Plant Mucilages. XLII.1) An Anti-complementary Mucilage from the Leaves of *Malva sylvestris* var. *mauritiana*

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A mucilage, designated as MSL-M, was isolated from the leaves of *Malva sylvestris* L. var. *mauritiana* Mill. It was homogeneous on electrophoresis and gel chromatography. Its intrinsic viscosity value in aqueous solution was 12.0, and its molecular weight was estimated to be about 6.0 x 10^6. The major constituent is an acidic polysaccharide composed of L-rhamnose: D-galactose: D-galacturonic acid: D-glucuronic acid in the molar ratio of 6:3:2:2. Methylation analysis of both the mucilage and the carboxylated-derived derivative, carbon-13 nuclear magnetic resonance and partial hydrolysis studies indicated its main structural features. It has considerable anti-complementary activity.

**Keywords** *Malva sylvestris* var. *mauritiana*; leaf; MSL-M; acidic polysaccharide; mucilage; intrinsic viscosity; polysaccharide structure; anti-complementary activity

The leaves of *Malva sylvestris* L. var. *mauritiana* Mill. form a crude drug used as an emollient, laxative and cough medicine. It is well known that the leaves of *Malva sylvestris* and *Malva sylvestris* var. *mauritiana* contain relatively large amounts of mucilages. Analytical data of component sugars of the mucilage from the former plant2,3) and that from the latter4) have been reported, but no structural study on the mucilages has been carried out so far. In the present paper, we report the isolation, structure, and anti-complementary activity of a new mucilage from the leaves of *Malva sylvestris* var. *mauritiana*.

The crude mucilage was isolated from the fresh leaves by extraction with cold water followed by precipitation with ethanol. The aqueous solution of the crude mucilage was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (carbonate form). After elution with water and 0.2 M ammonium carbonate, the eluate with 0.5 M ammonium carbonate was dialyzed and purified by successive gel chromatography with Sephadex S300, Toyopearl HW-75F, and Sephadex G-25.

The mucilage gave a single peak on gel chromatography with Toyopearl HW-75F. Further, it gave a single band on polyacrylamide gel disk electrophoresis (PAGE), after staining with periodic acid-Schiff (PAS) and Coomassie blue reagents. The mucilage showed [z]_d^24 + 34.0 (H_2O, c = 0.1), and its aqueous solution gave the intrinsic viscosity value of 12.0 at 30°C. Gel chromatography with standard dextrans gave a value of about 6.0 x 10^6 for the molecular weight. The substance is designated as MSL-M.

Quantitative analyses showed that MSL-M was composed of 94.4% polysaccharide and 5.0% peptide moieties. The polysaccharide contained 40.2% rhamnose, 22.2% galactose, 16.0% galacturonic acid and 16.0% glucuronic acid. Their molar ratio was 6:3:2:2.

The carboxyl groups of hexuronic acids in MSL-M were reduced with a carbodiimide reagent and sodium borohydride to give the corresponding neutral sugar residues.5) Methylation of MSL-M and the carboxyl-degraded derivative was performed with methylsulfanyl carbamion and methyl iodide in dimethyl sulfoxide.6) The methylated products were hydrolyzed, then converted into the partially methylated alditol acetates. Gas chromatography-mass spectrometry (GC-MS)7) revealed derivatives of 3,4-di-O-methyl-L-rhamnopyranose, 3-O-methyl-L-rhamnopyranose, 2,3,4,6-tetra-O-methyl-D-galactopyranose and 2,3,6-tri-O-methyl-D-galactopyranose as the products in a molar ratio of 5:1:1:2 from the original polysaccharide. Methyl ethers of the hexuronic acids were removed from the hydrolysis products of the methylated original sample by treatment with an anion-exchange resin. 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 5:1:2:1:2:2 from the carboxylated-reduced product.

These results suggested that the minimal repeating unit of the polysaccharide moiety of MSL-M was composed of six kinds of component sugar units as shown in Chart 1.

MSL-M was partially hydrolyzed with dilute sulfuric acid, then neutralized 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 some of the component monosaccharides, four oligosaccharides (I to IV) were obtained by stepwise elution with dilute formic acid, then purifier by rechromatography. Based on the results of sugar analysis and a comparison of their chromatographic properties, proton nuclear magnetic resonance (1H-NMR) spectra, and specific rotation values with those of authentic samples,8) I to IV were identified as the following four oligosaccharides (Chart 2).

