Carbohydrate Polymers 87 (2012) 1855–1862

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Chain structures of glucans from *Lentinus edodes* and their effects on NO production from RAW 264.7 macrophages

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ARTICLE INFO

Article history:
Received 11 July 2011
Received in revised form 6 October 2011
Accepted 9 October 2011
Available online 14 October 2011

Keywords:
Glucan
Lentinus edodes
Chain structure
Nitric oxide
Macrophage
RAW 264.7 cells

ABSTRACT

Four glucans (LNT-H, LNT-S, LNT-E, and LNT-B) were successively isolated from the fruiting bodies of *Lentinus edodes* by hot-water, sonication, enzyme, and NaOH. The chemical structures were characterized by GC, FTIR, and ^{13}C NMR, which demonstrated that the four samples are (1 → 3)-β-D-glucans with (1 → 6)-glucopyranoside side groups having different molecular weight. The results of viscometry and atomic force microscopy (AFM) proved that LNT-H, LNT-S, LNT-E, and LNT-B exist as stiff triple helices in water and random coil in dimethyl sulfoxide (DMSO). The morphology of the original triple helix was almost dominated by linear shape, but the renatured LNT-B contains some branched structures in AFM image. Among the four glucans, LNT-H, LNT-E, and LNT-B are clearly contaminated by endotoxin, while LNT-S significantly exhibits nitric oxide (NO) inhibition effect in LPS-stimulated RAW 264.7 macrophages in a dose-dependent manner. Therefore, LNT-S is probably used as a promising molecule which can be helpful to the diseases associated with NO overproduction. This is the first primary report dealing with the suppression of LPS-induced NO from macrophage RAW 264.7 cells by Lentinan.

1. Introduction

Mushroom is a kind of traditionally popular edible fungus with high nutritional value. Since Chihara, Maeda, Hamuro, Sasaki, and Fukuoka (1969; Sasaki & Takasuka, 1976) separated one (1 → 3)-β-D-glucan named as Lentinan with anti-tumor activity from *Lentinus edodes*, researches on polysaccharides have attracted much attention as functional food and anti-tumor drugs (Hamuro & Chihara, 1973; Maeda & Chihara, 1971, 1973; Maeda, Hamuro, & Chihara, 1971; Okuda, Yoshioka, Ikekawa, Chihara, & Nishioka, 1972). Later, many researchers extracted all sorts of polysaccharides from *Lentinus edodes*, and reported their antitumor activities and immunomodulating activities associated with T-cell mediating responses (Zakany, Chihara, & Fachet, 1980). It has been reported that molecular weight (Zakany et al., 1980), chain conformation (Suga, Shiio, Maeda, & Chihara, 1984) and water-solubility (Suga, Maeda, Uchida, Rokutanda, & Chihara, 1986) of polysaccharide are closely associated with their anti-tumor activity and immunomodulating activity. The solution properties including molecular weight, chain conformation and water-solubility etc. thus play an important role in clarification of the correlation between structures and bio-activities of Lentinan. More than 30 years ago, Bluhm and Sarko (1977) reported that there were 5 possible chain conformation of Lentinan in crystal including one single helix, two double helices and two triple helices by using X-ray diffraction. Among the five conformations, the right-hand triple helix with helical pitch of 0.29 nm was the most possible one. Later, other scientists also studied the secondary structure of Lentinan in gel status by using ^{13}C NMR (Saito, Ohki, & Sasaki, 1979; Saito et al., 1987; Saito, Yoshioka, Yakoi, & Yamada, 1990). To the best of our knowledge, the chain conformation of Lentinan in solution was scarcely reported besides our research.

Currently, Lentinan which is marketed as an anti-tumor drugs is mainly obtained by hot water-extraction (Sipka, Abel, Csongor, Chihara, & Fachet, 1985). By this method, the yield of Lentinan was very low (approximately 0.02%) resulting in the high cost. In our lab, an alternative method of NaOH (5%) aqueous solution-extraction after water-extraction was proposed (Zhang, Zhang, Cheng, & Zhang, 1999), which can largely enhance the yield of glucans (~6%) and sharply reduce the cost. This method is thus very promising to be used for Lentinan extraction in the future. The chemical structure was proved to be a (1 → 3)-β-D-glucan with (1 → 6) branching similar to the hot water-extracted one (so also nominated as Lentinan) by NMR, and the chain conformation of this glucan was deduced to be a triple helical conformation with helical pitch of 0.31 nm in aqueous solution (Zhang, Zhang, Zhou, Zhang, & Li, 2001), in accord with the result (0.29 nm) of hot water-extracted Lentinan by X-ray diffraction (Bluhm & Sarko, 1977). Moreover, we found that helix-coil conformation transition of the alkali-extracted Lentinan in NaOH aqueous solution occurred with triple

