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Fungal Polysaccharides. II.[1] Chemical Structure of the Polysaccharide from *Lampteromyces japonicus*

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The results of periodate oxidation, Smith-type degradation and methylation studies showed that the purified neutral polysaccharide from *Lampteromyces japonicus* (the component sugars; D-Man; 46%, D-Glc; 30%, D-Gal; 24%) contained mainly 1,6- and 1,2-linked mannopyranosyl residues, 1,3-linked glucopyranosyl residues and minorly 1,6-linked galactopyranosyl residues, and this glycan was branching mainly at C-3,6 position of glucose residues.

In recent years, it has been reported that the extracellular polysaccharides of the fungi belonging to Mucorales[3] and Imperfect fungi,[4] which are well known as pathogenic fungi, are heteroglycans. Besides, a heteroglycan has been isolated from *Tremella fuciformis*,[5] and the polysaccharides containing several kinds of sugars have been obtained from *Flammulina velutipes*[6] and *Pleurotus ostreatus*.[7]

In a previous work,[1] a polysaccharide was isolated from the fungi, *Lampteromyces japonicus*, and purified by deproteinization, removal of nucleic acid and diethylaminoethyl (DEAE) cellulose column chromatography. The purified neutral polysaccharide, which was eluted with water on DEAE cellulose, was composed of D-mannose, D-glucose and D-galactose (46%: 30%: 24%) and did not contain nitrogen, sulfur and phosphorus.

In this paper, the chemical structure of the neutral polysaccharide was examined by periodate oxidation, Smith-type degradation and methylation studies.

Results and Discussion

On periodate oxidation of the neutral polysaccharide, the amounts of periodate consumed and formic acid and formaldehyde liberated were 1.15, 0.48 and 0 mole per sugar unit, respectively.

Smith-type degradation[8] products were glycerol and oxidation-resistant glucose (the molar ratio, approximately 3.5: 1). Erythritol and oxidation-resistant sugar components other than glucose were negligible. These results suggest, at least, the presence of 1,2- and/or 1,6-linked hexopyranosyl residues and terminal hexopyranosyl residues (source of glycerol) and oxidation-resistant glucose residue such as 1,3-linked glucopyranosyl residue in the polysaccharide.

After the polysaccharide was methylated by the method of Hakomori,[9] hydrolysis was carried out with formic acid and successively with sulfuric acid. The O-methyl-monosac-

1) Part I: K. Fukuda, T. Uematsu, A. Hamada, S. Akiya, N. Komatsu, and S. Okubo, *Chem. Pharm. Bull.* (Tokyo), **23**, 1955 (1975).
2) Location: *Hatanodai, 1-5-8, Shinagawa-ku, Tokyo.*
3) T. Miyazaki and T. Irino, *Chem. Pharm. Bull.* (Tokyo), **20**, 330 (1972); *idem, ibid*, **19**, 1450 (1971).
4) T. Miyazaki and Y. Naoi, *Chem. Pharm. Bull.* (Tokyo), **22**, 1360 (1974); *idem, ibid.*, **23**, 157 (1975); *idem, ibid.*, **23**, 1752 (1975).
5) S. Ukai, K. Hirose, T. Kiho, and C. Hara, *Chem. Pharm. Bull.* (Tokyo), **22**, 1102 (1974).
6) Y. Yoshioka, T. Sano, and T. Ikekawa, *Chem. Pharm. Bull.* (Tokyo), **21**, 1772 (1973).
7) Y. Yoshioka, T. Ikekawa, M. Noda, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **20**, 1175 (1972).
8) J.K. Hamilton and F. Smith, *J. Am. Chem. Soc.*, **78**, 5907 (1956).
9) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

