Carbohydrate Research, 109 (1982) 290–294
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Note

Structural examination of an alkali-extracted, water-soluble heteroglycan of the fungus *Ganoderma lucidum**†

TOSHIO MIYAZAKI‡
Department of Microbial Chemistry, Tokyo College of Pharmacy, Hachiojishi, Tokyo 192-03 (Japan)
AND MOTOHIRO NISHIJIMA
Tokyo Metropolitan Research Laboratory of Public Health, Shinjuku-ku, Tokyo 160 (Japan)

(Received December 31st, 1981; accepted for publication in revised form, June 28th, 1982)

An earlier report[2] described the chemical structure of a water-soluble arabinoxyloglucan from the fruiting body of *Ganoderma lucidum*. This polysaccharide strongly inhibited the growth of Sarcoma 180. The isolation and partial characterization of an alkali-extracted, water-soluble, neutral heteroglycan from the same source are now described.

RESULTS AND DISCUSSION

An ethanol-precipitated fraction was obtained from the 0.1M sodium hydroxide extract of the fruiting body of *G. lucidum* in 1.6% yield. For examination of the carbohydrate portion, the precipitate was deproteinized by Pronase E and by the Sevag method, and the polysaccharide precipitated stepwise by ethanol. The major fraction, precipitated with 60% ethanol, was further purified by column chromatography on DEAE-cellulose with water and sodium hydrogencarbonate. The major fraction (eluted with water), **1**, gave a single spot on glass-fiber paper electrophoresis in borate buffer (26mM, pH 9.2), and was determined to be homogeneous by ultracentrifugal analysis.

The purified polysaccharide (**1**) had $[\alpha]_D^{20}$ −0.06° (*c* 1, water); gel filtration of **1** through Sephadex G-75 gave a narrow, single peak indicating a molecular weight of 38,000.

Component sugars of **1** were identified in an acid hydrolyzate as L-fucose, D-xylose, and D-mannose, in the molar ratio estimated by g.l.c. to be 1.0:1.0:1.0.

---

*Dedicated to Professor Sumio Umezawa on the occasion of his 73rd birthday and the 25th anniversary of the Microbial Chemistry Research Foundation.
†Studies on Fungal Polysaccharides, Part XXXII. For Part XXXI, see ref. 1.
‡To whom enquiries should be directed.

TABLE I

G.L.C. DATA FOR ALDITOL ACETATES DERIVED FROM METHYLATED **1**

Component	*Retention time*[a]	*Molar ratio*	*Mass fragmentation* (m/z)
1,5-Di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylgalactitol	0.65	1.0	43, 59, 101, 117, 131, 175
1,4,5-Tri-*O*-acetyl-2,3-di-*O*-methylxylitol or 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylxylitol	1.54	1.0	43, 87, 101, 117, 129, 189
1,3,4,5-Tetra-*O*-acetyl-2,6-di-*O*-methylmannitol	3.35	1.0	43, 45, 87, 101, 117, 129, 161, 189, 261

[a]Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as unity.

The polysaccharide **1** contained 97% of carbohydrate (as the component sugars) and was free from nitrogen (elemental analysis) and phosphorus (Fiske–SubbaRow method[3]). It is of interest that the component sugars of the water-extracted, main polysaccharide were glucose, xylose, and arabinose[2], those of the 0.01M sodium hydroxide-extracted, water-soluble fraction were glucose and mannose, and as just described, those of the 0.1M sodium hydroxide-extracted water-soluble fraction, **1**, were mannose, xylose, and fucose.

Compound **1** consumed 0.93 mol of periodate per sugar residue, releasing 0.24 mol of formic acid, but no formaldehyde. Smith degradation of **1** gave glycerol and mannitol, detected by g.l.c., in the molar ratio of 1.0:1.1.

Polysaccharide **1** was methylated by the Hakomori method[4] and then hydrolyzed, and the products were converted into their alditol acetates, which were analyzed by g.l.c., with 3% ECNSS-M and by g.l.c.–m.s., with 5% OV-225.

The results (Table I) suggested the presence of 1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylgalactitol, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylxylitol or 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylxylitol, and 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylmannitol, in the molar ratios of 1.0:1.0:1.0.

