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Plant Mucilages. IX.[1] The Location of the O-Acetyl Groups and the Nature of the Branches in Bletilla-glucomannan

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Bletilla-glucomannan, the mucous polysaccharide isolated from *Bletilla striata* Reichenbach fil., was found to contain 4.2% of O-acetyl group. The O-acetyl groups were located in position 3 of most of the glucose units. Methylation study provided the evidences that the polysaccharide is mainly composed of β-1→4 linked aldohexopyranose residues having a branched structure with 1→2 branch point at a part of mannose units, and mannose units occupy non-reducing terminal positions in the molecule.

The mucous polysaccharide from the tubers of *Bletilla striata* Reichenbach fil. named Bletilla-glucomannan was isolated and investigated in this laboratory.[1] The substance is composed of D-mannose and D-glucose in the molar ratio of 3:1. The measurement of osmotic pressure gave the value of 182000 as its molecular weight. Partial acid hydrolysis of it elucidated the structure to be mainly composed of β-1→4 linked aldohexopyranose residues. Periodate oxidation study also supported this conclusion, but both the value of formic acid liberation and the yield of mannose by Smith degradation suggested that the polysaccharide contains six aldohexose units per one end group on the average and a part of mannose residues occupies branching positions.

The present work was undertaken to identify and determine the acyl groups, and the location of them was elucidated. This paper is also concerned with the structural features of the polysaccharide, particularly the nature of the branches, revealed by methylation analysis.

As shown in Fig. 1, the infrared (IR) spectrum of Bletilla-glucomannan has the absorption bands of 1735 and 1250 cm^{-1} suggesting the presence of ester linkages in addition to the absorption of 890 cm^{-1} being due to β-glycosidic linkages. The acid hydrolysate of the polysaccharide was analyzed directly by gas–liquid chromatography (GLC) using 20% tetramethyl cyclobutanediol adipate-4% phosphoric acid column. It gave one peak, whose retention time was precisely equal to that of authentic sample of acetic acid, and the possibility of presence of other acids was eliminated. The acetyl content of the polysaccharide was determined to be 4.2% by GLC. This result corresponds to one acetyl group for every five aldohexose residues.

The presence of O-acetyl groups in glucomannans obtained from coniferous woods has already been known in literatures.[3–8] On the other hand, although there was a report[9] on acetylated salep glucomannan, very little was known about the nature of acetylated glucomannan isolated from the tubers of higher plants.

1) Part VIII: M. Tomoda, S. Nakatsuka, M. Tamai, and M. Nagata, *Chem. Pharm. Bull.* (Tokyo), **21**, 2667 (1973).
2) Location: *1-5-30, Shibakoen, Minato-ku, Tokyo, 105, Japan.*
3) H. Meier, *Acta Chem. Scand.*, **15**, 1381 (1961).
4) G. Katz, *Tappi*, **48**, 34 (1965).
5) W.S. Linnell, N.S. Thompson, and H.A. Swenson, *Tappi*, **49**, 491 (1966).
6) H.A. Swenson, *Tappi*, **51**, 141 (1968).
7) T. Koshijima and R. Tanaka, *Mokuzai Gakkaishi*, **16**, 399 (1970).
8) R. Tanaka and T. Koshijima, *Mokuzai Gakkaishi*, **18**, 403 (1972).
9) D.H. Juers, H.A. Swenson, and S.F. Kurath, *J. Polym. Sci.*, Part A-2, **5**, 361 (1967) [*C.A.*, **66**, 115912*d* (1967)].

In order to make clear the location of O-acetyl groups in Bletilla-glucomannan, the method originally developed by Bouveng[10] was employed. The sequence of reactions is illustrated in Chart 1. The polysaccharide (I) was swollen in dimethylformamide and treated with an excess of phenylisocyanate for conversion of the free hydroxyl groups to phenylcarbamate esters (II). Then the phenylcarbamate (II) was methylated with methyl iodide and silver oxide in dimethylformamide.[11] The O-acetyl groups were replaced by O-methyl groups and the phenylcarbamoyl groups were N-methylated to give the partially-O-methyl-glucomannan N-methylphenylcarbamate (III) by this procedure.[12] After removal of N-methylphenylcarbamoyl groups by reduction with lithium aluminum hydride in tetrahydrofuran, the resulting O-methyl derivative (IV) was hydrolyzed and the products were analyzed by paper partition chromatography (PPC), and by GLC of the alditol acetate after reduction and acetylation[13] of the hydrolysate. Besides mannose and glucose, a hexose methyl ether was detected and identified as 3-O-methyl-D-glucopyranose (V) by comparison with the synthetic specimen.

