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A MUCILAGE FROM *HIBISCUS MOSCHEUTOS* LEAVES*

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Key Word Index—*Hibiscus moscheutos*; Malvaceae; leaf; polysaccharide structure; hypoglycemic activity; mucilage.

Abstract—A mucilage, Hibiscus-mucilage ML, from leaves of *Hibiscus moscheutos* was found to be peptidoglycan with a molecular weight of *ca* 1800000. Its intrinsic viscosity in aqueous solution was 26.1. The major constituent is a partially acetylated, acidic polysaccharide composed of L-rhamnose: D-galactose: D-glucose: D-galacturonic acid: D-glucuronic acid in a molar ratio of 18:12:1:12:11. Chemical and physico-chemical studies indicated its main structural features. It had considerable hypoglycemic activity when administered to mice.

INTRODUCTION

Tomoda *et al.* recently reported the structure of a mucilage, 'Hibiscus-mucilage Mo', from the roots of *Hibiscus moscheutos* [1]. Although the leaves of this plant also contain large amounts of mucilage no previous structural studies have been carried out.

In the present paper, we report the isolation, structure and hypoglycemic activity of a new mucilage from the leaves of *H. moscheutos*.

RESULTS AND DISCUSSION

The crude mucilage was isolated from the fresh leaves of *H. moscheutos* by cold water extraction followed by precipitation with ethanol. The aqueous solution was applied to a DEAE-Sephadex A-25 column (carbonate form). After elution with water and 0.2 M ammonium carbonate, the eluate with 0.5 M ammonium carbonate was dialysed and purified by successive gel chromatography with Cellulofine GCL-2000-m and Sephadex G-25. The mucilage gave a single spot on zone electrophoresis with glass-fibre paper, and in addition, it gave a single band on PAGE, after staining with periodate–Schiff and Coomassie Blue reagents. Further, it gave a single peak on gel chromatography with Sephacryl S-400. The mucilage showed a positive specific rotation ($[\alpha]_D^{24}$ +54.4°) and its aqueous solution gave the high intrinsic viscosity value of 26.1 at 30°. Gel chromatography using standard dextrans gave a value of *ca* 1800000 for its M_r. The name 'Hibiscus-mucilage ML' is proposed for this substance.

Quantitative analyses showed that the mucilage was composed of a polysaccharide (90.6%) and a peptide moiety (8.6%). The polysaccharide was composed of L-rhamnose (26.7%), D-galactose (19.7%), D-glucose (1.7%), D-galacturonic acid (21.5%), D-glucuronic acid (19.7%) and *O*-acetyl groups (1.3%). Their molar ratio was 18:12:1:12:11:3. The amino acid composition was as follows (mol. %): Asp (13.5), Thr (8.2), Ser (5.2), Glu (14.7), Pro (9.2), Gly (16.7), Ala (9.5), Val (7.5), Ile (4.2), Leu (5.7), Phe (2.9) and Lys (2.7).

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 [2]. Both the original mucilage and the carboxyl-reduced derivative were methylated with methylsulphinyl carbanion and methyl iodide in dimethyl sulphoxide [3]. The methylated products were hydrolysed, then converted into the partially methylated alditol acetates. Methyl ethers of hexuronic acids were removed from the hydrolysis products of the methylated original mucilage by treatment with an anion-exchange resin. Gas chromatography/MS [4] showed the presence of 3,4-di-*O*-methyl rhamnose, 3-*O*-methyl rhamnose, 2,3,4,6-tetra-*O*-methyl galactose, 2,3,6-tri-*O*-methyl galactose and 2,3,6-tri-*O*-methyl glucose as the products, in a molar ratio of 16:2:2:10:1. The carboxyl-reduced derivative gave 3,4-di-*O*-methyl rhamnose, 3-*O*-methyl rhamnose, 2,3,4,6-tetra-*O*-methyl glucose, 2,3,4,6-tetra-*O*-methyl galactose, 2,3,6-tri-*O*-methyl galactose, 2,3,6-tri-*O*-methyl glucose and 2,6-di-*O*-methyl galactose in a molar ratio of 16:2:11:2:11:1:11.

