Constituents of the Seed of *Malva verticillata*. VI.[1] Characterization and Immunological Activities of a Novel Acidic Polysaccharide

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A novel acidic polysaccharide, designated as MVS-VI, was isolated from the seeds of *Malva verticillata* L. It was homogeneous on electrophoresis and gel chromatography, and its molecular mass was estimated to be 26000. It is composed of L-arabinose: D-xylose: D-galactose: D-glucose: L-rhamnose: D-galacturonic acid in the molar ratio of 30:15:20:3:2:10, in addition to small amounts of peptide moiety. Methylation analysis, carbon-13 nuclear magnetic resonance and periodate oxidation studies indicated its structural features to have mainly acidic α-arabino-3,6-β-galactan type structural units. MVS-VI showed significant reticuloendothelial system-potentiating activity in a carbon clearance test, and it possesses remarkable anti-complementary activity.

Keywords *Malva verticillata*; seed; MVS-VI; polysaccharide structure; acidic arabinogalactan; immunological activity; reticuloendothelial system; anti-complementary activity

In previous papers,[1-5] the isolation and structural features of three neutral polysaccharides (MVS-I, MVS-IIA and MVS-IIG), two acidic polysaccharides (MVS-IIIA and MVS-IVA) and the major peptidoglycan (MVS-V) from the seed of *Malva verticillata* L. (Malvaceae) were reported. These substances and the polysaccharide-rich fraction (MVS-V-CH) obtained from MVS-V were tested for anti-complementary and hypoglycemic activities.[6] Remarkable anti-complementary activities were observed for MVS-I and MVS-IIA, and MVS-I also showed remarkable hypoglycemic activity. We now report the isolation of a novel acidic polysaccharide from the water extract of this crude drug, and present its structural features and two immunological activities.

Material and Methods

Isolation of Polysaccharide The material was imported from China as described previously.[2] The seeds (200 g) were homogenized and extracted with hot water (2 l) under stirring for 1 h in a boiling water bath. After suction filtration, the filtrate was poured into two volumes of ethanol. The precipitate obtained was dissolved in water (200 ml) and applied to a column (5 × 78 cm) of diethylaminoethyl (DEAE)-Sephadex A-25 which had been pretreated as described in a previous report.[7] After elution with water (1760 ml), the column was eluted with 0.2 M ammonium carbonate. Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method.[8] The eluates obtained from tubes 135 to 161 were combined, dialyzed and concentrated, and then applied to a column (5 × 74 cm) of Sephacryl S-500. The column was pre-equilibrated with 0.1 M Tris–HCl buffer (pH 7.0) and eluted with the same buffer. Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method, and the eluates obtained from tubes 52 to 62 were combined, dialyzed and concentrated. The solution was applied to a column (5 × 78 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 35 were combined, concentrated and lyophilized. Yield, 95 mg (*ca.* 0.05%). This fraction (300 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (5 × 71 cm) of Toyopearl HW-60F, pre-equilibrated and eluted with the same buffer, and fractions of 20 ml were collected. The eluates obtained from tubes 33 to 48 were combined, dialyzed and concentrated. The solution was applied to a column (5 × 86 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 33 to 40 were combined, concentrated and lyophilized. MVS-VI (228 mg) was obtained as a white powder.

Polyacrylamide Gel Electrophoresis (PAGE) This was carried out in an apparatus with gel (7.5%) tubes (4 × 127 mm each) and 5 mM Tris–glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained by the periodate-Schiff (PAS) procedure and with Coomassie blue reagent. MVS-VI gave a distinct band at a distance of 53 mm from the origin. MVS-IIIA and -IVA moved 56 and 58 mm, respectively.

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, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol–sulfuric acid method. Standard pullulans (Shōwa Denkō Co.) having known molecular weights were run on the column to obtain a calibration curve.

Qualitative Analysis of Component Sugars Hydrolysis and cellulose thin-layer chromatography (TLC) of component sugars were performed as described in a previous report.[7] The configurations of component neutral sugars were identified by gas chromatography (GC) of trimethylsilylated α-methylbenzylaminoalditol derivatives.[9] GC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector.