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0144-8617/$ – see front matter © 2011 Published by Elsevier Ltd.
doi:10.1016/j.carbpol.2011.10.015

helix at NaOH concentration of less than 0.08 M and single coils at NaOH concentration higher than 0.08 M (Zhang, Zhang, & Xu, 2004). Notably, Lentinan used in our work were obtained by extracting from the fruiting bodies of *Lentinus edodes* with 1.25 M NaOH aqueous solution and neutralizing with acid and dialyzing against water. It means that the original triple helical structures were disrupted by NaOH with high concentration of 1.25 M in the extraction process, and the finally resulting triple helixes were renatured by neutralization and dialysis. It has been reported that the original triple helixes such as schizophyllan and scleroglucan exhibit linear morphology, but cyclic and branched species appeared and even dominated after denaturation–renaturation process (McIntire & Brant, 1998; McIntire, Penner, & Brant, 1995), which was dependent on the renaturation conditions such as temperature, solvent, concentration, and molecular weight (Falch, Elgsaeter, & Stokke, 1999; McIntire et al., 1995; Stokke, Elgsaeter, & Brant, 1991). In our previous work, the renatured triple helical Lentinan undergoing denaturation–renaturation in the extraction process obtained by Zhang et al. (2004) showed linear, cyclic, and branched species, whereas those obtained by Wang, Xu, and Zhang (2008) exhibited cycles as dominant ones. In order to explore what shape of the original triple helical structure of Lentinan, four processes including hot water-, ultrasonic-, enzymatic-, and NaOH-extractions were tried. The chain structures of the extracted polysaccharides in solution are presented in this paper.

Nitric oxide (NO) is one key molecule in the immunopharmacology, which has shown beneficial biological effects on a variety of normal cells mostly related to immunomodulatory or inflammatory or physiological processes (Davis, Martin, Turko, & Murad, 2001; Nathan, 1992). It is also an important cytotoxic mediator contributing to the antimicrobial and tumoricidal activity of the macrophages (Bogdan, 2001; Liew, 1995; MacMicking, Xie, & Nathan, 1997), and exerts a key role in the pathogenesis of many infectious and inflammatory diseases (Nathan, 1992). However, high level of NO generated by activated macrophages can induce diverse effects on host survival, ranging from direct cellular cytotoxicity (Feshel et al., 1995; Manjeet & Ghosh, 1999) to damage of components leading to mutagenesis (Bogdan, 2001; Kröncke, Fehsel, & Kolb-Bachofen, 1998; Wink et al., 1991). It has been reported that NO inhibition could markedly increase the survival rate in the cecal ligation and puncture (CLP) model (Hogaboam et al., 1998). Therefore, identification of novel pharmacological reagents which can suppress NO overproduction is of considerable medical interest. So far, there are few reports dealing with the influence of polysaccharides on the NO release from macrophages (Ruan, Su, Dai, & Wu, 2005). Based on the above, the effect of four polysaccharides extracted from *Lentinus edodes* on NO production from macrophage RAW 264.7 cells inactivated or activated by lipopolysaccharide (LPS) was primarily studied and discussed.

2. Experimental

2.1. Sample preparation

The dried fruiting bodies (250 g) of *Lentinus edodes*, a commercial product cultivated in Fujian province of China, were cut into small pieces and refluxed in ethyl acetate and then acetone for 8 h. After refluxing, the dried residues were dipped into 0.9% NaCl aqueous solution with continuous stirring at 25 °C for 24 h and centrifuged. This process was repeated three times, and the remaining residues were extracted according to the extraction order of 4000 mL of hot water (120 °C, three times), ultrasonic (2000 mL of water, 500 watts, 18 min, 25 °C), enzyme (3000 mL of cellulase/water solution with concentration of 0.067 mg/mL, pH = 4.5–5.0, 45 °C), and NaOH (1.25 M, 25 °C, overnight). The detailed procedures of extraction were described in Scheme 1. The collected supernatant in each step was treated with 30% H_2O_2 to decolorize and subjected to the Sevag's method (Sevag, 1934) to remove free proteins, followed by dialysis against distilled water (M_w cutoff = 8000) for 10 days. The solution was then filtered, concentrated by a rotary evaporator at reduced pressure below 45 °C, and precipitated into acetone. The precipitates were redissolved in distilled water and lyophilized to obtain white pure flakes coded according to above experiments as LNT-H, LNT-S, LNT-E, and LNT-B with yields of 0.25%, 0.44%, 0.36%, and 2.8%, respectively.

2.2. Fourier transform IR spectra measurements

Fourier transform infrared spectra (FTIR) of LNT-H, LNT-S, LNT-E, and LNT-B were recorded on a Nicolet 5700 spectrometer (Spectrum One, Thermo Nicolet Co., Madison, WI, USA) in a range from 4000 to 400 cm^{-1} using a KBr-pellet method. Moreover, two (1 → 3)-β-D-glucans schizophyllan (SPG) and curdlan (CUR) with known structure were also used for comparison.

2.3. Gas chromatography (GC)

LNT-H, LNT-S, LNT-E, and LNT-B were hydrolyzed to monosaccharides by 2 M sulfuric acid. The resulting monosaccharides were further reacted with acetic anhydride to prepare the monosaccharide alditol acetate derivatives according to the method of Blakeney, Harris, Henry, and Stone (1983). The alditol acetate derivatives were then analyzed by gas chromatography (GC). GC was conducted on a Hewlett–Packard 6910 gas chromatograph system. The detailed experimental conditions were as follows: H_2 (30 mL/min); O_2 (200 mL/min); N_2 (20 mL/min); the initial column temperature of 140 °C was held for 3 min, and the temperature was then increased at 7 °C min^{-1} to 240 °C and held at 240 °C for 10 min. The temperature of the injector was 250 °C, and that of the detector was 270 °C.

2.4. ^{13}C NMR

The ^{13}C NMR spectra of LNT-H, LNT-S, LNT-E, LNT-B, SPG, and CUR were recorded on a Mercury 600 NMR spectrometer (Varian Inc., Palo alto, CA, USA) by using a standard 5 mm probe and DMSO-d_6 as the solvent at 30 °C with acetone as internal standard (δ_H = 2.225, δ_C = 31.45 ppm). The polysaccharide concentration was adjusted to be ~3 wt%.

