

**Studies on Fungal Polysaccharide. XII.<sup>1)</sup> Water-soluble Polysaccharide of *Grifora umbellata* (FR.) PILÁT<sup>2)</sup>**

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Water-soluble polysaccharide of polyporus (sclerotium of *Grifora umbellata* (FR.) PILÁT) obtained by Cetavlon fractionation of crude polysaccharide is a glucan,  $[\alpha]_D -25.4^\circ$  ( $c=1$ , H<sub>2</sub>O). The results of periodate oxidation, Smith-type degradation, and methylation studies indicated that the glucan was branching at C-6 or C-3 position of glucose residue and possessing (1→3)-, (1→4)-, and (1→6)-linkages.

Polyporus ("Chorei" in Japanese) is the sclerotium of *Grifora umbellata* (FR.) PILÁT which belongs to Polyporaceae, Basidiomycetes, and is known as a diuretic in Chinese medicine. This fungus was taken up as a series of studies on fungal polysaccharide and its water-soluble polysaccharide was investigated.

Crude polysaccharide (GU-0) extracted from polyporus with hot water was further purified by Cetavlon fractionation.<sup>4)</sup> The purified polysaccharide (GU-1),  $[\alpha]_D -25.4^\circ$  ( $c=1$ , H<sub>2</sub>O), gave a glucose and a small amount of glucuronic acid by acid hydrolysis. GU-1 contained neither nitrogen nor phosphorus. In the infrared (IR) spectrum of GU-1, absorption maximum at 895 cm<sup>-1</sup> suggests a CH bending vibration in the β-glycosidic linkage (Chart 1).

Periodate oxidation<sup>5)</sup> of GU-1 resulted in the consumption of 1.09 mole of periodate with formation of 0.39 mole of formic acid per anhydroglucose unit and no production of formaldehyde.

The molar ratio of Smith-type<sup>6)</sup> degradation products, glucose, erythritol, and glycerol, was 1: 0.47: 0.90.

GU-1 was methylated by the Hakomori method<sup>7)</sup> and methanolysis of the methylated GU-1 was carried out. The presence of methyl 2,3,4,6-tetra-O-methyl-, methyl 2,3,4-tri-O-methyl-, methyl 2,3,6-tri-O-methyl-, and methyl 2,4,6-tri-O-methyl-D-glucopyranosides was confirmed, as shown in Fig. 1, by means of gas-liquid chromatography (GLC) of the methylated monosaccharides derived from the methylated GU-1.

After the methylated GU-1 was hydrolyzed with 90% formic acid and 1N sulfuric acid, the hydrolysate was separated into tetra-O-methyl-, tri-O-methyl-, and di-O-methyl-D-glucoses by means of thin-layer chromatography (TLC). The molar ratio of these glucoses (1.23: 2.63: 1) was estimated by titrimetry of the Willstatter-Shudel method.<sup>8)</sup> The di-O-methyl-D-glucose fraction separated by TLC was identified as 2,4-di-O-methyl-D-glucose by paper electrophoresis.

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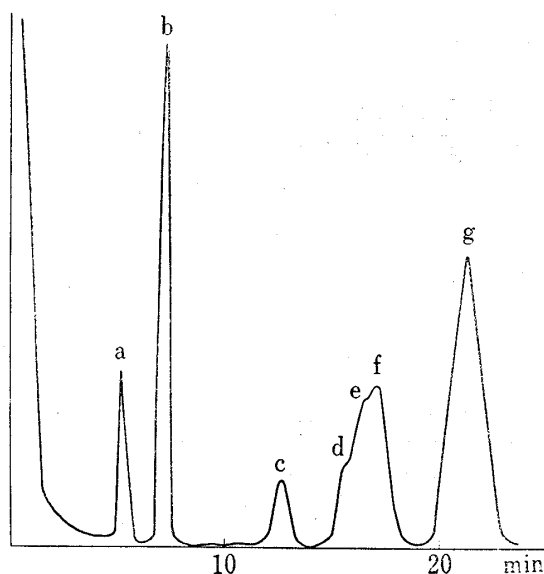


