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Plant Mucilages. XXIII.[1] Partial Hydrolysis of Abelmoschus-mucilage M and the Structural Features of Its Polysaccharide Moiety

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Partial acid hydrolysis of Abelmoschus-mucilage M, a representative mucilage isolated from the roots of *Abelmoschus manihot* Medicus, led to the isolation of three oligosaccharides. Analysis of components, reduction and methylation, and partial degradation studies showed that these oligosaccharides are *O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose, *O*-β-(D-glucopyranosyluronic acid)-(1→3)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose, and *O*-β-(D-glucopyranosyluronic acid)-(1→3)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl-(1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose. The structural features of the polysaccharide moiety in the mucilage are discussed.

Keywords——*Abelmoschus manihot*; Abelmoschus-mucilage M; partial acid hydrolysis; isolation of three oligosaccharides; reduction and methylation analysis; proton magnetic resonance; structures of three oligosaccharides; structural feature of polysaccharide moiety

The representative mucous substance obtained from the root of *Abelmoschus manihot* Medicus (=*Hibiscus manihot* L.), named Abelmoschus-mucilage M, has been isolated and investigated in this laboratory.[3] The substance is a complex of carbohydrate and peptide, and 82% consists of an acidic polysaccharide. The polysaccharide moiety is composed of L-rhamnose, D-galacturonic acid, and D-glucuronic acid in approximately equimolecular ratios. The reduction of carboxyl groups and methylation studies showed that the polysaccharide was composed of 1→2-linked L-rhamnopyranose units and 1→4-linked D-galactopyranosyluronic acid units having D-glucopyranosyluronic acid residues at position 3.

The present work was undertaken to isolate and identify three oligosaccharides obtained as the main products of partial hydrolysis of Abelmoschus-mucilage M. The structural features of the polysaccharide moiety are discussed.

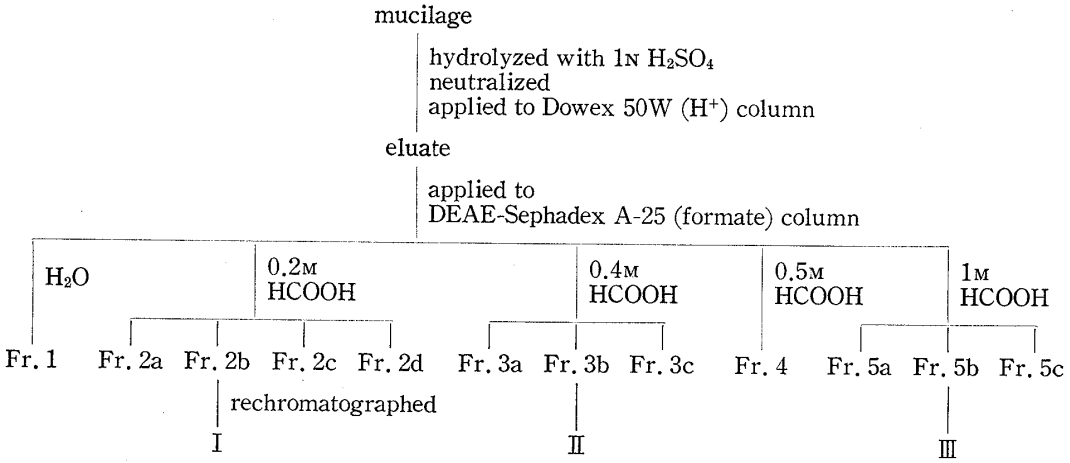

Chart 1. Isolation of Oligosaccharides

1) Part XXII: M. Tomoda and N. Satoh, *Chem. Pharm. Bull.* (Tokyo), **27**, 468 (1979).
2) Location: *1-5-30, Shibakōen, Minato-ku, Tokyo, 105, Japan.*
3) M. Tomoda and Y. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **25**, 3061 (1977).

The mucilage was hydrolyzed with 1 N sulfuric acid for 2 hr, then neutralized and applied to a column of Dowex 50W (H^+). The eluate with water was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (formate form), and three oligosaccharides (I to III) were obtained from the main fractions by stepwise elution with dilute formic acid. Among them, I was purified by rechromatography under the same conditions. The preparation of the partial hydrolysates is summarized in Chart 1.

The homogeneity of each oligosaccharide was checked by paper partition chromatography (PPC) and by paper electrophoresis. Table I gives the R_{Rha} values on PPC and the mobilities on paper electrophoresis.