charides formed were converted into alditol acetates and analyzed by the gas-liquid chromatography (GLC).[10] As shown in Fig. 1, the main products detected were 2,3,4,6-tetra-O-methyl-D-glucose or -D-mannose derivatives (I) (peak 1), 2,4,6-tri-O-methyl-D-glucose (III) or 3,4,6-tri-O-methyl-D-glucose or -D-mannose derivatives (IV) (peak 3), 2,3,4-tri-O-methyl-D-glucose or -D-mannose derivatives (V) (peak 4), 2,3,4-tri-O-methyl-D-galactose derivative (VI) (peak 5) and 2,4-di-O-methyl-D-glucose derivative (VII) (peak 6). In addition, 2,3,4,6-tetra-O-methyl-D-galactose derivative (II) (peak 2) and 3,4-di-O-methyl-D-galactose derivative (VIII) (peak 7) were also detected, though they were little in amount. The peak area ratios and the relative retention times are summarized in Table I. The retention times were consistent with those of authentic samples or the values in the literature.[10]

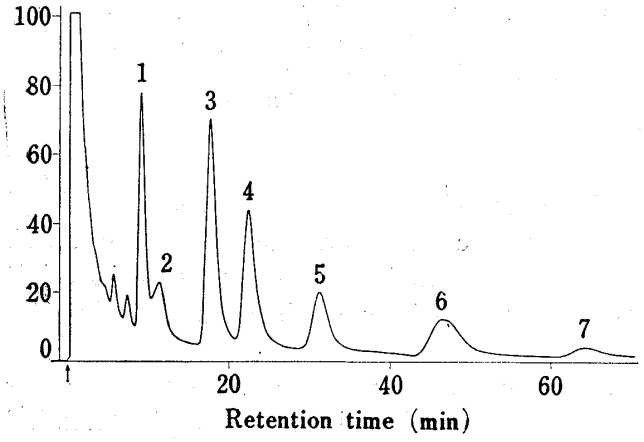

Fig. 1. Gas-Liquid Chromatography of O-Methylated Alditol Acetates obtained from the Methylated Polysaccharide

Each peak was explained in Table I. The conditions are described in the Experimental.

Since the two tri-O-methyl-D-hexitol derivatives in peak 3, 2,4,6-tri-O-methyl (III) and 3,4,6-tri-O-methyl-derivatives (IV), were not separated by GLC, gas chromatography-mass spectrometry (GC-mass)[11] was performed to distinguish between III and IV. The mass-spectrum of peak 3 (*m/e*; 43, 45, 87, 99, 101, 117, 129, 161, 189) exhibits the presence of both tri-O-methyl derivatives, because several characteristic fragments were found, *m/e* 117 for III and *m/e* 99 and 189 for IV.[11] Though the molar ratio of the two components in peak 3 are not determined by GC-mass, the results of periodate oxidation and Smith-type degradation suggest

Table I. Relative Retention Time of O-Methylated Alditol Acetates obtained from the Methylated Polysaccharide.

Peak number	Component (alditol acetate derivative)	Relative retention time	Ratio of peak area (%)
1	2,3,4,6-tetra-O-Me-D-Glc or -D-Man (I)	1.00	17.3
2	2,3,4,6-tetra-O-Me-D-Gal (II)	1.26	6.2
3	2,4,6-tri-O-Me-D-Glc (III) 3,4,6-tri-O-Me-D-Glc or -D-Man (IV)	1.93	28.0
4	2,3,4-tri-O-Me-D-Glc or -D-Man (V)	2.44	21.0
5	2,3,4-tri-O-Me-D-Gal (VI)	3.40	10.9
6	2,4-di-O-Me-D-Glc (VII)	5.08	13.3
7	3,4-di-O-Me-D-Gal (VIII)	7.00	3.3

that III and IV are in the ratio approximately 3:4. The products corresponding to peak 1, 2, 4 and 5 were also confirmed by GC-mass (peak 1, 2; *m/e*: 43, 45, 71, 87, 101, 117, 129, 145, 161, 205, peak 4, 5; *m/e*: 43, 87, 99, 101, 117, 129, 161, 189) and these mass spectra were coincident with those of authentic samples.

