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Plant Mucilages. XXXVII.[1] A Representative Mucilage, "Althaea-Mucilage RL," from the Leaves of *Althaea rosea*

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A representative mucilage, named Althaea-mucilage RL, was isolated from the leaves of *Althaea rosea* CAVAILLES. The final preparation was homogeneous as determined by ultracentrifugal analysis, electrophoresis, and gel chromatography. Its water solution gave an intrinsic viscosity value of 32.5. It was mainly composed of partially acetylated acidic polysaccharide, and its molecular weight was estimated to be about 1800000. The polysaccharide was composed of L-rhamnose: D-galactose: D-galacturonic acid: D-glucuronic acid: *O*-acetyl groups in the approximately molar ratio of 20 : 1 : 16 : 16 : 10. Methylation and partial hydrolysis studies made it possible to deduce the structural features of the polysaccharide moiety in the mucilage.

Keywords——*Althaea rosea*; leaf; mucilage; Althaea-mucilage RL; acetylated acidic polysaccharide; intrinsic viscosity; molecular weight; structural features

Recently, we have obtained a representative mucilage, "Althaea-mucilage R," from the roots of *Althaea rosea* CAVAILLES (hollyhock), and its structural features have been reported.[2] The root has been utilized as a crude drug with emollient and demulcent actions. In addition, the leaves of this plant have been used similarly. The leaves contain relatively large amounts of mucilages, but no structural study on the mucilages has been reported so far. The present paper deals with the isolation and structural features of a new representative mucilage from the leaves of *Althaea rosea*.

The fresh leaves were homogenized and extracted with cold water. The crude mucilage was obtained from the extract by addition of ethanol. The solution of the crude mucilage was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (carbonate form). After elution with 0.2 M ammonium carbonate, a pure mucilage was obtained from the eluate with 0.5 M ammonium carbonate.

The mucilage was homogeneous as examined by ultracentrifugal analysis (Fig. 1), cellulose acetate membrane electrophoresis, glass-fiber paper electrophoresis, and gel chromatography with Sephacryl S-400 (Fig. 2).

The mucilage showed a positive specific rotation ($[\alpha]_D^{24}$ +60.7° in H_2O, $c=0.1$), and its solution in water gave the high intrinsic viscosity value of 32.5 at 30 °C. The relative viscosity of the solution of the pure mucilage was about 5.6 times that of the crude mucilage. From both this result and the yield, it seems reasonable to assume that the pure mucilage is the representative mucous substance in the water extract from the leaves. Gel chromatography gave a value of about 1800000 for the molecular weight. The name "Althaea-mucilage RL" is proposed for this substance.

The mucilage was found to consist of L-rhamnose, D-galactose, D-galacturonic acid, D-glucuronic acid, *O*-acetyl groups, and protein. Quantitative determination showed that the mucilage contained 31.4% rhamnose, 1.8% galactose, 29.5% galacturonic acid, and 29.5% glucuronic acid, and that their molar ratio was 20.4 : 1.0 : 16.0 : 16.0. The acetyl content of the

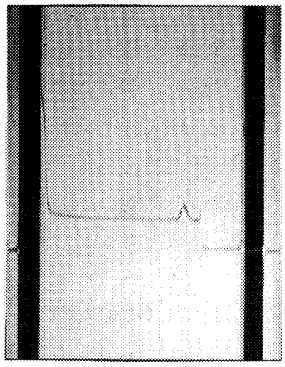

Fig. 1. Ultracentrifugal Pattern of Althaea-Mucilage RL

0.1% in H_2O, 20 °C, 34 min, 60000 rpm, Hitachi UCA-1A ultracentrifuge.

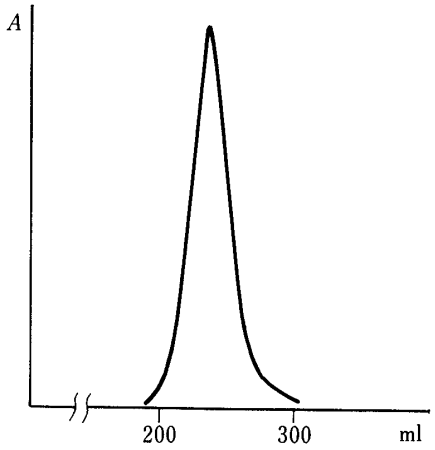

Fig. 2. Chromatogram of Althaea-Mucilage RL on Sephacryl S-400

TABLE I. Amino Acid Compositions (Molar Percent)

