Chemical Heterogeneity of β -D-Glucan in Sclerotia of *Grifora umbellata* (Fr.) Pilát†

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G. umbellata is a fungus, which is parasitic on an animate root of alder and Japanese oak. The fruit body and the sclerotia of the fungus can be used as a food and a herb medicine, respectively. In 1973, Miyazaki and Oikawa¹⁾ proposed a possible structure of a water-soluble polysaccharide from the sclerotia. However, the insoluble residue was found to contain a polysaccharide similar to the water-soluble one, though it can not be extracted with water. This paper is concerned with the structural outline deduced from methylation studies of the polysaccharides extracted successively from the sclerotia.

The sclerotia were collected in Hokkaido, Japan, in the fall of 1975. After being washed with water, they were pulverized and refluxed several times with 80% methanol to remove fatty materials. The defatted sclerotia (50 g) was extracted with water (600 ml) in an autoclave (1 kg/cm²) for 1 hr. The extraction was repeated until the supernatant showed negative α -naphthol reaction (Molisch test).

Four volumes of methanol was added to the supernatant to precipitate the polysaccharide, which was washed with methanol and ether, and dried *in vacuo* after being reprecipitated three times (F-1).

The residue was then extracted similarly in an autoclave with 10% aqueous zinc chloride. The Molisch-positive supernatants were combined, and dialyzed against running water for 4 days. A greyish precipitate formed during the dialysis was collected by centrifugation (F-2).

On addition of four volumes of methanol to the dialyzed solution, milk-white precipitate was obtained. It was washed with methanol and ether, and dried *in vacuo* after being reprecipitated three times (F-3).

The residue was thoroughly washed with water to

remove remaining zinc chloride and was extracted by stirring with 10% sodium hydroxide containing 5% urea for 24 hr at 5°C. The Molisch-positive supernatants were combined and neutralized with hydrogen chloride under cooling. Light-brown precipitate deposited was deionized by dialysis, washed with methanol and ether, and dried *in vacuo* (F-4).

A polysaccharide which was not precipitated by neutralization was recovered by addition of four volumes of methanol. It was washed with methanol and ether, and dried *in vacuo* after being reprecipitated three times (F-5).

A part of each fraction was dissolved in 100 parts of 1% sodium hydroxide, and to this solution 7% aqueous copper sulfate was added dropwise with stirring. The resulting copper-complex was collected by centrifugation and regenerated into the polysaccharide by the usual method (yield, 90~92%). Since F-2 was remarkably different from other fractions in total sugar content as well as in IR spectrum, no attempt was made to characterize the polysaccharide in F-2. However, other fractions showed the similar IR spectra, suggesting the occurrence of β -glycosidic linkage. On complete acid hydrolysis, these fractions yielded D-glucose and small quantities of D-galactose, D-mannose and unidentified sugars. Table I shows some properties of the fractions.

Partially purified polysaccharide (500 mg) was immersed in formamide (14 ml) and acetylated with acetic anhydride (4 ml) and pyridine (6 ml) in the usual way. The acetyl derivative thus obtained was stirred with acetone (200 ml) at room temperature for 1 hr, and then centrifuged. The supernatant was concentrated to about 50 ml and poured into water (500 ml). Resulting precipitate was collected by centrifugation, washed with methanol and ether, and dried *in vacuo*. The acetate which was insoluble in acetone was also collected by centrifugation. The ratios of acetone-soluble acetate to -insoluble one were 1.19 in F-1, ≈ 0 in F-3, 0.39 in F-4 and 0.28 in F-5, respectively. Thus, the acetate was composed of two components, but it was difficult to find significant differences in the IR spectra of both components.