Table I. R_{Rha} Values and Mobilities of Oligosaccharides

Oligosaccharides	PPC (R_{Rha})		Paper electrophoresis [distance (cm) from the origin]
	Solvent A	Solvent B	
I	0.37	0.67	+4.8
II	0.23	0.58	+7.3
III	0.07	0.27	+8.5

For the solvents and buffer, see "Experimental".

Cellulose thin-layer chromatography (TLC) of the hydrolysates of the oligosaccharides was carried out to identify their component sugars. The oligosaccharides were converted into the corresponding carboxyl-reduced oligosaccharides by reduction of the methyl ester methyl glycosides with sodium borohydride.[4] Quantitative determination of the component sugars was carried out by gas–liquid chromatography (GLC) of alditol acetates derived from the hydrolysates of the carboxyl-reduced oligosaccharides and by colorimetric methods. The results and specific rotations of the oligosaccharides in water are shown in Table II. All three oligosaccharides bear a L-rhamnose residue as a common reducing terminal.

Table II. Specific Rotations and Component Sugars of Oligosaccharides

Oligosaccharides	Specific rotations in water (final values)	Component sugars	Molar ratios	% of reducing terminal[a]
I	$[\alpha]_D^{20}$ +94.4° (c=0.3)	GalA: Rha	1.0: 1.0	45.4
II	$[\alpha]_D^{20}$ +83.8° (c=1.6)	GlcA: GalA: Rha	1.0: 1.0: 1.1	33.1
III	$[\alpha]_D^{20}$ +79.8° (c=1.0)	GlcA: GalA: Rha	1.0: 0.9: 1.1	17.0

Abbreviations: GlcA=D-glucuronic acid; GalA=D-galacturonic acid; Rha=L-rhamnose.
a) L-Rhamnitol in reduced oligosaccharides.

Table III. Products in Hydrolysates obtained from Methylated Carboxyl-reduced Oligosaccharides and Their Molar Ratios

Oligosaccharides	Products	Molar ratios
Carboxyl-reduced I	2,3,4,6-Me-Gal: 3,4-Me-Rha	1.1: 1.0
Carboxyl-reduced II	2,3,4,6-Me-Glc: 2,4,6-Me-Gal: 3,4-Me-Rha	1.0: 1.1: 0.9
Carboxyl-reduced III	2,3,4,6-Me-Glc: 2,4,6-Me-Gal: 2,6-Me-Gal: 3,4-Me-Rha	2.0: 1.0: 1.1: 2.1

Abbreviations: Me=methyl; Gal=D-galactose; Rha=L-rhamnose; Glc=D-glucose (*e.g.*, 2,3,4,6-Me-Gal=2,3,4,6-tetra-*O*-methyl-D-galactose).

4) R. Sømme, *Carbohydr. Res.*, **43**, 145 (1975).

Methylation of each carboxyl-reduced oligosaccharide was performed with methylsulfinylmethyl sodium and methyl iodide in dimethyl sulfoxide.[5] The fully methylated products were hydrolyzed and the hydrolysates were analyzed by gas-liquid chromatography–mass spectrometry (GLC–MS) after conversion into alditol acetates.[6] The products obtained by hydrolysis of the methylated carboxyl-reduced oligosaccharides are shown in Table III.

The reducing terminal rhamnose unit of each oligosaccharide was converted into the corresponding alditol by reduction with sodium borohydride. The proton magnetic resonance (PMR) spectra of the resulting non-reducing oligosaccharides showed an anomeric proton signal at δ 5.36 (1H, d, J=3 Hz) and a methyl signal of rhamnitol at δ 1.24 (3H, d, J=6 Hz) in the non-reducing derivative from I; two anomeric proton signals at δ 4.78 (1H, d, J=7 Hz) and δ 5.36 (1H, d, J=3 Hz), and a methyl signal at δ 1.22 (3H, d, J=6 Hz) in the non-reducing derivative from II; and four anomeric proton signals at δ 4.78 (2H, d, J=7 Hz), δ 5.20 (1H, d, J=2 Hz), δ 5.36 (1H, d, J=3 Hz), and δ 5.49 (1H, d, J=3 Hz), and a methyl signal at δ 1.22 (6H, d, J=6 Hz) in the non-reducing derivative from III. On the other hand, the mucilage was degraded by periodate oxidation followed by reduction with sodium borohydride. The product had $[\alpha]_D^{19}$ +84.7° (in H_2O, c=2.3), and showed an anomeric proton signal of D-galacturonic acid at branching points at δ 5.47 (1H, d, J=3 Hz). Neither L-rhamnose nor D-glucuronic acid was detected in the product. These data suggest that D-galacturonic acid residues in I, II, and III are α-linked (the signals at δ 5.36 [due to terminal and chain residues] and δ 5.49 [due to a branching point] in the PMR spectra), that D-glucuronic acid residues in II and III are β-linked (the signal at δ 4.78 in the PMR spectra), and that the intermediate L-rhamnose residue in III is α-linked (the signal at δ 5.20 in the PMR spectrum).[7–10]