	Althaea-mucilage RL	Althaea-mucilage R
Lysine	5.69	6.16
Histidine	1.59	1.86
Arginine	2.73	4.18
Aspartic acid	10.82	10.26
Threonine	5.58	7.52
Serine	6.61	7.21
Glutamic acid	13.67	10.24
Proline	8.77	5.46
Glycine	11.39	9.92
Alanine	9.91	10.00
Valine	7.06	6.63
Methionine	1.37	1.70
Isoleucine	4.44	4.55
Leucine	6.26	8.20
Tyrosine	1.48	2.02
Phenylalanine	2.62	4.08

mucilage was determined to be 4.3%. The determination of protein content was carried out by the method of Lowry *et al.*,[3] and a value of 4.4% was obtained. No compound other than carbohydrates and amino acids was detected in the hydrolysate of the mucilage. The amino acid composition after hydrolysis with 6 N hydrochloric acid is listed in Table I, together with that of Althaea-mucilage R.[2] There is no significant difference in amino acid composition between Althaea-mucilages RL and R, except for the values of arginine, proline, and phenylalanine.

The hexuronic acid residues in the mucilage were reduced with a carbodiimide reagent followed by treatment with sodium borohydride to give the corresponding neutral sugar units.[4] Methylation of the carboxyl-reduced mucilage was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide.[5] The fully methylated derivative was hydrolyzed with dilute sulfuric acid in acetic acid. The products were analyzed by gas-liquid chromatography-mass spectrometery (GLC-MS) after conversion into alditol acetates.[6] 3,4-Di-*O*-methyl-L-rhamnopyranose, 3-*O*-methyl-L-rhamnopyranose, 2,3,4,6-tetra-*O*-methyl-D-

glucopyranose, 2,3,4,6-tetra-*O*-methyl-D-galactopyranose, and 2,6-di-*O*-methyl-D-galactopyranose were identified as their alditol acetates in a molar ratio of 18.4 : 1.0 : 16.0 : 1.3 : 16.8.

The proton nuclear magnetic resonance (^{1}H-NMR) spectrum of the mucilage showed four anomeric proton signals at δ 4.59 (br s), δ 4.70 (br s), δ 5.01 (d, J=2 Hz), and δ 5.26 (d, J=2 Hz), and their integral ratio was 16 : 1 : 20 : 16. In addition, a methyl signal of rhamnose at δ 1.23 (d, J=5 Hz) and an acetyl signal at δ 1.89 (s) were observed. The signals at δ 4.59, 5.01, and 5.26 are due to β-D-glucuronic acid, α-L-rhamnose, and α-D-galacturonic acid residues, respectively.[7] The signal at δ 4.70 suggests that the D-galactose residues in the mucilage are β-linked.

The mucilage was hydrolyzed with 1 N sulfuric acid for 2 h, then neutralized and applied to a column of Dowex 50W (H^+). The eluate with water was applied to a column of DEAE-Sephadex A-25 (formate form). Six oligosaccharides (I to VI) were obtained by stepwise elution with dilute formic acid and purified by rechromatography. Based on the results of component sugar analysis and a comparison of their chromatographic properties, the ^{1}H-NMR spectra, and the values of specific rotation with those of authentic samples,[7,8] I to V were identified as the following five oligosaccharides (Chart 1).

