

Chemical Structure of the Water-Insoluble Polysaccharide Isolated from the Fruiting Body of *Ganoderma lucidum*

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ABSTRACT: Water-soluble and water-insoluble polysaccharide fractions were isolated from fruiting bodies of *Ganoderma lucidum* (rheishi) by extraction with phosphate buffer and 1N sodium hydroxide solutions at different temperatures and investigated by high-performance liquid chromatography (HPLC), one- and two-dimensional ^1H and ^{13}C NMR, infrared (IR) spectroscopy, and other techniques. This work was prompted by Sone *et al.*'s earlier conclusion that similarly extracted, water-insoluble polysaccharides are (1 \rightarrow 3)- β -D-glucans with very low degrees of branching though hardly hydrolyzed by the enzyme *exo*-(1 \rightarrow 3)- β -D-glucanase of *Basidiomycete* QM 806. HPLC on the acid hydrolysates from our extracts showed that the water-soluble fractions were minor constituents and mostly heteropolysaccharides, while the water-insoluble ones (*i.e.*, the alkali-extracts at 25 and 65°C) were essentially pure glucans. It was found from NMR and viscosity measurements in dimethylsulfoxide containing 0.25 M LiCl that these glucans consist of linearly linked (1 \rightarrow 3)- α -D-glucose residues and differ only in molecular weight. The (1 \rightarrow 3)- α -structure of the water-insoluble fractions was confirmed by IR and optical rotation and also by comparison with the NMR spectra taken for standard (1 \rightarrow 3)- β -D-glucan and (1 \rightarrow 3)- α -D-glucan samples.

KEY WORDS Polysaccharide / *Ganoderma lucidum* (Rheishi) / Chemical Structure / Nuclear Magnetic Resonance / (1 \rightarrow 3)- α -D-Glucan / (1 \rightarrow 3)- β -D-Glucan /

The fruiting body of *Ganoderma lucidum*, called lingzhi in China and rheishi in Japan, has long been used as Chinese medicine in Eastern countries. Its medicinal effects on cancer, hypertension, hepatitis, and hypercholesterolemia have been demonstrated by pharmacological studies in the last two or three decades.¹ On the other hand, physico-chemical properties of its constituent polysaccharides are as yet left almost unexplored. Although a few groups^{2–5} studied the chemical structures of the major constituents in relation to antitumor activity against Sarcoma 180 implanted in mice, the proposed polysaccharide structures are not conclusive for reasons given below.

Mizuno *et al.*^{2,4} extracted water-soluble and water-insoluble polysaccharides from the fruiting body of *Ganoderma lucidum*, and concluded from bioassay and various chemical analyses that a water-soluble (1 \rightarrow 3)- β -D-glucan with (1 \rightarrow 6)- β -linked glucose side chains and some acidic heteropolysaccharides are responsible for the antitumor activity of rheishi. Sone *et al.*⁵ drew the following conclusions from similar but more detailed analyses. 1. The major constituents of the extracts from rheishi are water-insoluble (1 \rightarrow 3)- β -D-glucans with very low degrees of branching. 2. These glucans have significantly lower antitumor activity than water-soluble (1 \rightarrow 3)- β -D-glucans with higher degrees of (1 \rightarrow 6)- β -branching (DB). 3. Most water-soluble components of rheishi are heteropolysaccharides, water-soluble (1 \rightarrow 3)- β -D-glucans being minor constituents. Sone *et al.* remarked, however, that the water-insoluble (1 \rightarrow 3)- β -D-glucans are hardly hydrolyzed by the enzyme *exo*-(1 \rightarrow 3)- β -D-glucanase of *Basidiomycete* QM 806. This seems incompatible with the (1 \rightarrow 3)- β -structure with a low DB, *i.e.*, the above conclusion 1. Further, the two conclusions 1 and 2 are not always consistent with each other, in that schizophyllan⁶ (water-soluble (1 \rightarrow 3)- β -D-glucan

with DB = 1/3) and curdlan⁷ (water-insoluble (1 \rightarrow 3)- β -D-glucan with DB = 0) are known to have equally high antitumor activities.