Based on the results of the methylation analysis and PMR spectra, we concluded that I and II are *O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose and *O*-β-(D-glucopyranosyluronic acid)-(1→3)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose, respectively. The specific rotation of I agreed with the reported value.[11,12] II was identified with the trisaccharide obtained by the partial hydrolysis of paniculatan,[13] the mucous polysaccharide from the inner bark of *Hydrangea paniculata*, on the basis of chromatographic behavior, PMR spectra, and specific rotation. However, there is a significant difference between the specific rotation of II and that reported in the literature.[14] This difference led to an erroneous conclusion regarding the configuration of the glycosidic linkage of D-glucuronic acid residues in paniculatan. We therefore wish to correct the structure of the trisaccharide described in the previous report;[13] that is, its D-glucuronic acid residue is not α-linked, but β-linked.

Marked production of II was observed on partial hydrolysis of III with 1 N sulfuric acid for 1 hr. The hydrolysate was analyzed by PPC and determined by GLC after conversion of the carboxyl-reduced derivatives into alditol acetates. In addition to II, the component monosaccharides, I, and *O*-β-(D-glucopyranosyluronic acid)-(1→3)-D-galactopyranosyluronic acid were produced, but II was the only trisaccharide in the product. The ratio of the yields of II, disaccharides, and monosaccharides was 5.2: 1.0: 2.2.

5) S. Hakomori, *J. Biochem.*, **55**, 205 (1964).
6) H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, **5**, 433 (1967).
7) M. Curvall, B. Lindberg, J. Lonngren, and W. Nimmich, *Carbohydr. Res.*, **42**, 73 (1975).
8) G.G.S. Dutton, K.L. Makie, and M.-T. Yang, *Carbohydr. Res.*, **65**, 251 (1978).
9) G.G.S. Dutton, K.L. Makie, A.V. Savage, and M.D. Stephenson, *Carbohydr. Res.*, **66**, 125 (1978).
10) J.-P. Joseleau, M. Lapeyre, M. Vignon, and G.G.S. Dutton, *Carbohydr. Res.*, **67**, 197 (1978).
11) G.O. Aspinall, J.W.T. Craig, and J.L. Whyte, *Carbohydr. Res.*, **7**, 442 (1968).
12) G.O. Aspinall, B. Gestetner, J.A. Molloy, and M. Uddin, *J. Chem. Soc.* (*C*), **1968**, 2554.
13) M. Tomoda and N. Satoh, *Chem. Pharm. Bull.* (Tokyo), **25**, 2910 (1977).
14) G.O. Aspinall and Nasir-ud-Din, *J. Chem. Soc.*, **1965**, 2710.

Based on the accumulated evidence described above, III was identified as *O*-β-(D-glucopyranosyluronic acid)-(1→3)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl-(1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose (Chart 2).