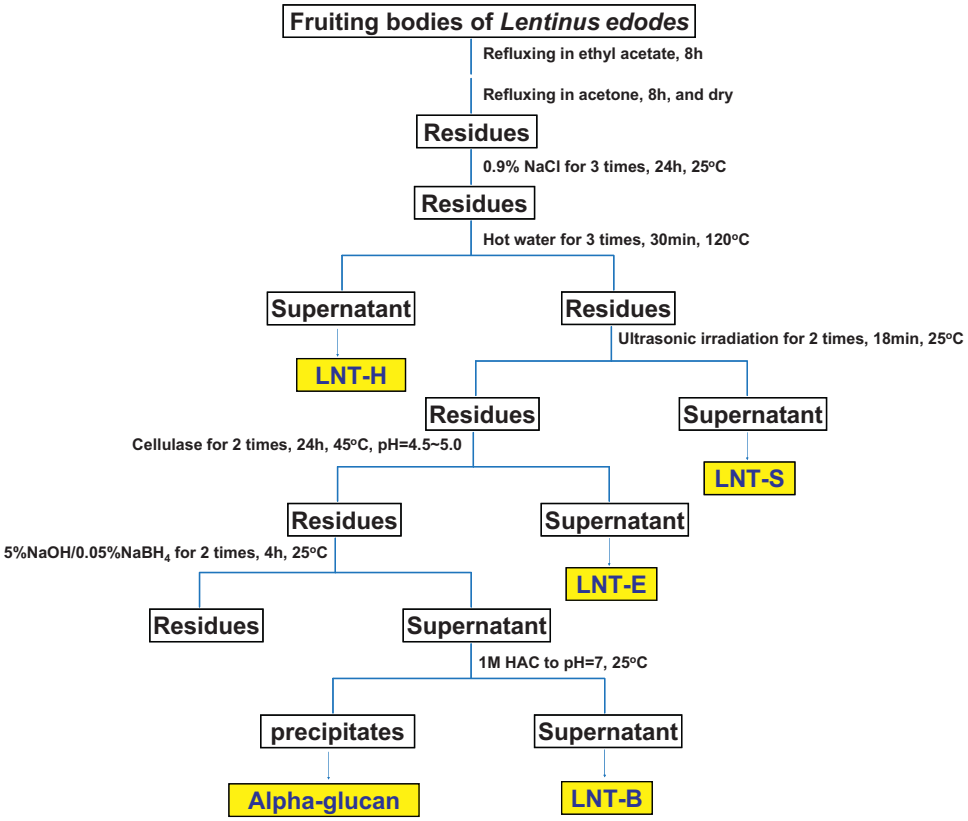
2.5. Intrinsic viscosity

Inherent viscosity ($\ln \eta_r/c$) and specific viscosities (η_{sp}/c) were measured at 25 °C on conventional Ubbelohde type capillary viscometer for four Lentinan samples in water and DMSO. Solutions were made by mixing the required amounts of Lentinan and water or DMSO in a flask and stirring gently for a few hours at room temperature. All the test solutions were maintained at a constant temperature within ±0.01 °C during the measurements, and the flowing time was measured to a precision of 0.1 s. The kinetic energy correction was always negligible. The data obtained for η_{sp} and η_r were treated by the following Huggins and Kraemer equations to determine $[\eta]$:

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

$$\frac{\ln \eta_r}{c} = [\eta] - (0.5 - k')[\eta]^2 c \quad (2)$$

where k' is Huggins coefficient which is a measure of pairwise hydrodynamic interactions between the macromolecules, and is



Scheme 1. Extraction process of the polysaccharides from the *Lentinus edodes*.