The present study was undertaken to check the chemical structures proposed by Sone *et al.*⁵ for major constituent polysaccharides from rheishi. We extracted polysaccharides from fruiting bodies of *Ganoderma lucidum* and investigated water-insoluble components, *i.e.*, alkali-extracts, by high-performance liquid chromatography, NMR, optical rotation, infrared spectroscopy, and other techniques.

EXPERIMENTAL

Isolation of Polysaccharides

Fruiting bodies of *Ganoderma lucidum* (2 kg) cultivated in Northeastern China were dried, crushed, and washed with hot ethylacetate and methanol over 4 h. They were immersed and homogenized in 90% aqueous ethanol overnight to remove soluble compounds of low molar mass.

Water-soluble polysaccharides were extracted stepwise from the fruiting bodies with a 0.2 M phosphate buffer solution (PBS) of pH = 7 at 25, 80, and 120°C. In each step, the PBS suspension was centrifuged, and to the supernatant was added a large quantity of ethanol (EtOH) to precipitate the polysaccharide. The precipitates were designated GL1, GL2, and GL3 in order of increasing extraction temperature. The residue obtained from the last PBS suspension was treated with 1N aqueous NaOH at 25 and 65°C to extract water-insoluble fractions GL4-1 and GL4-2, respectively; acetic acid (HOAc) was used to precipitate the polymers. These extraction procedures, similar to those reported by Sone *et al.*⁵ for rheishi and by Wang *et al.*⁸ for *Ganoderma tsugae*, are shown in Figure 1, along with the amounts

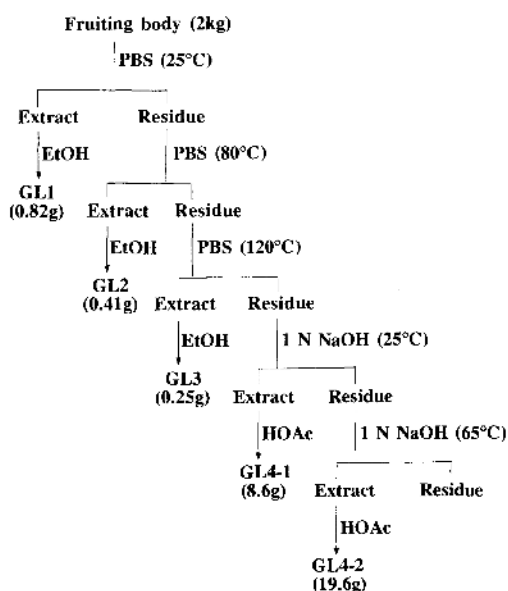


Figure 1. Extraction of polysaccharides from the fruiting body of *Ganoderma lucidum*. In each step, extraction was repeated three times with the indicated reagent of 5 L (*i.e.*, 15 L in amount).

of the respective fractions. It can be seen that the alkali-extracts, *i.e.*, the water-insoluble polysaccharides, were much richer than the water-soluble fractions. This is consistent with the finding of Song *et al.* mentioned in the Introduction.

All fractions except GL1 were treated by Sevag's procedure to remove proteins, and bleached by use of 20% aqueous hydrogen peroxide at 50°C. They were lyophilized after their aqueous solutions or suspensions had been dialyzed against water to remove low molar mass compounds. Ultraviolet (UV) spectroscopy (Shimadzu UV-160A) in water or 1N aqueous NaOH showed these fractions to have a single peak around 200 nm; no UV absorption was detected at 280 nm (for proteins) and 600 nm (for pigments). Fraction GL1 was mostly polysaccharide-protein complexes and not examined in this work.

At an early stage of this work, we found from viscosity measurements that 1N aqueous NaOH causes the water-insoluble fractions GL4-1 and GL4-2 to degrade very slowly at room temperature. We therefore searched for an alternative solvent suitable for purification and viscometry. Solubility tests showed that the two fractions dissolve in dimethylsulfoxide (DMSO) containing an appropriate amount of lithium chloride (LiCl) at room temperature and are stable in this mixed solvent system.

With 0.25M LiCl/DMSO (*i.e.*, DMSO containing 0.25M LiCl) at 25°C as the solvent, fractions GL4-1 and GL4-2 were further purified by the addition of acetone, a precipitant. The resulting fractions were reprecipitated from 0.25M LiCl/DMSO solutions into 80% aqueous acetone, washed with 50% aqueous acetone three times and anhydrous acetone six times, and dried in vacuum for 7 days.

