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STRUCTURE AND ANTICOMPLEMENTARY ACTIVITY OF AN ACIDIC POLYSACCHARIDE FROM THE LEAVES OF *Malva sylvestris* var. *mauritiana*

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ABSTRACT

A main acidic polysaccharide preparation was isolated from the leaves of *Malva sylvestris* L. var *mauritiana* Mill. It is composed of L-rhamnose, D-galactose, D-galacturonic acid, and D-glucuronic acid in the molar ratio of 22:6:22:11, and it contains 7.7% peptide. It was homogeneous by electrophoresis and gel chromatography, which gave a value of 11 000 as molecular weight. The structure of the polysaccharide component was elucidated by methylation analysis and partial hydrolysis. The substance showed considerable anticomplementary activity.

INTRODUCTION

The leaves of *Malva sylvestris* L. var. *mauritiana* Mill. is a crude drug used as an emollient, laxative, and cough medicine. Analytical data of component sugars of crude mucilages from this plant have been reported[1], but no structural study on the polysaccharide has been carried out so far. We now report the isolation, structural investigation, and anticomplementary activity of a main acidic polysaccharide obtained from the leaves.

RESULTS AND DISCUSSION

Fresh leaves were extracted with cold water, followed by precipitation with ethanol. The extract was purified by sequential ion-exchange chromatography with DEAE-Sephadex A-25, gel chromatography with Sephacryl S-300 and Sephadex G-25, and lyophilization. This product was designated as MSL-P. It gave a single band on poly(acrylamide) gel-disk electrophoresis. Both the periodate–Schiff reagent and the Coomassie Blue reagent revealed the band in the same position. Moreover, the product showed a single peak on gel chromatography with Sephacryl

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S-300. The polysaccharide had $[\alpha]_D^{24}$ + 58° (*c* 0.1, water), and its aqueous solution gave a low intrinsic viscosity value of 5.0 at 30°. Gel chromatography with standard pullulans gave a value of 11 000 for the molecular weight.

L-Rhamnose, D-galactose, D-galacturonic acid, and D-glucuronic acid were identified as component sugars of MSL-P in the proportions 28.9, 8.7, 34.9, and 17.4%, respectively, in addition to 7.7% of peptide. No compounds other than carbohydrates and amino acids were detected in the hydrolyzate.

The carboxyl group of hexuronic acids was reduced to give the corresponding neutral sugar residues[2]. Methylation of MSL-P and the carboxyl-reduced derivative, performed by the Hakomori method[3], followed by hydrolysis gave partially methylated alditol acetates, identified by g.l.c.–m.s.[4] as 3,4-di-*O*-methyl- and 3-*O*-methyl-rhamnose, and 2,3,4,6-tetra-*O*- and 2,3,6-tri-*O*-methyl-galactose in a molar ratio of 19:3:3:3. The methyl ethers of hexuronic acids were removed from the hydrolysis products of the methylated original sample by treatment with an anion-exchange resin. From the carboxyl-reduced methylated product, derivatives of 3,4-di-*O*-methyl- and 3-*O*-methyl-rhamnose, 2,3,4,6-tetra-*O*-methyl-glucose, and 2,3,4,6-tetra-*O*-methyl-, 2,3,6-tri-*O*-methyl-, and 2,6-di-*O*-methyl-galactose were obtained in a molar ratio of 19:3:11:3:14:11 (Table I).

MSL-P was partially hydrolyzed with dilute sulfuric acid, and the solution made neutral and treated with Dowex 50W (H^+). The water eluate was applied to a column of DEAE-Sephadex A-25 (HCO_3^-). In addition to small amounts of component monosaccharides, four oligosaccharides (**1–4**) were obtained by stepwise elution with dilute formic acid. Their structures were elucidated by analysis of the component sugar, and a comparison of their chromatographic properties, ^{1}H-n.m.r. spectra, and specific rotations with those of authentic samples[5] (Table II).