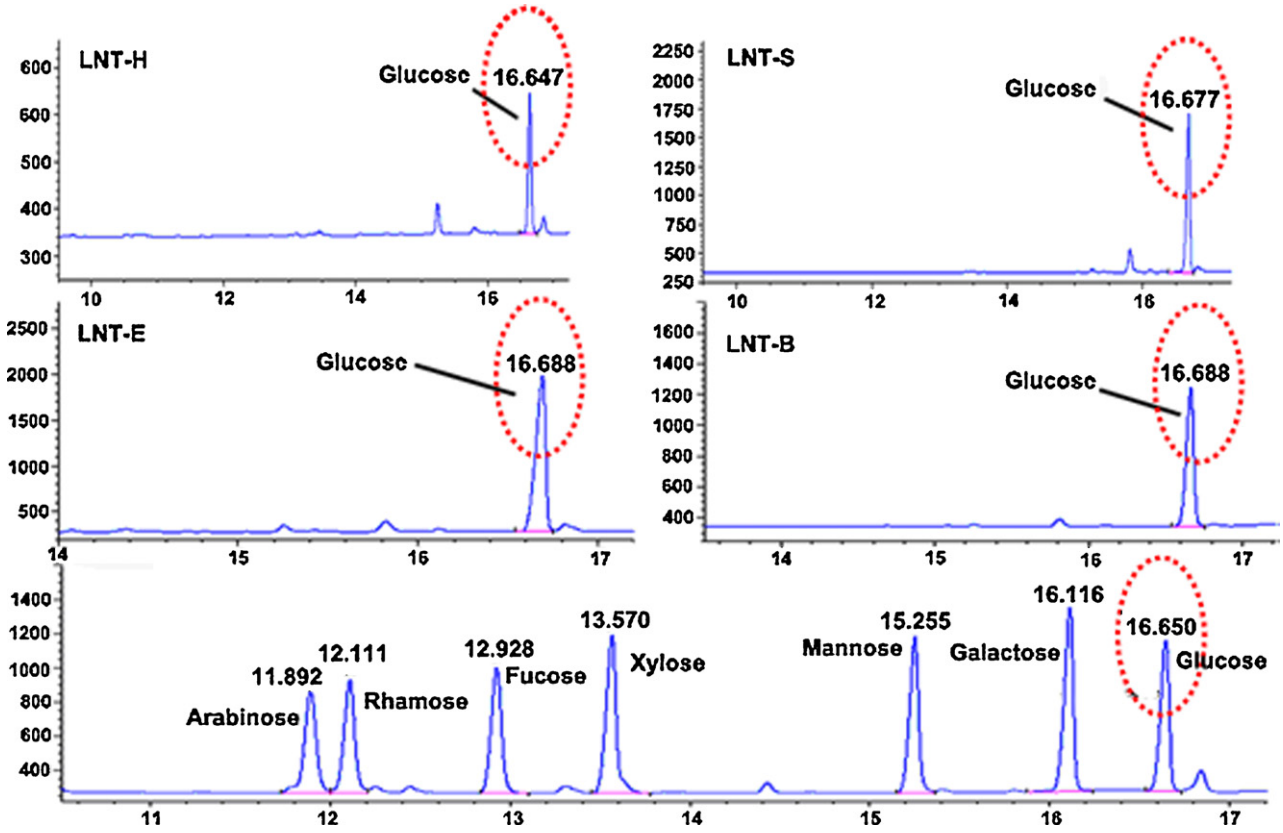


Fig. 1. GC profile of LNT-H, LNT-S, LNT-E, and LNT-B. The peaks of alditol acetates were labeled with corresponding standard monosaccharides including arabinose, rhamose, fucose, xylose, mannose, galactose, and glucose.

constant for the given polymer at desired condition; *c*, polysaccharide concentration (g/mL).

2.6. Atomic force microscopy (AFM)

The solutions were filtered through a 0.45 μm filter (NYL, 13 mm Syringe filter, Whatman Inc., USA), and diluted with deionized water to the polymer concentration of 5 μg/mL. A 10 μL drop was deposited onto freshly cleaved mica and allowed to dry in air for 1.5 h at room temperature in a small covered Petri dish prior to imaging with magnetically AC (MAC) mode AFM. The specimen was examined using a Picoscan atomic force microscopy (Molecular Imaging, Tempe, AZ, USA) in a MAC mode with commercial MAC lever II tips (Molecular Imaging, USA), with a spring constant of 0.95 N/m. A piezoelectric scanner with a range up to 6 μm was used for the image. The scanner was calibrated in the *xy* directions using a 1.0 μm grafting and in the *z* direction using several conventional height standards. The measurement was performed in air at ambient pressure and humidity and the image was stored as 256 × 256 array of points.

2.7. Cell culture

RAW 264.7 cells (a murine macrophage/monocyte-like cell line; American Type Culture Collection) were maintained in Dulbecco's modified eagle medium (DMEM, glutamine, high glucose) supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL) and 10% heat-inactivated Fetal bovine serum (FBS) at 56 °C for 30 min. Subculturing was done by dislodging the cells with trypsin (0.25%) and (EDTA·2Na)·2H_2O (0.02%), followed by centrifugation and seeding into the culture flask or dish, which was incubated at 37 °C under a humidified atmosphere of 95% air and 5% CO_2.

2.8. Nitric oxide (NO) determination

NO production was determined based on the amount of nitrite present in the conditioned media, a stable end product of NO, by the Griess reaction. Briefly, RAW 264.7 cells were seeded and pre-incubated for 24 h. The cells were rinsed with phosphate buffered solution (PBS) and exposed to LPS (100 ng/mL) and LNT-H, LNT-S, LNT-E, and LNT-B, respectively, in DMEM without FBS. After stimulation for desired time, each supernatant (100 μL) was mixed with an equal volume of Griess reagent (50 μL of 1% sulfanilamide in 5% phosphoric acid and 50 μL of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water) at room temperature. The absorbance was measured at 570 nm using the Wallac microplate reader (1420 ARVO Sx), and the concentration of NO was quantified with a standard curve generated with sodium nitrite in the range of 0–100 μM.

3. Results and discussion

3.1. Chemical structure characterization

The chemical structures of LNT-H, LNT-S, LNT-E, and LNT-B were characterized by GC, FTIR, and ^{13}C NMR, respectively. Fig. 1 showed the GC trace of the alditol acetate derivatives of the hydrolyzed polysaccharide products. Compared with standard monosaccharide derivatives (at the bottom of the figure), we found that all the four samples consist mainly of glucose besides a small quantity of mannose, indicating that the four polysaccharides from different extraction procedures are almost pure glucans.