Determination of Components Neutral sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described in a previous report.[10] Galacturonic acid was determined by the *m*-hydroxybiphenyl method.[11] Peptide determination was performed by the method of Lowry *et al.*[12]

Determination of *O*-Acetyl Groups The sample was hydrolyzed with 0.2 N hydrochloric acid and analyzed by GC using propionic acid as an internal standard as described previously.[13]

Determination of *O*-Methyl Groups in Methyl Esters This was performed by GC after saponification using ethanol as an internal standard as described previously.[14]

Nuclear Magnetic Resonance (NMR) NMR spectrum was 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 30 °C.

Reduction of Carboxyl Groups This was carried out with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate and sodium borohydride as described in a previous report.[15]

Methylation The sample (10 mg) was dissolved in dimethyl sulfoxide (1 ml), then finely powdered sodium hydroxide (100 mg) and methyl iodide (0.5 ml) were added to the solution. The whole was stirred at room temperature for 1 h. All procedures were carried out under nitrogen. Water and chloroform (5 ml each) were then added to the reaction mixture, and the whole was extracted five times with chloroform (5 ml each). The combined extract was washed three times with water (25 ml each), then dried over sodium sulfate. The filtrate was concentrated to dryness, and the residue was dissolved in chloroform–methanol mixture (2:1), then applied to a column (1 × 17 cm) of Sephadex LH-20. The column was eluted with the same solvent, and fractions of 1 ml were collected. The eluates obtained from tubes 3 to 6 were combined and concentrated. The infrared (IR) spectra of final residues showed no hydroxyl group absorption. Yield, 6.5 mg from the origin and 8.5 mg from the carboxyl-reduced product.

Analysis of 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.[16] The partially methylated alditol acetates obtained were analyzed by gas chromatography–mass spectrometry (GC-MS) using a fused silica capillary column (0.32 mm i.d. × 30 m) of SP-2330 (Supelco Co.) and with a programmed temperature increase of

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TABLE I. Relative Retention Times on GC and Main Fragments in MS of Partially Methylated Alditol Acetates

	Relative retention time[a)]	Main fragments (*m/z*)
1,4-Ac_2-2,3,5-Me_3-L-arabinitol	0.68	43, 45, 71, 87, 101, 117, 129, 161
1,3,5-Ac_3-2,4-Me_2-L-arabinitol	1.03	43, 87, 113, 117, 233
1,4,5-Ac_3-2,3-Me_2-L-arabinitol	1.14	43, 87, 101, 117, 129, 189
1,2,4,5-Ac_4-3-Me-L-arabinitol	1.52	43, 87, 129, 189
1,3,5-Ac_3-2,4-Me_2-D-xylitol	1.11	43, 87, 113, 117, 233
1,4,5-Ac_3-2,3-Me_2-D-xylitol	1.22	43, 87, 101, 117, 129, 189
1,2,5-Ac_3-3,4-Me_2-L-rhamnitol	0.95	43, 89, 129, 131, 189
1,2,4,5-Ac_4-3-Me-L-rhamnitol	1.30	43, 87, 101, 129, 143, 189, 203
1,5-Ac_2-2,3,4,6-Me_4-D-galactitol	1.09	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac_3-2,4,6-Me_3-D-galactitol	1.37	43, 45, 87, 101, 117, 129, 161
1,4,5-Ac_3-2,3,6-Me_3-D-galactitol	1.44	43, 45, 87, 99, 101, 113, 117, 233
1,5,6-Ac_3-2,3,4-Me_3-D-galactitol	1.60	43, 87, 99, 101, 117, 129, 161, 189
1,3,5,6-Ac_4-2,4-Me_2-D-galactitol	2.02	43, 87, 117, 129, 189
1,4,5-Ac_3-2,3,6-Me_3-D-glucitol	1.47	43, 45, 87, 99, 101, 113, 117, 233

a) Relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Abbreviations: Ac = acetyl; Me = methyl (*e.g.*, 1,4-Ac_2-2,3,5-Me_3- = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-).

