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Constituents of the Seed of *Malva verticillata*. III.[1] Characterization of the Major Pectic Peptidoglycan and Oligosaccharides

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The major pectic peptidoglycan, designated as MVS-V, was isolated from the seeds of *Malva verticillata* L. It was homogeneous on electrophoresis and gel chromatography. It was composed of about 43% polysaccharide and 57% protein moieties, and its molecular weight was estimated to be 22000. The polysaccharide moiety was isolated, and found to be composed of L-arabinose : D-xylose : D-galactose : L-rhamnose : D-galacturonic acid in the molar ratio of 6 : 5 : 3 : 8 : 24. One-sixth of the D-galacturonic acid residues exist as the methyl esters. *O*-Acetyl groups were identified, and the content amounted to 3.3% of the polysaccharide moiety. Methylation analysis of the carboxyl-reduced derivative, nuclear magnetic resonance, periodate oxidation, and partial hydrolysis studies enabled elucidation of the structural features of the polysaccharide moiety. It is a pectic substance having a rhamnogalacturonan backbone with arabinoxylogalactan side chains. Several oligosaccharides belonging to the raffinose family were also isolated and identified from the seeds.

Keywords——*Malva verticillata*; seed; peptidoglycan; MVS-V; polysaccharide moiety; pectic substance; structural feature; oligosaccharide; raffinose family

In the previous papers,[1,2] the isolation and structural features of the major and two minor polysaccharides from the seed of *Malva verticillata* L. (Malvaceae) were reported. We now report the isolation of a pectic peptidoglycan as the second major polymer in the water extract from this crude drug, and we present the results of a structural investigation of its polysaccharide moiety.

The crude precipitate was obtained from the hot water extract by addition of ethanol. The supernatant contains several oligosaccharides and an alditol as described later. An aqueous solution of the precipitate was applied to a column of diethylaminoethyl (DEAE)-Sephadex A-25 (carbonate form). The eluate with water contained neutral polysaccharides,[2] and some acidic polysaccharides were found in the eluate with 0.2 M ammonium carbonate. The purification and characterization of the latter substances are in progress. After elution with 0.2 M ammonium carbonate, the eluate with 0.5 M ammonium carbonate was dialyzed, concentrated and purified by gel chromatography with Sephadex G-25.

The substance gave a single peak on gel chromatography with Sephacryl S-400. In addition, it gave a single band on polyacrylamide gel disk electrophoresis (PAGE), after staining with periodate-Schiff (PAS) and Coomassie blue reagents. Both reagents revealed a clear band at the same position. It had $[\alpha]_D^{24}$ $-121.0°$ ($c=0.1$, H_2O). Gel chromatography with standard dextrans gave a value of 22000 for the molecular weight. The substance is designated as MVS-V.

Quantitative analyses showed that MVS-V was composed of 42.8% polysaccharide and 57.2% protein moieties. The polysaccharide contained 4.5% arabinose, 3.7% xylose, 6.4% rhamnose, 2.7% galactose, and 23.8% galacturonic acid. The molar ratio was 6 : 5 : 8 : 3 : 24. In addition, it contains *O*-acetyl and methyl ester groups as described later. The amino acid composition is given in Table I.

TABLE I. Amino Acid Composition of MVS-V (Molar Percent)

Aspartic acid	8.97	Methionine	3.39
Treonine	4.19	Isoleucine	2.14
Serine	5.63	Leucine	4.09
Glutamic acid	29.10	Tyrosine	2.09
Proline	3.59	Phenylalanine	1.94
Glycine	6.53	Lysine	5.43
Alanine	7.47	Histidine	2.19
Valine	2.89	Arginine	9.22
Cysteine	1.15		

Neither measurement of the nuclear magnetic resonance (NMR) spectrum nor methylation analysis of MVS-V gave good results. Thus, the carbohydrate moiety in MVS-V was isolated by treatment with a protease followed by gel chromatography with Sephadex G-25. The polysaccharide fraction obtained was composed of 89.6% carbohydrate moiety having the same sugar composition as that of MVS-V, and 10.4% peptide. This fraction is tentatively designated as MVS-V-CH.

