Structural Analysis of an Acidic Polysaccharide from *Ganoderma lucidum*[1] (Studies on Fungal Polysaccharides. XXXV)[2]

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A water soluble acidic polysaccharide GL-A, $[\alpha]_D^{20}$ −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-linked β-D-glucuronopyranosylglucosidic 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* (Polypolaceae), 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, $[\alpha]_D^{20}$ −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.[6,7] In the infrared (IR) spectrum of GL-A, absorbances at 920, 1430, 1610 cm^{-1} due to β-glycosidic 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 glucitols 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,

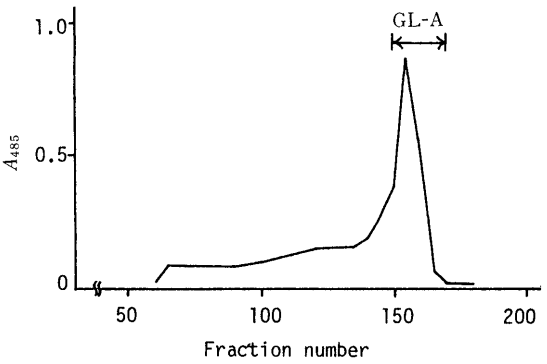

Fig. 1. Elution Profile of the 15% Ethanol-Precipitated Fraction on a Sephadex G-100 Column

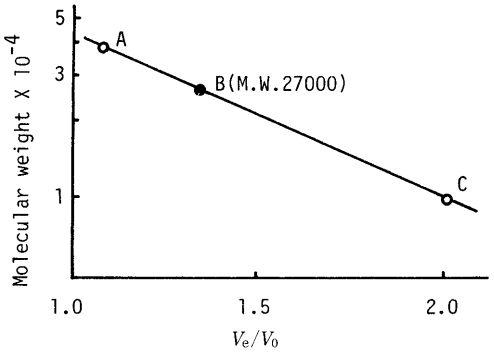

Fig. 2. Estimation of Molecular Weight of GL-A on Sephadex G-100
A, dextran T-40; B, GL-A; C, dextran T-10.

TABLE I. Molar Ratios of Alditol Acetates Derived from Methylated Glycans

Component	Molar ratio	
	GL-A	Reduced GL-A
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol	—	1
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol	1	3
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol	2	2.2

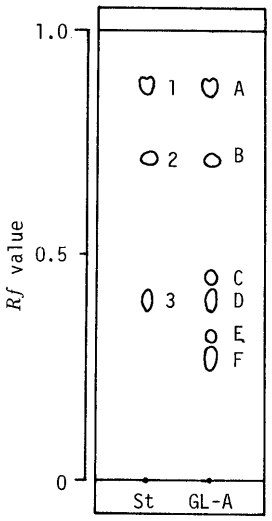

Fig. 3. Thin-Layer Chromatogram of GL-A-Hydrolyzate

Samples were developed on an HPTLC plate Si 50000 with the solvent system described in the text. Spots were detected by spraying with $AgNO_3$–NaOH and *p*-anisidine–HCl reagents. Standards: 1, D-glucuronolactone; 2, D-glucose; 3, D-glucuronic acid.

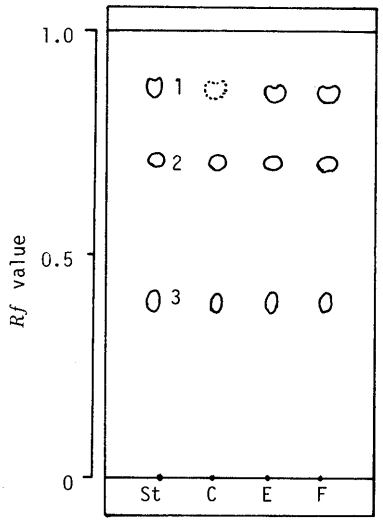

Fig. 4. Thin-Layer Chromatogram of the Hydrolyzate of Oligomers (Spots C, E and F)

Samples were developed on an HPTLC plate Si 50000 with the solvent system described in the text. Spots were detected by spraying with $AgNO_3$–NaOH reagent. Standards: 1, D-glucuronolactone; 2, D-glucose; 3, D-glucuronic acid.