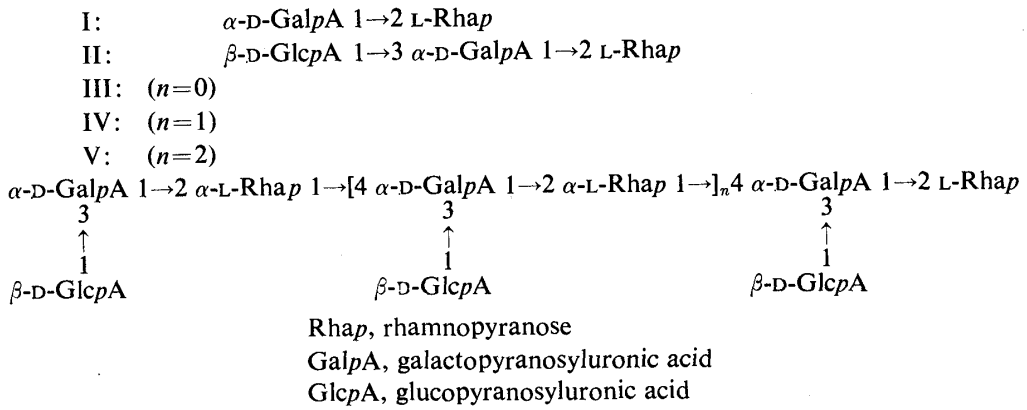

Chart 1. Structural Features of Oligosaccharides I, II, III, IV, and V

Oligosaccharide VI was composed of L-rhamnose and D-galacturonic acid in a molar ratio of 1 : 1. The determination of the reducing terminal was carried out by analysis of the hydrolysate of the corresponding alditol,[7] and the result indicated that VI has an L-rhamnose residue as a reducing terminal. The oligosaccharide was converted into the corresponding carboxyl-reduced derivative by reduction of the methyl ester methyl glycoside with sodium borohydride.[7] Methylation of the carboxyl-reduced derivative of VI was performed, and the product was hydrolyzed as described above. The hydrolysate was analyzed by GLC-MS after conversion into alditol acetates; 3,4-di-*O*-methyl-L-rhamnopyranose, 2,3,4,6-tetra-*O*-methyl-D-galactopyranose, and 2,3,6-tri-*O*-methyl-D-galactopyranose were identified as their alditol acetates in a molar ratio of 1.8 : 1.0 : 1.1. In addition, VI produced rhamnose, galacturonic acid, and oligosaccharide I on partial hydrolysis with 1 N sulfuric acid for 1 h. Based on the evidence described above, VI was identified as *O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranosyl-(1→4)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-L-rhamnopyranose.

All galactose residues were liberated from the mucilage under the partial hydrolysis conditions described above, as in the cases of Althaea-mucilage O,[8] and Okra-mucilage F[9] and R.[1] The glycosidic linkage of the galactose residues is much more easily cleaved than those of the other component sugars in the mucilage.[10] In conjunction with the results of methylation analysis, this finding suggests that a twentieth of the rhamnose residues in the

backbone chain possesses a galactose residue at position 4. A part (7%) of the rhamnose residues was also liberated by the partial hydrolysis.

Based on the accumulated evidence described above, it can be concluded that the polysaccharide moiety of the mucilage contains the units shown in Chart 2.

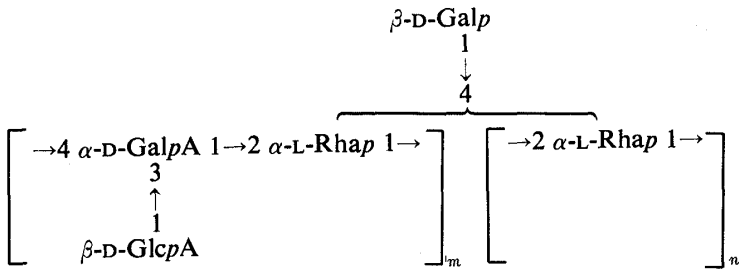

Chart 2. A Possible Structural Fragment of the Polysaccharide Moiety of Althaea-Mucilage RL

As already reported in previous papers, the component unit having the structure (1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-α-L-rhamnopyranose is common in the mucilages from the roots and leaves of *Althaea officinalis*,[8,11] and *Althaea rosea*,[2] the roots of *Abelmoschus manihot*,[7] *Abelmoschus glutinotextilis*,[12] *Abelmoschus esculentus*,[1] and the inner barks of *Hydrangea paniculata*.[13] It should be noted that the presence of 1,2-linked α-L-rhamnopyranosyl repeating units is a common characteristic of the structures of both Althaea-mucilage R[2] and Althaea-mucilage RL. However, Althaea-mucilage RL does not possess the characteristic heptasaccharide unit[2] seen in Althaea-mucilage R. Another structural difference between Althaea-mucilage R and Althaea-mucilage RL is the nature of the neutral sugar side chains. The former has the 1,4-linked galactosyl galactosyl glucose chain, while the latter possesses a galactose unit. In both mucilages, the side chains and residues link to position 4 of a part of the rhamnose units in the main chain, as in four other mucilages[1,2,8,9] obtained by us from plants in the Malvaceae family. The presence of only one galactose unit as neutral side units in Althaea-mucilage RL is unique, compared with the other mucilages which have been isolated from Malvaceae plants. In addition, Althaea-mucilage RL has the lowest degree of branching at rhamnose residues among all the mucilages having similar neutral sugar side chains. The results of detailed analysis of the structure will be reported in subsequent papers.