All the galactose residues and about one-third of the

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\begin{align*}
| & & & \rightarrow 2 \text{ L-Rhap} & \rightarrow (\text{two}) & \rightarrow 4 \text{ D-Galp} & \rightarrow 1 & \rightarrow \\
| & & & & & & & \\
| & (\text{one}) & \rightarrow 2 \text{ L-Rhap} & \rightarrow (\text{two}) & \rightarrow 4 \text{ D-Galp} & \rightarrow 1 & \rightarrow \\
| & & & & & & & \\
| & (\text{five}) & \rightarrow 2 \text{ L-Rhap} & \rightarrow (\text{one}) & \rightarrow 4 \text{ D-Galp} & \rightarrow 1 & \rightarrow \\
| & & & & & & & \\
| & (\text{two}) & \rightarrow 4 \text{ D-Galp} & \rightarrow 1 & \rightarrow & \text{Rhap, rhamnopyranose; Galp, galactopyranose; GalpA, galactopyranosyluronic acid; GlcP, glucopyranosyluronic acid} & \rightarrow \\
| & & & & & & & \\
| & & & & & & & \\
| & & & & & & & \\
| & & & & & & & \\
| & & & & & & & \\
\end{align*}
\]

**Chart 1. Component Sugar Residues in the Minimal Repeating Unit in the Structure of MSL-M**

a) Number of residues.

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rhamnose residues were liberated from the polysaccharide on partial hydrolysis. The combined yields of the fractions containing oligosaccharides II, III and IV were about 88% of the theoretical yield from a partial hydrolyzate of MSL-M. In addition to the results of methylation analysis, these facts suggest that one-sixth of the rhamnose residues in the backbone chain possesses a side chain composed of 1,4-linked galactopyranosyl units at position 4.

The $^{13}$C-NMR spectrum of the mucilage showed four signals due to anomeric carbons at δ 100.584, 101.205, 106.196 and 106.628 ppm. These signals were assigned to the anomeric carbons of α-d-galacturonic acid, α-l-rhamnose, β-d-galactose and β-d-glucuronic acid, respectively.

Based on the accumulated evidence described above, it can be concluded that the polysaccharide moiety of MSL-M contains the units shown in Chart 3. 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-rhamnopyranosyl residue having the structure (1→2)-linked α-l-rhamnopyranosyl rhamnopyranosyl units in the backbone chains, and of the residue having the structure (1→4)-linked β-d-galactopyranosyl galactopyranosyl units in the side chains is common in MSL-M and Okra-mucilage R obtained from the roots of Abelmoschus esculentus MOENCH. The proportion of rhamnose units in the backbone of MSL-M, however, is much higher than that of Okra-mucilage R.

The anti-complementary activity of MSL-M is shown in Fig. 1. MSL-M had potent activity, compared with the positive control (AR-4, an arabinogalactan fraction, from the root of Angelica acutiloba KITAGAWA).

Among a number of plant mucilages obtained from plants belonging to the Malvaceae family by Tomoda et al., Okra-mucilage R and Hibiscus-mucilages ML, SF and SL showed potent anti-complementary activities. These mucilages have the repeating structure (1→4)-[O-β-(d-glucopyranosyluronic acid)-(1→3)]-O-α-(d-galactopyranosyluronic acid)-(1→2)-O-α-l-rhamnopyranosyl in the main part of their backbone chains. In addition, they have the neutral sugar branch composed of (1→4)-linked β-d-galactopyranosyl units linking to position 4 of the rhamnose residues in the backbone in relatively high degree. MSL-M also possesses these structural characteristics. Their highly branched structures could be involved in the anti-complementary activity.

**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 JMN-GX 270 FT NMR spectrometer in heavy water containing 2,2-dimethyl-2-silapentane-5-sulfonate as an internal standard at 70°C. Infrared (IR) spectra were recorded on a JASCO IRA-2 infrared spectrophotometer. GC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector. GC-MS was performed with a JEOL JMS-GX mass spectrometer. Viscosity was determined with an Ubbelohde-type viscosimeter.

**Material**

The material was obtained at the end of June 1986 and 1987 from plants cultivated in Kyoto.