The FTIR spectra of LNT-H, LNT-S, LNT-E, and LNT-B together with the well known standards curdlan (CUR) and schizophyllan (SPG) are shown in Fig. 2. The four samples have very similar

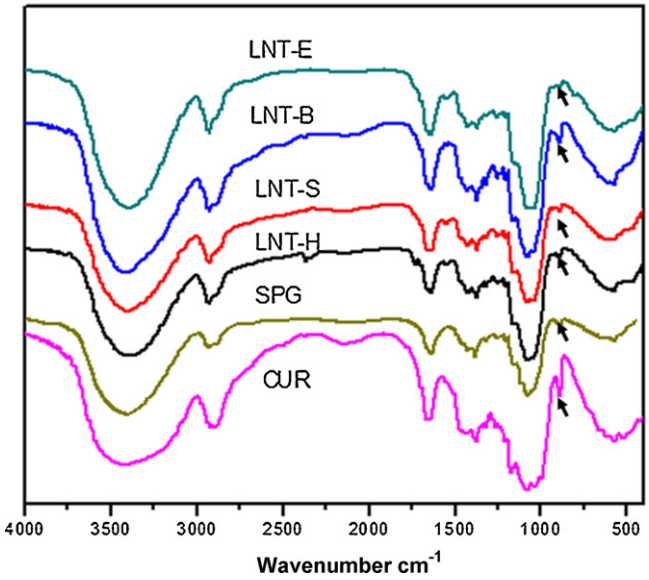


Fig. 2. FT-IR spectrum of LNT-H, LNT-S, LNT-E, LNT-B, SPG and CUR.

absorption peaks to those of CUR and SPG. The bands in the region of 3420 cm^{-1} is due to the O–H stretching vibration. The bands in the region of 2800–3000 cm^{-1} are due to C–H stretching vibration, and the bands in the region of 1650 cm^{-1} are due to associated water. The absorption peak around 1250 cm^{-1} was assigned to a C–O stretching in the ring. Peak at 1400 cm^{-1} was from a CH_2 vibration. Absorptions in the range of 1000–1200 cm^{-1} are suggested to be the stretching vibrations of C–C and C–O and the range between 1200 and 1500 cm^{-1} mainly by C–H deformation vibrations and C–OH bending vibrations (Xu, Chen, Wang, & Zhang, 2009). Absorptions at 890 cm^{-1} are typical for β-D-glucopyranose (Wang et al., 2009). Therefore, the β-configuration for the four

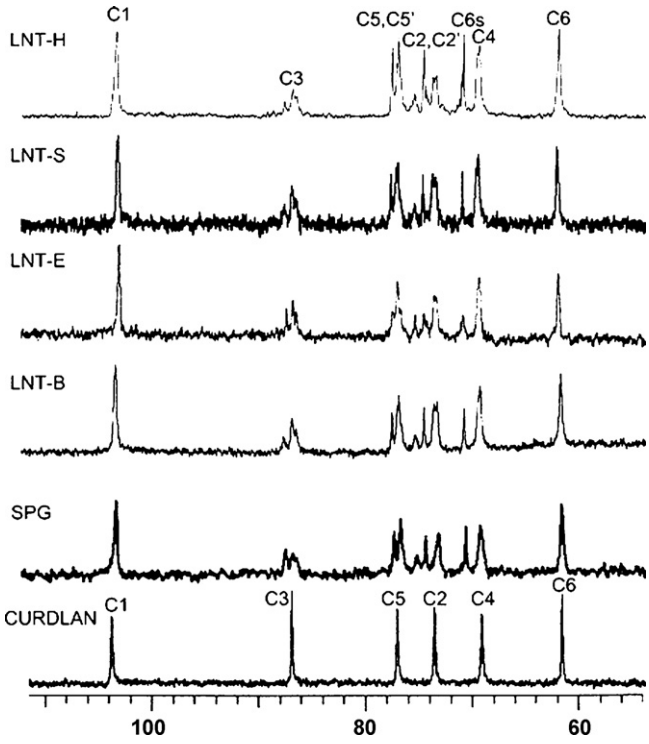


Fig. 3. 13 C NMR spectra of LNT-H, LNT-S, LNT-E, LNT-B, SPG, and CUR in DMSO-d6 at 25 °C.

glucans is concluded, which is further confirmed by the chemical shifts of anomeric carbon (C1) in following ^{13}C NMR spectra. The characteristic absorption peaks at 810 and 870 cm^{-1} of mannose are hardly seen, which is resulted from due to the trace of mannose.

The ^{13}C NMR spectra of LNT-H, LNT-S, LNT-E, LNT-B, CUR, and SPG in DMSO-d_6 are shown in Fig. 3. Clearly, the ^{13}C NMR spectra of four glucans are very similar to those of the well known (1 → 3)-β-D-glucans with side branches of (1 → 6) glucosyl units such as SPG. The assignments of ^{13}C NMR chemical shifts of all the samples including CUR and SPG are summarized in Table 1. Peaks at 103.6–103.7 (C1), 73.2–73.5 (C2), 87.0 (C3), 69.0–69.3 (C4), 76.8–77.0 (C5), and 61.6–61.7 (C6) ppm are typical signals of the carbon atoms of the (1 → 3)-β-D-glucan backbone, while peaks at 70.6–70.7 (C6s) ppm resulted from branching effect of the backbone, suggesting that the four glucans are branched at C6. The chemical shifts of the terminal glucose residue labeled ′ were also listed in Table 1. All the results indicated that LNT-H, LNT-S, LNT-E, and LNT-B belong to the same glucan family as SPG, having (1 → 3)-β-D-glucan backbone with (1 → 6) glucosyl side chain.

