Structural Analysis of an Acidic Polysaccharide from *Ganoderma lucidum*¹ (Studies on Fungal Polysaccharides. XXXV)²³

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A water soluble acidic polysaccharide GL-A, [α]D 20° = -1.2° (c = 4, water), M.W. 27000, was isolated from the fruit bodies of *Ganoderma lucidum* ("Rokkakushi," a kind of "Reishi," "Ling zhi cao") by alkali extraction. GL-A consists of D-glucose and D-glucuronic acid in a molar ratio of 1.0:2.4. Structural examination was carried out by methylation analysis and partial acid hydrolysis. It is concluded that GL-A has a linear structure consisting of 1,3- and 1,4-linked β-D-glucopyranosyl and 1,3,6-linked β-D-glucuronopyranosyl glucosidic residues.

Keywords: *Ganoderma lucidum*; Rokkakushi; Ling zhi cao; acidic polysaccharide; methylation analysis; Taylor-Conrad reduction; partial acid hydrolysis; glucuronopyranosyl glucopyranoside; polysaccharide structure; oligosaccharide unit

Fruit bodies of a fungus, *Ganoderma lucidum* (Polyporaceae), are well known as a crude drug "Reishi" (Chinese name "Ling zhi cao") used to treat hepatopathy, chronic hepatitis, nephritis, gastric ulcer, hypertension, arthritis, neurasthenia, insomnia, bronchitis, asthma and poisoning.³ Nowadays, in China, it is also used for leukopenia.⁴

As a part of our structural studies on the polysaccharides in fungal crude drugs, a water-soluble acidic polysaccharide component of the fungus "Rokkakushi," a kind of "Reishi," was investigated.

In order to remove the protein portion, the ethanol precipitate obtained from the non-dialyzable fraction of 0.5 m sodium hydroxide extract of the dried fruit bodies was treated with pronase and further deproteinized by the Sevag method,⁵ followed by stepwise precipitation with ethanol. The major fraction, precipitated with 15% ethanol, was further purified by Sephadex G-100 column chromatography with water (Fig. 1). The purified major fraction, GL-A, gave a single spot on glass-fiber paper electrophoresis (PE). GL-A, [α]D 20° = -1.2° (c = 4, water), contained 25% neutral sugar (as D-glucose)⁶ and 66% uronic acid (as D-glucuronic acid),⁷ and was free from nitrogen and phosphorus.⁸ The component sugars of GL-A were identified as D-glucose and D-glucuronic acid by gas liquid chromatography (GLC) of the acid hydrolyzate, and the molar ratio was estimated to be approximately 1:2.4 by colorimetry.⁹,¹⁰ In the infrared (IR) spectrum of GL-A, absorbances at 920, 1430, 1610 cm⁻¹ due to β-glucosidic linkages and carboxyl groups were observed. The molecular weight was estimated to be approximately 27000 by gel filtration (Fig. 2).

In the methylation analysis of GL-A, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl glucitol were identified by GLC. The molar ratio of 2,4,6- and 2,3,6-O-methylated alditol acetates was 1.0:2.0. Tetra- and di-O-methyl glucitol derivatives were not detected. After reduction of GL-A with the Taylor-Conrad procedure,⁹ 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl glucitol were identified. The molar ratio of 2,3,4,6-tetra-O-methyl, 2,4,6- and 2,3,6-tri-O-methylated alditol acetates was 1.0:3.0:2.2 (Table I). These results suggested the presence of 1,3- and 1,4-linked D-glucopyranosyl residues, and 1,3-linked and non-reducing terminal D-glucopyranosyluronic acid residues in GL-A. Also,

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**Fig. 1. Elution Profile of the 15% Ethanol-Precipitated Fraction on a Sephadex G-100 Column**

**Fig. 2. Estimation of Molecular Weight of GL-A on Sephadex G-100**

A, dextran T-40; B, GL-A; C, dextran T-10.

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**Table I. Molar Ratios of Alditol Acetates Derived from Methylated Glicans**

<table>
<thead>
<tr>
<th>Component</th>
<th>Molar ratio</th>
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<tbody>
<tr>
<td>GL-A</td>
<td>Reduced GL-A</td>
</tr>
<tr>
<td>1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl glucitol</td>
<td>—</td>
</tr>
<tr>
<td>1,3,5-Tri-O-acetyl-2,4,6-tri-O-methyl glucitol</td>
<td>3</td>
</tr>
<tr>
<td>1,4,5-Tri-O-acetyl-2,3,6-tri-O-methyl glucitol</td>
<td>2</td>
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the discrepancy of the molar ratios of glucuronic acid and glucose between GL-A and reduced GL-A suggested that fragmentation might occur during this reaction procedure.

GL-A gave D-glucurononlactone, D-glucose, D-glucuronic acid and three oligomers (spots C, E and F) having the Rf values 0.47, 0.34 and 0.29 in the thin layer chromatogram shown in Fig. 3) on partial hydrolysis with 0.5 M trifluoroacetic acid (TFA) at 100°C for 3 h. These oligomers gave a reddish brown color with p-anisidine reagent spray. These oligomer fractions which were isolated from the thin layer chromatography (TLC) plate gave D-glucose, D-glucuronic acid and D-glucuronolactone, respectively, on complete hydrolysis with 1 M TFA at 100°C for 4 h (Fig. 4). When glucuronolactone was converted to glucuronic acid, one of these oligomers (spot C) yielded glucuronic acid and glucose in the ratio of 1 : 1, and the other oligomers (spots E and F) afforded D-glucuronic acid and D-glucose in the ratio of 2 : 1. It is suggested that these oligomers are a disaccharide and two types of trisaccharide. From these results, it is proposed that GL-A consists of the structural units depicted in Fig. 5.

