Chemical Structure and Molecular Weights of α-(1→3)-β-D-Glucan from Lentinus edodes

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α-glucan (L-FV-II) and β-glucan (L-FV-I) were isolated from fruiting bodies of Lentinus edodes by extraction with 5% NaOH/0.05% NaBH₄, then precipitation with 1 M acetic acid. The analysis results of Smith degradation, high-performance liquid chromatography (HPLC), infrared spectra (IR), ¹³C NMR spectra, and optical rotation indicated that L-FV-II is α-(1→3)-β-D-glucan with slight (1→6) branch linkages, and L-FV-I is β-(1→3)-α-D-glucan containing 10% protein, and with branches. The weight-average molecular weight, Mₙ, number-average molecular weight, Mₓ, radii of gyration, <r>²<sup>1</sup>²</sup>, and second virial coefficients, A₂, of L-FV-II in aqueous 0.5 M urea/0.5 M NaOH (obtained from diluting its solution in 1.0 M urea/1.0 M NaOH) and 0.25 M LiCl in dimethylsulfoxide (DMSO)/0.25 M LiCl were measured by light scattering, membrane osmometry, and size exclusion chromatography (SEC). The values of Mₙ and Mₓ for L-FV-II in 0.5 M urea/0.5 M NaOH are 24.1 × 10⁴ and 10.9 × 10⁴, respectively, similar to those in DMSO/0.25 M LiCl. It was proved that strong intermolecular hydrogen bonds exist in L-FV-II, resulting in its water-insolubility, but there are not any aggregates or multiple-helix structure for L-FV-II in DMSO/0.25 M LiCl. The chain of α-(1→3)-β-D-glucan is more extended in DMSO/0.25 M LiCl than that in the aqueous solution.

Key words: α-(1→3)-β-D-glucan; Lentinus edodes; ¹³C NMR; molecular weight; solution property

Mushrooms have recently become attractive as a functional food and a source for the development of drugs.¹) β-(1→3)-β-D-glucan, named Lentinan as an antitumor polysaccharide, was first isolated from Lentinus edodes by Chihara et al.²,³) Following, antitumor polysaccharides from mushrooms were isolated, and proved to be an ideal immunotherapeutic, which can verify the phagocytic function of macrophages and the host defense mechanisms against tumor without detrimental effects.⁴,⁵) Normally, most of the antitumor polysaccharides have the basic β-glucan structure such as β-(1→3) linkages in the main chain of the glucan. However, obvious variations of antitumor polysaccharides are also noted. There are antitumor polysaccharides with other chemical structures such as α-glucan⁻<sup>⁶</sup> and α-D-glucan-protein.⁶) Shida et al.¹¹,¹²) have isolated an α-heterogalactan and α-(1→3)-β-D-glucan from the fruiting bodies of Lentinus edodes by extracting with 3% trichloroacetic acid and 1 M aqueous NaOH, respectively. The alkali-soluble glucan ([Ω]⁺<sup>20</sup> + 258°) has a slightly branched structure composed of (1→3)- and (1→4)-linked α-D-glucopyranose residues in the ratio 5.3:1. However, the molecular weight of the α-glucan from Lentinus edodes are almost unexplored.

Ogawa et al. found from an X-ray diffraction study that α-(1→3)-D-glucan chain conformation is nearly completely extended and is very close to a 2/1 helix in the solid state.¹³) Rees and Scott found that α-(1→3)-D-glucan is less stiff but more extend than β-(1→3)-D-glucan,¹⁴) while Brant et al. later deduced that α-(1→3)-D-glucan is more stiff than β-(1→3)-D-glucan.¹⁵) However, solution properties for α-(1→3)-β-D-glucan have seldom been published. It is well known that polysaccharides in solution have different chain conformations such as single helix,⁶) double helix,⁶) triple helix,⁶) random coil⁹) and aggregate,⁸) even if they are glucans. Therefore, it is important to identify polysaccharide structure, because the molecular weight, degree of branching, conformation, and chemical modification of the polysaccharides significantly affect their antitumor and immunomodulatory activities. These have prompted us to investigate the molecular weight and conformation of α-glucan. Recently, α-(1→3)-β-D-glucan from the fruiting body of Ganoderma lucidum was obtained, and its conformation was investigated.¹⁸,¹ Nin this study, an alkali-soluble polysaccharide from Lentinus edodes, α-glucan, was used to study the molecular weight and solution properties by light scattering, membrane osmometry, size exclusion chromatography (SEC), and viscometry.