By GC-mass it can not be decided whether parent sugar of the product I, IV or V was D-mannose or D-glucose. To get some information about the parent sugar of I produced

10) B. Lindberg, "Methods in Enzymology," Vol. XXVIII, ed. by V. Ginsburg, Academic Press, New York and London, 1972, p. 182.
11) H. Björndal, B. Lindberg, and S. Svensson, *Carbohyd. Res.*, **5**, 433 (1967).

from the non-reducing terminal residues, determination of permethylated glycosides was attempted by GLC. When authentic samples of methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside and methyl 2,3,4,6-tetra-O-methyl-α-D-mannopyranoside were applied on GLC analysis using a column of NPGS at 150°, no separation was observed.[12] On another liquid phase, ECNSS-M, the two permethylated glycosides were resolved at 100°, but not at 150° or 180°. On the best condition, therefore, permethylated glycoside mixture produced by methanolysis of the fully methylated polysaccharide was examined by GLC, indicating that the non-reducing terminal residues corresponding to peak 1 contained not only D-mannose but also D-glucose, and the amounts of the two hexoses might be almost the same. The chart is shown in Fig. 2. As shown in Table I, D-galactose was also confirmed to be one of the non-reducing terminal residues and comprised about 25% of all the non-reducing terminal residues. Therefore, the molar ratio of D-mannose, D-glucose and D-galactose in the non-reducing terminal residues was nearly 3:3:2.

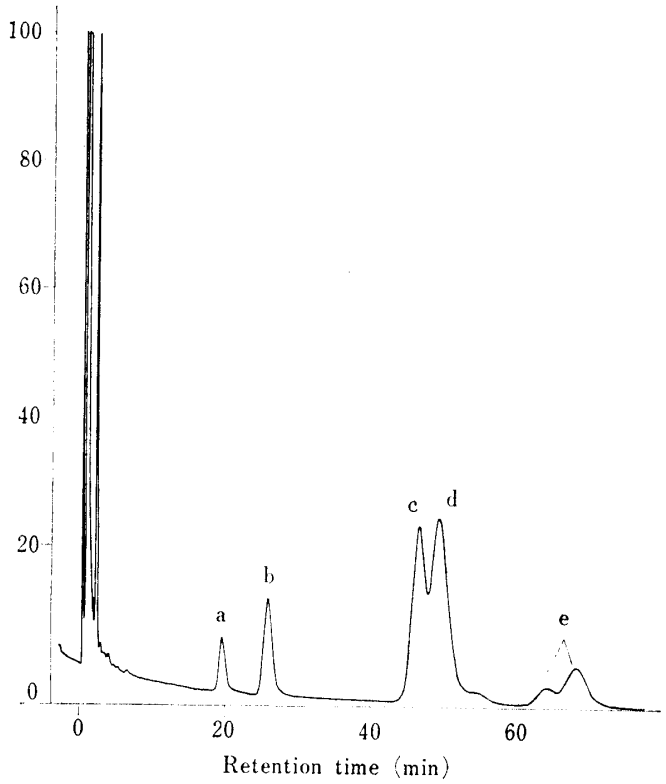

Fig. 2. Gas-Liquid Chromatography of Permethylated Glycosides produced from the Non-reducing Terminal Residues

The condition are described in the Experimental.
peak a : unknown
peak b : methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside
peak c : methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside
peak d : methyl 2,3,4,6-tetra-O-methyl-α-D-mannopyranoside
peak e : methyl 2,3,4,6-tetra-O-methyl-α-and-β-D-galactopyranoside

On the other hand, D-glucose residue, which comprises about 30% of hexoses,[1] accounts for I, III and VII, so that sugar in IV and V must be almost D-mannose. D-Galactopyranosyl derivatives found were VI, II and VIII, though last two derivatives were little in amount.

