Plant Mucilages. XL. 1) A Representative Mucilage, “Hibiscus-Mucilage SF,” from the Flower Buds of Hibiscus syriacus

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A representative mucilage, named Hibiscus-mucilage SF, was isolated from the white flower buds of Hibiscus syriacus L. It was homogeneous on electrophoresis and gel chromatography. Its intrinsic viscosity value in aqueous solution was 26.0. It is mainly composed of partially acetylated acidic polysaccharide of molecular weight 1050000, and is composed of L-rhamnose : D-galactose: D-galacturonic acid: D-glucuronic acid in the molar ratio of 36 : 36 : 33 : 22. Methylation analysis of both the mucilage and the carboxyl-reduced derivative, and partial hydrolysis studies enabled elucidation of the structural features.

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

The white flower bud of Hibiscus syriacus L. is an Oriental crude drug (Japanese name, Mokukinka) used as a demulcent and antidiarrheic. As constituents of the bud of this plant, saponarin, β-carotene and lutein, and an essential oil containing 65 components have been reported so far. In addition, it is well known that the bud contains relatively large amounts of mucilages, but no structural study on the mucilages has previously been reported. We have now isolated a representative mucilage from the white flower buds of this plant. Its properties and structural features are reported here.

The fresh buds were homogenized and extracted with cold water. The crude mucilage was precipitated from the extract by addition of ethanol, then dissolved again in water. The solution was treated with sodium lauryl sulfate and sodium chloride. After centrifugation, the supernatant obtained was poured into acetone. The precipitate obtained was dissolved in water and the solution was dialyzed and purified by gel chromatography with Sephadex G-25, then the eluate was lyophilized.

The mucilage gave a single spot on zone electrophoresis with glass-fiber paper, and it gave a single peak on gel chromatography with Sephacryl S-400. Further, it gave a clear band on polyacrylamide gel disk electrophoresis. Both periodate-Schiff reagent and the Coomassie blue reagent revealed the band in the same position. The mucilage had $[\alpha]_D^{25} + 30.0^\circ$ (0.1% NH₄OH, c = 0.1), and its aqueous solution gave the high intrinsic viscosity value of 26.0 at 30 °C. Gel chromatography with standard dextrans gave a value of about 1050000 for the molecular weight. The name “Hibiscus-mucilage SF” 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 20.2% rhamnose, 22.2% galactose, 22.5% galacturonic acid, and 15.2% glucuronic acid. The proton nuclear magnetic resonance ($^1$H-NMR) spectrum showed a signal at δ 2.13, suggesting the presence of O-acetyl groups. This was confirmed by gas chromatography (GC) of the hydrolyzate, and the acetyl content was determined to be 5.9%. The molar ratio of rhamnose:galactose:galacturonic acid:glucuronic acid: O-acetyl is 36:36:33:22:36. De-
termination of protein content was carried out by the method of Lowry et al.,5) and a value of 8.1% was obtained.

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.6) Methylation of the original mucilage and the carboxyl-reduced derivative was performed with methylsulfinyl carbamion and methyl iodide in dimethyl sulfoxide.7) The methylated products were hydrolyzed, and the hydrolyzates were converted into the partially methylated alditol acetates.8) Methyl ethers of hexuronic acids were removed from the hydrolyzate of the methylated original mucilage by treatment with an anion-exchange resin. Gas-liquid chromatography (GLC)-mass spectrometry (MS)9) revealed derivatives of 3,4-di-O-methyl-L-rhamnose, 3-O-methyl-L-rhamnose, 2,3,4,6-tetra-O-methyl-D-galactose, and 2,3,6-tri-O-methyl-D-galactose as the products in a molar ratio of 1:1:1 from the original mucilage. Alditol acetates of 3,4-di-O-methyl-L-rhamnose, 3-O-methyl-L-rhamnose, 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,6-tri-O-methyl-D-galactose, and 2,6-di-O-methyl-D-galactose were identified in a molar ratio of 18:18:22:18:29:22 from the carboxyl-reduced product.