HPLC

Each fraction (30 mg) was dissolved in 4N H₂SO₄ (5 cm³) and hydrolyzed in a sealed tube at 100°C for 24 h. The solution was neutralized by the addition of

BaCO₃, and the BaSO₄ produced was removed by centrifugation followed by filtration. After the solution had been concentrated to about 2 cm³, the sugar components were determined by high-performance liquid chromatography (HPLC) (Shimadzu LC-6A equipped with a refractive index detector and a μ -Bondapak-NH₂ column). A mixture of acetonitrile, water, and methanol (82:12:6 by volume) was used for the mobile phase.

Spectroscopy

One- and two-dimensional ¹H and ¹³C NMR measurements were made on a JEOL JNM-LA-500 spectrometer for the water-insoluble polysaccharide fractions GL4-1 and GL4-2 in 0.25 M LiCl/DMSO-*d*₆ (deuterated DMSO) at 60°C. The polymer concentration was adjusted to 5 wt% in all experiments.

A JASCO FT-IR 8300 spectrometer was used for infrared (IR) absorption spectroscopy. Test specimens were prepared by the KBr-disk method.

Specific rotations [α]_D at 589-nm wavelength were determined on a JASCO ORD/UV5 spectropolarimeter with 0.5N aqueous NaOH at 20°C as the solvent. A quartz cell of 10 cm in length was used, and the polymer mass concentration was adjusted to about 1 × 10⁻² g cm⁻³.

Viscometry and Light Scattering

The intrinsic viscosity [η] and Huggins' constant *k'* were determined for fractions GL4-1 and GL4-2 in 0.25 M LiCl/DMSO at 25°C using a conventional viscometer of the Ubbelohde type. The density of this mixed solvent was determined to be 1.104 g cm⁻³ at 25°C. DMSO was fractionally distilled under reduced pressure after being dehydrated with calcium hydride.

The higher molecular weight fraction GL4-2 (see below) in 0.25 M LiCl/DMSO at 25°C was studied by light scattering with vertically polarized incident light of 436-nm wavelength to estimate its molecular weight, using a Fica 50 light scattering photometer (see ref 9 for the experimental procedures). When an analyzer was set in the horizontal direction, depolarized scattering was detected. Though the origin of the optical anisotropy was not clear, the weight-average molecular weight *M_w* and the second virial coefficient *A₂* were corrected for this effect by the conventional method¹⁰; the correction was about 10% for the former.

Test solutions were heated at 65°C for 20 min and then optically clarified by filtration through Teflon Millipore filters (at room temperature) followed by centrifugation at about 2.5 × 10⁴ gravities for 2 h. We note that virtually no change in [η] at 25°C was observed before and after the heating.

The specific refractive index increment of fraction GL4-2 in 0.25 M LiCl/DMSO at 25°C and 436 nm was determined to be 0.0580 cm³ g⁻¹ for dialyzed solutions, using a modified Schulz-Cantow type differential refractometer. Dialysis was effected by the apparatus¹¹ described elsewhere.

RESULTS AND DISCUSSION

Carbohydrate Components

HPLC analysis of the acid hydrolysates from the ex-

tracts showed that the water-soluble fractions GL2 and GL3 are heteropolysaccharides containing D-glucose (only in GL2), D-galactose, D-mannose, D-xylose, L-fucose, and L-rhamnose, while the water-insoluble fractions GL4-1 and GL4-2 are almost pure glucans. D-glucuronic acid was detected in GL2 and GL3 by the carbazole reaction but neither in GL4-1 nor in GL4-2. These findings are quite similar to what was reported by Sone *et al.*⁵

In contrast to our and Sone *et al.*'s findings, the "water-insoluble" fractions extracted by Mizuno *et al.*⁴ with 5% aqueous NaOH at 30°C were heteropolysaccharides and contained a substantial amount (about 10%) of glucuronic acid. Though this inconsistency may come from a certain difference in the fruiting body and remains to be seen, the presence of the acid seems difficult to reconcile with the water-insolubility of the fractions.