Materials and Methods

Isolation of polysaccharides. Dry fruiting bodies of Lentinus edodes (1200 g), a commercial product cultivated in Fangxian (Hubei, China), was defatted with hot EtOAc and MeOH for 4 h, then cut into pieces and homogenized. The homogenized material was extracted in aq. 0.9% NaCl and centrifuged. The overall process is outlined in Scheme 1. Components of low molecular weight and dissociation protein in the polysaccharide solution were removed by dialysis and by repeated Sevag procedure, respectively. Each isolated polysaccharide was rotary evaporated under diminished pressure below 50°C, and then dried in a vacuum for a week. Water-insoluble L-FV-II (yield: 3.2%) and water-soluble L-FV-I (yield: 2.9%) are white and yellow powders, respectively, and were chosen for this study.

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HPLC. Samples L-FV-I and L-FV-II were hydrolyzed as in our previous work. 20 The sugar components were identified by high-performance liquid chromatography (HPLC) (Shimadzu LC-6A with a refractive index detector and a μ-Bondapak NH2 column). A mixture of acetonitrile, water, and methanol (85:10:5 by volume) was used for the mobile phase. To detect the components of protein in L-FV-I and L-FV-II, the amino acid analysis was done with an amino acid analyzer (Hitachi 835, Japan) in a 2.6 mm × 150 mm column, which was packed with Hitachi gel 26199 at 53°C. Ninhydrin was used to post-column derived and showed absorption at 440 and 570 nm. 19 It was shown that L-FV-I contains 10.4% protein inducing 17 kinds of amino acid, as Asp <1.13%, Glu 1.00%, Lue 1.25%, Val 0.85% etc., while the L-FV-II contains only 0.25% amino acid, is nearly pure polysaccharide.

Smith degradation. Samples L-FV-I and L-FV-II (120 mg) were oxidized with 10 μmol/L sodium metaperiodate (50 ml) at 6°C in the dark. After completed oxidation (120 h), ethylene glycol (10 ml) was added, and the mixture was stirred for 30 min, and then dialyzed. The dialyzate was concentrated to a small volume, then was reduced with sodium borohydride (200 mg) in the dark, with stirring at room temperature for 18 h. The excess of borohydride was decomposed by addition of 0.1 M acetic acid to pH 5.5, and the reduced polysaccharide was dialyzed to obtain glucan polyacohol. 200 mg of the dry polyacohol was hydrolyzed as in our previous work. After neutralization with BaCO3 and filtration, the filtrate was concentrated, and analyzed with HPLC. The mobile phase was the mixture of acetonitrile: water: methanol=90:10:5 (v/v).

Characterization. Infrared (IR) spectra of the L-FV-I and L-FV-II were done using a Nicolet FT-IR spectrometer. Test specimens were prepared by the KBr-disk method. High-resolution 13C NMR spectra were made on a Bruker ARX-400 NMR spectrometer at 400 MHz. The samples were dissolved in 0.5 M NaOD/D2O at 320 K, and the polysaccharides concentration was adjusted to 5 wt.% for NMR measurement. Specific rotation [α]D 20 at 589 nm wavelength was measured on an Automatic Polarimeter, (WZZ-2A model) with 0.5 M aqueous NaOH containing urea from 0 to 1 M at 20°C as solvent. The sample L-FV-II concentration was adjusted to about 1 × 10−2 g/ml.

Viscosity measurement. Viscosity of the L-FV-II solution were measured at 25 ± 0.1°C by using a modified capillary viscometer, which was a gift from the Institute of Industrial Science, Tokyo University. Mixtures of 0.5 M NaOH containing 0.0, 0.2, 0.5, 0.8, or 1.0 M urea were used as solvents. Huggins plots were used to estimate the intrinsic viscosity [η] in the aqueous solution. The L-FV-II solutions in aqueous 0.5 M NaOH containing 0, 0.2, 0.5, 0.8 or 1.0 M urea used in this work were all obtained from diluting its solutions in 1.0 M NaOH containing 0, 0.4, 1.0, 1.6 or 2.0 M urea.