All D-galactosyl residues were liberated from MSL-P under the conditions of hydrolysis described above. In conjunction with the results of methylation analysis, this observation suggested that ~13.5% of the rhamnosyl residues in the backbone-

TABLE I

METHYLATION ANALYSIS OF ORIGINAL POLYSACCHARIDE AND CARBOXYL-REDUCED PRODUCT

Methylated sugar	T[a]	*Molar ratio*		*Mode of linkage*[b]
		Original	*Carboxyl-reduced*	
3,4-Di-*O*-Me-Rha	0.95	19	19	→2)-Rha*p*-(1→
3-*O*-Me-Rha	1.28	3	3	→2,4)-Rha*p*-(1→
2,3,4,6-Tetra-*O*-Me-Glc	1.00	0	11	T-Glc*p*-(1→
2,3,4,6-Tetra-*O*-Me-Gal	1.09	3	3	T-Gal*p*-(1→
2,3,6-Tri-*O*-Me-Gal	1.44	3	14	→4)-Gal*p*-(1→
2,6-Di-*O*-Me-Gal	1.64	0	11	→3,4)-Gal*p*-(1→

[a]Retention time of the corresponding alditol acetate on a SP-2330 capillary column with a programmed temperature increase of 4°.min^{-1} from 160 to 220° at a He flow of 1 mL.min^{-1} relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]T denotes nonreducing terminal group.

TABLE II

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

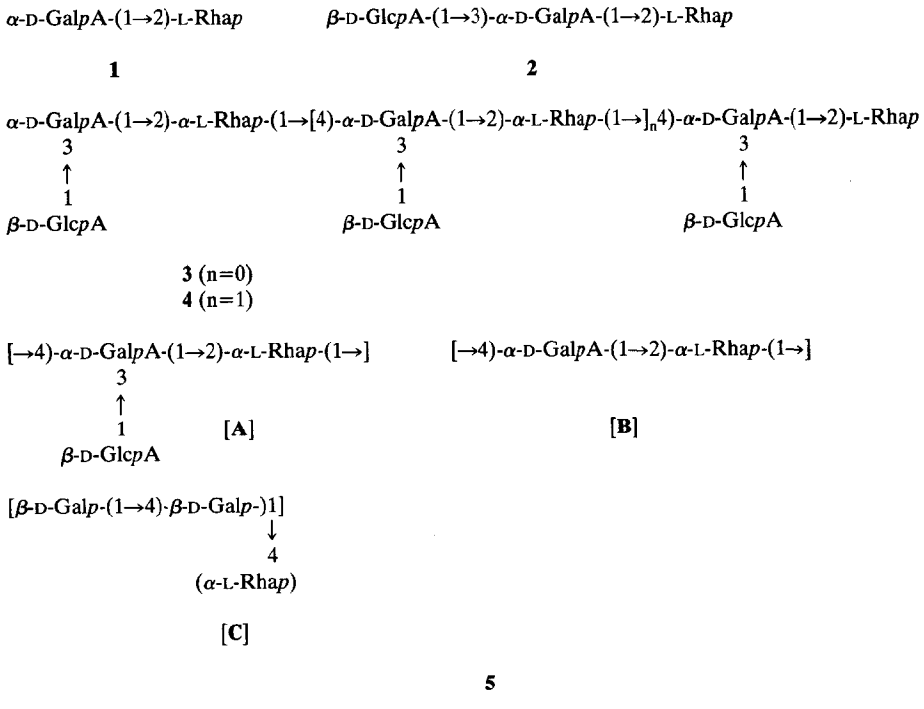
Oligosaccharide	*Sugar composition*	*Proportion of rhamnitol in reduced sample (%)*	$[\alpha]_D$	R_F[a]
1	GalA:Rha = 1:1	45	+93.0°	0.44
2	GlcA:GalA:Rha = 1:1:1	32	+84.5°	0.36
3	GlcA:GalA:Rha = 1:1:1	16	+81.0°	0.26
4	GlcA:GalA:Rha = 1:1:1	11	+78.0°	0.15

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

chain are substituted at O-4 with oligosaccharides containing 4-*O*-linked D-galactosyl residues.

The ^{13}C-n.m.r. spectrum of MSL-P showed four signals, at δ 100.354, 101.103, 105.872, and 106.115, that were assigned to C-1 of α-D-galacturonic acid, α-L-rhamnose, β-D-galactose, and β-D-glucuronic acid units, respectively[6,7].

The evidence just described suggested that the polysaccharide contains units (**A**, **B**, and **C**) having structure **5** (see Scheme 1). The backbone of the poly-



Scheme 1. Structural units of MSL-P.