These results suggested that the minimal repeating unit of the polysaccharide moiety of the mucilage was composed of eight sugar units as shown in Fig. 1.

The mucilage was partially hydrolysed with dilute sulphuric acid, then neutralized and treated with Dowex 50W (H^+). The eluate with water was applied to a DEAE-Sephadex A-25 column (formate form). In addition to some of the component monosaccharides, four oligosaccharides (1–4) were obtained by stepwise elution with dilute formic acid. Based on the results of sugar analysis and a comparison of chromatographic properties, ^{1}H NMR spectra and specific rotation values with those of authentic samples [5], the four oligosaccharides were identified as 1–4 (Fig. 2).

Most (90%) of the galactose residues and 30% of the rhamnose residues were liberated from the mucilage on partial hydrolysis. A small amount of glucose was also liberated. In addition to the results of methylation

* Part XLI in the series 'Plant Mucilages'. For Part XL, see ref. [1].

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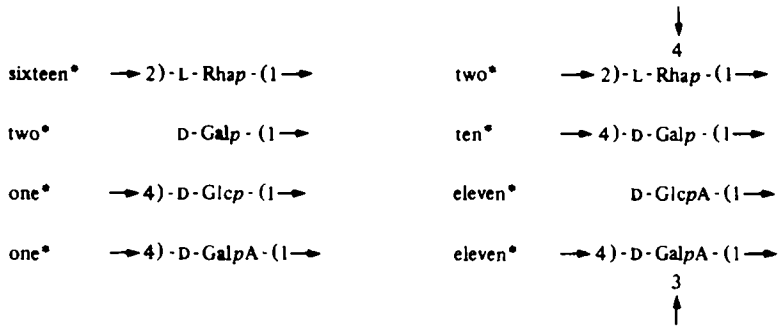


Fig. 1. Component sugar residues in the minimal repeating unit in the structure of Hibiscus-mucilage ML. *Number of residues.

1 α-D-Gal*p*A-(1→2)-L-Rha*p*

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

3 (*n* = 0)

4 (*n* = 1)

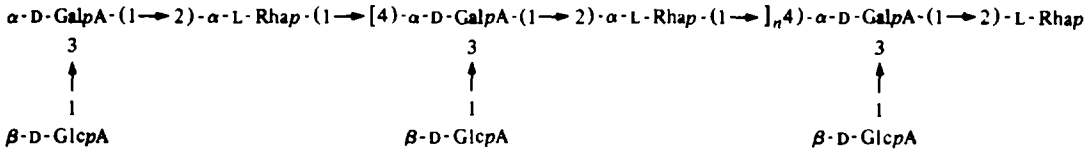
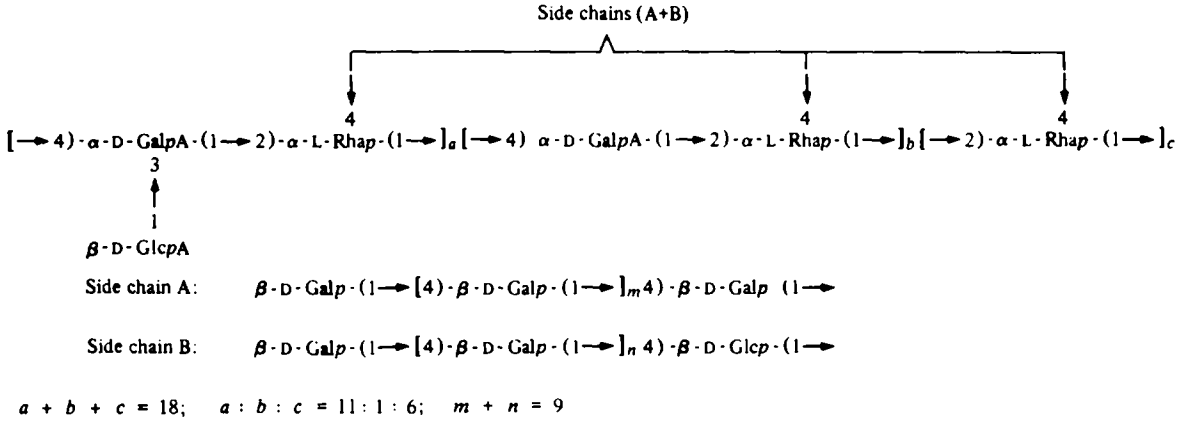


