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Plant Mucilages. XXXIX.[1] A Representative Mucilage, "Hibiscus-Mucilage SL," from the Leaves of *Hibiscus syriacus*

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A representative mucilage, named Hibiscus-mucilage SL, was isolated from the leaves of *Hibiscus syriacus* L. It was homogeneous on electrophoresis and gel chromatography. Its intrinsic viscosity value in aqueous solution was 26.5. It was mainly composed of acidic polysaccharide, and its molecular weight was estimated to be about 2000000. The polysaccharide is composed of L-rhamnose : D-galactose : D-galacturonic acid : D-glucuronic acid in the molar ratio of 8.0 : 1.1 : 8.0 : 4.0. Methylation analysis of both the mucilage and the carboxyl-reduced derivative, and partial hydrolysis studies enabled elucidation of the structural features.

Keywords——Hibiscus-mucilage SL; leaf; *Hibiscus syriacus*; intrinsic viscosity; acidic polysaccharide; component analysis; carboxyl reduction; methylation analysis; partial hydrolysis; structural feature

In the previous papers of this series, the isolation and structural features of four mucilages[2-5] from plants in the *Althaea* genus and of four mucilages[6-9] from plants in the *Abelmoschus* genus in the Malvaceae family were reported. Recently, we have obtained a representative mucilage from the roots of *Hibiscus moscheutos* L., and its structural features have been reported.[1] We now report the isolation and structural investigation of a representative mucilage from the leaves of *Hibiscus syriacus* L., as the second example of the mucilages from plants of the genus *Hibiscus*. The leaves contain relatively large amounts of mucilages, but no structural study on the mucilages has previously been reported.

The fresh leaves were homogenized and extracted with cold water. The crude mucilage was precipitated 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, the eluate with 0.5 M ammonium carbonate was dialyzed and purified by successive gel chromatography on Sephadex G-15, Sephacryl S-300 and Sephadex G-25.

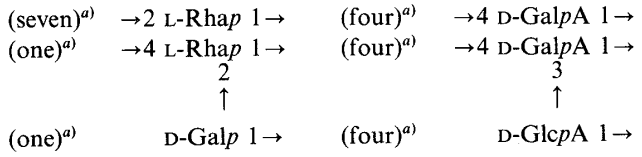
The mucilage gave a single spot on zone electrophoresis with glass-fiber paper, and in addition, it gave a single peak on gel chromatography with Sepharose CL-4B. The mucilage had $[\alpha]_D^{23}$ + 57.7 °(H_2O, c = 0.1), and its aqueous solution gave the high intrinsic viscosity value of 26.5 at 30 °C. The relative viscosity of the solution of the pure mucilage was about 18 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 with standard dextrans gave a value of about 2000000 for the molecular weight. The name "Hibiscus-mucilage SL" is proposed for this substance.

As component sugars of the mucilage, rhamnose, galactose, galacturonic acid, and glucuronic acid were identified. Quantitative determination showed that the mucilage contained 31.6% rhamnose, 4.8% galactose, 38.0% galacturonic acid, and 19.0% glucuronic acid, and that their molar ratio was 8.0 : 1.1 : 8.0 : 4.0. Determination of protein content was carried out by the method of Lowry *et al.*,[10] and a value of 1.8% was obtained. The nuclear

magnetic resonance (NMR) spectrum and infrared (IR) spectrum of the mucilage showed no acetyl signals.

The carboxyl groups of hexuronic acids in the mucilage were reduced with a carbodiimide reagent and sodium borohydride to give the corresponding neutral sugar residues.[11] Methylation of the original mucilage and the carboxyl-reduced derivative was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide.[12] The fully methylated products were hydrolyzed, and the hydrolyzates were converted into the partially methylated alditol acetates.[13] Methyl ethers of the hexuronic acids were removed from the hydrolysis products of the methylated original mucilage by treatment with an anion-exchange resin. Gas-liquid chromatography (GLC)-mass spectrometry (MS)[14] revealed derivatives of 3,4-di-*O*-methyl-L-rhamnopyranose, 3-*O*-methyl-L-rhamnopyranose, and 2,3,4,6-tetra-*O*-methyl-D-galactopyranose as the products in a molar ratio of 7.5 : 0.9 : 1.0 from the original mucilage. Alditol acetates of 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, 2,3,6-tri-*O*-methyl-D-galactopyranose, and 2,6-di-*O*-methyl-D-galactopyranose were identified in a molar ratio of 7.4 : 0.9 : 3.5 : 0.9 : 4.6 : 4.0 from the carboxyl-reduced product.

