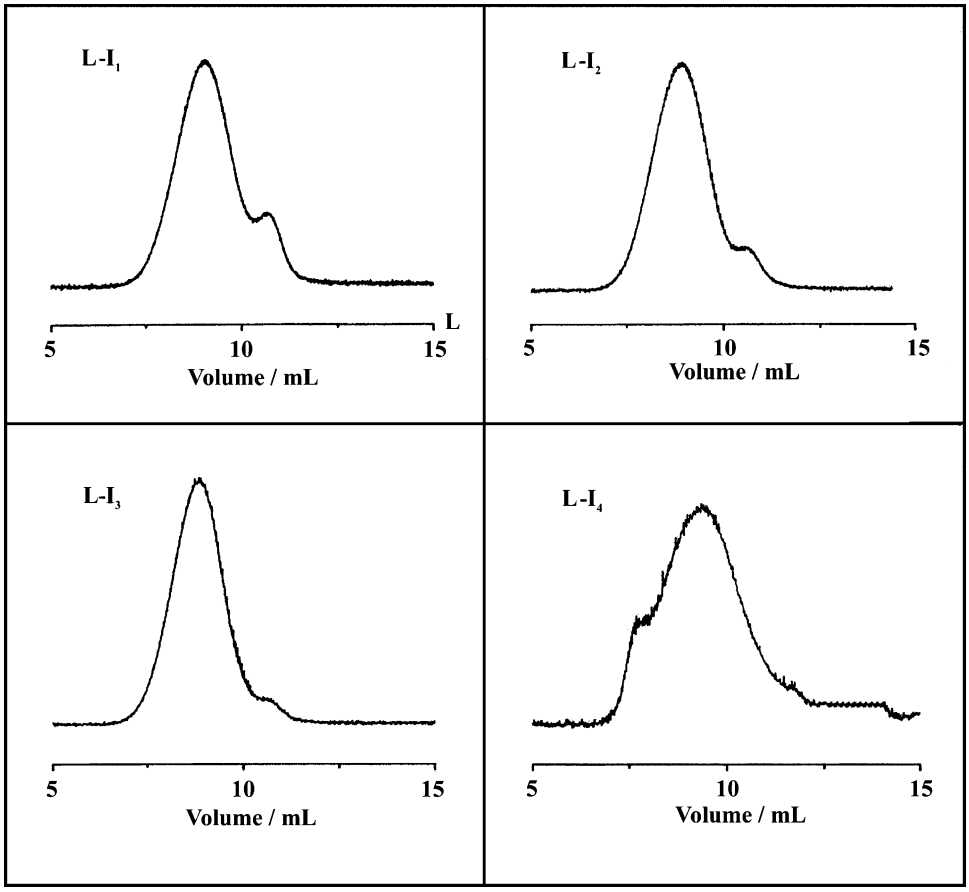

Fig. 4. SEC chromatograms of the samples L-I_1, L-I_2, L-I_3 and L-I_4 in DMSO at 25 °C detected by LLS.

Table 1
The experimental results of M_w, [η], protein content and yield of (1→3)-β-D-glucans from different *Lentinus Edodes*

Sample	In 0.2 M NaCl			In DMSO			$M_{w,aq}/M_{w,DMSO}$	$[\eta]_{aq.}/[\eta]_{DMSO}$	Content of protein, (%)	Yield, (%)
	$M_{w,aq}\times 10^{-4}$	$<s^2>_z^{1/2}$ (nm)	(η) (mL/g)	$M_{w,DMSO}\times 10^{-4}$	$<s^2>_z^{1/2}$ (nm)	(η) (mL/g)				
L-I_1	147.9	88.1	1050	49.0	42.6	224	3.0	4.6	5.8	10.0
L-I_2	157.6	112.7	1123	53.3	40.7	288	3.0	4.0	5.5	3.5
L-I_3	151.9	94.3	1162	46.5	56.1	275	3.3	4.1	4.6	8.1
L-I_4	167.6	119.3	1250	51.2	53.4	222	3.3	5.6	15.2	3.6

$<s^2>_z^{1/2}$ and M_w for the samples in DMSO. The slopes calculated from main peak 1 in Fig. 3 are in the range of 0.81–0.89 in aqueous NaCl, and those in Fig. 4 are 0.47–0.54 in DMSO. The slope is related to the chain conformation, and the values obtained here are corresponding to the stiff and flexible chain, respectively. In our laboratory, the slope of $<s^2>_z^{1/2}-M_w$ for triple-helical β-(1→3)-D-glucans has been determined by LLS to be 0.80 (Zhang et al., 2001). It is well known that the slope of $<s^2>_z^{1/2}-M_w$ for single flexible chain lies in the range from 0.5 to 0.6. As shown in Fig. 5, the dashed line represents values of flexible pullulan in water (Kato, Okamoto, Tokuya, & Takahashi, 1982). The data points for the glucans in DMSO lie in the range for flexible polysaccharides, indicating a flexible chain. In view of these results, the four glucans from different strains mainly exist as triple helical chains in aqueous solution and as single flexible chains in DMSO.