From these results, it is concluded that the neutral polysaccharide from *L. japonicus* contains mainly 1,6- and 1,2-linked mannopyranosyl residues, 1,3-linked glucopyranosyl residues and 1,6-linked galactopyranosyl residues, including a branched structure which contains mainly 1,3,6-linked glucopyranosyl residues.

Further it is required to elucidate the main core and branching chain of the polysaccharide by partial acid hydrolysis. These points will be presented in the series.

Experimental

Periodate Oxidation——The purified polysaccharide from *L. japonicus* (16.5 mg) was oxidized in 50 ml of 0.018 M $NaIO_4$ at room temperature in the dark. A blank solution containing no polysaccharide was processed similarly. An aliquot was taken at different periods, and $NaIO_4$ consumption was determined by ultra violet absorption method.[13] The number of moles of $NaIO_4$ consumed per hexose unit of polysaccharide was as follows: 1.15 (24 hr), 1.15 (30 hr), 1.36 (7 days). The formation of HCOOH was determined by titration with 0.01 N NaOH, after excess $NaIO_4$ was decomposed by ethylene glycol. The value of HCOOH per

12) M. Tomoda, S. Nakatsuka, M. Tamai, and M. Nagata, *Chem. Pharm. Bull.* (Tokyo), **21**, 2667 (1973).
13) G.O. Aspinall and R.J.Ferrier, *Chem. Ind.* (London), **1956**, 1216.

hexose unit: 0.48 (24 hr), 0.48 (30 hr), 0.53 (7 days). The formation of HCHO was determined by the acetyl acetone method.[14] The value for HCHO was as same as blank.

Smith-type Degradation of Periodate-Oxidized Polysaccharide[8]——The polysaccharide (22.0 mg) was oxidized for 48 hr as described above, excess $NaIO_4$ was decomposed by the addition of 1 ml of ethylene glycol. The reaction mixture was dialyzed in a Visking cellulose tube against a tap water for 17 hr, the internal solution was concentrated to 5 ml *in vacuo*, and the resultant polyaldehyde was reduced by stirring with 30 mg of $NaBH_4$ at room temperature for 17 hr. Excess of $NaBH_4$ was decomposed with AcOH. The reaction mixture was dialyzed in a Visking cellulose tube against a tap water, the internal solution was concentrated, and the residue was heated with 1 N H_2SO_4 (4 ml) at 100° for 5 hr. The hydrolysate was neutralized with $BaCO_3$, $BaSO_4$ formed was removed by filtration, the filtrate was concentrated and reduced with $NaBH_4$. After addition of AcOH, treatment with Amberlite MB-3 and concentration, boric acid was codistilled with methonal. The product was treated with acetic anhydride–pyridine, 1:1 (2 ml), at 100° for 2 hr. The acetylation mixture was first diluted with water, concentrated to dryness, dissolved in acetone and injected into the column of the gas-liquid chromatograph.

GLC was carried out using a HITACHI K-53 model with nitrogen as carrier gas (2 kg/cm²) on a stainless steel column (2 m × 0.3 cm) containing 3% of ECNSS-M on Gas Chrom Q (100-120 mesh), at the gradient temperature, 150°—200° (2°/min, after it arrived at 200°, it was maintained at isothermal temp.). The retention times of the standards, glycerol acetate, erythritol acetate, mannitol acetate and glucitol acetate, were 6.0, 17.0, 55.0 and 67.0 min. The products obtained by Smith type degradation showed two peaks corresponding to glycerol and glucose. The ratio of the peak area was approximately 3:1 (glycerol:glucose). Response factors estimated by the peak area of glycerol and glucose per mole were approximately 0.87:1.00. Accordingly the molar ratio was approximately 3.5:1 (glycerol:glucose).