The carbon 13 nuclear magnetic resonance (^{13}C-NMR) spectrum of MVS-V-CH showed five signals due to anomeric carbons at δ 110.216, 105.306, 104.658, 101.852, and 101.178 ppm. These were assigned to the anomeric carbons of α-L-arabinofuranose,[3] β-D-galactopyranose,[2,4] β-D-xylopyranose,[4] α-L-rhamnopyranose,[5] and α-D-galactopyranosyluronic acid.[5] In addition, the ^{13}C-NMR spectrum showed signals at δ 55.580 and 29.652 ppm. They suggest the presence of *O*-methyl groups as carboxylic acid methyl esters and *O*-acetyl groups in agreement with those of Angelica-pectin A.[6] The presence of these groups in MVS-V was confirmed by gas chromatography (GC) of the hydrolyzate, and the methoxyl and the acetyl contents were determined to be 0.7% and 1.4%, respectively. Thus one per six galacturonic acid residues in MVS-V exists as methyl esters.

The carboxyl groups of galacturonic acids in MVS-V-CH were reduced with a carbodiimide reagent and sodium borohydride to give the corresponding neutral sugar residues.[7] Methylation of the carboxyl-reduced drivative was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide.[8] The methylated product was hydrolyzed, then converted into the partially methylated alditol acetates. Gas-liquid chromatography (GLC)-mass spectrometry (MS)[9] revealed derivatives of 2,3,5-tri-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-L-arabinose, 2,3-di-*O*-methyl-D-xylose, 3,4-di-*O*-methyl-L-rhamnose, 3-*O*-methyl-L-rhamnose, 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 as the products in a molar ratio of 3:3:3:6:2:1:20:2. Methylation analysis of the original MVS-V-CH gave poor results as in the case of Angelica-pectin A.[6]

MVS-V was subjected to periodate oxidation followed by reduction with sodium borohydride. The component sugar analysis of the product showed that about a quarter of the rhamnose units and two-thirds of the galactose units survived after periodate oxidation. No arabinose, xylose, or galacturonic acid was found in the product. The periodate oxidation of the deacetylation product of MVS-V gave similar results.

These results suggested that the minimal repeating unit of the polysaccharide moiety of MVS-V is composed of eight kinds of component sugar units as shown in Chart 1.

MVS-V was partially hydrolyzed with dilute trifluoroacetic acid. After removal of the acid by evaporation, an aqueous solution of the residue was applied to a column of DEAE-Sephadex A-25 (formate form). In addition to the component monosaccharides, an oligosaccharide was obtained by stepwise elution with dilute formic acid. Based on the results of component sugar analysis, and a comparison of chromatographic properties, proton nuclear

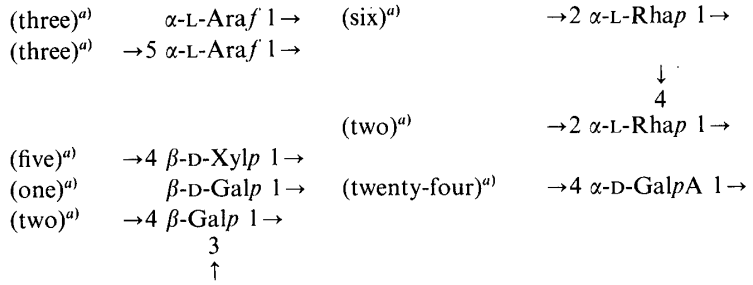

Chart 1. Component Sugar Residues in the Minimal Unit in the Structure of MVS-V

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

magnetic resonance (^{1}H-NMR) spectrum and the specific rotation value with those of an authentic sample,[10-12] the oligosaccharide was identified as 2-*O*-α-(D-galactopyranosyluronic acid)-L-rhamnopyranose.

Based on the accumulated evidence described above, it can be concluded that the polysaccharide moiety of MVS-V resembles that of pectic substances. D-Galacturonic acid residues in it exhibit one-sixth carboxyl-methyl esterification. It has a main chain consisting of α-1→4-linked D-galacturonic acid residues which are interspersed with α-1→2-linked L-rhamnose residues, and a quarter of the rhamnose residues occupy branching points bearing side chains at position 4, as in typical pectins.[13,14]

In general, other neutral sugar residues are present in side chains in pectic substances. The yield of 2,3-di-*O*-methyl-D-xylose was lower than the expected value in the methylation analysis, but no other derivative of xylose was found by GLC-MS. The result of periodate oxidation also supports the presence of 1,4-linked D-xylopyranose units. A slightly lower value of 2,3,6-tri-*O*-methyl-D-galactose suggests that some of the galacturonic acid residues in MVS-V-CH were lost during the process of carboxyl-reduction.[6] The presence of a large amount of peptide moiety is characteristic of MVS-V. However, it has no gel-forming power in aqueous solution. The results of detailed analysis of the structure will be reported in subsequent papers.

