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THE CARBOHYDRATE STRUCTURE OF A MUCILAGE FROM THE ROOTS OF *Hibiscus moscheutos* L.[†,*]

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ABSTRACT

A representative mucilage, named Hibiscus-mucilage Mo, was isolated from the roots of *Hibiscus moscheutos* L.; it was homogeneous by ultracentrifugal analysis, electrophoresis, and gel chromatography. Its intrinsic viscosity in aqueous solution was 32.2. It is a partially acetylated, acidic polysaccharide of molecular weight ~1,900,000, and is composed of L-rhamnose:D-galacturonic acid:D-glucuronic acid in approximately equimolar ratio. Methylation of the carboxyl-reduced derivative and partial hydrolysis studies indicated a repeating unit of the following structure.

$$\begin{array}{l} \rightarrow 4)\text{-}\alpha\text{-D-Gal}p\text{A-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow \\ \qquad\qquad\quad 3 \\ \qquad\qquad\quad \uparrow \\ \qquad\qquad\quad 1 \\ \qquad\quad \beta\text{-D-Glc}p\text{A} \end{array}$$

INTRODUCTION

Many mucilages are found in various plants in the Malvaceae family. The roots and leaves of some plants have been used as emollients, demulcents, and as cough medicines. Further, some mucilages have been used as sizes and as a plasma expander. In the previous papers of this series, the isolation and structural features of representative mucilages from the roots[2] and leaves[3] of *Althaea officinalis* L., the roots[4] of *Abelmoschus manihot* Medicus, the roots[5] of *Abelmoschus glutinotextilus* Kagawa, the immature fruits[6] and roots[7] of *Abelmoschus esculentus* Moench, and the roots[8] and leaves[1] of *Althaea rosea* Cavailles have been reported from our laboratory.

These studies are concerned with the mucilages from plants in the *Althaea*

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and *Abelmoschus* genera, but no structural study on the mucilages from plants in the *Hibiscus* genus has been reported thus far. We now report the isolation and structural investigation of a representative mucilage from the roots of *Hibiscus moscheutos* L.

EXPERIMENTAL

Material. — The material was obtained at the beginning of September 1984 from plants cultivated in Saitama prefecture, Japan. The fresh roots contained 85.2% water.

Isolation of the mucilage. — The fresh roots (100.6 g) were homogenized and extracted with water (500 mL) under stirring for 3 h at room temperature. After centrifugation, the supernatant solution was treated with two volumes of ethanol. The precipitate was lyophilized, and a light-brown powder (yield, 10.6% from the dried material) was obtained as a crude mucilage. A part of this mucilage (160 mg) was dissolved in 0.01% sodium sulfate (80 mL), and 10% cetyltrimethylammonium bromide (1.6 mL) was added. The resultant precipitate was separated by centrifugation, and dissolved in 2M sodium chloride (240 mL). The solution was centrifuged to remove small amounts of impurities. The supernatant solution was poured into two volumes of ethanol, and the resulting precipitate was dissolved in water, and treated again with ethanol. The precipitate was dissolved in water and one half of the solution was applied to a column (5 × 87 cm) of Sephadex G-25. The column was eluted with water, and 20-mL fractions were collected and analyzed by the phenol–sulfuric acid method[9]. Carbohydrate-containing fractions were combined, concentrated, and lyophilized. The pure mucilage was obtained as a grayish-white powder (yield, 17.5% from the crude mucilage).

Cellulose acetate membrane electrophoresis. — This was performed as described in a previous report[6] with 0.08M pyridine–0.04M acetic acid (pH 5.4) at 420 V for 40 min and 0.1M NaOH:0.025M $Na_2B_4O_7 \cdot 10\ H_2O$ (1:10, pH 9.3) at 420 V for 1 h. The membrane was stained with Toluidine Blue. The mucilage gave a single spot at distances of 3.6 cm in the acidic buffer and of 4.0 cm in the alkaline buffer from the center toward the anode.