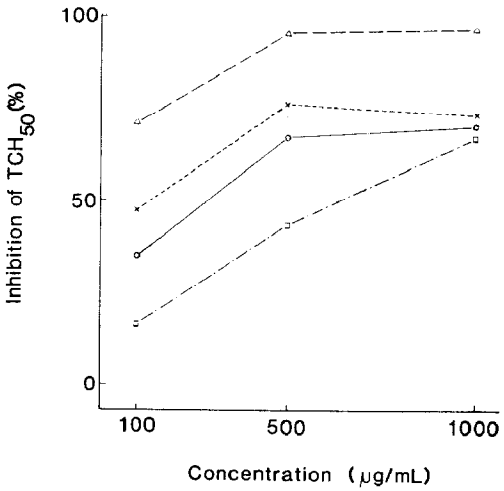


Fig. 1. Anticomplementary activity of MSL-P. (○) MSL-P, (△) Hibiscus-mucilage SF polysaccharide, (x) AR-4 (positive control), and (□) okra-mucilage F polysaccharide.

saccharide is composed of alternating 4-*O*-linked α-D-galactosyluronic acid and 2-*O*-linked α-L-rhamnosyl residues (**1**). Approximately 50% of the 4-*O*-linked D-galactosyluronic acid residues are also substituted at O-3 with terminal, non-reducing D-glucopyranosyluronic acid groups (**2**, **3**, and **4**). Some (~13.5%) of the 2-*O*-linked L-rhamnosyl residues are also substituted at O-4 with oligosaccharides containing 4-*O*-linked D-galactosyl residues.

MSL-P has potent anti-complementary activity (Fig. 1), which was nearly equal to that of the positive control (AR-4, an arabinogalactan fraction, from the root of *Angelica acutiloba* Kitagawa[8]).

Among numerous plant mucilages obtained by Tomoda and assoc. from plants belonging to the Malvaceae family, Hibiscus-mucilage SF polysaccharide from the flower bud of *Hibiscus syriacus* L. possesses a backbone and side-chains similar to those of MSL-P[9]. The presence of β-D-galactopyranosyl-(1→4)-D-galactopyranose side-chains at O-4 of a part of the L-rhamnosyl residues of the backbone is common for MSL-P and Hibiscus-mucilage SF polysaccharide. The latter substance showed an anticomplementary activity[10] greater than that of MSL-P (Fig. 1). The ratio of units **A** to units **B** (**5**) is 1:1 in MSL-P and 2:1 in Hibiscus-mucilage SF polysaccharide. In the polysaccharide[11] of okra-mucilage F, the mucilage obtained from the immature fruit of *Abelmoschus esculentus* Moench, the backbone is solely made up of units **B**, and the side-chains are similar to those of MSL-P and Hibiscus-mucilage SF polysaccharide. Okra-mucilage F showed an activity[10] lower than that of MSL-P (Fig. 1). Therefore, the presence of unit **A** in the backbone may contribute to the activity. In addition to this factor and the role of the molecular weight, a difference in the branching degree on the structures may be involved in the anticomplementary activity[12].

EXPERIMENTAL

General methods. — Optical rotations were measured with a Jasco DIP-140 automatic polarimeter. N.m.r. spectra were recorded with a Jeol JMN-GX 270 FT NMR spectrometer for solutions in D_2O containing sodium 4,4-dimethyl-4-silapentane-1-sulfonate as an internal standard at 70°. G.l.c. was carried out on a Shimadzu GC-7AG gas chromatograph equipped with an H_2 flame-ionization detector. G.l.c.–m.s. 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, Japan.

Isolation of the polysaccharide. — 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 solution was treated with ethanol (2 vols.). The resulting precipitate (9 g) was dissolved in water (450 mL) and applied to a column (5 × 75 cm) of DEAE-Sephadex A-25 (Pharmacia Co.). DEAE-Sephadex was pretreated as described in a previous report[13]. After elution with water (1540 mL) and 0.2M $(NH_4)_2CO_3$ (1520 mL), the column was eluted with 0.5M $(NH_4)_2CO_3$. Fractions (20 mL) were collected and analyzed by the phenol–H_2SO_4 method[14]. The eluates obtained from tubes 44 to 68 were combined, dialyzed against distilled water, and concentrated. The solution was applied to a column (5 × 82 cm) of Sephacryl S-300, eluted with 0.1M Tris·HCl buffer (pH 7.0), and fractions (20 mL) were collected and analyzed as described above. The eluates obtained from tubes 42 to 71 were combined, dialyzed, and concentrated. The solution was applied again to a column (5 × 80 cm) of Sephacryl S-300, eluted with 0.1M Tris·HCl buffer (pH 7.0), and fractions (20 mL) were collected and analyzed as described above. The eluates obtained from tubes 31 to 53 were combined, dialyzed, and concentrated. The solution was applied to a column (2.6 × 93 cm) of Sephadex G-25. The column was eluted with water and fractions (10 mL) were collected. The eluates obtained from tubes 31 to 35 were combined, concentrated, and lyophilized to give MSL-P (78.5 mg) as a white powder.