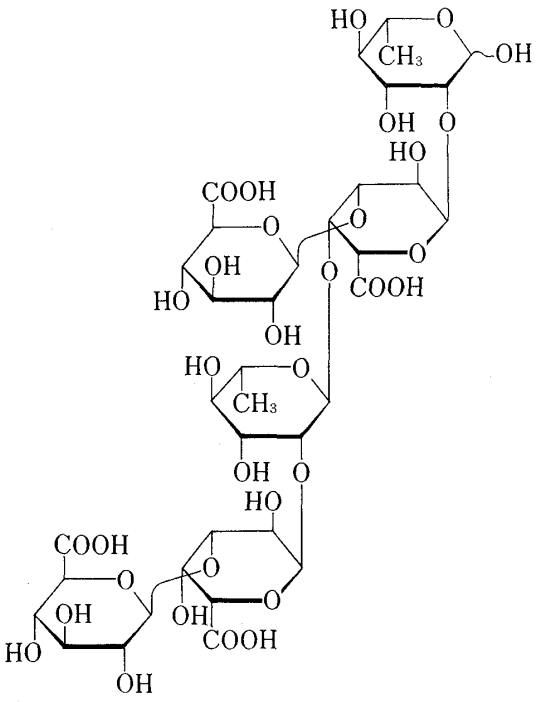

Chart 2. Structure of Oligosaccharide III

The combined yields of II and III were more than half of the total mono- and oligosaccharides obtained from a partial hydrolysate of Abelmoschus-mucilage M. Consequently, it can be concluded that the structure of III represents the fundamental unit of the polysaccharide moiety of the mucilage. This conclusion is supported by the results of methylation analysis of the original mucilage as described in the previous report.[3] The present work has thus elucidated the sequence of the component sugars and the configurations of the glycosidic linkages.

The component unit having the structure II is common in the mucilages from the roots of *Abelmoschus manihot* and *Althaea officinalis*[15,16] and the inner barks of *Hydrangea paniculata*.[13] However, only Abelmoschus-mucilage M possesses a simple repeating structure in its polysaccharide moiety.

Experimental

Solutions were concentrated at or below 40° with rotary evaporators under reduced pressure. Optical rotation was measured with a JASCO DIP-SL automatic polarimeter. Infrared (IR) spectra were recorded on a JASCO IRA-2 infrared spectrophotometer. GLC was carried out on a Hitachi 063 gas chromatograph equipped with a hydrogen flame ionization detector. GLC–MS was performed with a JEOL JGC-20K gas chromatograph and a JEOL JMS-D100 mass spectrometer. PMR spectra were recorded with a JEOL MH-100 NMR spectrometer in heavy water at 70°.

Partial Hydrolysis and Isolation of Oligosaccharides——The mucilage (0.49 g) was suspended in 1 N sulfuric acid (50 ml) and heated under reflux at 100° for 2 hr. The solution was neutralized with barium carbonate, and after filtration, the filtrate was passed through a column (1 × 12 cm) of Dowex 50W-X8 (H^+). The eluate with water was concentrated and applied to a column (2 × 18 cm) of DEAE-Sephadex A-25 (formate form, Pharmacia Co.). The column was eluted successively with water (65 ml), 0.1 M formic acid (80 ml), 0.2 M formic acid (260 ml), 0.4 M formic acid (325 ml), 0.5 M formic acid (155 ml), and 1 M formic acid (320 ml). Fractions of 5 ml were collected and analyzed by the phenol-sulfuric acid method.[17] The eluates obtained from the column were divided into twelve groups: Frac. 1, tubes 6 to 9; Frac. 2a, tubes 30 to 32; Frac. 2b, tubes 33 to 41; Frac. 2c, tubes 42 to 62; Frac. 2d, tubes 63 to 76; Frac. 3a, tubes 86 to 94; Frac. 3b, tubes 95 to 114; Frac. 3c, tubes 115 to 142; Frac. 4, tubes 157 to 171; Frac. 5a, tubes 186 to 194; Frac. 5b, tubes 195 to 215; Frac. 5c, tubes 216 to 241. The yields were 21.3 mg in Frac. 1, 1.5 mg in Frac. 2a, 12.5 mg in Frac. 2b, 11.6 mg in Frac. 2c, 5.6 mg in Frac. 2d, 2.7 mg in Frac. 3a, 34.8 mg in Frac. 3b, 11.4 mg in Frac. 3c, 2.8 mg in Frac. 4, 1.3 mg in Frac. 5a, 48.0 mg in Frac. 5b, and 10.3 mg in Frac. 5c. Frac. 2b was rechromatographed using the same column. After elution with 0.1 M formic acid (100 ml) and 0.2 M formic acid (200 ml), the fractions (5 ml each) from tubes 29 to 35 provided I (yield, 8.8 mg). II and III were obtained directly from Frac. 3b and 5b, respectively. On PPC, the main spots in Frac. 3a and 3c corresponded to II, and the main spots in Fracs. 5a and 5c corresponded to III. III was obtained as a water-soluble colorless powder, mp 172—176° (dec.), by lyophilization. *Anal.* Calcd. for $C_{36}H_{54}O_{33}$: C, 42.61; H, 5.36. Found: C, 42.55; H, 5.23.