Experimental

Solutions were concentrated at or below 40 °C with rotary evaporators under reduced pressure. Optical rotations were measured with a JASCO DIP-140 automatic polarimeter. Infrared (IR) spectra were recorded on a JASCO IRA-2 infrared spectrophotometer. ^{1}H-NMR spectra were recorded on a JEOL JNM-GX 270 FT NMR spectrophotometer in heavy water containing acetone as an internal standard at 70 °C. GLC was carried out on a Shimadzu GC-7AG 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 spectrophotometer. Amino acids were determined with a Hitachi KLA-5 amino acid analyzer. Viscosity was determined with an Ubbelohde-type viscosimeter.

Isolation of the Mucilage——The material was obtained in July 1983 from plants cultivated in Saitama prefecture. The fresh leaves (370 g), which contained 81.6% water, were homogenized and extracted with water (3700 ml) under stirring for 1 h at room temperature. After centrifugation, the supernatant was poured into two volumes of ethanol, then centrifuged. The yield of the crude mucilage was 1.2%. A fifth of the crude mucilage was dissolved in water and applied to a column (5 × 78 cm) of DEAE-Sephadex A-25 (Pharmacia Co.). DEAE-Sephadex was pretreated as described in a previous report.[10] After elution with water (2200 ml) and 0.2 M ammonium carbonate

(4700 ml), the column was eluted with 0.5 M ammonium carbonate. Fractions of 20 ml were collected and analyzed by the phenol-sulfuric acid method.[14] The eluates obtained from tubes 53 to 68 were combined, concentrated and poured into ethanol. The precipitate was dissolved in water, then dialyzed against distilled water and lyophilized. Althaea-mucilage RL (106 mg) was obtained as a white powder.

Cellulose Acetate Membrane Electrophoresis——This was carried out as described in a previous report[12] at 420 V for 1 h with 0.08 M pyridine–0.04 M acetic acid buffer (pH 5.4). The sample gave a single spot at a distance of 5.1 cm from the center toward the anode.

Glass-Fiber Paper Electrophoresis——This was performed as described in a previous report[15] at 570 V for 1 h on Whatman GF 83 glass-fiber paper with 0.025 M borax: 0.1 N sodium hydroxide buffer (10 : 1, pH 9.3). The sample gave a single spot at a distance of 2.1 cm from the center toward the cathode.

Gel Chromatography——The sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (2.6 × 96 cm) of Sephacryl S-400. Elution was carried out by the descending method with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol-sulfuric acid method. Standard dextrans having known molecular weights were run on the column to obtain a calibration curve.

Qualitative Analysis of Components——This was carried out by cellulose thin-layer chromatography (TLC) of the acid hydrolysate of the mucilage as described in a previous report.[10]

Determination of Components——Neutral sugars in the original and the carboxyl-reduced mucilages were analyzed by GLC after conversion into alditol acetates as described in a previous report.[7] Rhamnose was also determined by the thioglycolic acid method,[15] and hexuronic acids in the original mucilage were estimated by a modification of the carbazole method.[16] Determination of *O*-acetyl groups was performed by GLC of the hydrolysate as described in a previous report.[17]

Reduction of the Mucilage——The mucilage (50 mg) was suspended in water (25 ml), then 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (0.5 g) was added. The pH of the reaction mixture was maintained at 4.75 by titration with 0.1 N hydrochloric acid under stirring for 2 h, then 2 M sodium borohydride (6 ml) was added gradually to the reaction mixture during 4 h while the pH was maintained at 7.0 by titration with 4 N hydrochloric acid under stirring at room temperature. The solution was dialyzed against distilled water overnight, then the non-dialyzable fraction was concentrated to 20 ml. The product was reduced four times more under the same conditions. The final non-dialyzable fraction was centrifuged, then the supernatant was concentrated and applied to a column (5 × 80 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluate obtained from tubes 22 to 36 were combined and lyophilized. Yield, 18.2 mg.

Methylation——Sodium hydride (30 mg) was mixed with dimethyl sulfoxide (3 ml) in an ultrasonic bath for 30 min, followed by stirring at 70 °C for 1 h. Then the mixture was added to a solution of the sample (10 mg) in dimethyl sulfoxide (4 ml). The reaction mixture was stirred at room temperature for 4 h, then methyl iodide (3 ml) was added, and the whole was stirred overnight at room temperature. All procedures were carried out under nitrogen. After addition of water (20 ml), the reaction mixture was extracted five times with chloroform (20 ml each). The combined extract was washed five times with water (100 ml each), then dried over sodium sulfate, and the filtrate was concentrated to dryness. The residue was methylated three times more under the same conditions. The final residue was dissolved in chloroform–methanol mixture (2 : 1), and applied to a column (1 × 20 cm) of Sephadex LH-20. The column was eluted with the same solvent, and fractions of 1 ml were collected. The eluates obtained from tubes 3 to 10 were combined and concentrated. The IR spectrum of the final residue showed no hydroxyl group absorption. Yield, 7.6 mg.