Glass-fiber paper electrophoresis. — This was performed with Whatman GF 83 glass-fiber paper in the manner described in a previous report[2a], with the same alkaline buffer as already mentioned, at 570 V for 90 min. The sample gave one spot 1.6 cm from the origin toward the cathode. *p*-Anisidine–sulfuric acid reagent was used for the detection.

Polyacrylamide gel electrophoresis. — This was performed in an apparatus equipped with gel[10] tubes (5 × 135 mm each) and 0.05M Tris–glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained for carbohydrate by the PAS procedure, and stained for protein with Coomassie Blue. The sample gave a clear band with the former reagent 3.0 cm from the origin and gave a faint coloration with the latter reagent at the same position.

Molecular weight. — The mucilage (3 mg) was dissolved in 0.1M Tris–HCl buffer (pH 7.0) and applied to a column (2.6 × 90 cm) of Sephacryl S-400 pre-equilibrated with the same buffer, that was then eluted with the same solvent. Fractions of 5 mL were collected and analyzed by the phenol–sulfuric acid method. The column was calibrated by using standard dextrans having known molecular weights. The fraction numbers of peaks of dextrans T2000, T500, T70, and the mucilage were 40, 55, 75, and 41.

Hydrolysis and t.l.c. of component sugars. — These were performed as described in a previous report[2a].

Determination of component sugars. — Neutral sugars in the original and carboxyl-reduced mucilages were analyzed by g.l.c. with a column (3 mm × 2 m) packed with 3% OV225 on Gaschrom Q at 220°, after conversion of the hydrolyzates into alditol acetates as described in a previous report[4b]. Rhamnose was also determined by the thioglycolic acid method[11], and hexuronic acids were estimated by a modification of the carbazole method[12].

Determination of O-acetyl groups. — The sample was hydrolyzed with 0.2M hydrochloric acid and subjected to g.l.c. with a column (3 mm × 2 m) packed with 5% Thermon-1000–0.5% H_3PO_4 on Chromosorb W at 120° as described in a previous report[13].

Reduction of carboxyl groups. — An aqueous solution of the mucilage was treated with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene-sulfonate, hydrochloric acid, and sodium borohydride[14]. The reaction was repeated three times, then the final product was purified by gel filtration with Sephadex G-15 as described in a previous report[8].

Methylation analysis. — Methylation was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide[15]. The reaction was repeated twice as described in a previous report[8]. The final product, which showed no i.r. absorption for hydroxyl groups, was hydrolyzed with dilute sulfuric acid in acetic acid, and then reduced and acetylated in the manner described in a previous report[6] G.l.c.–m.s. of partially methylated alditol acetates was performed with a column (3 mm × 2 m) packed with 3% OV225 on Gaschrom Q at 200°. Mass spectra were recorded on a JEOL JMS-D100 instrument.

Partial hydrolysis. — The mucilage (400 mg) was suspended in 0.5M sulfuric acid and heated for 2 h at 90°. The resulting precipitate was removed by centrifugation, although it contained the same component sugars as those of the mucilage. The supernatant solution was made neutral with barium carbonate, and, after filtration, the filtrate was passed through a column (1 × 15 cm) of Dowex 50WX8 (H^+). The eluate with water was concentrated and lyophilized (yield, 180 mg), and then an aqueous solution of the lyophilizate was applied to a column (1 × 18 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted successively with water (50 mL), 0.1M formic acid (90 mL), 0.2M formic acid (60 mL), 0.4M formic acid (110 mL), 0.6M formic acid (150 mL), 0.8M formic acid (200 mL), and 1.5M formic acid (200 mL). Fractions of 5 mL were collected and analyzed by the

