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Polysaccharides in Fungi. VI.[1] The Locations of the *O*-Acetyl Groups in Acidic Polysaccharides of *Tremella fuciformis* Berk.[2]

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The locations of *O*-acetyl groups in the acidic polysaccharides AC and BC isolated from the fruit bodies of *Tremella fuciformis* Berk were determined by employing an improved procedure. The results indicated that the *O*-acetyl groups in AC and BC are located at positions 4, 6, and both 4 and 6 of a part of the mannose residues, and at positions 2, 4, and both 2 and 4 of a part of the glucuronic acid residues. AC and BC differ significantly in that the amount of the *O*-acetyl groups linked to the mannose residues in BC was higher than that in AC.

Our improved procedure should be widely applicable for determining the locations of *O*-acyl groups on acidic polysaccharides.

Keywords——*Tremella fuciformis* Berk; polysaccharide; location of *O*-acetyl groups; *O*-acetyl groups on glucuronic acid residues; β-elimination; reduction and methylation analysis; structural features of acidic polysaccharides

In the previous papers[3] of this series, we have reported the structural features of the acidic polysaccharides AC and BC prepared from aqueous extract of the fruit bodies of *Tremella fuciformis* Berk. The basic structures are quite similar to each other, though one of the main differences between AC and BC is in *O*-acetyl content (AC: 5.5%; BC: 9.9%).[3a]

On the other hand, the previous papers[3b,c] indicated that acidic polysaccharides from *Tremella* and *Cryptococcus* species are similar in structure, and showed some chemical taxonomic relationship between the two species in the cross reaction with antisera to type II pneumococcal capsular polysaccharide. These polysaccharides contained *O*-acetyl groups, though the positions are still unkown. Knowledge of the locations of *O*-acetyl groups in these polysaccharides would be useful in connection with the chemotaxonomy of these fungi. The present work was undertaken to elucidate the locations of the *O*-acetyl groups in our acidic polysaccharides.

The locations of *O*-acetyl groups in acidic polysaccharides had been studied in a few polysaccharides from *Klebsiella* species by Lindberg *et al.*[4] and Bebault *et al.*[5] We have attempted to elucidate the locations of *O*-acetyl groups in our polysaccharides AC and BC according to the procedures of these workers,[4,5] but failed to obtain satisfactory results. Further, Bhattacharjee *et al.*[6] reported that a similar study of the acidic polysaccharide from *Cryptococcus neoformans* was unsuccessful. The reason for the failure may be attributed to β-elimination of uronic acid residues,[7] which occurred under basic conditions (*e.g.* in the presence of dimsyl or methoxide anion) in the methylation of the acetated polysaccharide derivatives. In order to avoid the β-elimination, we carried out carboxyl-reduction of the acetalated polysaccharide derivatives prior to the methylation. We were then able to determine the locations of the *O*-acetyl groups in AC and BC as follows. The free hydroxyl groups were protected as acetals with methyl vinyl ether in the presence of *p*-toluenesulfonic acid in dimethyl sulfoxide,[8] and the carboxyl groups were converted into the methyl esters by treatment with diazomethane. The products (AC-1 and BC-1) were purified by gel filtration on Sephadex LH-20, followed by methyl ester-reduction and deacetylation with lithium aluminium hydride in tetrahydrofuran. The acetalated neutral polysaccharides (AC-2 and BC-2) were methylated by the method of Hakomori,[9] fractionated on a column of silica gel to isolate methylated neutral polysaccharides (AC-3 and BC-3), and then hydrolyzed. The hydrolysates were analyzed as alditol acetates by gas–liquid chromatography (GLC) and gas-liquid chromatography-

Table I. Relative Retention Times on GLC and Prominent Peaks (*m/e*) in MS of Methylated Alditol Acetates

Peak number	Sugar[a] (as alditol acetate)	Relative retention time[b]	Prominent peaks (*m/e*)	Molar ratios AC	Molar ratios BC
I	2,4,6-Me–Glc	1.86	43, 45, 87, 101, 117, 129, 161	0.3	0.5
II	Fuc	2.18	43, 99, 103, 115, 128, 145, 157, 170, 187	0.7	0.4
III	4,6-Me–Man	3.07	43, 45, 87, 101, 129, 161, 261	2.7	5.5
IV	2,6-Me–Glc	3.42	43, 45, 87, 117, 129	1.9	1.3
V	4,6-Me–Glc	3.61	43, 45, 87, 101, 129, 161, 261	3.1	3.3
VI	6-Me–Man	4.10	43, 45, 87, 115, 129	6.5	10.5
VII	Xyl	4.50	43, 85, 103, 115, 128, 145, 158, 187, 217	10.0	10.0
VIII	6-Me–Glc	5.18	43, 45, 87, 115, 129	3.8	3.0
IX	4-Me–Man	8.06	43, 85, 87, 99, 127, 129, 189, 261	3.1	4.4
X	Man	8.78	43, 85, 97, 103, 115, 128, 139, 145	21.7	15.0
XI	Glc	11.60	157, 170, 187, 217, 259, 289	2.5	0.7
	Unkown	2.62		—[c]	—[c]

a) 2,4,6-Me-Glc=2,4,6-tri-*O*-methyl-D-glucose, *etc.*
b) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol
c) The intensity of the peak was very weak.