3.2. Chain conformation

The Huggins constant k' and intrinsic viscosity $[\eta]$ of four samples in water and dimethyl sulfoxide (DMSO) were estimated according to Huggins and Kraemer equations (1) and (2), and the results are illustrated in Table 2. It is well known that the Huggins constant provides an indication of the hydrodynamic interactions of the polymer with the solvent (Grigorescu & Kulicke, 2000). The k' usually has values roughly between 0.3 and 0.8 with values of 0.3–0.5 for a polymer in good solvents and 0.5–0.8 for polymers in theta solvents (Goh, Hemar, & Singh, 2005; Graessley, 1974). The k' values are 0.3–0.5 for four samples in water and DMSO, indicating that they can be molecularly dispersed in good solvent. Therefore, aggregates hardly coexist in these solutions. Usually, intrinsic viscosity reflects the hydrodynamic volume of the polymer in solution. Obviously, the $[\eta]$ values of four samples in water are much higher than those in DMSO, in which the $[\eta]$ values are comparative to those of random coils such as polystyrene (Einaga, Miyaki, & Fujita, 1979) and amylose (Nakanishi, Norisuye, Teramoto, & Kitamura, 1993). This result demonstrated that all the four samples adopt a more ordered chain conformation in water than those in DMSO.

AFM permits the nondestructive imaging of particularly soft biological surfaces, and has been widely used to study the molecular and supramolecular structures of biological macromolecules (McIntire & Brant, 1998, 1997; McIntire et al., 1995; Morris, 1994; Shao & Yang, 1995; Vuppu, Garcia, & Vernia, 1997). In this work, we also used AFM to characterize the morphology of the four samples. Fig. 4 shows the AFM images of the four samples dissolved in pure water. The AFM topographic images of all the samples reveal almost rod-like structures. By contrast with each other, it is very clear that LNT-S has the shortest chain length due to sonication degradation. LNT-H and LNT-S are more extended than LNT-E. LNT-B has relatively more branches than LNT-H, LNT-S, and LNT-E, which can be explained as LNT-B is a renatured glucan as mentioned in the Introduction. The measured mean length (L) of the four samples calculated by computer averaged over hundreds of molecules are listed in Table 2. According to Mark–Houwink equation of Lentinan in aqueous solution obtained by Zhang, Li, Xu, and

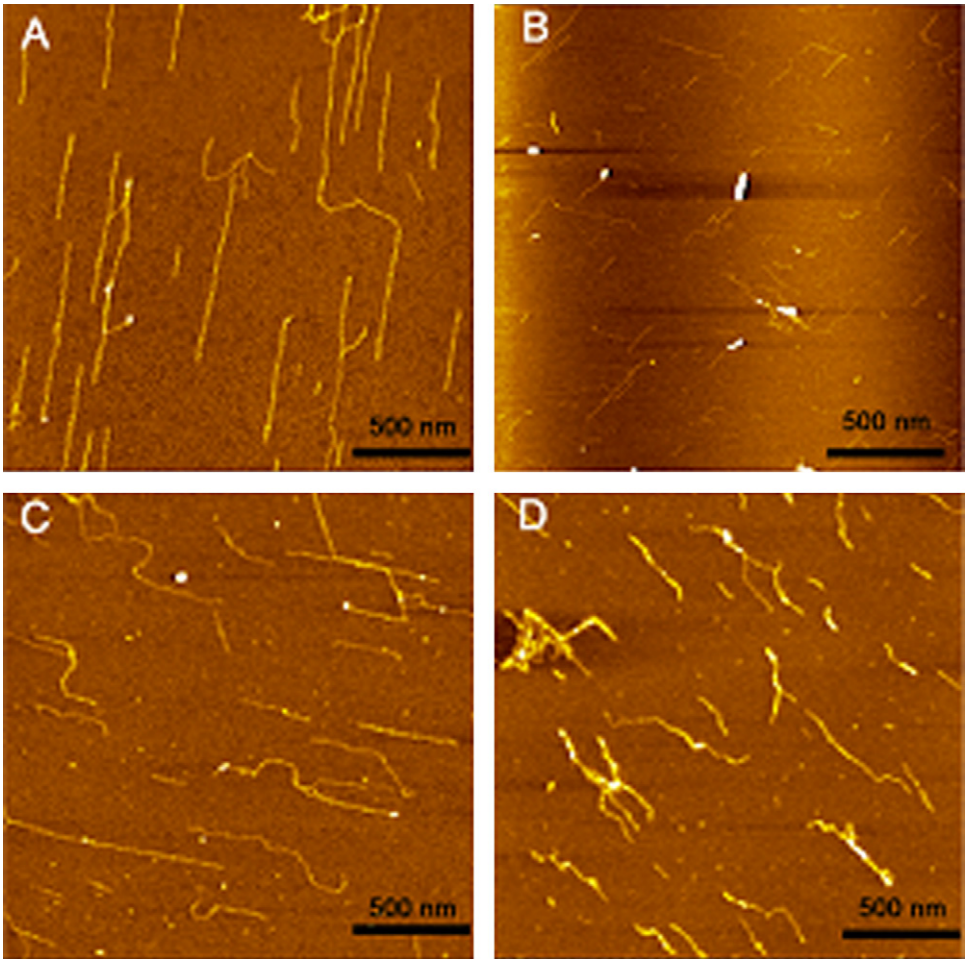


Fig. 4. The AFM images of LNT-H (A), LNT-S (B), LNT-E(C), and LNT-B (D) on micas.

Table 1
^{13}C NMR chemical shifts (δ/ppm) of four samples LNT-H, LNT-S, LNT-E, LNT-B and two reference standards SPG and CUR in DMSO-d_6 at 25 °C. ′ stands for the terminal Glc.