Fig. 1. GLC of Methylated monosaccharides Derived from GU-1

- a : methyl 2,3,4,6-tetra-O-methyl- $\beta$ -  
 b : methyl 2,3,4,6-tetra-O-methyl- $\alpha$ -  
 c : methyl 2,3,4-tri-O-methyl- $\beta$ -  
 d : methyl 2,4,6-tri-O-methyl- $\beta$ -  
 e : methyl 2,3,6-tri-O-methyl- $\beta$ -  
 f : methyl 2,3,4-tri-O-methyl- $\alpha$ -  
 g : methyl 2,3,6-tri-O-methyl- $\alpha$ -  
 methyl 2,4,6-tri-O-methyl- $\alpha$ -

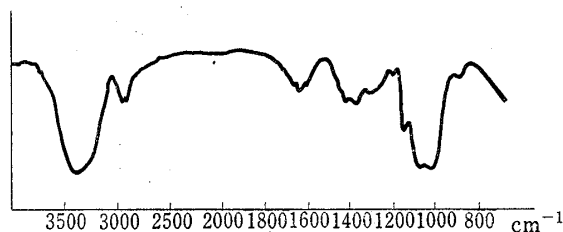


Chart 1. IR Spectrum of GU-1

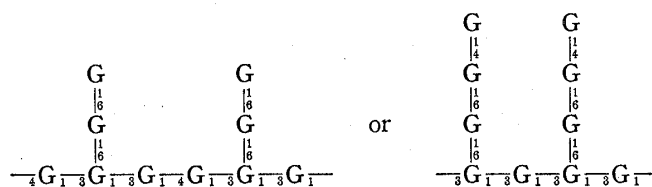


Fig. 2. Most Probable Structure of GU-1

Thus, GU-1 obtained by Cetavlon fractionation<sup>4)</sup> of the extract from sclerotium is thought to mainly consist of  $\beta$ -D-glucose on the basis of specific rotation and IR spectrum (Chart 1).

The results of GLC of the methanolysate and TLC of the hydrolysate from methylated GU-1 showed that GU-1 possessed 1,6-, 1,4-, and 1,3-glycosidic linkages and branched at C-3 or C-6 position of glucose residue.

The structure shown in Fig. 2 has a high possibility as the structure of GU-1 according to the molar ratio of glycerol, erythritol, and glucose obtained by the Smith-type degradation.<sup>6)</sup> Because, if GU-1 possesses one of these structures, theoretical number of moles of periodate consumed and formic acid formed would be 1 and 0.4 mole, respectively, and these values correspond with the experimental number of moles of periodate oxidation<sup>5)</sup> at 48 hr, *i.e.*, 1.09 and 0.39 mole. Further, as to Smith-type degradation<sup>6)</sup> products, the theoretical molar ratio of GU-1 (1:0.5:1) is similar to the experimental data (0.90:0.47:1).

In the proposed structures shown in Fig. 2, arrangement of 1,6-, 1,4-, and 1,3-linkages are changeable with one another, but these structures might be reasonable in the water-solubility<sup>9)</sup> or in consideration of the structure of the polysaccharide II of *Coriolus versicolor*<sup>10)</sup> or extracellular glucan of *Coriolus versicolor*<sup>11)</sup> or Shizophyllan.<sup>12)</sup> Moreover, GU-1 is also similar to the polysaccharide of fraction A of *Lyophyllum ulmarium*<sup>13)</sup> with respect to 1,6-, 1,4-, and 1,3-glycosidic linkages.

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Relationship between the chemical structure and antitumor effect of GU-1 *in vivo* are in progress.