Mizuno *et al.*² also isolated almost pure, water-soluble glucans. To see whether our water-soluble fraction GL2 contains a pure glucan, it was divided into five parts by gel permeation chromatography. Except for the third (central) fraction, the fractions were all heteropolysaccharides as reported by Sone *et al.*⁵ However, the central fraction was a pure glucan with a fairly large $[\eta]$ of $600 \text{ cm}^3 \text{ g}^{-1}$ (without correction for shear rate) in 0.5 M aqueous NaCl at 25°C. Although such a large $[\eta]$ suggests that the polymer is semiflexible, no further characterization was feasible because of the very limited quantity.

Structure of Water-Insoluble Polysaccharides

One-dimensional ^1H and ^{13}C NMR spectra showed that the water-insoluble glucans GL4-1 and GL4-2 are chemically identical and have no side chain. The intrinsic viscosities for GL4-1 and GL4-2 in 0.25 M LiCl/DMSO at 25°C were $37 \text{ cm}^3 \text{ g}^{-1}$ ($k' = 0.45$) and $102 \text{ cm}^3 \text{ g}^{-1}$ ($k' = 0.34$), respectively. Thus these two fractions differ only in molecular weight. Our light scattering experiment yielded $M_w = 1.95 \times 10^5$ and $A_2 = 1.33 \times 10^{-4} \text{ mol cm}^3 \text{ g}^{-2}$ for the higher molecular weight fraction GL4-2.

Figure 2 shows the HMQC (heteronuclear multi-quantum coherence) NMR spectrum (for GL4-1), which maps the correlation between the directly bonded C and H atoms, thus distinguishing the protons directly and indirectly linked to a C atom. The peaks for C-6 and H-6 can readily be assigned, since only C-6 is directly bonded to two H atoms. Figure 3 shows the DQF (double-quantum filter) spectrum, which presents the correlation among the H atoms connected within three chemical bonds. The spectra in these two figures allow us to assign all the peaks for H, C, and OH with the aid of the already assigned C-6 and H-6 peaks. The results for the H and C atoms are summarized in Table I. The absence of OH-3 in the DQF spectrum (Figure 3) clearly shows the fraction to be a (1→3)-D-glucan. The chemical shift of 5.1 ppm for H-1 is much closer to that expected for α -glucose than for β -glucose (about 5.2 ppm for α -glucose and 4.6 ppm for β -glucose in D_2O).¹² This indicates that our fraction is not a (1→3)- β -D-glucan but should be a (1→3)- α -D-glucan.

To confirm this, we made additional NMR measurements on a commercially available curdolan sample (Wako Pure Chemical Ind.; Lot No. RSN 9512) under the same

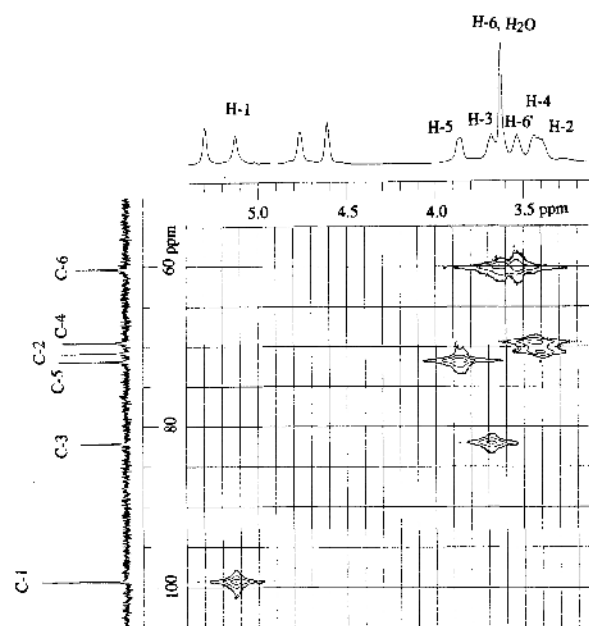


Figure 2. NMR spectrum (HMQC) for water-insoluble fraction GL4-1 in 0.25 M LiCl/DMSO- d_6 at 60°C.