Light scattering. The light-scattering intensities were obtained with a dynamic light-scattering spectrometer (DLS-700, Otsuka Electronics Co.) at 633 nm in an angular range from 30 to 150° and 15° intervals at 25°C. The L-FV-II was scintillated in 0.5 M urea/0.5 M NaOH aqueous solution or dimethylsulfoxide (DMSO) containing 0.25 M LiCl (DMSO/0.25 M LiCl) for 24 h. Optical clarification of the solution was done by using a sand filter, with subsequent filtration through a 0.2 μm pore size filter (M-HJY) into the scattering cell. The refractive index increments (dn/dc) was measured with a double-beam differential refractometer (DRM-1020, Otsuka Electronics Co.) at 633 nm and 25°C. The polysaccharide in 0.5 M urea/0.5 M NaOH aqueous solution was dialyzed against solvent for 72 h, and the value of dn/dc was 0.179 g−1 cm3.

Size exclusion chromatography. A HPLC instrument (Waters Co.) with a TSK GMH column (300 mm × 7.5 mm), 717 plus autosampler, 600 pump, 410 differential refractometer, and 2010 Millennium Workstation was used for analytical SEC experiment. The pullulan standards (P-10, P-50, P-100, P-400, and P-800) for the calibration curve and two samples of glucan (14.4 × 104 and 56.3 × 104 of Mw; 9.6 × 104 and 37.1 × 104 of Mn), which were a gift from the National Research Center for Certified Reference Materials in Beijing, for band broadening correction to obtain factor G were done on the HPLC apparatus. The eluent was DMSO/0.25 M LiCl, and the flow-rate was 1.0 ml/min at 40°C. The calibration curves obtained from pullulan standards were represented as following:

\[ \log M = 11.96 - 0.886 Ve \]  

where Ve is the elution volume. The factor G of the band broadening correction for the SEC column was measured to be 1.54, therefore values of Mw and Mn were calculated by:

\[ M_w = M_{n,SEC} / G \]  
\[ M_n = M_{n,SEC} \cdot G \]
The SEC software was used for data treatment.

The sample L-FV-II was dissolved in DMSO/0.25 M LiCl to prepare about 0.01 gml⁻¹ concentration, and stored in a refrigerator for three days before measurement. The injected volume was 50 μl.

Membrane osmometry. Osmotic pressures (π) of L-FV-II in DMSO/0.25 M LiCl and 0.5 M urea/0.5 M NaOH aqueous solution were measured with an improved Bruss membrane osmometer with a regenerated cellulose semi-permeable membrane with pore size of 8 nm prepared in our laboratory. Dynamic osmometry was used to measure osmotic pressures. The densities for solvents at 30°C were 1.0232 gcm⁻³ for 0.5 M urea/0.5 M NaOH; 1.0954 gcm⁻³ for DMSO/0.25 M LiCl. The number-average molecular mass Mₐ and second virial coefficient A₂ were evaluated from osmotic pressure π for five concentrations c using the relationship:

\[(π/c)¹/²= (RT/Mₐ)¹/²(1+0.5A₂c/Mₐc) \]  

where \(R\) is the gas constant, and \(T\) is the absolute temperature (K).

Results and Discussion

Component and structure

The IR spectra for L-FV-I, L-FV-II, pullulan, and L-FV-II film, which was prepared by coating a solution of L-FV-II in DMSO/0.25 M LiCl to KBr disk, are shown in Fig. 1. It indicated that L-FV-II and L-FV-II film have absorption peaks at 926, 844, and 822 cm⁻¹, characteristic of α-(1→3)-β-glucan, while L-FV-I has an absorption peak at 890 cm⁻¹ for the β-configuration of β-glucan. Pullulan, α-(1→6)-glucan, had IR absorption at 929 and 850 cm⁻¹, indicative of the α-configuration, but no peak at 822 cm⁻¹, which has been associated only with (1→3)-linkages. The –OH stretching vibration band of L-FV-II was broadened and shifted to a lower wavenumber (at 3300 cm⁻¹) compared with L-FV-I, pullulan, and L-FV-II film, suggesting that strong intermolecular hydrogen bonds exists in L-FV-II, but decreased in L-FV-II film. It was indicated that DMSO/0.25 M LiCl broke inter- and intramolecular hydrogen bonds of polysaccharide, so that the hydrogen bonds in the L-FV-II film were significantly weakened, similar to water-soluble polysaccharides L-FV-I and pullulan. The new absorption peaks at 1318 cm⁻¹ (S=O), 3000 cm⁻¹ and 954 cm⁻¹ (–CH₃) for the L-FV-II film are caused by DMSO remaining in the film. The water-insolubility of L-FV-II is due to a strong interaction between interchain hydrogen bonding. The IR results indicated that the DMSO/0.25 M LiCl could break intermolecular hydrogen bonds, and easily dissolve L-FV-II polysaccharide.