Fig. 2. Structural features of oligosaccharides 1–4.



$a + b + c = 18$; $a : b : c = 11 : 1 : 6$; $m + n = 9$

Fig. 3. Structural units of Hibiscus-mucilage ML.

analysis, this suggests that one-ninth of the rhamnose residues in the main chain are substituted in the 4-position with galactose and glucose residues.

^{1}H NMR spectrum of the mucilage showed four anomeric hydrogen signals at δ4.69 (*d*, J = 7 Hz), 4.71 (*d*, J = 7 Hz), 5.00 (*d*, J = 2 Hz) and 5.15 (*br s*). Among these, it was evident that those at δ4.69 and 4.71 were attributable to anomeric protons of β-D-glucuronic acid, β-D-galactose and β-D-glucose, those at δ5.00 and 5.15 to anomeric protons of α-L-rhamnose and α-D-galacturonic acid [5, 6]. Based on the accumulated evidence, it may be concluded that the polysaccharide moiety of the mucilage contains the units shown in Fig. 3.

Most of the backbone chains consist of alternating (1 → 4)-linked α-D-galactopyranosyluronic acid and (1 → 2)-linked α-L-rhamnopyranose in mucilages obtained from members of the Malvaceae [5–14]. In addition, the component unit 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 and major in all but Okra-mucilage F [6]. Althaea-mucilages OL [8], R [9] and RL [10], Abelmoschus-mucilage M [11], Okra-mucilage R [13] and Hibiscus-mucilage SF [7] possess (1 → 2)-linked α-L-rhamnopyranose repeating units in their backbone chains. On the other hand, the majority of Malvaceae

mucilages have branches composed of (1 → 4)-linked β-D-aldohexopyranose units at position 4 of some of the rhamnose residues in the backbone chains. Among them, Hibiscus-mucilage ML and Althaea-mucilage R [9] are the only example having a (1 → 4)-linked β-D-glucopyranose unit as the reducing terminal of side chains.

When administered i.p. (10, 30, 100 mg/kg), Hibiscus-mucilage ML reduced the blood glucose concentration dose-dependently (after 7 hr: 91, 74, 49%, after 24 hr: 100, 92, 68% of the control). The majority of Malvaceae mucilages have shown significant hypoglycemic activity on i.p. injection to normal mice [15] and so it was not surprising that Hibiscus-mucilage ML also showed remarkable hypoglycemic activity, and its effect was typically dose-dependent.

EXPERIMENTAL

Plant Material. Leaf material of *H. moscheutos* was obtained at the end of August 1985 from plants cultivated in Saitama prefecture, Japan. The fresh leaves contained 75.5% H_2O.