3.2. In vivo antitumor activity

The results from in vivo assay of the antitumor activities of the tested samples are listed in Table 2. The L-I_2, L-I_3 and L-I_4 samples at high dose exhibit an inhibition ratio higher than 30%, especially L-I_3, which exhibits more effectiveness

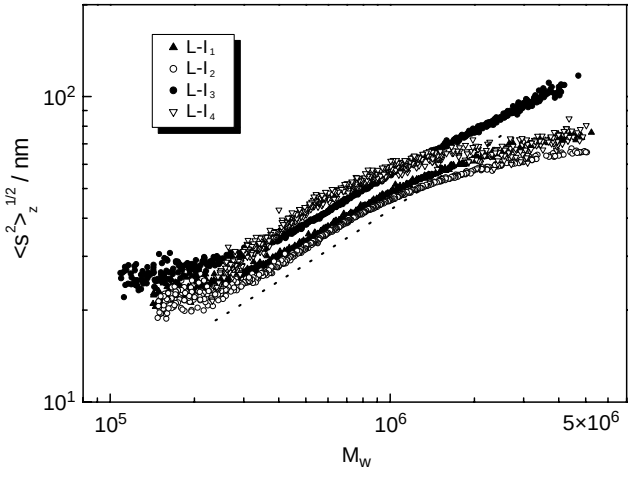

Fig. 5. M_w dependence of $<s^2>_z^{1/2}$ for the samples in DMSO at 25 °C. Dashed line presents values calculated from data of flexible Pullulan in water at 25 °C.

(50–70%) than 5-Fu. 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 did not have the same toxicity as 5-Fu, which kills normal cells as well as cancer cells. The differences in antitumor activity among the various β-glucan are probably as a result of their different origins and the different strains, as well as molecular weight and bound protein content. Interestingly, the inhibition ratio of the LNP-I_1, LNP-I_2, LNP-I_3 and LNP-I_4 samples without bound protein obviously decreases, compared with the native glucans, suggesting protein are favorable in improvement of antitumor activity. The maximum of ξ value of L-I_3 reaches 70.0% higher than those of others, indicating an effect of the strain.

Table 2
Antitumor activities against Sarcoma 180 Solid tumor of the samples L-I_1, L-I_2, L-I_3, L-I_4 and LNP-I_1, LNP-I_2, LNP-I_3, LNP-I_4

Sample	Structural feature	Dose (mg $kg^{-1}\times$ days)	Inhibition ratio (%)	Enhancement ratio of body weight (%)
Control				44.2
FU-5		20×7	48.6	39.1
L-I_1	With bound protein	20×7	12.1	55.0
		60×7	47.8	29.1
L-I_2	With bound protein	20×7	25.9	41.3
		30×7	40.7	34.7
		60×7	54.5	31.4
L-I_3	With bound protein	20×7	50.0	42.7
		30×7	63.0	37.1
		60×7	70.0	27.4
L-I_4	With bound protein	20×7	20.3	33.3
		30×7	31.8	46.1
		60×7	37.1	45.0
Control				45.4
FU-5		20×7	51.7	35.6
LNP-I_1	Without bound protein	20×7	7.5	57.8
		60×7	17.1	58.6
LNP-I_2	Without bound protein	20×7	0.6	52.2
		30×7	20.7	56.3
		60×7	30.4	45.3
LNP-I_3	Without bound protein	20×7	12.1	50.9
		30×7	17.2	37.1
		60×7	41.4	45.0
LNP-I_4	Without bound protein	20×7	9.8	49.6
		30×7	19.5	46.7
		60×7	37.6	45.4

Table 3
Antitumor activities against Sarcoma 180 solid tumor of the samples LU-I_2, LU-I_3, LSC-I_2 and LSC-I_3

Sample	Conformation	Dose (mg $kg^{-1}\times d$)	Inhibition ratio (%)	Enhancement ratio of body weight (%)
Control				43.36
FU-5		20×7	42.22	32.86
LU-I_2	Triple helix	20×7	20.0	40.8
	with low M_w	60×7	24.2	43.7
LSC-I_2	Single flexible	20×7	0.6	24.4
		60×7	16.7	34.1
LU-I_3	Triple helix	20×7	20.7	41.2
	with low M_w	60×7	28.2	32.0
LSC-I_3	Single flexible	20×7	2.2	45.4
		60×7	4.2	33.8