HPLC of L-FV-I and L-FV-II indicated that L-FV-I and L-FV-II mainly consist of β-glucose. Their Smith degradation and acid hydrolysis products are shown in Fig. 2. The HPLC results demonstrate that degradation products for L-FV-I were components of glucose together with glycerol, but those for L-FV-II were glucose with a trace of glycerol. These results suggest that the major linkage of L-FV-I is (1→3), with an 8% proportion of (1→6) linkages. L-FV-II consists of a backbone α-(1→3) residues of glucose with a few (1→6) branch linkages (2%).

Figure 3 illustrates the ¹³C NMR spectra of L-FV-II and L-FV-I in NaOD/D₂O solution. The peaks at 101.7
and 100.6(C-1), 83.2(C-3), 72.8(C-5), 71.1(C-2), 70.6(C-4), and 61.1(C-6) ppm are signals of the α-(1→3)-d-glucan, similar to those of *Ganoderma lucidum* α-(1→3)-d-glucan, which were well assigned by one- and two-dimensional NMR spectra. The chemical shifts of L-FV-I are in good agreement with the values of β-(1→3)-d-glucan, namely *Lentinan* after treatment with 8 M urea. 

The small peaks at 103.3(β-C-1), 102.3(α-C-1(1→6)), 86.8(β-C-3), 76.3(β-C-5), 73.5(β-C-2), 68.5(β-C-4), 66.7(α-C-6(1→6)), 62.7(α-C-6(1→6)), and 60.5(β-C-6) ppm in the L-FV-II spectrum indicated that L-FV-II was mostly α-(1→3)-d-glucan with a few (1→6) branch linkages, and a small amount of β-(1→3)-d-glucan from L-FV-I.

The value of [α]_D^20 for L-FV-II in aq. 0.5 M NaOH at 20°C is 193.5° cm^2 g⁻¹. The large positive value for L-FV-II is comparable to α-(1→3)-d-glucan from *Agrocybe cylindracea* (195° cm^2 g⁻¹ in aq. 1 M NaOH) reported by Kiho et al., while [α]_D^20 for β-(1→3)-d-glucan curdlan was -13° cm^2 g⁻¹ under the same conditions.

**Solution behaviors**

Figure 4 shows the urea concentration c_urea dependence of [α]_D^20 for L-FV-II in 0.5 M NaOH aqueous solution containing urea. With increasing c_urea from 0.3 to 0.6 M, [α]_D^20 undergo sharp decreases. This finding implies that the solvent-induced change in L-FV-II is accompanied by a decrease in dimensions of the polysaccharide chain.

Interestingly, the different curves of η_0/c – c of the L-FV-II in 0.5 M NaOH containing 0.0 M, 0.2 M, 0.5 M, 0.8 M, and 1.0 M urea aqueous solution at 25°C were observed (Fig. 5). When the glucan concentration comes down to extremely dilute region, upward and downward curves were obtained in 0.0 M, 0.2 M and 0.8 M, 1.0 M urea, respectively. These abnormal viscosity behaviors may be caused by interaction between NaOH, urea, and chains of L-FV-II polysaccharide. The almost straight line in 0.5 M urea/0.5 M NaOH, indicating the abnormal effect of solvents on the chains was offset. [η] for L-FV-II in DMSO/0.25 M LiCl is evidently higher than those values of schizophyllan ([η] = 109 cm⁻¹ g) with a similar molecular weight, indicating that L-FV-II chain is more extended than β-(1→3)-d-glucan. This is consistent with the theoretical predictions.