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

\[
\begin{align*}
\text{(eighteen)} & \quad \text{D-Galp} \quad 1 \rightarrow \quad \text{(twenty-two)} & \quad \text{D-GlcPA} \quad 1 \rightarrow \\
\text{(eighteen)} & \quad \rightarrow 4 \quad \text{D-Galp} \quad 1 \rightarrow \quad \text{(eleven)} & \quad \rightarrow 4 \quad \text{D-GlpA} \quad 1 \rightarrow \\
\text{(eighteen)} & \quad \rightarrow 4 \quad \text{L-Rhap} \quad 1 \rightarrow \quad \text{(twenty-two)} & \quad \rightarrow 4 \quad \text{D-GlpA} \quad 1 \rightarrow \\
\text{(eighteen)} & \quad \rightarrow 2 \quad \text{L-Rhap} \quad 1 \rightarrow \quad 3 \\
\end{align*}
\]

\[\text{Galp, galactopyranose; Rhap, rhamnopyranose; GlcPA, glucopyranosyluronic acid; GalpA, galactopyranosyluronic acid}\]

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

\(a)\) Number of residues.

The mucilage was partially hydrolyzed with dilute sulfuric acid, and then neutralized and treated with Dowex 50W (H⁺). The eluate with water was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (formate form). In addition to a part of the component monosaccharides, three oligosaccharides (I to III) 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 ¹H-NMR spectra, and their values of specific rotation with those of authentic samples,10) I to III were identified as the following three oligosaccharides (Chart 2).

All galactose residues were liberated from the mucilage under the conditions of partial hydrolysis described above. In conjunction with the results of methylation analysis, this finding suggests that a half of the rhamnose residues in the backbone chain possesses a 1→4
galactosyl galactose chain at position 4. A part (5.3%) of the rhamnose residues was also liberated by partial hydrolysis. The value of specific rotation of the galactose fraction was consistent with the D configuration.

The $^1$H-NMR spectrum of the mucilage showed four anomeric proton signals at $\delta$ 4.62 (d, $J = 7$ Hz), $\delta$ 4.71 (d, $J = 7$ Hz), $\delta$ 4.98 (d, $J = 2$ Hz), and $\delta$ 5.18 (br s). Their integral ratio was 3 : 2 : 3 : 3. The signals at $\delta$ 4.71, 4.98, and 5.18 were assigned to the anomeric protons of $\beta$-D-glucuronic acid, $\alpha$-L-rhamnose, and $\alpha$-D-galacturonic acid, respectively. Thus, it can be concluded that the anomeric signal at $\delta$ 4.62 is due to $\beta$-D-galactose residues.

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

$$[\beta\text{-D-Galp} 1 \rightarrow 4 \beta\text{-D-Galp}]_d$$

$$\rightarrow4 \alpha\text{-D-GalpA} 1 \rightarrow 2 \alpha\text{-L-Rhap} 1 \rightarrow$$

$$\text{ } 3$$

$$\text{ } 1$$

$$\beta\text{-D-GlcpA}$$

$$a:b:c:d = 22:11:3:18$$

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

The component unit having the repeating structure $(1 \rightarrow 4)$-$[O-\beta-(D-glucopyranosyluronic acid)-(1 \rightarrow 3)]-O-\alpha-(D-galactopyranosyluronic acid)-(1 \rightarrow 2)$-$\alpha$-$L$-rhamnopyranose is common and major in all mucilages except Okra-mucilage obtained by us from plants in the Malvaceae family. Okra-mucilage F from the immature fruits of *Abelmoschus esculentus* has a backbone chain consisting of the $(1 \rightarrow 4)$-$O-\alpha-(d$-galactopyranosyluronic acid)-(1 $\rightarrow 2)$-$\alpha$-$L$-rhamnopyranose unit. This unit was also found in the backbone chains of Althaea-mucilage OL from the leaves of *Althaea officinalis*, Abelmoschus-mucilage G from the roots of *Abelmoschus glutinotextilis*, and Hibiscus-mucilage SL from the leaves of *Hibiscus syriacus*, in addition to Okra-mucilage F. $\alpha$-$1 \rightarrow 4$-Linked L-rhamnopyranosyl L-rhamnopyranose residues were found in the backbone chains of Althaea-mucilage OL and Althaea-mucilages R and RL from the roots and the leaves of *Althaea rosea*, Abelmoschus-mucilage M from the roots of *Abelmoschus manihot*, and Okra-mucilage R from the roots of *Abelmoschus esculentus*. In the former studies on the mucilages from the plant sources in the Malvaceae family, Althaea-mucilage OL was the sole example having the three types of units in the backbone chain. Thus Hibiscus-mucilage SF is the second example having the three units shown in Chart 3 in its main chain.