mass spectrometry (GLC–MS). The alditol acetates were identified by comparing the retention times in GLC and the mass spectra with those of authentic samples or with the values in the literature.[10] Table I shows the results of the methylation analysis.

The glucuronic acid residues which were substitiuted wth *O*-acetyl groups should be detected as the 6-*O*-methyl-D-glucose derivative (VIII), and the identification of 2,4,6-tri-*O*-methyl-D-glucose (I), 2,6-di-*O*-methyl-D-glucose (IV) and 4,6-di-*O*-methyl-D-glucose (V) indicated that *O*-acetyl groups were located on positions both 2 and 4, position 2, and posituion 4 of the glucuronic acid residues in AC and BC. The substitution of *O*-acetyl groups at positions both 2 and 4, and 4 of the glucuronic acid residues now explains the detection of glucuronic acid and erythrono-γ-lactone among the Smith degradation products of the native polysaccharides AC and BC in the previous paper.[3b] The identification of 4,6-di-*O*-methyl-D-mannose (III), 6-*O*-methyl-D-mannose (VI) and 4-*O*-methyl-D-mannose (IX) indicated that positions both 4 and 6, position 6, and position 4 of the mannose residues were *O*-acetylated. The percentages of the total mannose residues were approximately 8, 19 and 9% in AC, and 15, 30 and 12% in BC, respectively. The absence of the methylated xylose derivative indicated that none of the xylose residues were substituted with *O*-acetyl groups, and the peaks of non-methylated glucose derivative (XI) and fucose derivative (II) presumably arose from glucose and fucose as minor component sugars of AC and BC.

The conclusions to be drawn from these results are as follows. The locations of *O*-acetyl groups in AC and BC are essentially the same. The groups are linked to position 4, position 6, and positions both 4 and 6 of some of the mannose residues, and to position 2, position 4, and positions both 2 and 4 (a small amount) of some of the glucuronic acid residues. Furthermore, the content of the mannose residues containing *O*-acetyl groups in BC is higher than that in AC.

From the structural features previously proposed and the present results, a possible repeating unit of BC which includes *O*-acetyl groups is shown in Chart 1; the unit of AC is somewhat different from that of BC in that the amounts of the *O*-acetyl groups located at position 6, and positions both 4 and 6 of mannose residues in AC are less than those in BC.

In this manner, we succeeded in elucidating the locations of the *O*-acetyl groups on glucuronic acid and other sugar residues of acidic polysaccharides without the occurrence of β-elimination. The glucuronic acid residues were converted into glucose residues by carboxyl-reduction and identified as the 6-*O*-methyl glucose derivatives by subsequent methylation analysis.