15) M. Tomoda, S. Kaneko, M. Ebashi, and T. Nagakura, *Chem. Pharm. Bull.* (Tokyo), **25**, 1357 (1977).
16) M. Tomoda, N. Satoh, and K. Shimada, *Chem. Pharm. Bull.* (Tokyo), submitted.
17) J.E. Hodge and B.T. Hofreiter, "Methods in Carbohydrate Chemistry," Vol. I, ed. by R.L. Whistler and M.L. Wolfrom, Academic Press, New York and London, 1962, p. 388.

PPC, TLC, and Paper Electrophoresis——PPC was carried out with Tōyō-Roshi No. 51 paper by a descending method, and TLC was performed using Avicel SF cellulose. The following three solvent systems were used; A, AcOEt: pyridine: AcOH: H_2O (5: 5: 1: 3); B, AcOEt: AcOH: HCOOH: H_2O (18: 3: 1: 4); C, BuOH: pyridine: H_2O (6: 4: 3). Electrophoresis was carried out with Tōyō-Roshi No. 51 paper (12×38 cm long) and 0.025 M borax: 0.1 N sodium hydroxide buffer (10: 1, pH 9.2), at 570 volts for 2 hr. Samples were applied in a line at the center. The inside of the apparatus was cooled with dry ice. Sugars were visualized with *p*-anisidine hydrochloride[18] and silver nitrate[19] reagents. The *Rf* values of the component sugars in TLC were the same as those described in the previous report.[13]

Determination of Component Sugars——Each sample (1 mg) was mixed with methanol (1 ml) and Dowex 50W-X8 (H^+) (10 mg), then heated at 67° for 24 hr in a sealed tube. After filtration and washing with water and methanol, followed by evaporation to dryness, the reaction product was again subjected to similar treatment. The product was dissolved in water (1 ml), then reduced with sodium borohydride (10 mg) at room temperature for 18 hr, and treated with Dowex 50W-X8 (H^+) up to pH 4. The filtrate was evaporated down and boric acid was removed by repeated addition and evaporation of methanol. The product was hydrolyzed with 2 N sulfuric acid at 100° for 6 hr followed by neutralization with Dowex 2 (OH^-). The filtrate was reduced with sodium borohydride for 1 hr. After neutralization with Dowex 50W-X8 (H^+), boric acid was removed as described above. The product was acetylated with acetic anhydride-pyridine mixture (1: 1) at 100° for 40 min. After concentration of the solution, the residue was dissolved in chloroform-methanol mixture (1: 1) and subjected to GLC. GLC was carried out under the condition A, using a column (0.3 cm×2 m long spiral glass) packed with 3% OV 225 on Gaschrom Q (100 to 120 mesh) at 210° with a nitrogen flow of 60 ml per min. Xylose was used as an internal standard. The retention times (min) of rhamnitol acetate, galactitol acetate, glucitol acetate, and xylitol acetate were 5.2, 19.0, 21.2, and 8.9. Rhamnose was also determined by the thioglycolic acid method,[20] and hexuronic acids in the original oligosaccharides were estimated by a modified carbazole method.[21] For determination of the reducing terminal, each sample was reduced with sodium borohydride, then hydrolyzed and acetylated as described above. Rhamnitol acetate in the products was determined by GLC under the condition A.

Methylation of Carboxyl-reduced Oligosaccharides——The carboxyl groups in each sample (5 mg) were reduced as described above. The carboxyl-reduced oligosaccharide obtained was dissolved in dimethyl sulfoxide (1 ml). Sodium hydride (10 mg) was mixed with dimethyl sulfoxide (2 ml) at 70° for 1 hr, then the mixture was added to the sample solution. The reaction mixture was stirred at room temperature for 6 hr, then methyl iodide (1 ml) was added and the mixture was stirred overnight at room temperature. All procedures were carried out under nitrogen. After dilution with water (10 ml), the mixture was extracted with chloroform (10 ml each) five times. The combined extract was washed with water (50 ml each) five times, then dried over sodium sulfate, and the filtrate was evaporated to dryness. The residue was methylated three more times under the same conditions. The IR spectrum of the final residue showed no absorption near 3400 cm^{-1}.