On the other hand, the supernatant obtained by adding ethanol to the hot water extract of the material seeds was evaporated. The residue was dissolved in water and applied to a column of Sephadex G-15, and six fractions were obtained by elution with water. Each fraction was examined by thin-layer chromatography (TLC) and by high-performance liquid chromatography (HPLC). Stachyose, raffinose, and sucrose were found as oligosaccharides, and the former two were identified by enzymatic degradation followed by TLC and HPLC analyses. In addition, D-fructose and D-glucitol were identified as a monosaccharide and an alditol by TLC, HPLC, and GLC of their derivatives. Their yields from the seed were 0.7% for stachyose, 3.1% for raffinose, 1.5% for sucrose, 0.4% for fructose, and 0.6% for glucitol. Thus it was revealed that the seed of *Malva verticillata* contains several oligosaccharides belonging to the "raffinose family," and that raffinose is the chief component in them.

Experimental

Solutions were concentrated at or below 40 °C with rotary evaporators under reduced pressure. Optical rotation was 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 30 °C. Infrared (IR) spectra were measured with a JASCO IRA-2 infrared spectrophotometer. GC was carried out on a Shimadzu GC-7AG gas chromatograph equipped with a hydrogen flame ionization detector. GLC-

MS was performed with a JEOL JMS-GX 303 mass spectrometer. HPLC was carried out on a Hitachi L-6200 liquid chromatograph equipped with a Shodex SE-51 RI detector.

Isolation of Proteoglycan——The material was imported from China as described previously.[2] The seeds (200 g) were homogenized and extracted with hot water (2000 ml) under stirring for 1 h in a boiling water bath. After suction filtration, the filtrate was poured into two volumes of ethanol. After centrifugation and drying, the precipitate was dissolved in water (200 ml). The solution was applied to a column (5 × 78 cm) of DEAE-Sephadex A-25 which had been pretreated as described in a previous report.[15] After elution with water (1760 ml) and 0.2 M ammonium carbonate (1920 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.[16] The eluates obtained from tubes 216 to 246 were combined, dialyzed and concentrated, and half the concentrate was applied to a column (5 × 79 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 39 were combined, concentrated and lyophilized. MVS-V was obtained as a pale yellow powder (yield, 353 mg, from 200 g of seeds).

Gel Chromatography——A sample (3 mg) was dissolved in 0.1 M Tris–HCl buffer (pH 7.0), and applied to a column (2.6 × 92 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 dextrans having known molecular weights were run on the column to obtain a calibration curve.

PAGE——This was carried out in an apparatus with gel tubes (4 × 130 mm each) and 0.005 M Tris–glycine buffer (pH 8.3) at 5 mA/tube for 40 min. Gels were stained for carbohydrate using the PAS procedure, and for protein with Coomassie blue reagent. MVS-V gave a clear band at a distance of 4.6 cm from the origin.

Qualitative Analysis of Component Sugars——Hydrolysis and cellulose TLC of component sugars were performed as described in a previous report.[15] The configurations of component sugars were proved by GLC of trimethylsilylated α-methylbenzylaminoalditol derivatives.[17]

Determination of Components——A sample was hydrolyzed, reduced and acetylated as described in a previous report.[10] The derivatives were analyzed for neutral component sugars by GLC with a column (3 mm × 2 m long spiral glass) packed with 3% OV 225 on Gaschrom Q (100 to 120 mesh) at 220 °C with a helium flow of 50 ml per min. Allose was used as an internal standard. Galacturonic acid was estimated by a modification of the carbazole method.[18]

Protein determination was performed by the method of Lowry *et al.*[19] Amino acids were determined by the method of Bidlingmeyer *et al.*[20] after hydrolysis with 6 N hydrochloric acid.