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-glucuronolactone, 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.

Glc A_p–, Glc A_p–(1→4)–Glc_p–, or Glc A_p–(1→3)–Glc_p–,
–Glc A_p–(1→3)–Glc A_p–(1→3)–Glc_p–,
–Glc A_p–(1→3)–Glc A_p–(1→4)–Glc_p–

Fig. 5. Most Probable Structural Units of GL-A

Glc_p, D-glucopyranosyl; Glc A_p, D-glucuronopyranosyluronic acid.

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.[6] 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*.

Protease Digestion A solution of the precipitate (15 g) in water (50 ml) was adjusted to pH 7.0 with 1% sodium hydroxide, and pronase E (150 mg, Kaken Kagaku Co., Ltd., Tokyo) was added. The mixture was stored at 37 °C for 5 d, and then dialyzed against running water for 3 d, concentrated to 100 ml, and shaken vigorously for 0.5 h with chloroform–butanol (4 : 1, v/v; 30 ml).[5] The procedure was repeated until the gelatinous precipitate was no longer formed. After centrifugation, the supernatant solution was concentrated under reduced pressure at 35 °C, and 4 vol. of ethanol was added. The precipitate (3.2 g) was collected by centrifugation, and dried *in vacuo*.

Purification The crude polysaccharide solution was fractionated by the stepwise addition of ethanol to 15%, 30%, 60% and 90%. Each precipitate was collected by centrifugation and dried *in vacuo*. The yields of these precipitates were 31.1%, 5.4%, 28.5% and 0.4%, respectively. An aqueous solution of the 15% ethanol-precipitated fraction (560 mg) was applied to a column (2.6 × 100 cm) of Sephadex G-100. Elution was carried out with water at a flow rate of 20 ml/h. An aliquot (0.2 ml) of each fraction was mixed with 2.0 ml of water, 1 ml of 5% phenol and 5 ml of sulfuric acid, and the optical density was read at 485 nm on a colorimeter.[6] The major fraction was collected and concentrated to a small volume *in vacuo*, and 3 vol. of ethanol was added. The collected precipitate was washed with ethanol, acetone and ether, and dried *in vacuo*. The yield of GL-A was 0.13% in the crude drug.

Investigation of GL-A Properties: The neutral sugar content of GL-A was estimated by the phenol–sulfuric acid method.[6] 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.[8] 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[12] and *p*-anisidine hydrochloride.[10] 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 1 M TFA (1 ml) at 100 °C for 8 h. After evaporation to dryness, the hydrolyzate was derived to alditol acetates in the usual way,[11] and analyzed with a Hitachi 163 gas chromatograph using a glass column (0.3 × 200 cm) of 5% (w/w) ECNSS-M on Chromosorb W (AW-DMCS, 80—100 mesh), at 180 °C under N_2 at a flow rate of 50 ml/min. GL-A reduced by the method of Taylor and Conrad[9] was also analyzed as described above. Uronic acid was estimated by the carbazole method[7] 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) carbodiimide (EDC). As the reaction proceeded, the pH of the reaction mixture was maintained at 4.75 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 borohydride solution was added slowly to the reaction mixture at room termperature. The pH rose rapidly to 7.0 as a result of destruction of borohydride at the acid pH and was maintained at this pH with 4 M hydrochloric acid. The borohydride 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)[9] were methylated individually by the method of Hakomori (twice)[13] 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 borohydride at room temperature for 8 h to the corresponding alditols, and then acetylated as described above. GLC of the partially *O*-methylated alditol 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 N_2 at a flow rate of 50 ml/min. GLC-mass spectroscopy (GLC-MS) of the partially *O*-methylated alditol 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: 1,5-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.
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