4 °C per min from 160 to 220 °C at a helium flow of 1 ml per min. GC-MS was performed with a JEOL JMS-GX mass spectrometer. 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 I.

Periodate Oxidation The sample (102.3 mg) was oxidized with 0.05 M sodium metaperiodate (50 ml) at 5 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[17)] The oxidation was completed after 3 d. The reaction mixture was successively treated with ethylene glycol (1 ml) at 5 °C for 1 h and sodium borohydride (0.3 g) at 5 °C for 16 h, then adjusted to pH 5.0 by addition of acetic acid. The solution was concentrated and applied to a column (5 × 87 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 33 to 37 were combined, concentrated and lyophilized. Yield, 79 mg. Determination of the components was carried out as described above.

Phagocytic Activity This was measured as described in a previous report.[18)] The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (50 mg/kg body weight) once a day. The phagocytic index, *K*, was calculated by means of the following equation:

$$K = (\ln OD_1 - \ln OD_2)/(t_2 - t_1)$$

where OD_1 and OD_2 are the optical densities at times t_1 and t_2, respectively. Results were expressed as the arithmetic mean ± S.D. of five male mice (ICR-SPF).

Anti-complementary Activity Gelatin–veronal-buffered saline (pH 7.4) containing 500 μM Mg^{2+} and 150 μM Ca^{2+} (GVB^{2+}) was prepared,[19)] 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 at 37 °C for 30 min and the residual total hemolytic complement (TCH_{50}) was determined by a method using immunoglobulin M (IgM)–hemolysin-sensitized sheep erythrocytes at 1×10^8 cells/ml. NHS was incubated with water GVB^{2+} to provide a control. The activity of the sample was expressed as the percentage inhibition of the TCH_{50} of the control.

Results

The crude precipitate was obtained from the seeds of *Malva verticillata* by hot water extraction followed by addition of ethanol. An aqueous solution of the precipitate was applied to a column of DEAE-Sephadex A-25 (carbonate form). The eluate with water afforded three neutral polysaccharides,[2,3)] and the eluate with 0.2 M ammonium carbonate was dialyzed and subjected to gel chromatography on Sephacryl S-500. High molecular weight fraction afforded two acidic polysaccharides, MVS-IIIA[1)] and MVS-IVA,[5)] and a relatively low molecular weight fraction was dialyzed and purified by successive gel chromatography with Toyopearl HW-60F and Sephadex G-25. A novel acidic polysaccharide designated as MVS-VI was obtained. The isolation method of the polysaccharides is summarized in Fig. 1.

MVS-VI gave a single band on PAGE, and gave a single peak on gel chromatography. It had $[\alpha]_D^{24}$ −47.4° (H_2O, $c = 0.2$). Gel chromatography gave a value of 2.6×10^4 for the molecular mass.

MVS-VI is composed of L-arabinose, D-xylose, D-galactose, D-glucose, L-rhamnose, D-galacturonic acid and a peptide moiety. Quantitative analyses showed that it contained 30.8% arabinose, 15.6% xylose, 25.1% galactose, 3.7% glucose, 2.3% rhamnose, 13.9% galacturonic acid and 7.1% peptide moiety. The molar ratio of these component sugars was 30 : 15 : 20 : 3 : 2 : 10.

The carbon-13 NMR (^{13}C-NMR) spectrum of MVS-VI showed seven signals due to anomeric carbons at δ 100.42, 101.15, 101.96, 104.31, 106.14, 107.01 and 110.16 ppm. The signals at δ 100.42 and 101.96 ppm were attributable to anomeric carbons of α-D-galactopyranosyluronic acid and α-L-rhamnopyranose,[20)] those at δ 101.15, 104.31, 106.14 ppm to anomeric carbons of α-D-glucopyranose, β-D-xylopyranose and β-D-galactopyranose,[21)] and those at δ 107.01 (quite minor) and 110.16 (major) ppm to anomeric carbons of α-L-arabinopyranose and α-L-arabinofuranose.[22)] In addition, the ^{13}C-NMR spectrum showed the signals of *O*-acetyl groups at δ 21.72 and 176.37 ppm and the signal of *O*-methyl groups as carboxylic acid methyl esters at δ 57.01 ppm. The presence of these groups was also confirmed by GC of the hydrolyzate, and quantitative analyses showed that MVS-VI contained 1.4% acetyl and 0.3% methoxyl groups. About 12% of the galacturonic acid residue exist as methyl esters.