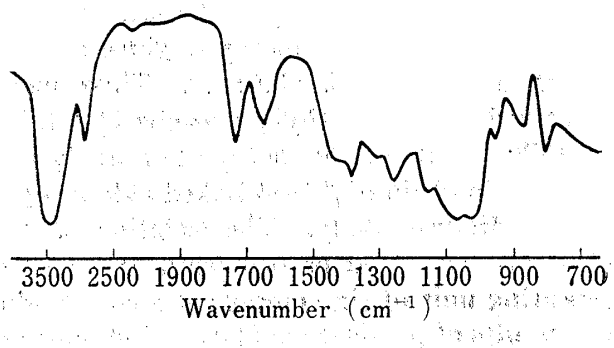

Fig. 1. IR spectrum of Bletilla-glucomannan

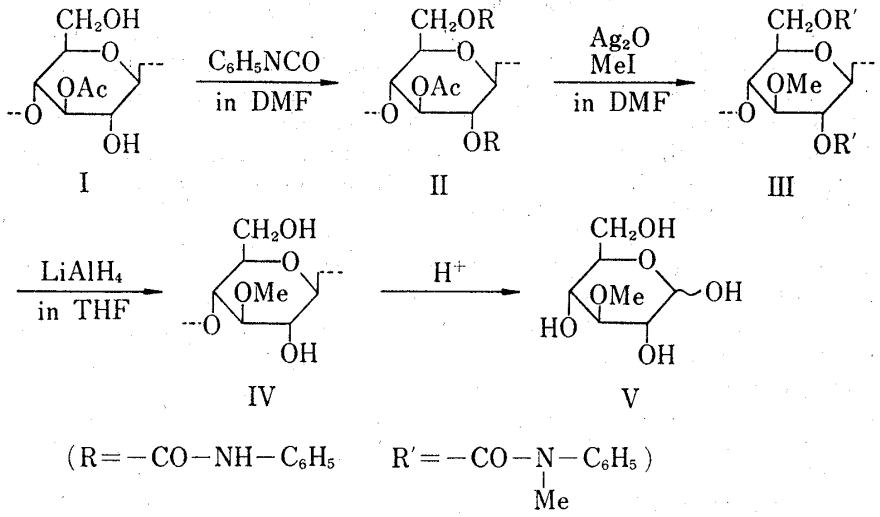

Chart 1

Owing to this result, it is able to conclude that the O-acetyl groups are attached to position 3 of the most of D-glucopyranose units in Bletilla-glucomannan. The value of quantitative analysis indicated that about 80% of glucose residues in the polysaccharide possess 3-O-acetyl groups.

The methylation of Bletilla-glucomannan was performed with sodium methylsulfinyl-carbanion and methyl iodide in dimethylsulfoxide.[14] The fully methylated product was hydrolyzed with formic acid and dilute sulfuric acid. The products were separated by PPC, then analyzed by GLC after conversion to alditol acetates.[13] As the hydrolysis products

10) H.O. Bouveng, *Acta Chem. Scand.*, **15**, 96 (1961).
11) R. Kuhn, H. Trischmann, and I. Löw, *Angew. Chem.*, **67**, 32 (1955).
12) Y. Nishikawa, T. Takeda, S. Shibata, and F. Fukuoka, *Chem. Pharm. Bull.* (Tokyo), **17**, 1910 (1969).
13) H. Björndal, B. Lindberg, and S. Svensson, *Acta Chem. Scand.*, **21**, 1801 (1967).
14) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).

of the methylated polysaccharide, 2,3,4,6-tetra-O-methyl-D-mannose, 2,3,6-tri-O-methyl-D-mannose, 2,3,6-tri-O-methyl-D-glucose and 3,6-di-O-methyl-D-mannose were obtained in a molar ratio of 1.7: 5.1: 3.0: 2.2. These methyl derivatives of component sugars were also identified as their methyl glycosides by GLC.

The results of the methylation analysis provided the evidences that the polysaccharide has a main chain of β-1→4 linked aldohexopyranose residues, as already suggested by partial acid hydrolysis study. The isolation of 3,6-di-O-methyl-D-mannose showed the branched structure with 1→2 branch point at a part of mannose residues, occuring with an average repeating unit of six component sugar residues. And this value is in good agreement with the results of periodate oxidation and Smith degradation.