Sample	Chemical shift (ppm)												
	C1	C2	C3	C4	C5	C6	C6s	C1′	C2′	C3′	C4′	C5′	C6′
LNT-H	103.6	73.2	86.9	69.1	76.8	61.6	70.6	103.6	74.4	77.4	69.1	76.8	61.6
LNT-S	103.7	73.5	87.1	69.2	76.9	61.7	70.7	103.7	74.5	77.6	69.2	76.9	61.7
LNT-E	103.7	73.5	87.0	69.1	77.0	61.6	70.7	103.7	74.5	77.6	69.1	77.0	61.6
LNT-B	103.7	73.2	87.0	69.2	76.9	61.7	70.7	103.7	74.5	77.6	69.2	76.9	61.7
SPG	103.7	73.2	87.0	69.3	76.9	61.6	70.7	103.7	74.4	77.0	69.3	76.9	61.6
CUR	103.7	73.5	86.9	69.0	77.0	61.5							

Table 2
The experimental results of $[\eta]$ and k' in DMSO, the viscosity-average molecular weight M_η. contour length L, and molar mass per contour length M_L of LNT-H, LNT-S, LNT-E, and LNT-B in water at 25 °C.

Sample	$[\eta]$ (mL g^{-1})		$[\eta]_{in\ water}/[\eta]_{in\ DMSO}$	k'		$M_\eta \times 10^{-4}$ (g/mol)	L (nm)	M_L (nm^{-1})
	DMSO	Water		$k'_{in\ DMSO}$	$k'_{in\ water}$			
LNT-H	78.9	810	10.3	0.31	0.43	109	700	1557
LNT-S	45.8	448	9.8	0.36	0.32	68	300	2267
LNT-E	101.1	1377	13.6	0.33	0.41	167	1000	1670
LNT-B	62.1	633	10.2	0.30	0.47	90	500	1800

Zeng (2005), the viscosity-average molecular weight M_η of the four samples was estimated as shown in Table 2. LNT-E has the highest molecular weight, while LNT-S by sonication extraction shows the lowest molecular weight due to degradation during sonication. On the basis of M_η and L, the molar mass per contour length M_L ($M_L = M_\eta/L$) is found to be similar to the proposed triple helical structure (Bluhm, Deslandes, Marchessault, Pérez, & Rinaudo, 1982; Norisuye, Yanaki, & Fujita, 1980; Stokke & Brant, 1990; Yanaki, Norisuye, & Fujita, 1980; Zhang et al., 2001), suggesting that all the glucans isolated from *Lentinus edodes* by different methods have similar triple helical structure with different molecular weight.

3.3. Effect of the glucans on NO production in LPS-stimulated RAW 264.7 macrophages

Fig. 5A shows the effect LNT-H, LNT-S, LNT-E, and LNT-B on NO production in LPS-stimulated macrophage RAW 264.7 cells. It can be seen that LPS induced an amount of NO secretion as a positive control, and that LNT-H, LNT-E, and LNT-B also induced NO production except LNT-S. When the cells were co-stimulated with LPS plus four glucans, only LNT-S significantly suppressed NO secretion (down regulation ~40%). It is not known that LNT-H, LNT-E, and LNT-B themselves can activate RAW 264.7 cells to produce NO or they were contaminated by endotoxin. Polymyxin B (PMB) is known to be a potent antibiotic, a natural peptide, which can bind to the major component lipid A of the endotoxin (Cardoso et al., 2007). Based on it, we used PMB to check if LNT-H, LNT-E, and LNT-B were contaminated by endotoxin. As shown in Fig. 5B, the NO production caused by the activation of LNT-E on RAW 264.7 cells was inhibited by PMB in a dose dependent manner. Similar inhibition effects were also observed in the cells treated with LNT-H and LNT-B (data not shown), which indicates that the LNT-H, LNT-E and LNT-B were really contaminated by endotoxins. As for LNT-S, it did not induce NO production compared with PBS stimulation, and we thus can draw a conclusion that there is not markedly detectable endotoxin in LNT-S in the present experiment.

Among the four glucans, LNT-S especially caught our eyes not because it did not induce NO production but it could suppress NO production from LPS-activated macrophages. So we checked the co-stimulation time and concentration dependence of NO inhibition in RAW 264.7 cells. The results show that LNT-S markedly inhibited LPS-induced NO production at time points of 24 h and 30 h by ~40% (Fig. 6A) and the inhibition effect exhibits a strong dose dependent manner (Fig. 6B and C). Based on the results, LNT-S is a promising molecule which might be helpful to the diseases associated with NO overproduction. The detailed mechanism of NO inhibition by LNT-S will be elucidated in the near future.