Analysis of Methylated Products——The product (2 mg) was successively treated with 90% formic acid (0.2 ml) at 100° for 2.5 hr and 0.5 N sulfuric acid (0.3 ml) at 100° for 19 hr. After neutralization with Dowex 2 (OH^-), the hydrolysate was reduced with sodium borohydride, then acetylated as described above. GLC of partially methylated alditol acetates was carried out under the condition A′, using the same column as under the condition A, but at 180°. GLC–MS was carried out under the condition A′ using helium as a carrier gas. The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and their main fragments in the mass spectra are shown in Table IV.

Table IV. Relative Retention Times on GLC and Main Fragments in MS of partially Methylated Alditol Acetates

	Relative retention times[a] Condition A′	Main fragments (*m/e*)
1,2,5-Ac-3,4-Me-L-Rhamnitol	0.88	43, 89, 129, 131, 189
1,5-Ac-2,3,4,6-Me-D-Glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,5-Ac-2,3,4,6-Me-D-Galactitol	1.16	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac-2,4,6-Me-D-Galactitol	1.88	43, 45, 87, 101, 117, 129, 161
1,3,4,5-Ac-2,6-Me-D-Galactitol	2.90	43, 45, 87, 117, 129

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Ac=acetyl; Me=methyl (*e.g.*, 1,2,5-Ac-3,4-Me=1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-).

18) L. Hough and J.K.N. Jones, "Methods in Carbohydrate Chemistry," Vol. I, ed. by R.L. Whistler and M.L. Wolfrom, Academic Press, New York and London, 1962, p. 28.
19) W.E. Trevelyan, D.P. Procter, and J.S. Harrison, *Nature* (London), **166**, 444 (1950).
20) M.N. Gibbons, *Analyst* (London), **80**, 268 (1955).
21) T. Bitter and H. Muir, *Anal. Biochem.*, **4**, 330 (1962).

Partial Hydrolysis of III and Analysis——III (1 mg) was dissolved in 1 N sulfuric acid (0.1 ml) and heated at 100° for 1 hr. After neutralization with barium carbonate, followed by treatment with Dowex 50W-X8 (H^+), the hydrolysate was analyzed by PPC with solvent A under 48 hr development at 22°. The R_{Rha} values of L-rhamnose, D-glucuronic acid, I, II, and *O*-β-(D-glucuronic acid)-(1→3)-D-galacturonic acid were 1.00, 0.52, 0.49, 0.28, and 0.23. On the other hand, the hydrolysate was reduced with sodium borohydride. After neutralization with Dowex 50W-X8 (H^+) and removal of boric acid, the product was treated with methanol and Dowex 50W-X8 (H^+) at 67° for 24 hr, followed by reduction with sodium borohydride as described above. The alditols of the carboxyl-reduced products obtained were acetylated with acetic anhydride-pyridine mixture, then subjected to GLC. GLC was carried out under the condition B, using a column (0.3×40 cm long spiral stainless steel) packed with 2% OV 101 on Uniport HP (80 to 100 mesh) and with a programmed temperature increase of 5° per min from 120° to 320° at a nitrogen flow of 60 ml per min. The retention times (min) of the acetates of L-rhamnitol, D-glucitol, reduced I, 3-*O*-β-D-glucosyl D-galactitol, and reduced II were 3.2, 6.8, 18.3, 21.3, and 29.3.

O-β-(D-Glucopyranosyluronic acid)-(1→3)-D-galactopyranosyluronic Acid——This disaccharide was produced by partial acid hydrolysis of II, then isolated by preparative PPC with solvent A, and identified by component sugar analysis and by methylation analysis after conversion to the carboxyl-reduced derivative as described above.

Periodate Oxidation followed by Reduction——The mucilage (200 mg) was oxidized with 0.05 M sodium metaperiodate (100 ml) at 5° in the dark. The oxidation was completed after seven days. The periodate consumption was measured by a spectrophotometric method.[22] The sample consumed 1.3 mol of periodate per anhydro component sugar unit. The residue of the reaction mixture was treated with ethylene glycol (0.4 ml) for 1 hr, then reduced with sodium borohydride (1 g) at 5° for 16 hr. After addition of acetic acid up to pH 5, the solution was dialyzed against running water for two days. The solution was concentrated and applied to a column (5×87 cm) of Sephadex G-15. The column was eluted with water, and fractions of 20 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from tubes 27 to 33 were combined, evaporated down and lyophilized. Yield, 117 mg. The galacturonic acid content in the product was 25.3%.

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22) *a*) J.S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954); *b*) G.O. Aspinall and R.J. Ferrier, *Chem. Ind.*, **1957**, 1216.