Analysis of the Methylated Products——The product was hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report.[12] GLC-MS was carried out under the same

TABLE II. Relative Retention Times on GLC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention time[a]	Main fragments (*m/z*)
1,2,5-Ac-3,4-Me-L-Rhamnitol	0.88	43, 89, 129, 131, 189
1,2,4,5-Ac-3-Me-L-Rhamnitol	1.59	43, 87, 101, 129, 143, 189, 203
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
1,4,5-Ac-2,3,6-Me-D-Galactitol	1.98	43, 45, 87, 99, 101, 113, 117, 233
1,3,4,5-Ac-2,6-Me-D-Galactitol	2.78	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-).

Table III. Specific Rotations, Sugar Compositions, and *Rf* Values of Oligosaccharides

Oligosaccharide	$[\alpha]_D^{23}$ in H_2O	Sugar composition	TLC (*Rf*)
I	+93.2°	GalA : Rha = 1 : 1	0.45
II	+85.4°	GlcA : GalA : Rha = 1 : 1 : 1	0.35
III	+82.1°	GlcA : GalA : Rha = 1 : 1 : 1	0.24
IV	+78.0°	GlcA : GalA : Rha = 1 : 1 : 1	0.14
V	+73.1°	GlcA : GalA : Rha = 1 : 1 : 1	0.06
VI	+96.0°	GalA : Rha = 1 : 1	0.32

conditions as in a previous report.[17] The relative retention times of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol in GLC and their main fragments in the mass spectra are listed in Table II.

Partial Hydrolysis and Isolation of Oligosaccharides——The mucilage (68 mg) was suspended in 1 N sulfuric acid (7 ml) and heated at 90 °C for 2 h. After neutralization with barium carbonate, the filtrate was passed through a column (1 × 6 cm) of Dowex 50W- × 8 (H^+). The eluate with water was concentrated and lyophilized (yield, 36 mg), then an aqueous solution of the lyophilisate was applied to a column (1 × 3.5 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted successively with water (15 ml), 0.1 M formic acid (30 ml), 0.2 M formic acid (25 ml), 0.4 M formic acid (30 ml), 0.6 M formic acid (50 ml), 0.8 M formic acid (55 ml), and 1 M formic acid (70 ml). Fractions of 5 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from the column were divided into seven groups: frac. 1, tubes 1 and 2; frac. 2, tubes 4 to 8; frac. 3, tubes 10 to 14; frac. 4, tubes 15 to 18; frac. 5, tubes 22 to 27; frac. 6, tubes 34 to 39; frac. 7, tubes 45 to 51. The yields were 2.8 mg for frac. 1, 2.1 mg for frac. 2, 1.5 mg for frac. 3, 3.4 mg for frac. 4, 4.3 mg for frac. 5, 4.1 mg for frac. 6, and 2.6 mg for frac. 7. Frac. 1 contained galactose and rhamnose in a ratio of 1.0 : 1.4. Frac. 2 was purified on a column of Sephadex G-15, and fracs. 3, 4, 5, 6, and 7 were each purified on a column of Sephadex G-25 as described in a previous report.[2] Oligosaccharides, I, II, III, IV, and V were obtained from fracs. 2, 4, 5, 6, and 7, respectively. The yields were 1.5 mg for I, 2.5 mg for II, 3.2 mg for III, 2.5 mg for IV, and 2.1 mg for V. Oligosaccharide VI was obtained from frac. 3 in a yield of 1.2 mg.

Analysis of the Oligosaccharides——Analysis of component sugars was carried out as described in a previous report.[7] TLC was performed on Merck precoated Kieselgel 60 plates using *n*-butanol–acetic acid–water (2 : 1 : 1, v/v) as a developing solvent. Detection was done by spraying 0.2% orcinol in 20% sulfuric acid followed by heating at 110 °C for 5 min. The results are listed in Table III.

Partial Hydrolysis of Oligosaccharide VI——VI was hydrolyzed with 1 N sulfuric acid at 90 °C for 1 h. After neutralization, the products were analyzed by TLC as described above, and by GLC of the trimethylsilyl derivatives in the manner described in a previous report.[2]

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