**Isolation of the Mucilage**

The fresh leaves (350 g) were homogenized and extracted with water (3500 ml) under stirring for 1 h at room temperature. After centrifugation, the supernatant was poured into two volumes of ethanol. The precipitate was dissolved in water (450 ml) and applied to a column (5 x 75 cm) of DEAE-Sephadex A-25 (Pharmacia Co.). DEAE-Sephadex was prewetted as described in a previous report. After elution with water (1540 ml) and 0.2 M ammonium carbonate (1520 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. The eluates obtained from tubes 44 to 68 were combined, dialyzed against distilled water and concentrated. The solution was applied to a column (5 x 82 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 27 to 42 were combined, dialyzed and concentrated. The solution was applied to a column (2.6 x 94 cm) of Toyopearl HW-25F. Elution was carried out with 0.1 M Tris-HCl buffer (pH 7), and fractions of 10 ml were collected and analyzed.

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**Chart 2. Structural Features of Oligosaccharides I—IV**

**Chart 3. A Possible Structural Fragment of the Polysaccharide Moiety of MSL-M**

**Fig. 1. Anti-complementary Activity of MSL-M**

- O: MSL-M, x: AR-4 (positive control).
analyzed as described above. The eluates obtained from tubes 23 to 35 were combined, dialyzed and concentrated. The solution was applied to a column (2.6 x 93 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 22 to 27 were combined, concentrated and lyophilized. MSL-M (518 mg) was obtained as white powder.

Polyacrylamide Gel Electrophoresis This was carried out in an apparatus with gel tubes (4 x 130 mm each) and 0.005 M Tris-glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained by the PAS procedure and with Coomassie blue reagent. MSL-M gave a clear band at a distance of 69 mm from the origin.

Gel Chromatography The sample (3 mg) was dissolved in 0.1 M Tris-HCl buffer (pH 7) and applied to a column (2.6 x 95 cm) of Toyopearl HW-75F, pre-equilibrated and developed 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. Fraction number of the peaks of dextrans 2.0 x 10^6, 2.7 x 10^5, 1.5 x 10^5, MSL-M and native dextran (void, Tokyo Kasei Co.) were 53, 63, 65, 48 and 41.

Qualitative Analysis of Component Sugars Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described in a previous report. The configurations of component sugars were identified by GC of the trimethylsilylated 2-methylbenzylamino- dilol derivatives.

Determination of Components Neutral sugars in the original and the carboxyl-reduced mucilages were analyzed by GC after conversion of hydrolyzates into alditol acetates as described in a previous report. GC was carried out with a fused silica capillary column (0.53 mm i.d. x 15 m) of SP-2330 (Supelco Co.) and with a programmed temperature increase of 3 °C per min from 160 to 200 °C at a helium flow of 10 ml per min. Allose was used as an internal standard. Rhamnose was also determined by the thioglycolic acid method, and hexuronic acids in the original mucilage were estimated by a modification of the carbazole method. Peptide determination was performed by the method of Lowry et al.

Reduction of Carboxyl Groups This was carried out with 1-cyclohexyl-3,4-morpholinomethyl-carboxymethylo-p-toluenesulfonate and sodium borohydride in a previous report. The reaction was repeated twice more under the same conditions. Yield was 12 mg from 36 mg of the sample.

Methylation Analysis Methylation was performed with methylsulfonil carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report. The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report. The partially methylated alditol acetates obtained were analyzed by GC-MS using a fused silica capillary column (0.32 mm i.d. x 30 m) of SP-2330 (Supelco Co.) and with a programmed temperature increase of 4 °C per min from 160 to 220 °C at a helium flow of 1 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 GC and the main fragments in MS are listed in Table II.