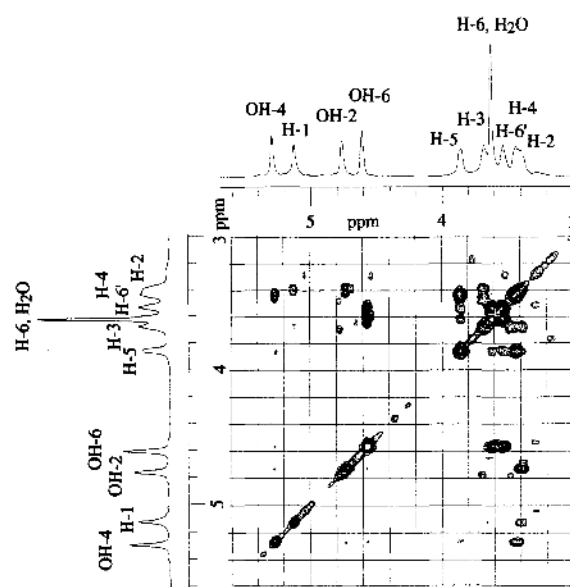


Figure 3. NMR spectrum (DQF) for water-insoluble fraction GL4-1 in 0.25 M LiCl/DMSO- d_6 at 60°C.

conditions as for our water-insoluble fractions; note that curdolan also dissolves in mixtures of DMSO and LiCl.¹³ The HMQC spectrum obtained is shown in Figure 4. The peaks have been assigned with the aid of the corresponding DQF spectrum (not shown here), but in actuality, the assignments of C-4 and C-5 have relied on the literature.¹⁴ Apparently, the HMQC spectrum for curdolan differs from the corresponding spectrum for GL4-1 in Figure 2. In particular, the difference in the chemical shift for H-1 between the two glucans is distinct, substantiating that our polysaccharide cannot be a (1→3)- β -D-glucan. The coupling constant 3J (H-1, H-2) for curdolan was estimated to be 7.5 Hz, a value expected for β -glucose.¹² That for our glucan could not be estimated since possibly the 3J (H-1, H-2) value expected for α -glucose is as small as 3 to 4 Hz.¹² We further took

Table I. Chemical shifts from ^{13}C and ^1H NMR spectra for the water-insoluble rheishi glucan in 0.25 M LiCl/DMSO- d_6 at 60°C

Assignment	Chemical shift ^a	Assignment	Chemical shift ^a
C-1	99.3	H-1	5.13
C-2	70.9	H-2	3.40
C-3	82.2	H-3	3.68
C-4	69.5	H-4	3.44
C-5	71.9	H-5	3.86
C-6	60.4	H-6	3.64
		H-6'	3.53

^a in ppm.

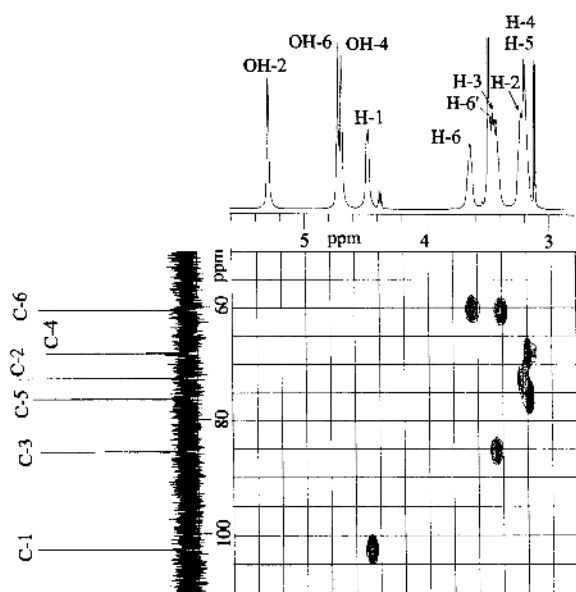


Figure 4. NMR spectrum (HMQC) for curdlan in 0.25 M LiCl/DMSO- d_6 at 60°C.