phenol–sulfuric acid method. The eluates obtained were divided into eight groups: Frac. 1, tubes 2–7; Frac. 2, tubes 18–21; Frac. 3, tubes 29–31; Frac. 4, tubes 35–39; Frac. 5, tubes 46–58; Frac. 6, tubes 76–88; Frac. 7, tubes 112–132; and Frac. 8, tubes 157–164. Rhamnose and hexuronic acids were obtained from Fracs. 1 and 3, and their yields were 15.7 and 2.5 mg, respectively. Fracs. 4–8 were each purified on a column of Sephadex G-25 as described in a previous report[8]. Oligosaccharides **1, 3, 4**, and **5** were obtained from Fracs. 2, 6, 7, and 8, respectively. Oligosaccharide **2** was obtained from Fracs. 4 and 5. The yields were 6.2 mg for **1**, 35.2 mg for **2**, 28.9 mg for **3**, 25.6 mg for **4**, and 7.8 mg for **5**.

RESULTS AND DISCUSSION

The mucilage was isolated from the fresh roots by sequential cold-water extraction, ethanol precipitation, selective precipitation with cetyltrimethylammonium bromide, ethanol precipitation, gel chromatography with Sephadex G-25, and lyophilization.

The mucilage was homogeneous as determined by ultracentrifugal analysis (Fig. 1), and gave a single spot on zone electrophoresis with a cellulose acetate membrane and with glass-fiber paper. In addition, it gave a clear band on polyacrylamide gel disk electrophoresis. Both the periodate–Schiff reagent and the Coomassie Blue reagent revealed the band in the same position. Further, it gave a single peak on gel chromatography with Sephacryl S-400. The mucilage had $[\alpha]_D^{25}$ +48.5° (*c* 0.1, M sodium hydroxide), and its aqueous solution gave the high intrinsic viscosity value of 32.2 at 30°. Gel chromatography with standard dextrans gave a value of ~1,900,000 for the molecular weight. The name "Hibiscus-mucilage Mo" is proposed for this substance.

As component sugars of the mucilage, rhamnose, galacturonic acid, and glucuronic acid were identified. The carboxyl groups of hexuronic acids in the mucilage were reduced to give the corresponding neutral sugar residues[14]. The

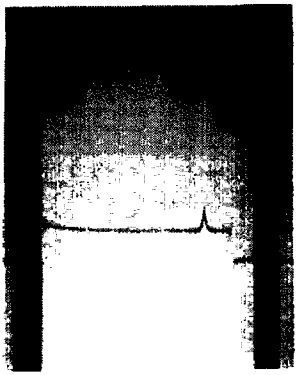

Fig. 1. Ultracentrifugal pattern of Hibiscus-mucilage Mo., measured with a Hitachi UCA-1A ultracentrifuge in a 0.1% solution containing 1% NH_4OH at 60,000 r.p.m. for 34 min.

carboxyl-reduced derivative thus obtained contained component sugars in almost equimolar proportions. Quantitative determination showed that the mucilage contained 22.5% rhamnose, 24.5% galacturonic acid, and 24.5% glucuronic acid. The i.r. spectrum showed absorption bands at 1250 and 1725 cm^{-1}, suggesting the presence of ester linkages. The acid hydrolyzate gave a single peak with a retention time equal to that of acetic acid by g.l.c. The acetyl content of the mucilage was determined to be 8.0%. The mucilage contained 2.7% nitrogen. The amino acid composition after hydrolysis with 6M hydrochloric acid is listed in Table I. No compound other than carbohydrates and amino acids was detected in the hydrolyzate.

The carboxyl-reduced mucilage was methylated by the Hakomori method[15]. The methylated product was hydrolyzed, and the products were converted into the partially methylated alditol acetates[16]. G.l.c.–m.s.[17] revealed derivatives of 3,4-di-*O*-methylrhamnose, 2,3,4,6-tetra-*O*-methylglucose, and 2,6-di-*O*-methylgalactose as the products in a molar ratio of 1.0:0.9:1.1 (Table II).