Partial Hydrolysis and Isolation of Oligosaccharides The mucilage (40 mg) was suspended in 1 N sulfuric acid (4 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 x 5 cm) of Dowex 50WX8 (H+). The eluate with water was concentrated and lyophilized (yield, 28 mg), then an aqueous solution of the lyophilized was applied to a column (1 x 10 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted successively with water (25 ml), 0.1 M formic acid (65 ml), 0.4 M formic acid (75 ml), 0.6 M formic acid (155 ml) and 0.8 M formic acid (195 ml). Fractions of 5 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from the column were divided into six groups: Frac. 1, tubes 1 to 3; frac. 2, tubes 12 to 18; frac. 3, tubes 20 to 22; frac. 4, tubes 32 to 44; frac. 5, tubes 66 to 77; frac. 6, tubes 106 to 123. The yields were 8.8 mg for frac. 1, 1.8 mg for frac. 2, 1.9 mg for frac. 3, 4.8 mg for frac. 4, 3.8 mg for frac. 5 and 2.6 mg for frac. 6. Fraction 1 contained rhamnose and galactose, and frac. 3 contained galacturonic acid and glucuronic acid. Fraction 2 was purified on a column of Sephadex G-15, and fracs. 4, 5 and 6 were each purified on a column of Sephadex G-25 as described in a previous report. Oligosaccharides I, II, III and IV were obtained from fracs. 2, 4, 5 and 6, respectively. The yields were 1.4 mg for I, 3.9 mg for II, 2.9 mg for III and 1.9 mg for IV.

Analysis of the Oligosaccharides Analysis of component sugars was performed as described in a previous report. 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 in Table III.

Measurement of Anti-complementary Activity Gelatin-veronal-buffered saline (pH 7.4) containing 500 µM Mg^2+ and 120 µM Ca^2+ (GVB^2- was prepared) and normal human serum (NHS) was obtained from a healthy adult. Various dilutions of the samples in water (50 µl) were incubated with 50 µl of NHS and 50 µl of GVB^2- for 24 h at 37 °C. The mixtures were incubated at 37 °C for 30 min and the residual total hemolytic complement (TCH50) was determined by a method using IgM-hemolysin-sensitized sheep erythrocytes at 1 x 10^5 cells/ml. NHS was incubated with water and GVB^2- to provide a control. The activity of the sample was expressed as the percentage inhibition of the TCH50 of the control.

Acknowledgement We are grateful to Mr. F. Kawanishi, Kyoto Herbal Garden, Pharmacognosy Laboratories, Takeda Chemical Industries, Ltd., for providing the material plants, and Prof. M. Tomita, School of Pharmaceutical Sciences, Showa University, for the determination of amino acids. We also thank Misses A. Kawan and Y. Sakabe for their technical assistance.

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**Table I. Amino Acid Composition of MSL-M (Molar Percent)**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Molar Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>11.42</td>
</tr>
<tr>
<td>Threonine</td>
<td>5.72</td>
</tr>
<tr>
<td>Serine</td>
<td>5.51</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>11.72</td>
</tr>
<tr>
<td>Proline</td>
<td>8.56</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.84</td>
</tr>
<tr>
<td>Alanine</td>
<td>11.27</td>
</tr>
<tr>
<td>Valine</td>
<td>9.25</td>
</tr>
</tbody>
</table>

**Table II. Relative Retention Times (Rf) on GC and Main Fragments in MS of Partially Methylated Alditol Acetates**

<table>
<thead>
<tr>
<th>Alditol Acetate</th>
<th>Rf (m/z)</th>
<th>Main Fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2,5-Ac-3,4-Me-t-rhamnitol</td>
<td>0.95</td>
<td>43, 89, 129, 131, 189</td>
</tr>
<tr>
<td>1,2,4,5-Ac-3,4-Me-t-rhamnitol</td>
<td>1.28</td>
<td>43, 87, 101, 129, 143, 189, 203</td>
</tr>
<tr>
<td>1,5-Ac-2,3,4,6-Me-t-glucitol</td>
<td>1.00</td>
<td>43, 45, 71, 87, 101, 117, 129, 145, 161, 205</td>
</tr>
<tr>
<td>1,2,4,5-Ac-3,4-Me-t-galactitol</td>
<td>1.09</td>
<td>43, 45, 71, 87, 101, 117, 129, 145, 161, 205</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>[α]D in H2O</th>
<th>Sugar Composition</th>
<th>TLC (Rf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>+93.0</td>
<td>GlA: Rha = 1:1</td>
<td>0.44</td>
</tr>
<tr>
<td>II</td>
<td>+84.5</td>
<td>GlC: GlA: Rha = 1:1:1</td>
<td>0.36</td>
</tr>
<tr>
<td>III</td>
<td>+81.0</td>
<td>GlC: GlA: Rha = 1:1:1</td>
<td>0.26</td>
</tr>
<tr>
<td>IV</td>
<td>+78.0</td>
<td>GlC: GlA: Rha = 1:1:1</td>
<td>0.15</td>
</tr>
</tbody>
</table>

References