Poly(acrylamide) gel electrophoresis. — The electrophoresis was performed in an apparatus equipped with gel tubes (4 × 130 mm each) and 5mM Tris–glycine buffer (pH 8.3) at 5 mA per tube for 40 min. Gels were stained by the periodate–Schiff procedure, and with the Coomassie Blue reagent. The sample gave a clear band at a distance of 69 mm from the origin.

Molecular weight. — A sample (3 mg) was dissolved in 0.1M Tris·HCl buffer (pH 7.0), and applied to a column (2.6 × 95 cm) of Sephacryl S-300 pre-equilibrated and developed with the same solvent. Fractions (5 mL) were collected and analyzed by the phenol–H_2SO_4 method. Standard pullulans having known molecular weights (Shodex standard P-82) were applied to the column to obtain a calibration curve.

Qualitative analysis of component sugars. — Hydrolysis and cellulose t.l.c. of

component sugars were performed as described in a previous report[13]. The configurations of the component sugars were identified by g.l.c. of the per(trimethylsilyl) ethers of (α-methylbenzyl)aminoalditol derivatives[15].

Determination of components. — Neutral sugars in the original and carboxyl-reduced polysaccharides were analyzed by g.l.c. with a fused silica capillary column (0.53 mm × 15 m) of SP-2380 and with programmed temperature-increase of $3°.min^{-1}$ from 160 to 200° at a He flow of 10 mL min^{-1}, after conversion of the hydrolyzates into alditol acetates as described in a previous report[16]. L-Rhamnose was also determined by the thioglycolic acid method[17], and hexuronic acids were estimated by a modification of the carbazole method[18]. Peptide determination was performed by the method of Lowry *et al.*[19].

Reduction of carboxyl groups. — An aqueous solution of the sample was treated with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, HCl, and $NaBH_4$ as described in a previous report[20]. The treatment was repeated twice under the same conditions.

Methylation analysis. — Methylation was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report[20]. The final product, which showed no i.r. absorption for hydroxyl groups, was hydrolyzed with dilute H_2SO_4 in acetic acid, and then reduced and acetylated in the manner described in a previous report[11]. G.l.c.–m.s. of partially methylated alditol acetates was performed with a fused silica capillary column (0.32 × 30 m) of SP-2330 and with a programmed temperature-increase of $4°.min^{-1}$ from 160 to 220° at a He flow of 1 $mL.min^{-1}$.

Partial hydrolysis. — A sample (33 mg) was suspended in 0.5M H_2SO_4 and heated in a boiling water bath for 2 h. After neutralization with $BaCO_3$, followed by filtration, the filtrate was passed through a column (1 × 5 cm) of Dowex 50W-X8 (H^+). The eluate with water was concentrated and lyophilized (yield, 26 mg). An aqueous solution of the lyophilizate was applied to a column (1 × 10 cm) of DEAE-Sephadex A-25 (HCO_3^-). The column was eluted successively with water (20 mL), and 0.1M (60 mL), 0.2M (95 mL), 0.4M (80 mL), and 0.6M formic acid (95 mL). Fractions (5 mL) were collected and analyzed by the phenol–H_2SO_4 method. The eluates obtained were divided into six groups: Tubes 1–3 (Fraction 1), 10–14 (2), 15–17 (3), 22–24 (4), 36–39 (5), and 53–55 (6). Neutral monosaccharides (5.8 mg) and hexuronic acids (2.1 mg) were obtained from Fractions 1 and 3, respectively. Fractions 2 and 4–6 were each purified on a column of Sephadex G-25 as described in a previous report[21]. Oligosaccharides **1** (1.5 mg), **2** (3.7 mg), **3** (2.0 mg), and **4** (0.4 mg) were obtained from Fractions 2, 4, 5, and 6, respectively.

Determination of anticomplementary activity. — A gelatin, veronal-buffered saline solution (pH 7.4) containing 500μM Mg^{2+} and 150μM Ca^{2+} (GVB^{2+}) was prepared[8], 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+}. The mixtures were incubated for 30 min at 37°, and the residual total hemolytic complement (TCH_{50}) was determined by a method using

IgM-hemolysin-sensitized sheep erythrocytes at 1×10^8 cells.mL^{-1}. NHS was incubated with water and GVB^{2+} as control. The activity of the sample was expressed as the percentage inhibition of the TCH_{50} of the control.

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