These results suggested that the minimal repeating unit of the polysaccharide moiety of Hibiscus-mucilage SL is composed of six kinds of component sugar units as shown in Chart 1.



Rha*p*, rhamnopyranose; Gal*p*, galactopyranose;
Gal*p*A, galactopyranosyluronic acid;
Glc*p*A, glucopyranosyluronic acid

Chart 1. Component Sugar Residues in the Minimal Repeating Unit in the Structure of Hibiscus-Mucilage SL

a) Number of residues.

The mucilage was partially hydrolyzed with dilute sulfuric acid, and then neutralized and treated with Dowex 50 W (H^+). The eluate with water was applied to a column of DEAE-Sephadex A-25 (formate form). In addition to small amounts of the component monosaccharides, five oligosaccharides (I to V) were obtained by stepwise elution with dilute formic acid, then purified by rechromatography. Based on the results of component sugar analysis and a comparison of their chromatographic properties, their ^{1}H-NMR spectra, and their values of specific rotation with those of authentic samples,[2b,5] I to V was identified as the following five oligosaccharides (Chart 2).

All galactose residues were liberated from the mucilage under the conditions of partial hydrolysis. In addition to the results of methylation analysis, this fact suggests that one-eighth

I: α-D-Gal*p*A 1→2 L-Rha*p*
II: α-D-Gal*p*A 1→2 α-L-Rha*p* 1→4 α-D-Gal*p*A 1→2 L-Rha*p*
III: β-D-Glc*p*A 1→3 α-D-Gal*p*A 1→2 L-Rha*p*
IV: ($n=0$)
V: ($n=1$)

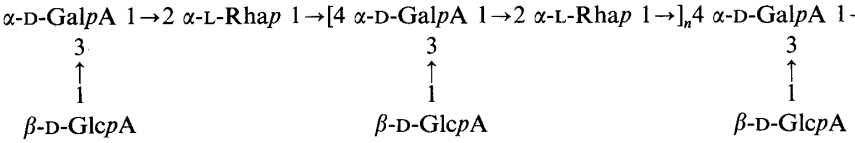


Chart 2. Structural Features of Oligosaccharides I—V

of the rhamnose residues in the backbone chain possesses a galactose residue at position 4. The value of specific rotation of the galactose fraction was consistent with the D configuration.

The glycosidic linkage of the galactose residues is much more easily cleaved than those of the other component sugars in the mucilage.[2a] The mucilage was treated with hot dilute trifluoroacetic acid to obtain the main fraction having the backbone structure and no galactose. The ^{13}C-NMR spectrum of the mucilage showed four anomeric signals at δ 107.57, 106.68 (β-D-glucuronic acid), 101.10 (α-L-rhamnose) and 100.34 (α-D-galacturonic acid).[15] By a comparison of the ^{13}C-NMR spectra of the original mucilage and the product obtained by the acid treatment, it can be concluded that the anomeric signal at δ 107.57, which is observed only in the former, is due to β-D-galactose residues.

Based on the accumulated evidence described above, it may be concluded that the polysaccharide moiety of the mucilage contains the units shown in Chart 3.

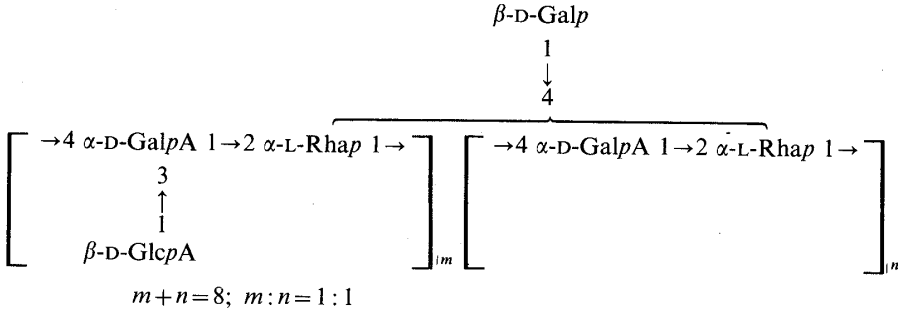


Chart 3. A Possible Structural Fragment of the Polysaccharide Moiety of Hibiscus-Mucilage SL