Isolation of the mucilage. The fresh leaves (440 g) were homogenized and extracted with H_2O (4400 ml) with stirring for 90 min at room temp. After centrifugation, the supernatant was treated with 2 vols of EtOH. The ppt was dissolved in H_2O (500 ml), and applied a 5 i.d. × 76 cm DEAE-Sephadex A-25 column (Pharmacia Co.). DEAE-Sephadex was pretreated as described in a previous report [16]. After elution with H_2O (3000 ml) and 0.2 M $(NH_4)_2CO_3$ (2580 ml), the column was eluted with 0.5 M $(NH_4)_2CO_3$. 20 ml Fractions were collected and analysed by the PhOH–H_2SO_4 method [17]. The eluates obtained from tubes 46 to 76 were combined, dialysed against distilled H_2O, concd and applied to a 5 i.d. × 80 cm Cellulofine GCL-2000-m column (Seikagaku Kōgyo Co.). Elution was carried out with 0.05 M phosphate buffer (pH 7.5) containing 0.1 M NaCl, and 20 ml fractions collected. The eluates from tubes 29 to 44 were combined, dialysed, concd and applied to a 5 i.d. × 84 cm Sephadex G-25 column, which was eluted with H_2O and 20 ml fractions collected. The eluates from tubes 31 to 50 were combined, concd and lyophilysed. Hibiscus-mucilage ML (130 mg) was obtained as a white powder.

Glass-fibre paper electrophoresis. This was performed on Whatman GF 83 glass-fibre paper (12 i.d. × 38 cm) with 0.1 M NaOH–0.025 M $Na_2B_4O_7 \cdot 10H_2O$ (1:10, pH 9.3) at 570 V for 1 hr [16]. The mucilage gave a single spot 14.2 cm from the origin toward the cathode.

PAGE. This was carried out in apparatus equipped with gel [18] tubes (0.4 i.d. × 14.2 cm) and 0.005 M Tris–glycine buffer (pH 8.3) at 5 mA/tube for 1 hr. Gels were stained for carbohydrate using the PAS procedure, and stained for protein with Coomassie Blue reagent.

Determination of M_r. The sample (5 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a 2.6 i.d. × 94 cm Sephacryl S-400 column, pre-equilibrated and developed with the same buffer. 5 ml Fractions were collected and analysed by the PhOH–H_2SO_4 method. The M_r was calculated by comparison of the V_e of the sample with the V_es of standard dextrans.

Determination of the components. The sample was hydrolysed with 1 M H_2SO_4 at 100° for 6 hr and analysed by cellulose TLC [16], then the product was reduced, acetylated and analysed for component sugars by GC [11]: 3% OV 225 on Gaschrom Q (100–120 mesh; 0.3 i.d. × 200 cm, spiral glass), 220°, He 50 ml/min. Rhamnose was also determined by the thioglycolic acid method [19], and hexuronic acids were estimated by a modification of the carbazole method [20].

For the determination of *O*-acetyl groups, the sample was hydrolysed with 0.2 M HCl and subjected to GC with a column (0.3 i.d. × 200 cm) packed with 5% Thermon-1000–0.5% H_3PO_4 on Chromosorb W at 120° as described in a previous report [21].

Protein determination was by the method of Lowry *et al.* [22] and the amino acid composition determined with an amino acid analyser after hydrolysis with 6 M HCl at 110° for 24 hr.

Reduction of carboxyl groups. The mucilage (70 mg) was dissolved in H_2O (20 ml), then 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene-sulphonate (0.7 g) was added. The pH of the reaction mixture was maintained at 4.75 by titration with 0.1 M HCl with stirring for 2 hr, then 2 M $NaBH_4$ (7 ml) was added gradually to the reaction mixture during 4 hr while the pH was maintained at 7.0 by titration with 4 M HCl with stirring at room temp. The soln was dialysed against distilled H_2O overnight and the non-dialysable fraction concd to 20 ml. The product was reduced a further 3 times using the same methods. The final non-dialysable fraction was concd and applied to a 5 i.d. × 83 cm Sephadex G-25 column, which was eluted with H_2O, and 20 ml fractions collected. The eluates from tubes 30 to 40 were combined and lyophilyzed. Yield, 17.2 mg.