The results from in vivo assay of the antitumor activities of the LNP-I_2, LNP-$_3$,LSC-I_2 and LSC-I_3, LU-I_2 and LU-I_3 samples are listed in Table 3. This indicates that the antitumor activities against S-180 tumor cells of the degraded samples significantly decrease. The antitumor activities for the polysaccharides are in the order of native β-glucan > LNP > LU > LSC. The results reveal that antitumor activities of the polysaccharides are strongly related with their molecular weight, content of bound protein and chain conformation. The single chain β-glucans (LSC-I_2 and LSC-I_3) hardly exhibits antitumor activities. Therefore, the triple helix conformation of (1→3)-β-D-glucan plays an important role in the enhancement of the antitumor activities. Especially, the protein bounded on the polysaccharides is favorable to enhancing the bioactivity.

3.3. In vitro antitumor activity

The inhibition ratios to the proliferation of S-180 cell by different concentrations (5000, 500, 50 and 5 μg mL^{-1}) of the L-I_1, L-I_2, L-I_3 and L-I_4 samples are shown in Fig. 6. Basically, the four samples exhibit the inhibition against S-180 cell growth at the four concentration levels tested. The four samples also showed a dose–response relationship in inhibiting the S-180 cells proliferation. It is noted that L-I_3 exhibit the highest inhibition ratio of 65, 57, 31 and 24% to the proliferation of S-180 cell line at the concentrations of 5000, 500 50 and 5 μg mL^{-1}, respectively. Interestingly, the polysaccharides containing small amount of bound protein have much higher in vitro inhibition ratio than L-I_4 containing relatively more bound proteins at corresponding concentrations.

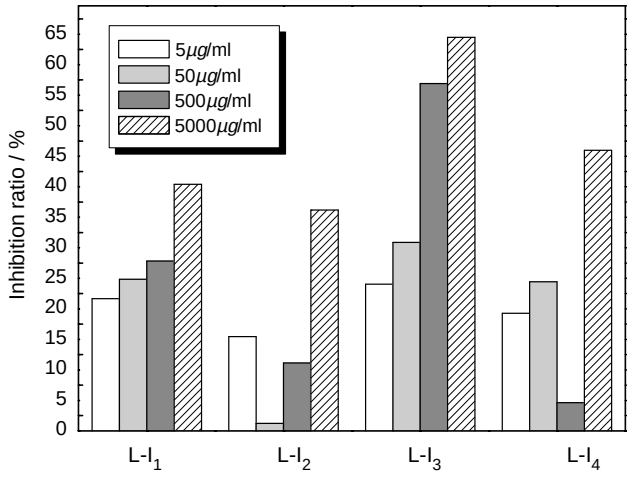

Fig. 6. Inhibition ratio of proliferation of Sarcoma 180 cells at different concentrations of the β-glucan L-I_1, L-I_2, L-I_3 and L-I_4.

4. Conclusion

The water-soluble L-I_1, L-I_2, L-I_3 and L-I_4 samples were isolated from four kinds of fruiting bodies of *Lentinus Edodes* with different strain. The samples are protein bound β-(1→3)-D-glucans with the protein content of 4.6, 5.8, 5.5, 15.2%, and apparent-mean M_w of 147.9×10^4, 157.6×10^4, 151.9×10^4, and 167.6×10^4, respectively. Analysis of the M_w drop by a factor of 3 from in aqueous solution to in DMSO as well as the relationships between M_w and $< s^2 >_z^{1/2}$ from the main peak in SEC-LLS chromatogram revealed that the predominant species of the (1→3)-β-D-glucan exist as triple-stranded helical chains in 0.2 M aqueous NaCl and as single flexible chains in DMSO. The results from in vivo and vitro antitumor activity against the growth of S-180 solid tumor for the polysaccharides show that the native triple helical β-(1→3)-D-glucans containing protein exhibit obvious antitumor bioactivities, whereas those having single flexible chains hardly exhibit antitumor activities. Therefore, the triple helical conformation plays an important role in the enhancement of the antitumor activities. Maximum inhibition ratio of the L-I_1 sample achieves 70%, indicating an effect of the strain Fcro 2. The antitumor activities of the samples LNP and LU were much lower than those of the native ones, suggesting that the antitumor activity of polysaccharide is also related to their molecular weight and content of the bound protein.

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