**Molecular weights**

It is well known that the polysaccharides generally have a tendency to aggregate in solution because of the
result, was used as a solvent to compare with 0.5 M urea/0.5 M NaOH aqueous solution in the measurements of molecular weight. Figure 6 illustrates the Zimm plot for sample L-FV-II, where K is the light scattering constant; \( R_\theta \) is the reduced Rayleigh ratio at angle \( \theta \). The measured values of \( M_n \), radii of gyration \( \langle s^2 \rangle^{1/2} \) and second virial coefficients \( A_2 \) of L-FV-II in 0.5 M urea/0.5 M NaOH and DMSO/0.25 M LiCl, respectively at 25°C are summarized in Table 1. The plots of \( \langle \pi/c \rangle^{1/2} \) against \( c \) for L-FV-II in two kinds of solvents are shown in Fig. 7, and \( M_n \) and \( A_2 \) are also listed in Table 1. The values of \( M_n \) and \( M_a \) in 0.5 M urea/0.5 M NaOH are in good agreement with those in DMSO/0.25 M LiCl, indicating absence of aggregates or multiple-helix structure in the solution. The \( [\eta] \) of L-FV-II in DMSO/0.25 M LiCl was higher than that in 0.5 M NaOH/0.5 M urea. This probably attributable to the increase in solvent affinity for the sugar units by DMSO, and thus the \( \alpha-(1\rightarrow3) \)-d-glucan chains are much expanded, thereby increasing the \( [\eta] \) values. In contrast, the values of \( [\eta] \) for triple-stranded helix schizophyllan in DMSO were markedly smaller than in water, because of the conformation change of the ordered triple stranded helix to a single coil.18

The SEC chromatogram of L-FV-II on TSK GMHx column with DMSO/0.25 M LiCl as the mobile phase at 40°C is shown in Fig. 8. It gives a sharper peak, indicating there are not any aggregates for L-FV-II in DMSO/0.25 M LiCl. This strongly supports the conclusion from molecular weights.

### Table 1. Experimental Results of Viscosity at 25°C and Molecular Weights for L-FV-II

<table>
<thead>
<tr>
<th>Solvents</th>
<th>( [\eta] ) (cm(^2) g(^{-1}))</th>
<th>( \langle s^2 \rangle^{1/2} ) (nm)</th>
<th>( A_2 \times 10^4 ) (cm(^2) mol g(^{-1}))</th>
<th>( M_a \times 10^{-4} )</th>
<th>( A_2 \times 10^4 ) (cm(^2) mol g(^{-1}))</th>
<th>( M_a/M_n )</th>
<th>( M_n \times 10^{-4} )</th>
<th>( M_a \times 10^{-4} )</th>
<th>( M_n/M_a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 M urea/0.5 M NaOH</td>
<td>135</td>
<td>24.1</td>
<td>51</td>
<td>10.9</td>
<td>4.3</td>
<td>2.2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>DMSO/0.25 M LiCl</td>
<td>186</td>
<td>28.3(^1)</td>
<td>46(^2)</td>
<td>11.8</td>
<td>3.9</td>
<td>2.4</td>
<td>24.4</td>
<td>9.6</td>
<td>2.5</td>
</tr>
</tbody>
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\(^1\) The values were measured by a DAMN Light scattering detector using a microbatch set up at Wyatt Technology corporation.
Conclusion

Two kinds of α- and β-glucan co-exist in the extract with 5% NaOH/0.05% NaBH₄ aqueous solution from fruiting bodies of *Lentinus edodes*. L-FV-II was obtained through precipitation with 1 M acetic acid from the extract above, and identified to be α-(1→3)-d-glucan with a few (1→6) branch linkages, and the most abundant polysaccharide component. L-FV-I consists mainly of a backbone chain of β-(1→3)-d-glucose residues with (1→6) branching, and is water-soluble. The aqueous 0.5 M urea/0.5 M NaOH is a good solvent for L-FV-II, in which normal viscosity was observed. Strong intermolecular hydrogen bonds exist in L-FV-II, resulting in its water-insolubility, but there are not any aggregates or multiple-helix structures for L-FV-II in DMSO/0.25 M LiCl. The values of Mₐ and Mₘ for L-FV-II in 0.5 M urea/0.5 M NaOH (obtained from diluting its solution in 1.0 M urea/1.0 M NaOH) and in DMSO/0.25 M LiCl were 24.1 x 10⁶ and 10.9 x 10⁶, 28.3 x 10⁶ and 11.8 x 10⁶, respectively.

Acknowledgments

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References