NMR spectra for two enzymatically prepared (1 \rightarrow 3)- α -D-glucan samples furnished by Professor S. Kitamura of Kyoto Prefectural University; the values of $[\eta]$ for these samples in 0.25 M LiCl/DMSO at 25°C were 30 and 106 cm³ g⁻¹. The NMR spectra obtained were perfectly identical to those for the two fractions GL4-1 and GL4-2, thus confirming that the water-insoluble polysaccharide extracted from rheishi is a linear (1 \rightarrow 3)- α -D-glucan.

The values of $[\alpha]_D$ for our glucan and curdlan in 0.5N aqueous NaOH at 20°C were 170 and -13 deg cm² g⁻¹, respectively. This characteristic difference also distinguishes between the α - and β -type structures. Two points are to be noted here. First, the large positive $[\alpha]_D$ value for our glucan is comparable to that (195 deg cm² g⁻¹ in 1N aqueous NaOH) reported for a (1 \rightarrow 3)- α -D-glucan from *Agrocybe cylindracea* by Kiho *et al.*¹⁵ Secondly, the normality 0.5N of aqueous NaOH is high enough to break the possible helical conformation of curdlan¹⁶ and hence $[\alpha]_D$ of -13 deg cm² g⁻¹ for that glucan may be considered to contain no substantial contribution from the helical structure.

Sone *et al.*⁵ determined the chemical structures of water-insoluble fractions by gas-liquid chromatography, methylation, Smith degradation, and IR. Their results except from IR showed the backbone structures of the polysaccharide fractions to be composed of (1 \rightarrow 3)-D-

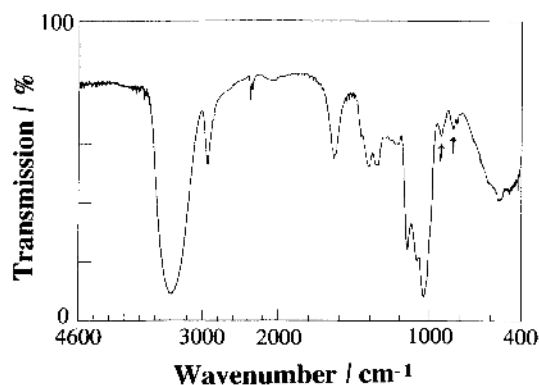


Figure 5. Infrared spectrum for fraction GL4-1. Arrows indicate absorption peaks at 849 and 927 cm⁻¹.

glucose residues, as consistent with our NMR analysis. Their conclusion on the β -type came entirely from IR spectra reported to have an absorption peak at 890 cm⁻¹ characteristic of a (1 \rightarrow 3)- β -D-glucan.^{17,18}

The IR spectrum for our glucan is shown in Figure 5. It has absorption peaks at 849 and 927 cm⁻¹ characteristic of α -D-glucans^{15,17,18} but no discernible peak at 890 cm⁻¹. Thus, Sone *et al.*'s IR observation implies that their fractions contained (1 \rightarrow 3)- β -D-glucans. However, since these glucans were hydrolyzed only to 2.6–5.5% by *exo*-(1 \rightarrow 3)- β -D-glucanase of *Basidiomycete* QM 806, their β content must be such low.

CONCLUDING REMARKS

We showed by high performance liquid chromatography, NMR, optical rotation, and infrared spectroscopy that the water-insoluble polysaccharide extracted from the fruiting body of *Ganoderma lucidum* with 1N aqueous NaOH is a linear (1 \rightarrow 3)- α -D-glucan. This is consistent with the low degree of tumor inhibition found by Sone *et al.*⁵ for water-insoluble glucans, since the (1 \rightarrow 3)- α -glucan from *Agrocybe cylindracea* has a low antitumor activity against Sarcoma 180.¹⁵ Thus, water-soluble fractions must be responsible for the antitumor activity of rheishi. Rheishi has other medicinal effects,¹ so that it is intriguing to test the alkali-extract for those effects.

Except for the crystalline structure determined by X-ray diffraction,¹⁹ little is known about the physico-chemical properties of a linear (1 \rightarrow 3)- α -D-glucan. This is probably because this type of glucan is seldom available though it is present in a number of fungi in crystalline form.²⁰ The present study shows that almost pure, linear (1 \rightarrow 3)- α -D-glucan samples can be obtained from the fruiting body of *Ganoderma lucidum* as described here.

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