The presence of the component residue having the structure (1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranose is common in most mucilages[1-7,9] obtained by us from plants in the Malvaceae family, whereas Okra-mucilage F[8] from the immature fruits of *Abelmoschus esculentus* has no branch at any of the D-galacturonic acid residues in the main chain. The unit in backbone chain consisting of alternating (1→4)-linked α-D-galactopyranosyluronic acid and (1→2)-linked α-L-rhamnopyranose was also found in Althaea-mucilage OL[3] from the leaves of *Althaea officinalis* and Abelmoschus-mucilage G[7] from the roots of *Abelmoschus glutinotextilis*, in addition to Okra-mucilage F. The rates of the partial lack of glucuronic acid branches at position 3 of galacturonic acid residues in the main chain were one-eleventh in Altheae-mucilage OL and one-fourth in Abelmoschus-mucilage G. The presence of both the component residue having the structure (1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranose and the residue having the structure (1→4)-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranose in similar amounts in the main chain is characteristic of Hibiscus-mucilage SL. From the results of partial hydrolysis, it can be concluded that the sequence of these two units is not a regular repetition. The presence of galactose branches at position 4 of a part of the rhamnose residues in the main chain is common in Abelmoschus-mucilage RL[5] and Hibiscus-mucilage SL. 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. NMR spectra were recorded on a JEOL JNM-GX 270

FT NMR spectrometer in heavy water containing sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 50 °C. IR spectra were recorded on a JASCO IRA-2 infrared spectrophotometer. 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 spectrometer. Viscosity was determined with an Ubbelohde-type viscosimeter.

Material——The material was obtained at the end of August 1985 from plants cultivated in Saitama Prefecture. The fresh leaves contained 67.0% water.

Isolation of the Mucilage——The fresh leaves (300 g) were homogenized and extracted with water (3000 ml) under stirring for 1 h at room temperature. After centrifugation, the supernatant was poured into two volumes of ethanol. The precipitate was lyophilized; the yield of the crude mucilage was 1.8%. The crude mucilage was dissolved in water and applied to a column (4 × 33 cm) of DEAE-Sephadex A-25 (Pharmacia Co.). DEAE-Sephadex was pretreated as described in a previous report.[2a] After elution with water (1380 ml) and 0.2 M ammonium carbonate (1200 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.[16] The eluates obtained from tubes 21 to 79 were combined, dialyzed against distilled water and concentrated. The solution was applied to a column (5 × 83 cm) of Sephadex G-15. Fractions of 20 ml were collected with water, and the eluates obtained from tubes 25 to 54 were combined and concentrated. The solution was applied to a column (5 × 84 cm) of Sephacryl S-300. Elution was carried out with 0.1 M Tris–HCl buffer (pH 7), and fractions of 20 ml were collected and analyzed as described above. The eluates obtained from tubes 26 to 33 were combined, dialyzed and concentrated. The solution was applied to a column (5 × 81 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 29 to 42 were combined, concentrated and lyophilized. Hibiscus-mucilage SL (156 mg) was obtained as a white powder.

Glass-Fiber Paper Electrophoresis——This was performed as described in a previous report[17] on Whatman GF 83 glass-fiber paper at 570 V with the following two buffers: A, 0.08 M pyridine–0.04 M acetic acid (pH 5.4); B, 0.025 M borax: 0.1 N sodium hydroxide (10 : 1, pH 9.3). The sample gave a single spot at distances of 3.5 cm in buffer A (1.5 h) and 3.8 cm in buffer B (1 h) from the origin toward the cathode.

Gel Chromatography——The sample (2 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7) and applied to a column (2.6 × 92 cm) of Sepharose CL-4B. Elution was carried out 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 hydrolyzate of the mucilage as described in a previous report.[2a]

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.[6b] Rhamnose was also determined by the thioglycolic acid method,[18] and hexuronic acids in the original mucilage were estimated by a modification of the carbazole method.[19] Amino acids were determined with a Hitachi KLA-5 amino acid analyzer after hydrolysis with 6 N hydrochloric acid, and the composition is listed in Table I.

Reduction of the Mucilage——The mucilage (36 mg) was dissolved in water (21 ml), then 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (0.7 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 (7 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 twice more under the same conditions. The final non-dialyzable fraction was concentrated and applied to a column (5 × 78.5 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 29 to 43 were combined and lyophilized. Yield, 12.3 mg.