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

α-D-Gal*p*A-(1→2)-L-Rha*p* β-D-Glc*p*A-(1→3)-α-D-Gal*p*A-(1→2)-L-Rha*p*

1 **2**

α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→[4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]$_n$4)-α-D-Gal*p*A-(1→2)-L-Rha*p*

3 3 3
↑ ↑ ↑
1 1 1
β-D-Glc*p*A β-D-Glc*p*A β-D-Glc*p*A

3 (n = 0)
4 (n = 1)
5 (n = 2)

Based on the accumulated evidence described here, it may be concluded that the polysaccharide moiety of the mucilage is built up from repeating units of the following structure.

→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→
3
↑
1
β-D-Glc*p*A

TABLE I

AMINO ACID COMPOSITION AFTER HYDROLYSIS WITH HYDROCHLORIC ACID

Amino acid	*Mole percent*	*Amino acid*	*Mole percent*
Lysine	7.94	Glycine	14.06
Histidine	3.05	Alanine	10.39
Arginine	3.46	Valine	7.33
Aspartic acid	8.96	Methionine	0
Threonine	5.91	Isoleucine	3.52
Serine	7.74	Leucine	5.91
Glutamic acid	13.24	Phenylalanine	3.18
Proline	5.30	Tyrosine	0

TABLE II

METHYLATION ANALYSIS OF CARBOXYL-REDUCED POLYSACCHARIDE

Methylated sugar[a]	T[b]	*Molar ratio*	*Mode of linkage*
3,4-Rha	0.89	1.0	→2)-Rha*p*-(1→
2,3,4,6-Glc	1.00	0.9	T-Glc*p*-(1→[c]
2,6-Gal	2.77	1.1	→3,4)-Gal*p*-(1→

[a]3,4-Rha = 3,4-di-*O*-methyl rhamnose, *etc.* [b]Retention time of the corresponding alditol acetate on a 3% OV-225 column at 200° with He flow of 60 mL per min relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c]T denotes non-reducing terminal.

TABLE III

SUGAR COMPOSITIONS, SPECIFIC ROTATIONS, AND R_F VALUES OF PARTIAL HYDROLYZATES

Oligosaccharide	*Sugar composition*	*Rhamnitol in reduced sample*	$[\alpha]_D$	R_F[a]
1	GalA:Rha = 1:1	45.4%	+93.2°	0.45
2	GlcA:GalA:Rha = 1:1:1	31.8%	+85.4°	0.35
3	GlcA:GalA:Rha = 1:1:1	15.9%	+82.1°	0.24
4	GlcA:GalA:Rha = 1:1:1	10.5%	+78.0°	0.14
5	GlcA:GalA:Rha = 1:1:1	8.3%	+73.1°	0.06

[a]R_F value on t.l.c. with Merck precoated Kieselgel 60 plates, using 2:1:1 (v/v) 1-butanol–acetic acid–water as the developing solvent.

As already reported in previous papers[1–8], most of the backbone chains consist of alternating (1→4)-linked α-D-galactopyranosyluronic acid and (1→2)-linked α-L-rhamnopyranose in the mucilages we have obtained from plants in the Malvaceae family. Among them, Althaea-mucilages OL[3], R[8], and RL[1] possess (1→2)-linked rhamnosylrhamnose residues in part of their main chains. In addition, the component residue having the repeating structure (1→4)-[*O*-β-(D-glucopyranosyluronic acid)-(1→3)]-*O*-α-(D-galactopyranosyluronic acid)-(1→2)-*O*-α-L-rhamnopyranose is common in all but Okra-mucilage F[6], although the partial

lack of glucuronic acid branches at position 3 of the D-galacturonic acid in the main chains are found in both Althaea-mucilage OL[3] and Abelmoschus-mucilage G[5]. On the other hand, the majority of them, namely, Okra-mucilages F[6] and R[7], and Althaea-mucilages O[2], R[8], and RL[1], have branches composed of (1→4)-linked aldohexopyranose units at position 4 of a part of the L-rhamnose residues in the main chains. Thus Hibiscus-mucilage Mo possesses a simple repeating-structure in its polysaccharide moiety similarly to that of Abelmoschus-mucilage M[4], and a fairly high acetyl content is a characteristic of the former.

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