Detailed investigations by partial enzymic hydrolysis and by partial acetolysis are now under progress to reveal the sequences of linkages of component sugar residues in the whole molecule.

Experimental

Solutions were concentrated at or below 40° with rotary evaporators under reduced pressure. IR spectra were measured with Hitachi model EPI-G3 infrared spectrophotometer. GLC was carried out by the use of Hitachi model 063 gas chromatograph equipped with hydrogen flame ion detector.

Determination of O-Acetyl Groups——The IR spectrum of the polysaccharide showed the absorption bands of ester. IR ν_{max}^{KBr} cm^{-1}: 1735, 1250 (ester), 890 (β-glycosidic linkage).

The sample (2.1 mg) was hydrolyzed with 1N hydrochloric acid (0.05 ml) containing propionic acid (0.1 mg) as an internal standard in a sealed tube at 100° for 2 hr. The hydrolysate was applied directly to GLC. GLC was carried out under condition A, a column (0.3 cm × 2 m long spiral stainless steel) packed with 20% tetramethyl cyclobutanediol adipate–4% phosphoric acid on Chromosorb W (80 to 100 mesh) at 120° with a flow of 20 ml per min of N_2; t_R, acetic acid 6.0; propionic acid (internal standard) 9.4.

Preparation of Phenylcarbamate——The sample (200 mg) was swollen in dimethylformamide (7 ml) and then phenylisocyanate (0.7 ml) was added. After refluxing on a boiling water-bath for 8 hr, the reaction mixture was poured into ethanol. The resulting precipitate was collected by suction filtration, washed with ethanol and ether, then dried. Two more repetition of the similar reaction yielded white powder (412 mg). IR ν_{max}^{KBr} cm^{-1}: 1735 (acetyl ester), 1715 (ester-amide), 1602 (phenyl).

Methylation of Phenylcarbamate——The phenylcarbamate (400 mg) was dissolved in dimethylformamide (16 ml), then methyl iodide (2.4 ml) and silver oxide (1.6 g) were added successively under stirring. After refluxing at 45° for 6 hr, the reaction mixture was stirred overnight at room temperature. The reaction was performed in a dark. After filtration, methyl iodide (2 ml) and silver oxide (1.6 g) were added again into the filtrate, then the reaction was repeated similarly three times. The final filtrate was poured into ethanol and the resulting precipitate was collected by centrifugation, washed with ethanol and ether, then dried. The absence of O-acetyl groups in the N-methylphenylcarbamate was verified by GLC after acid hydrolysis as described above.

Reduction of N-Methylphenylcarbamate——The N-methylphenylcarbamate (450 mg) was dissolved in tetrahydrofuran (4 ml) and lithium aluminum hydride (30 mg) was added gradually under stirring. The reaction mixture was stirred overnight at room temperature, then refluxed at 70° for 1 hr with continuous stirring. After cooling, additional lithium aluminum hydride (30 mg) was added and the mixture was stirred at room temperature for two days. After the dropwise addition of water (0.6 ml), the reaction mixture was acidified to pH 6 with 5% phosphoric acid and stirred for 1 hr. The gray precipitate was collected by filtration, washed successively with water, ethanol and ether. Yield, 213 mg.

Hydrolysis and Analysis of the O-Methyl Derivative——The partially methylated polysaccharide was hydrolyzed with 2N sulfuric acid at 100° for 6 hr, then neutralized with barium carbonate. The hydrolysate was applied to PPC with Tôyô-Roshi No. 50 and the solvent system of AcOEt: HCOOH: H_2O (3: 1: 1). The *Rf* values (0.52, 0.35, and 0.30) of the three spots detected with benzidine reagent[15] were identical with those of authentic 3-O-methyl-D-glucose, which was synthesized by means of methylation of 1,2: 5,6-di-O-isopropylidene-D-glucose,[16] and D-mannose and D-glucose.

On the one hand, the hydrolysate (2 mg) was reduced in water (5 ml) with sodium borohydride (10 mg) for 2 hr. After neutralization with Dowex 50 W (H^+), the filtrate was evaporated and boric acid was removed by the repeated addition and evaporation of methanol. Then the product was acetylated with acetic an-

15) J.S.D. Bacon and J. Edelman, *Biochem. J.*, **48**, 114 (1951).
16) O.T. Schmidt, *Methods in Carbohyd. Chem.*, **2**, 321 (1963).