Experimental
Isolation of Crude Polysaccharide from Ganoderma lucidum The dried fruit bodies of G. lucidum (450 g) were extracted with distilled water for 8 h at 100°C, and this extraction was repeated until the extract was negative to the phenol–sulfuric acid reagent. The hot-water insoluble material was extracted exhaustively with 0.5 M sodium hydroxide (10 times) at room temperature, and after centrifugation, the supernatant was neutralized with acetic acid, then dialyzed in Visking Cellophane tubing against water for 2 d. The internal solution was concentrated to a small volume in vacuo, and then the precipitate formed on the addition of ethanol was collected by centrifugation, washed with ethanol, acetone and ether, and dried in vacuo.

Investigation of GL-A Properties: The neutral sugar content of GL-A was estimated by the phenol–sulfuric acid method. Optical rotation was determined with a JASCO automatic polarimeter. The IR spectrum was recorded with a Hitachi 215 spectrometer. GL-A was hydrolyzed with 6 M hydrochloric acid at 100°C, and the hydrolyzate was analyzed with a Hitachi 835 amino acid analyzer. Phosphorus was estimated by the method of Fiske and SubbaRow. PE of GL-A was carried out using GF83 glass-fiber paper (Whatman) with 0.026 M borate buffer (pH 9.2), 1.0 M acetate buffer (pH 4.0) and 0.5 M phosphate buffer (pH 7.0); the spray reagents were alkaline silver nitrate and p-anisidine hydrochloride. The molecular weight of GL-A was estimated by use of a Sephadex G-100 column.

Component Sugar: GL-A (10 mg) was hydrolyzed with 1N TFA (1 ml) at 100°C for 8 h. After evaporation to dryness, the hydrolyzate was derivatized to acetol acetates in the usual way, and analyzed with a Hitachi 163 gas chromatograph using a glass column (0.3 x 200 cm) of 5% (W/w) ECNSS-M on Chromosorb W (AW-DMCS, 80-100 mesh), at 180°C under N2 at a flow rate of 50 ml/min. GL-A reduced by the method of Taylor and Conrad was also analyzed as described above. Uronic acid was estimated by the carbazole method with D-glucuronic acid as the standard.

Reduction of GL-A by the Method of Taylor and Conrad: A solution containing GL-A (20 mg in 10 ml of water) was treated with 10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC). As the reaction proceeded, the pH of the reaction mixture was maintained at 4.73 with
0.1 M hydrochloric acid. All reactions were allowed to proceed for 2 h. After hydrogen ion uptake had ceased, an aqueous 2 M sodium borohydrate solution was added slowly to the reaction mixture at room temperature. The pH rose rapidly to 7.0 as a result of destruction of borohydrate at the acid pH and was maintained at this pH with 4 M hydrochloric acid. The borohydrate solution was added with a hypodermic syringe. Then reduced GL-A was dialyzed in Visking Cellophane tubing against water for 24 h. The internal solution was concentrated to dryness in vacuo.

Methylation Analysis of GL-A and Reduced GL-A: GL-A and its reduced product (method of Taylor and Conrad) were methylated individually by the method of Hakomori (twice) until they showed no significant IR absorption due to hydroxyl groups at 3500 cm⁻¹. Each methylated polysaccharide was heated with 90% formic acid at 100 °C for 4 h. Formic acid was distilled off, and the residue was hydrolyzed with 1 M TFA at 100 °C for 8 h followed by evaporation to dryness. The resulting partially O-methylated sugars were reduced with sodium borohydrate at room temperature for 8 h to the corresponding alditois, and then acetylated as described above. GLC of the partially O-methylated alditois acetates was carried out by using a glass column (0.3 × 200 cm) packed with 5% (w/w) silicone OV-225 on Gas-Chrom Q, and analyzed at 180 °C under N₂ at a flow rate of 50 ml/min. GLC-mass spectroscopy (GLC-MS) of the partially O-methylated alditois acetates was carried out on the same column under the same conditions. The electron impact mass spectra were recorded by a JEOL JMS-D 300. In GLC-MS, the following results were observed: 1,3,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-glucitol, m/z: 43, 45, 71, 87, 101, 117, 129, 145, 161, 205, 1,3,5-Tri-O-acetyl-2,4,6-tri-O-methyl glucitol, m/z: 43, 45, 87, 101, 117, 129, 161, 1,4,5-Tri-O-acetyl-2,3,6-tri-O-methyl glucitol, m/z: 43, 45, 87, 99, 101, 113, 117, 233.

Partial Acid Hydrolysis of GL-A: Partial acid hydrolysis of GL-A (5 mg) was carried out with 0.5 M TFA (1 ml) at 100 °C for 3 h, and the hydrolyzate was evaporated to a syrup under reduced pressure below 40 °C. TLC analysis of the syrup using ethyl acetate–pyridine–acetic acid–water (5:5:1:3, v/v) and high performance thin-layer chromatography (HPTLC) plates Si 50000 (Merck) gave six spots as revealed by spraying with the p-anisidine hydrochloride reagent and heating at 100 °C for 10 min. These oligomers were isolated from the TLC plate, extracted with water, and hydrolyzed with 1 M TFA (1 ml) at 100 °C for 4 h. Each hydrolyzate was analyzed as described above. The spots were detected by a Shimadzu CS-910 dual-wavelength TLC scanner after spraying of the plates with the alkaline silver nitrate reagent.

References and Notes
1) A part of this work was presented at the 106th Annual Meeting of the Pharmaceutical Society of Japan, Chiba, April 1986.
5) M. G. Sevag, Biochem. Z., 273, 419 (1934).