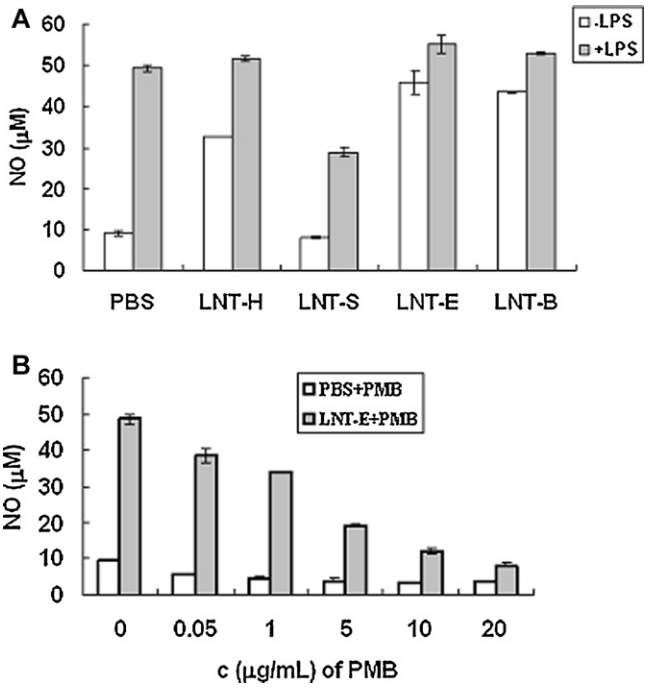


Fig. 5. NO production in macrophage RAW 264.7 cells. (A) RAW 264.7 cells were seeded (2×10^5 cells/well) in 48-well plate and incubated for 24 h, then treated with LPS (100 ng/mL), LNT-H, LNT-S, LNT-E, and LNT-B with final concentration of 100 μg/mL. After a 48-h stimulation, the conditioned medium was collected and used to determine NO concentration by the Griess reagent. (B) RAW 264.7 cells were seeded (2×10^5 cells/well) in 48-well plate and incubated for 24 h, and treated with polymyxin B (PMB) with different concentration for 30 min. LPS (100 ng/mL) and LNT-E (100 μg/mL) were then added into the well, and stimulated the cells for 48 h. Finally, the conditioned medium was collected to determine NO concentration by the Griess reagent. Each value in (A) and (B) represents the mean ± S.E. of three independent experiments.

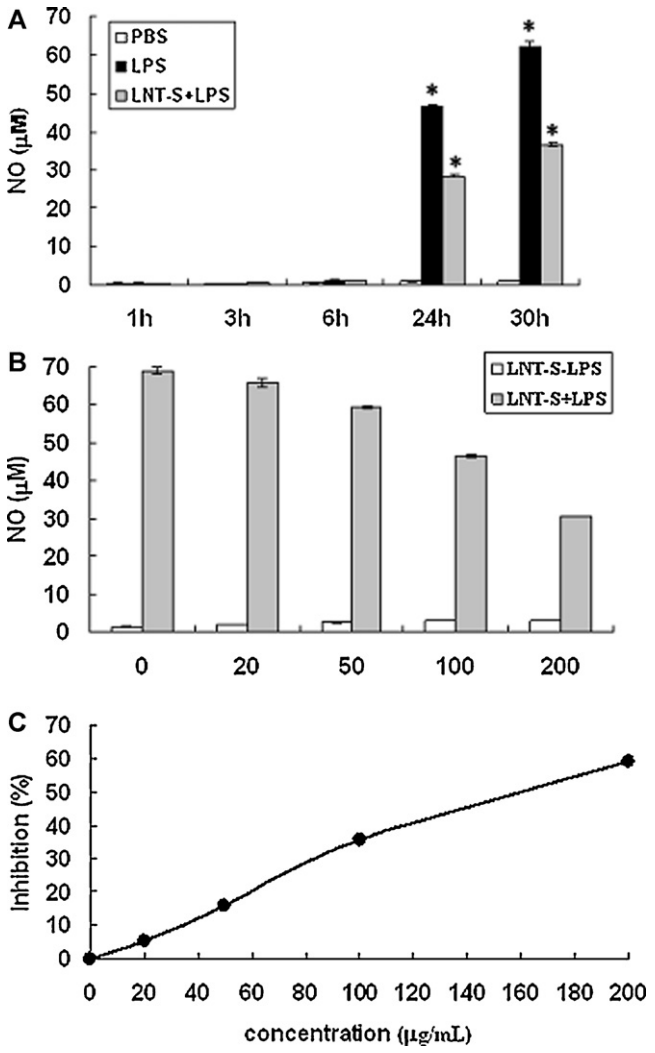


Fig. 6. NO production in macrophage RAW 264.7 cells. (A) RAW 264.7 cells were seeded (5×10^5 cells/well) in 24-well plate and incubated for 24 h. The cells were then co-treated with LPS (100 ng/mL) and LNT-S (200 μg/mL). After stimulation for desired time as indicated in the figure, the conditioned medium was collected to determine NO concentration by the Griess reagent. (B and C) RAW 264.7 cells were seeded (5×10^6 cells) in cell culture dish (Φ 60) and incubated for 24 h. The cells were then co-treated with LPS (100 ng/mL) and LNT-S (different concentration). After a 24-h stimulation, the conditioned medium was collected to determine NO concentration by the Griess reagent, and the inhibition rate of NO was calculated. Each value in (A) and (B) represents the mean ± S.E. of three independent experiments. Student's *t*-test was used to determine significant differences. $^*p < 0.05$ vs control.

4. Conclusion

In summary, four glucans LNT-H, LNT-S, LNT-E, and LNT-B which were successively extracted by four different method exhibited similar chemical structures and stiff chain conformation but different molecular weight. It is very interesting that only LNT-S contains no detectable endotoxin and shows significant NO inhibition effect. To exploit its application in medicinal industry, more detailed studies are needed in our future work.

Acknowledgements

We gratefully acknowledge the financial supports from the National Natural Science Foundation (20874078), Youth Technology Chenguang Project of Wuhan (200950431193), and Japan Scociety for the Promotion of Science Postdoctoral Fellowship (20.08431, to X. Xu).

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