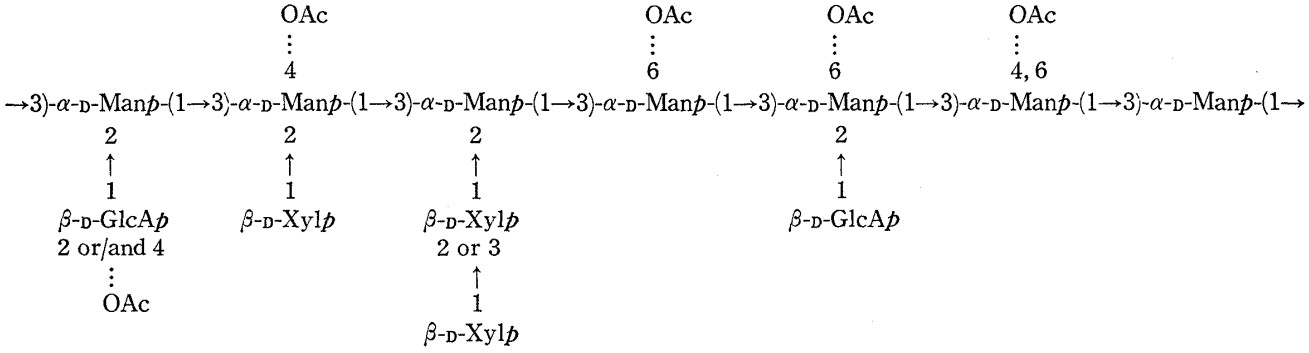

Chart 1. A Possible Repeating Unit of BC

Our improved procedure should be applicable for determining the locations of *O*-acetyl groups in acidic polysaccharides composed of uronic acid and other sugar residues, except for the aldose corresponding to the carboxyl-reduced sugar derived from the uronic acid. However if lithium aluminium deuteride is used in the carboxyl-reduction instead of lithium aluminium hydride, our procedure would also be useful for studies of a variety of acidic polysaccharides, since the aldose could be discrminated from the deuterated aldose (C-6) obtained by the carboxyl-reduction with lithium aluminium deuteride by GLC–MS analysis. Work on the modified procedure is in progress.

Experimental

Infrared (IR) spectra were recorded on a JASCO IRA-1 spectrometer. GLC was carried out on a JEOL JGC-1100 gas chromatograph equipped with a hydrogen flame ionization detecter. GLC–MS was performed with a JEOL JMS-D 300 gas chromatograph and mass spectrometer.

Treatment with Methyl Vinyl Ether and Diazomethane——Each sample (AC a nd BC) (20 mg) was dissolved in dimethyl sulfoxide (7 ml), and then *p*-toluenesulfonic acid (10 mg) and methyl vinyl ether (condensed at −10°, 2 ml) were added. The mixture was stirred for 4 hr at 15°, then excess methyl vinyl ether was evaporated off under reduced pressure, and diazomethane in anhydrous ether (10 ml) was added. The reaction mixture was stirred for 2 hr at room temperature, and then volatile compounds were evaporated off under reduced pressure. The resulting mixture was fractionated on a column (2.4 × 30 cm) of Sephadex LH-20 equilibrated with anhydrous acetone. The eluate corresponding to the first yellow band was collected and concentrated to dryness. The final product showed no hydroxyl absorption band in the IR spectrum.

Deacetylation and Reduction of the *O*-Acetyl-*O*-(1-methoxyethyl) Polysaccharide Methyl Ester (AC-1 and BC-1)——A solution of each product (AC-1 and BC-1) (35 mg) in tetrahydrofuran (5 ml) was treated with a 1% suspension of $LiAlH_4$ in tetrahydrofuran (10 ml), and then the mixture was stirred for 1 hr at room temperature, followed by refluxing for 3 hr. The excess hydride was destroyed by dropwise addition of H_2O with stirring in a cold water bath. The mixture was filtered and the residue was extracted with MeOH and $CHCl_3$. The filtrate and combined extracts were evaporated to dryness. In the IR spectrum, the product showed hydroxyl absorption, but no carboxyl absorption band.

Methylation of the *O*-(1-Methoxyethyl) Neutral Polysaccharide (AC-2 and BC-2)——Methylation of each product (AC-2 and BC-2) (15 mg) was accomplished by the method of Hakomori[9] as described in our previous paper.[3b] The partially acetalated, partially methylated neutral polysaccharide was purified on a column (2.4 × 30 cm) of silica gel (Wako gel C-200). The column was eluted with $CHCl_3$–EtOAc–EtOH (45: 4: 1), and then $CHCl_3$–EtOH (9: 1). The eluate with the second solvent was tested by the Molisch reaction,[11] and was collected and evaporated to dryness. No absorption near 3400 cm^{-1} was observed in the IR spectrum.

Analysis of the *O*-Methyl-*O*-(1-methoxyethyl) Neutral Polysaccharide (AC-3 and BC-3)——A part of each product (AC-3 and BC-3) was successively treated with 90% HCOOH (2 ml) at 100° for 10 hr and 0.5 N H_2SO_4 (2 ml) at 100° for 5 hr. After neutralization with $BaCO_3$, the hydrolysates were reduced with $NaBH_4$, and acetylated with Ac_2O–pyridine (1: 1) at 100° for 2 hr, then subjected to GLC and GLC–MS as described in our previous papers.[3b,c] GLC was performed on a glass column (0.3 cm × 2 m) packed with 3% ECNSS–M on Gaschrom Q (100 to 120 mesh) at 176° at a flow rate of 2.2 kg/cm^2 of N_2. GLC–MS was carried out on the above column (0.2 cm × 1 m) at 180° at a flow rate of 0.8 kg/cm^2 of helium. The mass spectra were recorded at an ionizing potential of 70 eV, an ionizing current 50 μA and a temperature of the ion source of 230°.

Table I shows the relative retention times of the methylated sugars as alditol acetates with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and presents the mass spectral data.

References and Notes

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