Methylation analysis. NaH (15 mg) was mixed with DMSO (3 ml) in an ultrasonic bath for 30 min. The mixture was stirred at 70° for 1 hr and added to the sample (10 mg) in DMSO (1 ml). After stirring for 4 hr at room temp., MeI (3 ml) was added, and the whole stirred overnight at room temp. All procedures were carried out under N_2. The reaction mixture was poured into H_2O (20 ml) and extracted (× 5) with $CHCl_3$ (20 ml each). The combined extract was washed (× 5) with H_2O (100 ml each), then dried (Na_2SO_4), and the filtrate concd to dryness. The residue was methylated (× 4) under the same conditions. The final residue was dissolved in $CHCl_3$–MeOH (2:1), and applied to a 1 i.d. × 18 cm Sephadex LH-20 column, which was eluted with the same solvent and 1 ml fractions collected. The eluates from tubes 5 to 8 were combined and concd to dryness. The final products (9.4 mg from the original; 11.5 mg from the carboxyl-reduced derivative) showed no hydroxyl absorption in their IR spectra. The products were hydrolysed with dilute H_2SO_4 in HOAc, then reduced and acetylated as described in a previous report [6]. The partially methylated alditol acetates obtained were analysed by GC/MS using a 3% OV 225 column as described above at 200°, He 60 ml/min. The RR_fs 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 MS are shown in Table 1.

Partial hydrolysis. The mucilage (57 mg) was suspended in 0.5 M H_2SO_4 (5 ml) and heated at 95° for 2 hr. The resulting ppt was removed by centrifugation and the supernatant neutralized with $BaCO_3$. After filtration, the filtrate was passed through a column (1 i.d. × 16 cm) of Dowex 50WX8 (H^+). The eluate with H_2O was concd and lyophilyzed. Yield, 36.9 mg. An aq soln was applied to a 1 i.d. × 17 cm DEAE-Sephadex A-25 column (formate form), which was eluted successively with H_2O (30 ml), 0.1 M HCOOH (90 ml), 0.2 M HCOOH (60 ml), 0.4 M HCOOH (65 ml), 0.6 M HCOOH (50 ml), and 1 M HCOOH (80 ml). 5 ml Fractions were collected and analysed by the PhOH–H_2SO_4 method. The eluates obtained were divided into 6 groups: Frac. 1, tubes 2–5; Frac. 2, tubes 7–24; Frac. 3, tubes 32–36; Frac. 4, tubes 42–46; Frac. 5, tubes 52–56; and Frac. 6, tubes 62–70. Rhamnose and galactose were obtained from Frac. 1 (yield, 14.3 mg). Fracs 2–6 were each purified on columns of Sephadex G-15 and G-25 as described in a previous report [9]. Oligosaccharides **1**, **3** and **4** were obtained from Fracs 2, 5 and 6, respectively. Oligosaccharide **2** was obtained from Fracs 3 and 4. The yields were 2.6 mg for **1**, 2.2 mg for **2**, 2.0 mg for **3**, and 1.7 mg for **4**.

Measurement of hypoglycemic activity. Male mice (Std:ddY strain, 25–30 g) were used in groups of five and given food and

Table 1. RR_ts on GC and main fragments in the mass spectra of partially methylated alditol acetates

Acetate	RR_t	Main fragments (m/z)
1,2,5-Ac-3,4-Me-L-Rhamnitol	0.89	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	1.14	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,2,4,5-Ac-3-Me-L-Rhamnitol	1.58	43, 87, 101, 129, 143, 189, 203
1,4,5-Ac-2,3,6-Me-D-Galactitol	1.95	43, 45, 87, 99, 101, 113, 117, 233
1,4,5-Ac-2,3,6-Me-D-Glucitol	2.03	43, 45, 87, 99, 101, 113, 117, 233
1,3,4,5-Ac-2,6-Me-D-Galactitol	2.72	43, 45, 87, 117, 129

drinking water freely. The mucilage was dissolved in physiological saline soln and injected (i.p.). Blood was drawn periodically from the orbital sinus by micro-haematocrit tubes. The glucose level of plasma obtained by centrifugation of blood was measured with a glucose analyser by the glucose oxidase method. Data are expressed as mean ± s.e. One-way analysis of variance was used to evaluate the results.

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