The carboxyl groups of galacturonic acid residues in the polysaccharide were reduced to give the corresponding neutral sugar residues.[23)] Both the original polysaccharide and the carboxyl reduced derivative were methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.[24)] The methylated products were hydrolyzed, then converted into the partially methylated alditol acetates. The hexuronic acid methyl ether was removed from the hydrolysis products of the methylated native polysaccharide by treatment with anion-exchange resin. GC-MS[25)] showed the presence of 2,3,5-tri-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 3-*O*-methyl-L-arabinose, 2,4-di-*O*-methyl-D-xylose, 2,3-di-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose, 2,4-di-*O*-methyl-D-galactose, 2,3,6-tri-*O*-methyl-D-glucose, 3,4-di-*O*-methyl-L-rhamnose and 3-*O*-methyl-L-rhamnose as the products from the methylated original polysaccharide in a molar ratio

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a) dialyzed and applied to Sephadex G-25 column

Fig. 1. Isolation of MVS-VI

(ten)[a]		α-L-Ara*f* 1→	(one)[a]		β-D-Gal*p* 1→
(one)[a]	→3	α-L-Ara*p* 1→	(eight)[a]	→3	β-D-Gal*p* 1→
(sixteen)[a]	→5	α-L-Ara*f* 1→	(one)[a]	→4	β-D-Gal*p* 1→
(three)[a]	→5	α-L-Ara*f* 1→	(three)[a]	→6	β-D-Gal*p* 1→
		2 ↑			↓ 6
(six)[a]	→3	β-D-Xyl*p* 1→	(seven)[a]	→3	β-D-Gal*p* 1→
(nine)[a]	→4	β-D-Xyl*p* 1→	(three)[a]	→4	α-D-Glc*p* 1→
(one)[a]	→2	α-L-Rha*p* 1→	(ten)[a]	→4	α-D-Gal*p*A 1→
		↓ 4			
(one)[a]	→2	α-L-Rha*p* 1→			

Chart 1. Component Sugar Residues in the Minimal Unit in the Structure of MVS-VI

a) Number of residues. Ara*f*, arabinofuranose; Ara*p*, arabinopyranose; Xyl*p*, xylopyranose; Rha*p*, rhamnopyranose; Gal*p*, galactopyranose; Glc*p*, glucopyranose; Gal*p*A, galactopyranosyluronic acid.

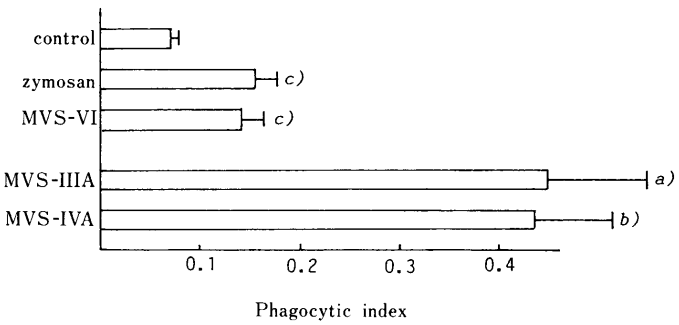

Fig. 2. Effects of MVS-VI, MVS-IIIA and MVS-IVA on Carbon Clearance Index in ICR Mice

Significantly different from the control, *a*) $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

of 10:1:16:3:6:9:1:8:1:3:7:3:1:1, and as the products from the carboxyl-reduced derivative in a molar ratio of 10:1:16:3:6:9:1:8:11:3:7:3:1:1. These results indicated that the minimal unit of MVS-VI is composed of fifteen kinds of component sugar units, as shown in Chart 1.