The presence of $\beta$-$1 \rightarrow 4$-linked D-galactopyranosyl D-galactopyranose side chains at position 4 of a part of the rhamnose residues in the main chain is common in Hibiscus-mucilage SF, Althaea-mucilage OL, and Okra-mucilage F. We have obtained a hexasaccharide, a nonasaccharide, and a dodecasaccharide having the repeating structure of the trisaccharide unit, $(1 \rightarrow 4)$-$[O-\beta-(D-glucopyranosyluronic acid)-(1 \rightarrow 3)]-O-\alpha-(D-galactopyranosyluronic acid)-(1 \rightarrow 2)$-$\alpha$-$L$-rhamnopyranose, in addition to the trisaccharide and the disaccharide, $\alpha$-$D$-galactopyranosyluronic acid-$\alpha$-$L$-rhamnopyranose, by partial hydrolysis of Althaea-mucilage OL and most of the other mucilages from plants in the Malvaceae family. However, in the case of Hibiscus-mucilage SF, neither the nonasaccharide nor the dodecasaccharide was found in the partial hydrolyzate. Thus, the trisaccharide unit must be present in a dispersed condition as its
monomer or dimer in the backbone chain of Hibiscus-mucilage SF, and such a state in the main chain appears to be characteristic of this substance.

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 70 °C. GC and GLC were 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 a plant cultivated in Saitama prefecture. The fresh buds contained 86.3% water.

Isolation of the Mucilage—The fresh buds (50 g) were homogenized and extracted with water (500 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 1.0%. The crude mucilage was dissolved in water (500 ml) and 5% sodium lauryl sulfate (50 ml) was added, followed by sodium chloride (2.92 g) at 5 °C. After centrifugation, the supernatant was poured into two volumes of acetone. The precipitate was dissolved in water, then dialyzed against distilled water and concentrated. The solution was applied to a column (5 × 84 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 30 to 54 were combined, concentrated and lyophilized. Yield, 60 mg. Hibiscus-mucilage SF was obtained as a white powder.

Glass-Fiber Paper Electrophoresis—This was performed as described in a previous report(19) 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 M sodium hydroxide (10:1, pH 9.3). The sample gave a single spot at distances of 2.4 cm in buffer A (1.5 h) from the origin toward the anode and 3.5 cm in buffer B (45 min) from the origin toward the cathode.

Polyacrylamide Gel Electrophoresis—This was performed in an apparatus with gel tubes (4 × 145 mm each) and 0.05 M Tris–glycine buffer (pH 8.3) at 5 mA/tube for 1 h. Gels were stained for carbohydrate by the periodic acid–Schiff (PAS) procedure, and stained for protein with Coomassie blue. The sample gave a clear band with both reagents at a distance of 6.8 cm from the origin.

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 × 94 cm) of Sephacryl S-400. 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—Hydrolysis, isolation and cellulose thin-layer chromatography (TLC) of component sugars were carried out as described in a previous report.21

Determination of Components—Neutral sugars in the original and the carboxyl-reduced mucilages were analyzed by GLC after hydrolysis and conversion into alditol acetates as described in a previous report.22 Rhamnose was determined by the thioglycolic acid method, and hexuronic acids in the original mucilage were estimated by a modification of the carbazole method.23

Determination of O-Acetyl Groups—The sample was hydrolyzed with 0.2 N hydrochloric acid and subjected to GLC with a column (3 mm × 2 m long spiral glass) packed with 5% Thermon-1000–0.5% phosphoric acid on Chromosorb W at 120 °C as described in a previous report.24

Reduction of Carboxyl Groups—The mucilage (70 mg) was dissolved in water (50 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, then the non-dialyzable fraction was concentrated to 50 ml. The product was reduced five times more under the same conditions. The final non-dialyzable fraction was concentrated and applied to a column (5 × 84 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 27 to 44 were combined and lyophilized. Yield, 15.8 mg.