### Experimental

**Isolation of Water-soluble Polysaccharide**—The sclerotium of *Grifora umbellata* (Fr) PILÁT (100 g), which was crushed into small pieces and treated with cold MeOH previously, was further ground in a mortar and soaked in distilled water (500 ml) overnight. The soaked sclerotium was extracted three times with distilled water on a boiling water bath for 2 hr. After each extract was filtered while hot, the filtrate was dialyzed against running water for 2 days and concentrated *in vacuo*. The concentrated filtrate was added with EtOH to give the crude polysaccharide (GU-0) as a brown precipitate. The precipitate was collected by centrifugation, washed with EtOH, acetone and ether and dried *in vacuo*. The yield of GU-0 was 0.75% of the original sclerotium. Sugar content of GU-0 was 77.9% by  $C_6H_5OH-H_2SO_4$  method.<sup>14)</sup>

**Component Sugar of GU-0**—On acid hydrolysis (1N  $H_2SO_4$ , 100°, 5 hr) of GU-0 (*ca.* 10 mg), glucose and small amounts of galactose, mannose, xylose, and glucuronic acid were detected on the filter paper (Toyo Roshi No. 50) using the solvent systems, (A) AcOEt-pyridine- $H_2O$  (10:4:3), and (B) AcOEt-pyridine-AcOH- $H_2O$  (5:5:1:3).  $AgNO_3-NaOH$ <sup>15)</sup> and *p*-anisidine-HCl<sup>16)</sup> were used as the spray reagents.

**Purification of GU-1 by Cetavlon Fractionation**<sup>4)</sup>—After part of GU-0 slightly soluble in  $H_2O$  was removed by centrifugation, GU-0 was dissolved in 0.4% of  $Na_2B_4O_7 \cdot 10H_2O$  solution and 10% of cetyltrimethylammonium bromide was added until no more precipitate was formed. The supernatant fraction from the Cetavlon treatment was precipitated with EtOH, the precipitate was collected, and washed with EtOH several times. The precipitate was dissolved in  $H_2O$ , the solution was acidified weakly with AcOH and dialyzed against running water. The dialysate was concentrated *in vacuo* and the concentrate was precipitated with EtOH. The precipitate was collected, washed with EtOH, acetone and ether and dried *in vacuo*. This process was repeated, until the precipitate (GU-1) gave glucose and a small amount of glucuronic acid on acid hydrolysis.

**Properties of GU-1**—GU-1, which was decationized by Amberlite IR 120 resin ( $H^+$ ) and then dialyzed against distilled water, contained 91.8% of total sugar (by  $C_6H_5OH-H_2SO_4$  method<sup>14)</sup> as glucose) and was free from P (by Fiske-Subbarow method<sup>17)</sup>) and N (elemental analysis). GU-1 showed no coloration with  $I_2$  and showed  $[\alpha]_D -25.4^\circ$  ( $c=1$ ,  $H_2O$ ). IR  $\nu_{max}^{KBr}$   $cm^{-1}$ : 895 ( $\beta$ -glycosidic linkage). Zone electrophoresis of GU-1 gave a single peak using Pevicon C-870 (MS kiki Co., Ltd.), 1% of  $Na_2B_4O_7 \cdot 10H_2O$  solution and detection by  $C_6H_5OH-H_2SO_4$ . Content of D-glucuronic acid, 2.6%, was determined by carbazol- $H_2SO_4$ <sup>18)</sup> after acid hydrolysis (1N  $H_2SO_4$ , 100°, 6 hr) and then paper chromatography using solvent system (A).

**Periodate Oxidation**<sup>9)</sup> of GU-1—GU-1 (19.3 mg) was oxidized in 50 ml of 0.0176M  $NaIO_4$  at room temperature in the dark. A blank solution containing no glucan was processed similarly.  $NaIO_4$  consumption and formation of HCOOH and HCHO were determined by the procedures of Maraprade,<sup>19)</sup> Whistler,<sup>20)</sup> and of O'Dea and Gibbons,<sup>21)</sup> respectively. The number of moles of  $NaIO_4$  consumed per anhydroglucose unit of the polysaccharide was as follows: 0.30 (1 hr), 0.62 (3 hr), 0.76 (6 hr), 0.90 (9 hr), 1.07 (24 hr), 1.09 (48 hr). The corresponding number of moles of HCOOH produced was 0.088 (1 hr), 0.20 (3 hr), 0.24 (6 hr), 0.29 (9 hr), 0.24 (24 hr), 0.39 (48 hr). HCHO was not produced.