MVS-VI was deacetylated,[26] and both the original polysaccharide and the *O*-deacetylated product were separately subjected to periodate oxidation followed by reduction with sodium borohydride. The periodate oxidation-reduction product from the original sample contained 4.4% arabinose, 6.3% xylose, 18.7% galactose and 1.2% rhamnose. Neither glucose nor galacturonic acid was found in this product. The deacetylated product gave similar results to those of the original sample.

The effect of MVS-VI on the reticuloendothelial system (RES) was demonstrated by a modification[18] of the *in vivo* carbon clearance test[27] using zymosan as a positive control. As shown in Fig. 2, the phagocytic index was significantly

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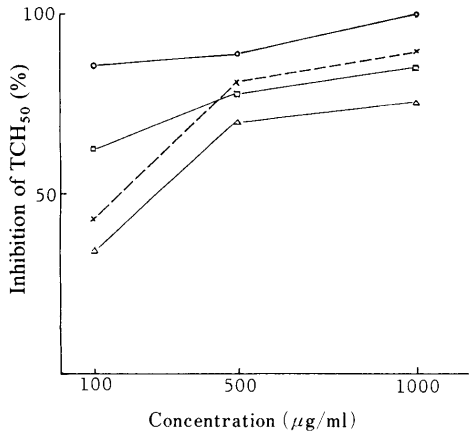

Fig. 3. Anti-complementary Activity of MVS-VI, MVS-IIIA and MVS-IVA

○, MVS-VI; △, MVS-IIIA; □, MVS-IVA; ×, NART-2,4.

increased, suggesting the activation of RES by i.p. injection of MVS-VI.

The anti-complementary activity of MVS-VI is shown in Fig. 3. MVS-VI had remarkable activity compared with the positive control (NART-2,4, the crude polysaccharides, from the root of *Angelica acutiloba* KITAGAWA[19]).

Discussion

We have reported the RES activities of MVS-IIIA and MVS-IVA,[1,5] the other two acidic polysaccharides obtained from the seed of *Malva verticillata*. These substances are mainly made up of arabino-3,6-galactan structure with α-1,3-linked L-arabinopyranosyl, β-1,4-linked D-xylosyl and α-1,4-linked D-galacturonan units, and MVS-IVA possesses additional α-1,2-linked L-rhamnosyl units. They showed remarkable RES-potentiating activity in the carbon clearance test, while the activity of MVS-VI was relatively low. MVS-VI possesses the same structural units as those in MVS-IIIA and MVS-IVA, and further, it has additional β-1,4- and β-1,6-linked D-galactosyl, β-1,3-linked D-xylosyl, α-1,4-linked D-glucosyl, α-2,4-branched L-rhamnosyl and the characteristic α-2,5-branched L-arabinosyl units. In MVS-IIIA and MVS-IVA, arabinogalactan moieties occupy approximately 85% and 79% of the polysaccharides, respectively. In MVS-VI, however, the ratio between arabinogalactan units and the other component sugar residues is about 3:2. In addition, the ratio of α-1,3-linked L-arabinopyranose units in the whole L-arabinose residues is much lower in MVS-VI, than in MVS-IIIA and MVS-IVA. These differences in their structural features may influence the RES-potentiating activity.[28]

On the other hand, MVS-IIIA and MVS-IVA had potent anti-complementary activities,[6] which were at almost the same level as that of the positive control, AR-arabino-galactan mixture from the root of *Angelica acutiloba*.[19] In this case, MVS-VI showed especially remarkable anti-complementary activity. Its value is superior to those of MVS-IIIA and MVS-IVA,[6] and its complicated structure could be involved in the anti-complementary activity.[29]

The yield of MVS-VI is approximately five times those of MVS-IIIA and MVS-IVA, thus MVS-VI is the main acidic polysaccharide in the material seed. The removal of peptide moiety in MVS-V contributed to the rise in immunological activity,[6] so it can be concluded that the activity is attributable to the polysaccharide moiety.

Acknowledgement This work was supported in part by The Science Research Promotion Fund of the Japan Private School Promotion Foundation. Technical assistance of Misses A. Shioya and M. Hoshikawa is gratefully acknowledged.

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