Methylation—This was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report.13 The methylation was repeated four times under the same conditions. Yields were 15.5 mg from 12 mg of the carboxyl-reduced product and 3 mg from 10 mg of 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.18 GLC and GLC-MS of partially methylated alditol acetates were performed with 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/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
Partial Hydrolysis and Isolation of Oligosaccharides

The mucilage (100 mg) was suspended in 1 N sulfuric acid (20 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 (1 × 5 cm) of Dowex 50WX8 (H+). The eluate with water was concentrated and lyophilized (yield, 40 mg), then an aqueous solution of the lyophilizate was applied to a column (1 × 8 cm) of DEAE-Sephadex A25 (formate form). The column was eluted successively with water (25 ml), 0.1 M formic acid (75 ml), 0.2 M formic acid (65 ml), and 0.3 M formic acid (45 ml). Fractions of 5 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from the column were divided into four groups: Frac. 1, tubes 1 to 3; frac. 2, tubes 10 to 17; frac. 3, tubes 21 to 33; frac. 4, tubes 34 to 42. The yields were 22.8 mg for frac. 1, 13.9 mg for frac. 2, 1.0 mg for frac. 3, and 0.3 mg for frac. 4. Frac. 1 contained galactose and rhamnose in a ratio of 19:1. Frac. 2 was dissolved in water and applied to a column (5 × 84 cm) of Sephadex G-25. The column was eluted with water and fractions of 5 ml were collected. The eluates obtained from the column were divided into two groups: Frac. a, tubes 125 to 138; frac. b, tubes 141 to 160. The yields were 2.8 mg for frac. a and 6.3 mg for frac. b. Oligosaccharides I and II were obtained from fracs. b and a, respectively. Oligosaccharide III was obtained from fracs. 3 and 4 after purification on a column of Sephadex G-25 as described in a previous report.13) Yield, 1.0 mg.

Analysis of the Oligosaccharides

Analysis of component sugars was performed as described in a previous report.13) 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 as described in a previous report.13) The results are listed in Table II.

Amino Acid Composition

Amino acids were determined by the method of Bidlingmeyer et al.25) after hydrolysis with 6 N hydrochloric acid, and the composition found is given in Table III.

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**Table I.** Relative Retention Times on GLC and Main Fragments in MS of Partially Methylated Alditol Acetates

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Relative retention time (min)</th>
<th>Main fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2,5-Ac-3,4-Me-1-Rhamnitol</td>
<td>0.89</td>
<td>43, 89, 129, 131, 189</td>
</tr>
<tr>
<td>1,2,4,5-Ac-3,4-Me-1-Rhamnitol</td>
<td>1.58</td>
<td>43, 87, 101, 129, 143, 189, 203</td>
</tr>
<tr>
<td>1,5-Ac-2,3,4,6-Me-1-Glucitol</td>
<td>1.00</td>
<td>43, 45, 71, 87, 101, 117, 129, 145, 161, 205</td>
</tr>
<tr>
<td>1,5-Ac-2,3,4,6-Me-1-Galactitol</td>
<td>1.14</td>
<td>43, 45, 71, 87, 101, 117, 129, 145, 161, 205</td>
</tr>
<tr>
<td>1,4,5-Ac-2,3,6-Me-1-Galactitol</td>
<td>1.95</td>
<td>43, 45, 87, 99, 101, 113, 117, 233</td>
</tr>
<tr>
<td>1,3,4,5-Ac-2,6-Me-1-Galactitol</td>
<td>2.72</td>
<td>43, 45, 87, 117, 129</td>
</tr>
</tbody>
</table>

*a* Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-n-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 II.** Specific Rotations, Sugar Compositions, and *Rf* Values of Oligosaccharides

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>[α]D25° in H2O</th>
<th>Sugar composition</th>
<th>TLC (<em>Rf</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+93.2°</td>
<td>GalA: Rha = 1:1</td>
<td>0.44</td>
</tr>
<tr>
<td>II</td>
<td>+84.4°</td>
<td>GlcA: GalA: Rha = 1:1:1</td>
<td>0.36</td>
</tr>
<tr>
<td>III</td>
<td>+81.0°</td>
<td>GlcA: GalA: Rha = 1:1:1</td>
<td>0.26</td>
</tr>
</tbody>
</table>

**Table III.** Amino Acid Composition of Hibiscus-Mucilage SF (Molar Percent)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Molar Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>7.39</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.67</td>
</tr>
<tr>
<td>Serine</td>
<td>5.98</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>9.10</td>
</tr>
<tr>
<td>Proline</td>
<td>5.21</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.40</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.09</td>
</tr>
<tr>
<td>Valine</td>
<td>7.48</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.56</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>6.41</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.44</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.08</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.10</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.34</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.63</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.10</td>
</tr>
</tbody>
</table>
Acknowledgement We are grateful to Prof. M. Tomita, School of Pharmaceutical Sciences, Showa University, for the determination of amino acids. Financial support from the Ministry of Education, Science and Culture of Japan in the form of a Grant-in-Aid for Scientific Research is also gratefully acknowledged.

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