Table I. Relative Retention Times of Methylated Products

	Condition B[a] (3% ECNSS-M)	Condition C[b] (15% BDS)	Condition D[b] (5% NPGS)
2,3,4,6-Tetra-O-methyl-1,5-di-O-acetyl-D-mannitol	0.99		
2,3,6-Tri-O-methyl-1,4,5-tri-O-acetyl-D-mannitol	2.10		
2,3,6-Tri-O-methyl-1,4,5-tri-O-acetyl-D-glucitol	2.43		
3,6-Di-O-methyl-1,2,4,5-tetra-O-acetyl-D-mannitol	4.38		
Methyl 2,3,4,6-tetra-O-methyl-D-mannoside		1.34, 1.86	1.42, 2.23
Methyl 2,3,6-tri-O-methyl-D-mannoside		3.62, 4.24	3.39, 4.53
Methyl 2,3,6-tri-O-methyl-D-glucoside		3.68, 4.07	3.36, 4.28
Methyl 3,6-di-O-methyl-D-mannoside		8.90	7.80, 9.14

a) for partially methylated alditol acetates; 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-glucitol=1.00
b) for methyl glycosides of partially methylated aldoses; methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside=1.00

hydride–pyridine mixture (1: 1, 2 ml) at 100° for 10 min. After evaporation of the solution, the residue was dissolved in chloroform–methanol mixture (1: 1) and applied to GLC. GLC was carried out under condition B, a column (0.3 cm × 2 m long spiral stainless steel) packed with 3% ECNSS-M on Gaschrom Q (100 to 120 mesh) at 180° with a flow of 40 ml per min of N_2. The result revealed that the product has 3-O-methyl-1,2,4,5,6-penta-O-acetyl-D-glucitol as the sole partially methylated alditol acetate, whose relative retention time to 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-glucitol was 8.95.

Methylation of Bletilla-glucomannan——Sodium hydride (200 mg) was mixed with dimethyl sulfoxide (7 ml) and the mixture was stirred at 70° for 1 hr. The sample (100 mg) was dissolved in dimethyl sulfoxide (20 ml) and the solution was added into this mixture. After stirring at 70° for 20 min, the reaction mixture was cooled to room temperature, then methyl iodide (10 ml) was added and the mixture was stirred overnight at room temperature. All procedures were carried out in nitrogen atmosphere. After dilution with water (200 ml), the mixture was extracted with chloroform (200 ml) three times. The combined extract was washed with water (500 ml) three times, then dried over sodium sulfate and the filtrate was evaporated. The residue was methylated two more times under the same condition. The IR spectra of the final product had no absorption near 3400 cm^{-1}.

Hydrolysis and Analyses of the Methylated Product——The fully methylated polysaccharide (100 mg) was dissolved in 90% formic acid (12 ml) and the solution was heated in a sealed tube at 90° for 16 hr. After cooling, the solution was evaporated and formic acid was removed by the repeated addition and evaporation of methanol. The residue was hydrolyzed in 0.5N sulfuric acid (30 ml) at 100° for 3 hr and the solution was neutralized with barium carbonate. The filtrate was evaporated and applied to PPC with Tôyô-Roshi No. 50 and the solvent system of BuOH: EtOH: H_2O (4:1 :5, upper layer). A tetramethyl hexose was extracted from a part showing *Rf* value of 0.85, two trimethyl hexoses were extracted from parts showing *Rf* values of 0.75 and 0.73, and a dimethyl hexose was extracted from a part showing *Rf* value of 0.55.

A part of the methylated sugars was dissolved in water and reduced with sodium borohydride to alditol then acetylated with acetic anhydride–pyridine mixture as described above. GLC of partially methylated alditol acetates was carried out under condition B.

The other part of the methylated sugars was methanolized with 3% methanolic HCl in a sealed tube at 70° for 16 hr. After removal of HCl by the repeated addition and evaporation of methanol, GLC of methyl glycosides of partially methylated aldoses was carried out under following two conditions: C, a column (0.3 cm × 2 m long spiral stainless steel) packed with 15% Poly-butane 1,4-diol succinate on Chromosorb W (80 to 100 mesh) at 175° with a flow of 20 ml per min of N_2; D, a column (0.3 cm × 2 m long stainless steel) packed with 5% Neopentylglycol succinate on Chromosorb G (60 to 80 mesh) at 150° with a flow of 20 ml per min of N_2. Table I shows relative retention times of the products to 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-glucitol (for partially methylated alditol acetates) and to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside (for methyl glycosides of partially methylated aldoses).