**Smith-type Degradation**<sup>9)</sup> of GU-1—Smith-type degradation<sup>9)</sup> of GU-1 (30 mg) was carried out after GU-1 was oxidized for 48 hr as described above. The excess of  $NaIO_4$  was decomposed by the addition of 1.5 ml of ethylene glycol. The reaction mixture was dialyzed against running water overnight, and the internal solution was concentrated to a small volume (*ca.* 5 ml) *in vacuo*. The oxidized GU-1, polyaldehyde, was reduced by stirring with 30 mg of  $NaBH_4$  overnight. After excess of  $NaBH_4$  was destroyed with AcOH, the reaction mixture was dialyzed against running water, the internal solution was concentrated to dryness *in vacuo*, and hydrolyzed with 1N  $H_2SO_4$  on a boiling water bath for 6.5 hr. The hydrolysate was neutralized with  $BaCO_3$  and filtered, the filtrate was concentrated to a small volume *in vacuo* and examined by paper chromatography using the solvent system of AcOEt-pyridine- $H_2O$  (10:4:3). Each area of three spots, *i.e.*, glucose, erythritol, and glycerol, was cut into small pieces and extracted with distilled water. Each extract was determined by the O'Dea-Gibbons method and 293  $\mu g$  of glycerol, 205  $\mu g$  of erythritol, and 636  $\mu g$  of glucose were found.

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**Methylation of GU-1 by the Hakomori Method<sup>7)</sup>**—GU-1 (30 mg) was dissolved in 3.75 ml of Me<sub>2</sub>SO. NaH (100 mg) washed with petr. ether and 6 ml of Me<sub>2</sub>SO were stirred at 65–70° vigorously to produce MeSOCH<sub>2</sub>Na<sup>+</sup>. A mixture of the solution of GU-1 and 2.25 ml of MeSOCH<sub>2</sub>Na<sup>+</sup> solution were stirred for 1 hr and MeI was added. The reaction mixture was stirred overnight and was dialyzed for 24 hr after removal of the excess of MeI *in vacuo*. The internal solution was concentrated to dryness and dried *in vacuo*. These procedures of methylation were repeated three times until no absorption of OH group was confirmed on IR spectrum. The CHCl<sub>3</sub> solution of methylated GU-1 was precipitated with hexane and the precipitate was methanolized with 0.5N MeOH-HCl (2 ml) in a boiling water bath for 10 hr.

**GLC of Methanolysate of Methylated GU-1**—After removal of MeOH and HCl, the methanolysate was dissolved in a minimum amount of Me<sub>2</sub>O. GLC was carried out using a Simadzu DC-IC unit, equipped with a flame ionization detector. Conditions: Column temperature, 172°, detector temperature, 182°, on butane-1,3-diol succinate (BDS) column. N<sub>2</sub> flow rate, 50 ml/min; air pressure, 0.8 kg/cm<sup>2</sup>.

**TLC and Paper Electrophoresis of Hydrolysate of Methylated GU-1**—The methylated GU-1 was hydrolyzed with 90% HCOOH in a boiling water bath for 10 hr and, after removal of HCOOH, the residue was further hydrolyzed with 1N H<sub>2</sub>SO<sub>4</sub> for 4 hr. The hydrolysate was neutralized with BaCO<sub>3</sub>, BaSO<sub>4</sub> was filtered off, and the filtrate was concentrated to a syrup. The methylated monosaccharides in the syrup were separated into tetra-O-methyl-, tri-O-methyl-, and di-O-methyl-D-glucoses by TLC using the solvent system of acetone-benzene (1:1). Each part of separated monosaccharides was extracted with CHCl<sub>3</sub>, acetone, and MeOH. After removal of the extraction solvent, each monosaccharide was dissolved in distilled water and titrated by the Willstätter-Shudel method.<sup>8)</sup> The molar ratio of tetra-, tri-, and di-O-methyl-D-glucoses was 1.23:2.63:1. Paper electrophoresis of the separated di-O-methyl-D-glucose, using 0.5% of Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O solution for 2.5 hr gave 0.05 MG value and it was 2,4-di-O-methyl-D-glucose. Reference<sup>22,23)</sup>: Di-O-methylglucoses have following Mc values; Mc < 0.05 (2,4-), 0.135 (2,3-), 0.28 (3,4-), 0.546 (3,6-), and 0.185 (4,6-).

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