Methylation——Sodium hydride (30 mg) was mixed with dimethyl sulfoxide (1 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 (6 mg) in dimethyl sulfoxide (1 ml). The reaction mixture was stirred at room temperature for 4 h, then methyl iodide (3 ml) was

TABLE I. Amino Acid Composition of Hibiscus-Mucilage SL (Molar Percent)

Lysine	6.24	Glycine	10.53
Histidine	1.72	Alanine	10.27
Arginine	3.99	Valine	7.56
Aspartic acid	9.89	Methionine	1.58
Threonine	6.92	Isoleucine	3.89
Serine	7.69	Leucine	7.77
Glutamic acid	9.30	Tyrosine	2.01
Proline	7.13	Phenylalanine	3.51

added, and the whole was stirred overnight at room temperature. All procedures were carried out under nitrogen. After dialysis against distilled water, the non-dialyzable fraction was evaporated to dryness. The residue was methylated once more under the same conditions. Water (15 ml) was added to the reaction mixture, and the whole was extracted five times with chloroform (15 ml each). The combined extract was washed five times with water (75 ml each), then dried over sodium sulfate, and the filtrate was concentrated to dryness. The residue was methylated once more in the case of the carboxyl-reduced product, and methylated three times more in the case of the original mucilage 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 4 to 8 were combined and concentrated. The IR spectra of the final residues showed no hydroxyl group absorption. Yield, 5.7 mg from the carboxyl-reduced product and 3.6 mg from the original mucilage.

Analysis of the Methylated Products——The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report.[8] GLC and GLC-MS of partially methylated alditol acetates were performed on a column (3 mm × 2 m long spiral glass) packed with 3% OV 225 on Gaschrom Q (100 to 120 mesh) at 200 °C with a helium flow of 60 ml per min. 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 (100 mg) was suspended in 1 N sulfuric acid (10 ml) and heated in a boiling water bath for 2 h. After neutralization with barium carbonate, followed by filtration, the filtrate was passed through a column (0.7 × 5 cm) of Dowex 50WX8 (H^+). The eluate with water was concentrated and lyophilized (yield, 49 mg), then an aqueous solution of the lyophilizate was applied to a column (1 × 7.5 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted successively with water (45 ml), 0.1 M formic acid (95 ml), 0.2 M formic acid (50 ml), 0.3 M formic acid (80 ml), 0.4 M formic acid (65 ml), and 0.6 M formic acid (90 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 2 to 4; frac. 2, tubes 13 to 17; frac. 3, tubes 18 to 22; frac. 4, tubes 32 to 34; frac. 5, tubes 41 to 48; frac. 6, tubes 49 to 51; frac. 7, tubes 70 to 75. The yields were 10.0 mg for frac. 1, 3.1 mg for frac. 2, 2.2 mg for frac. 3, 1.7 mg for frac. 4, 8.9 mg for frac. 5, 1.4 mg for frac. 6, and 1.3 mg for frac. 7.

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	0.89	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	0.44
II	+96.0°	GalA : Rha = 1 : 1	0.32
III	+85.4°	GlcA : GalA : Rha = 1 : 1 : 1	0.36
IV	+82.1°	GlcA : GalA : Rha = 1 : 1 : 1	0.26
V	+78.0°	GlcA : GalA : Rha = 1 : 1 : 1	0.15

Frac. 1 contained rhamnose and galactose, and frac. 3 contained glucuronic acid. Frac. 2 was purified on a column of Sephadex G-15, and fracs. 4, 5, 6, and 7 were each purified on a column of Sephadex G-25 as described in a previous report.[4] Oligosaccharides I, II, III, IV, and V were obtained from fracs. 2, 4, 5, 6, and 7, respectively. The yields were 2.4 mg for I, 1.4 mg for II, 7.2 mg for III, 1.1 mg for IV, and 1.0 mg for V.

Analysis of the Oligosaccharides——Analysis of component sugars was performed as described in a previous report.[6b] TLC was carried out 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 inTable III.

Elimination of Galactose by Partial Hydrolysis——The mucilage (13.4 mg) was dissolved in water (1.3 ml), then 0.2 M trifluoroacetic acid (1.3 ml) was added. The mixture was heated in a boiling water bath for 30 min. After evaporation to dryness, the acid was removed by repeated addition and evaporation of methanol. The residue was dissolved in water, and applied to a column (5 × 83.5 cm) of Sephadex G-25. The column was eluted with water. Fractions of 20 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from tubes 30 to 33 were combined, concentrated and lyophilized. No galactose was detected in the product by TLC of its hydrolyzate. Yield, 12 mg.

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