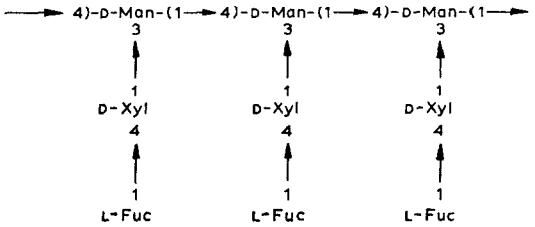
Partial hydrolysis of **1** with 0.1M trifluoroacetic acid for 1 h at 100° released fucose and xylose (mol. ratio 2.4:1.0) as monosaccharides; after heating for 2 h, the ratio changed to 1.2:1.0.

Methylation analysis of the non-dialyzable fraction, obtained by heating for 2 h, showed the presence of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol, 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methylmannitol, and 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol, in the ratio of 4.5:1.3:1.0. No di-*O*-methylated xylitol derivative could be detected. These results strongly suggest, therefore, that fucosyl residues are located as terminal positions of the side chains, each xylopyranosyl residue is linked directly to mannopyranosyl and fucopyranosyl residues, and furthermore, the core portion consists of a (1→4)-linked mannopyranosyl main chain.

The linkage to the xylopyranosyl residues might be (1→4), as indicated by the production of a large proportion of glycerol in the Smith degradation.

Thus, **1** appears to have a highly branched structure involving 1,3,4-tri-substituted D-mannopyranosyl and (1→4)-linked D-xylopyranosyl residues; the non-reducing end-groups consist of L-fucopyranosyl groups.

In view of these results, it is reasonable to conclude that **1** consists of structural units of the following type.



EXPERIMENTAL

Heteroglycan from Ganoderma lucidum. — *A. Isolation.* Cut into small pieces, dried fruiting bodies (100 g) of the fungus were stirred with distilled water for 8 h at 100°. The hot-water-insoluble material was extracted with 0.01M sodium hydroxide at room temperature. The residue was further extracted with 0.1M sodium hydroxide at room temperature for 6 h, the extraction was repeated several times, and the supernatant solution was made neutral with acetic acid and then dialyzed (Visking cellophane tubing) against running water for 2 days. After centrifugation, the supernatant solution was concentrated *in vacuo*. The precipitate (~1.6 g) formed on the addition of ethanol was collected by centrifugation, washed with ethanol, acetone, and ether, and dried *in vacuo*. The yield of 0.01M-sodium hydroxide extract was 0.8% of the fruiting bodies.

B. Protease digestion. A solution of the crude, 0.1M-sodium hydroxide extracted polysaccharide (1.6 g) in water (250 mL) was adjusted to pH 7.2 with aqueous 1% sodium hydroxide, Pronase E (100 mg, Kaken Kagaku Co. Ltd., Tokyo) was added, and the mixture was stored for 3 days at 37°, and then dialyzed against running water for 3 days, concentrated to ~50 mL, and shaken vigorously for 0.5 h with 4:1 chloroform–1-butanol (30 mL) by Sevag's procedure. The procedure was repeated until a gelatinous precipitate was no longer formed. After centrifugation, the supernatant solution was concentrated under diminished pressure at 35°, and 4 vol. of ethanol were added to the concentrate. The resulting precipitate (1.5 g) was collected by centrifugation, and dried *in vacuo*.

C. Purification. A solution of the crude polysaccharide was fractionated by stepwise addition of ethanol to 30, 60, and 90% concentration. Each precipitate was collected by centrifugation and dried *in vacuo*. The yields of these precipitates were 10.5, 74.8, and 4.2%, respectively. An aqueous solution of the main fraction, (60%

ethanol-precipitated, 100 mg), was applied to a column (3.5 × 50 cm) of DEAE-cellulose (OH^- form), and the column was eluted stepwise with water, 0.05 and 0.3M sodium hydrogencarbonate, and finally with 0.1M sodium hydroxide, at 100 mL/h. Each fraction was assayed by the anthrone reagent[5], dialyzed (Visking cellophane tubing) against running water for 3 days and then against distilled water, and concentrated *in vacuo* to low volume. Ethanol (10 vol.) was added, and each resulting precipitate was collected by centrifugation, and treated as already described. The yields were as follows: water eluate (**1**), 55.1 mg; 0.05M salt eluate, 13.0 mg; 0.3M eluate, 8.0 mg, and 0.1M sodium hydroxide eluate, 14.8 mg. The yield of the purified, major fraction (**1**) was 0.62% of the fruiting body. Compound **1** was subjected to chromatography on a column (2.6 × 9.5 cm) of Sephadex G-75, eluted with water, after calibration with standard dextrans.