The Methylation of the Polysaccharide——The methylation of the polysaccharide was carried out by Hakomori's method.[9] To obtain a methylsulfinyl carbanion solution, NaH (0.3 g) which washed with heptan was mixed with 5 ml of $(CH_3)_2SO$ and the mixture was heated at 50° for 45 min with stirring. The polysaccharide (200 mg) was dissolved with 15 ml of $(CH_3)_2SO$ at 60° for 1 hr with stirring. To this solution was added a methylsulfinyl carbanion solution (4 ml) with stirring at room temperature. All the procedures were carried out in nitrogen atmosphere. After 4 hr, 0.6 ml of CH_3I was added dropwise to the reaction mixture with stirring at room temperature and the mixture was stirred overnight. The methylation product was dialyzed with a tap water overnight, extracted with $CHCl_3$ and evaporated to dryness. Methylated polysaccharide showed no significant absorption band of OH in the 3500 cm^{-1} region in its IR spectrum in nujol.

GLC and GC-mass of O-Acetyl-O-methyl Alditols obtained from the Methylated Polysaccharide——The methylated polysaccharide (*ca.* 5 mg) was heated with 90% HCOOH (6 ml) in a boiling water bath for 5 hr. HCOOH was distilled off and the residue was further hydrolysed with 1 N H_2SO_4 for 16 hr at 100° in a sealed tube. The reaction mixture was neutralized with $BaCO_3$, and after passing through in a small column of Amberlite MB-3, the clear filtrate was concentrated. Then the mixture of O-methyl sugars was reduced with $NaBH_4$ for 2 hr. After addition of AcOH, treatment with Amberlite MB-3 and concentration, boric acid was removed by codistillation with MeOH and the product was acetylated with acetic anhydride–pyridine, 1:1, at room temprature overnight. The acetylation mixture was diluted with water and concentrated to dryness and dissolved in acetone. GLC was carried out using a HITACHI K-53 model at the condition of gas flow rate of helium 2 kg/cm², H_2 1 kg/cm², air 1.5 kg/cm² on a stainless steel column (2 m × 3 mm) containing 3% of ECNSS-M on Gas Chrom Q (100—120 mesh) at column temp.; 180°, injected temp.; 240°. The result is shown in Fig. 1 and Table I. The retention times of authentic samples, 1,5-di-O-acetyl-2,3,4,6 tetra-O-methyl glucitol, 1,3,5-tri-O-acetyl-2,4,6-tri-O-methyl glucitol and 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl mannitol, were 9.2, 17.7 and 22.4 min (relative retention time, 1.00; 1.93; 2.44).

GC-mass spectra were determined with JEOL JMS-OISG-2 model, equipped with a stainless steel column containing 3% ECNSS-M on Gas Chrom Q (100—120 mesh). Authentic samples, 1,5-di-O-acetyl 2,3,4,6-tetra-O-methyl glucitol: *m/e* 43, 45, 71, 87, 101, 117, 129, 145, 161, 205; 1,5,6,-tri-O-acetyl-2,3,4,-tri-O-methyl mannitol: *m/e* 43,87, 99, 101, 117, 129, 161, 189.

GLC of Methyl O-Methylated Glycosides——The methylated polysaccharide (3 mg) was converted into methyl glycosides by heating with 5% MeOH–HCl (3 ml) in a sealed tube for 5 hr at 100°. The permethylated glycosides which were produced from the non-reducing terminal residues, were determined by GLC using a Shimazu GC-4CM model at the following conditions, N_2; 1.2 kg/cm²(*ca.*70 ml/min), H_2; 0.6 kg/cm², air; 1.0 kg/cm², column temp; 100°, injected temp; 230°, on a glass column (2 m × 3 mm) packed with 3% ECNSS-M. Authentic samples; methyl-2,3,4,6-tetra-O-methyl-β-D-glucoside (26.0 min), -α-D-glucoside (46.5 min), methyl-2,3,4,6-tetra-O methyl-α-D-mannoside (49.5 min).

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14) T. Nash, *Biochem. J.*, 55, 416 (1953).