Acetone-soluble and -insoluble acetates (*ca.* 100 mg) were individually dissolved in dimethylsulfoxide and methylated by the method of Hakomori.²⁾ The methylated polymer was extracted with chloroform and the extract was evaporated to dryness. The final product was dissolved in tetrahydrofuran and added slowly to three volumes of petroleum ether to yield a precipitate which was dried *in vacuo* (yield 40~50%). The methylated polysaccharide (5~10 mg) was treated with 70% sulfuric acid (0.8 ml) for 11 hr at room temperature, and then refluxed for 7 hr after being diluted to 8 ml. Partially methylated monosaccharides recovered in the usual way were converted into the corresponding alditol acetates by the usual method and

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TABLE I. SOME PROPERTIES OF EACH POLYSACCHARIDE-FRACTION EXTRACTED FROM THE SCLEROTIA

Fraction	Yield ^a (g)	N (%)	Sugar ^b content (%)	Constitutional sugar ^c			[α] _D ^d
				D-Glu	D-Gal	D-Man	
F-1	1.40	0.64	66	100	1	2	—
F-2	5.50	0.17	29	—	—	—	-16°
F-3	0.85	0.08	58	100	1	4	+14°
F-4	2.70	0.46	52	100	—	10	+32°
F-5	20.00	0.15	78	100	1	9	+40°

^a From 50 g of the defatted sclerotia.^b Determined as D-glucose after partial purification *via* Cu-complex.^c Determined as trimethylsilyl derivatives by gas chromatography.^d Determined with 0.5% solution in 5% aqueous sodium hydroxide.

TABLE II. GAS CHROMATOGRAPHY OF ALDITOL ACETATES DERIVED FROM THE METHYLATED POLYSACCHARIDES

Alditol acetate of	Relative retention time ^a	Molar ratio ^b			
		F-1	F-3	F-4	F-5
2,3,4,6-Tetra-O-methyl-D-glucose	1.00 (1.00)	1.00	1.00	1.00 (1.00)	1.00 (1.00)
2,4,6-Tri-O-methyl-D-glucose	1.80 (1.95)	1.70	1.98	34.90 (7.80)	1.85 (1.44)
2,3,4-Tri-O-methyl-D-glucose	2.24 (2.49)	0.48	0.17	2.04 (1.48)	0.18 (0.28)
2,3,6-Tri-O-methyl-D-glucose	— (2.50)	+	—	+(+)	+(-)
2,4-Di-O-methyl-D-glucose	4.23 (5.10)	0.86	0.91	0.46 (0.71)	0.86 (0.91)

^a Values when analyzed on a 3 m column of 3% ECNSS-M at 180°C; Numeral in parenthesis is reference value (B. Lindberg, "Methods in Enzymology," Vol. 28, ed. by V. Ginsburg, Academic Press, New York, 1972, p. 178).^b Numeral in parenthesis is what obtained from the acetone-insoluble acetate. A mark of + stands for being detected in a small quantity.

then submitted to a combined gas chromatograph-mass spectrometer, Hitachi RMU-6MG. The compound was separated on an ECNSS-M column at 170°C and identified by the mass spectrum.

In some fractions, the mass spectrum of a peak having a retention time of 2.24 suggested contamination of a small quantity of 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol in 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol; these compounds have almost the same retention times on the column. In such a case, presence of such compound was consequently confirmed by gas chromatography using a column of NPGA as methyl glycoside after a part of the hydrolyzate was treated with methanolic hydrogen chloride. The results are summarized in Table II.

It is possible to conclude from the present results that the common structural features of the polysaccharides in the sclerotia are the back-bone mainly composed of β -(1 $\rightarrow$ 3)-linked D-glucose residues and β -(1 $\rightarrow$ 6)-linkages occurring both at branch point and in linear part. However, it is not clear whether linear β -(1 $\rightarrow$ 6)-linkage is present in the main chain or in the side chain. In addition, presence of a polysaccharide containing (1 $\rightarrow$ 4)-linkage in the sclerotia is also suggested, though the amount of such linkage is assumed

to be very small.

Interestingly, the polysaccharide (F-4) which was extracted with sodium hydroxide and precipitated by neutralization has a long average chain length and a large amount of (1 $\rightarrow$ 6)-linkage as compared with other polysaccharides. On the other hand, the polysaccharide (F-5) which was not precipitated by neutralization seems to have a similar structure to that of the water-soluble polysaccharide (F-1). It appears, therefore, that chain length and proportion of (1 $\rightarrow$ 6)-linkage might be related to the conformation and consequently to the solubility of the β -(1 $\rightarrow$ 3)-D-glucan in water. However, with respect to the solubility of the acetate in acetone, F-1 and -5 are remarkably different from each other. From the present results, it is not clear what factor actuated the acetate to become insoluble in acetone.

Detailed structure of the polysaccharide is under investigation.

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