Investigation of **1**. — *A. Component sugars.* A solution of **1** (10 mg) in trifluoroacetic acid (3 mL) was heated in a sealed tube for 8 h at 100°, and then evaporated, and the residue was dissolved in water (~20 mL). Sodium borohydride (~20 mg) was added to the solution, and the mixture was stirred overnight. Sugars were converted into their corresponding alditol acetates by a procedure already described[2].

Component sugars of the minor fractions eluted by salt solutions, and of the 0.01M sodium hydroxide-extracted fraction, were glucose (main) and mannose, those of the 30%-ethanol precipitate were glucose (main), mannose, xylose, and arabinose, and those of the 90%-ethanol precipitate were mannose, xylose, fucose, and glucose.

B. Properties. Paper electrophoresis of **1** in 26mM borate buffer (pH 9.2) showed a single spot (detected with the Molisch reagent).

C. Periodate oxidation. A solution of **1** (52.0 mg) in distilled water (25 mL) was made up to 50 mL with 0.22M sodium periodate (4 mL) and distilled water. The mixture was kept in the dark at room temperature. The consumption of oxidant and the amounts of formic acid and formaldehyde released were determined on samples (3 mL) as previously described[2]. Periodate consumption and production of formic acid reached maximum values of 0.93 and 0.24 mol per sugar residue, respectively, after 36–48 h.

D. Smith degradation. Compound **1** (52.0 mg) was oxidized with periodate as described in *C*. The excess of periodate was reduced with ethylene glycol (0.4 mL); after 48 h, the solution was dialyzed against running water for 24 h and then concentrated to ~20 mL, and sodium borohydride (~100 mg) was added. The mixture was stirred overnight, acidified with acetic acid, dialyzed against running water for 2 days, and evaporated. The syrupy residue was hydrolyzed with M trifluoroacetic acid (3 mL) for 6 h at 100°. The hydrolyzate was evaporated *in vacuo*, and, after reduction and acetylation as already described, subjected to g.l.c.

E. Methylation. Methylation was performed conventionally. The methylated **1**, which showed no significant i.r. absorption for hydroxyl groups at 3500 cm^{-1}, was heated with 90% formic acid for 4 h at 100°. Formic acid was distilled off, and the residue was hydrolyzed with 0.5M sulfuric acid for 8 h. Reduction and acetylation

were then performed as previously described[2]. The resulting alditol acetates were subjected to g.l.c. and g.l.c.–m.s., under conditions previously described[2].

F. Partial acid hydrolysis. Partial acid hydrolysis of **1** (50 mg) was performed with 0.1M trifluoroacetic acid (4 mL) for 1 and 2 h at 100°. Each hydrolyzate was dialyzed against distilled water through Visking cellophane tubing. The external solution was evaporated *in vacuo* to a syrup that was examined by g.l.c. Monosaccharides in each dialyzable fraction and their molar ratios were as follows: for 1 h, fucose and xylose 2.4:1.0; 2 h, fucose, xylose, and mannose 1.2:1.0:trace.

The internal solution was evaporated *in vacuo*, the residue methylated by the Hakomori method, and the product then treated as just described. The resulting alditol acetates were identified by g.l.c.–m.s. 1,5-Di-*O*-acetyl-2,3,6-tri-*O*-methyl- and 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-mannitol and 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylxylitol were detected in the molar ratios of 4.5:1.3:1.0.

REFERENCES

1 H. TSUCHIHASHI, T. YADOMAE, AND T. MIYAZAKI, *Trans. Mycol. Soc. Jpn*, 23 (1982) 29–35.
2 T. MIYAZAKI AND M. NISHIJIMA, *Chem. Pharm. Bull.*, 29 (1981) 3611–3616.
3 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 81 (1929) 629–679.
4 S.-I. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208
5 J. E. HODGE AND B. T. HOFREITER, *Methods Carbohydr. Chem*, 1 (1962) 389–390.