Isolation of the Polysaccharide Moiety——A sample (102 mg) was dissolved in water (5 ml) and heated at 100 °C for 10 min, then the solution was cooled to room temperature and 0.2 M Tris–HCl buffer containing 0.004 M calcium chloride (pH 7.9, 5 ml) was added. Actinase E (Kaken Kagaku Co., 10 mg) was added to the resulting solution, which was then incubated at 40 °C with a few drops of toluene for 72 h. After heating at 80 °C for 10 min followed by addition of Actinase E (10 mg), the incubation was continued for 48 h under the same conditions. The solution was heated at 80 °C for 10 min, then centrifuged at room temperature. The supernatant was applied to a column (5 × 85 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method. The eluates obtained from tubes 30 to 38 were combined, concentrated and lyophilized. Yield, 50 mg.

Reduction of Carboxyl Groups——A sample (100 mg) was dissolved in water (30 ml), then 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (1 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

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

	Relative retention time[a]	Main fragments (*m/z*)
1,4-Ac-2,3,5-Me-L-Arabinitol	0.69	43, 45, 71, 87, 101, 117, 129, 161
1,4,5-Ac-2,3-Me-L-Arabinitol	1.13	43, 87, 101, 117, 129, 189
1,4,5-Ac-2,3-Me-D-Xylitol	1.21	43, 87, 101, 117, 129, 189
1,2,5-Ac-3,4-Me-L-Rhamnitol	0.95	43, 89, 129, 131, 189
1,2,4,5-Ac-3-Me-L-Rhamnitol	1.28	43, 87, 101, 129, 143, 189, 203
1,5-Ac-2,3,4,6-Me-D-Galactitol	1.09	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,4,5-Ac-2,3,6-Me-D-Galactitol	1.43	43, 45, 87, 99, 101, 113, 117, 233
1,3,4,5-Ac-2,6-Me-D-Galactitol	1.64	43, 45, 87, 117, 129

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-2,3,5-Me- = 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-).

(10 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 30 ml. The product was reduced four times more under the same conditions. The final non-dialyzable fraction was concentrated and applied to a column (5 × 79 cm) of Sephadex G-25, which was eluted with water, and fractions of 20 ml were collected. Fractions 29 to 37 were combined and lyophilized. Yield, 48.4 mg.

Methylation——This was performed with methylsulfinyl carbanion and methyl iodide in dimethyl sulfoxide as described in a previous report.[21] The methylation was repeated five times under the same conditions. Yield was 9 mg from 15 mg of the sample.

Analysis of the Methylated Products——The product was hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated in the manner described in a previous report.[22] GLC-MS of partially methylated alditol acetates was performed with a fused silica capillary column (0.32 mm i.d. × 30 m) of SP 2330 (Supelco Co.) and with 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 GLC and their main fragments in MS are listed in Table II.

Periodate Oxidation——A sample (11 mg) was oxidized with 0.05 M sodium metaperiodate (5 ml) at 5 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[23] The oxidation was completed after 3 d, and the maximal value of consumption was 0.6 mol per mol of anhydrosugar unit. The reaction mixture was successively treated with ethylene glycol (0.1 ml) at 5 °C for 1 h and sodium borohydride (30 mg) at 5 °C for 16 h, then adjusted to pH 5 by addition of acetic acid. The solution was applied to a column (2.6 × 91 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 19 to 21 were combined, concentrated and lyophilized. Yield, 7 mg.

Deacetylation——This was carried out as described in a previous report.[24]

Determination of *O*-Acetyl and *O*-Methyl Groups——These were performed by GLC[25] and gas-solid chromatography (GSC) of the hydrolyzates as described in a previous report.[6]

Partial Hydrolysis and Isolation of an Aldobiouronic Acid——A sample (250 mg) was dissolved in 2 M trifluoroacetic acid (25 ml) and heated under reflux at 100 °C for 3 h. The solution was evaporated for the removal of acid, then the residue was dissolved in water and applied to a column (2 × 10 cm) of DEAE-Sephadex A-25 (formate form). The column was eluted successively with water (70 ml) and 0.1 M formic acid (350 ml). Fractions of 10 ml were collected and analyzed by the phenol–sulfuric acid method. Fraction 1 was obtained from tubes 3 to 6, and fr. 2 from tubes 25 to 39. Neutral component sugars were found in fr. 1 (yield, 40 mg). Fraction 2 was applied to a column (5 × 88 cm) of Sephadex G-25, pre-equibrated and developed with 0.04 M pyridine–0.02 M acetic acid buffer (pH 5.4). Fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method. The aldobiouronic acid was obtained from tubes 55 to 57, and galacturonic acid was obtained from tubes 60 to 61. The yields were 11.7 and 11.1 mg, respectively.

Analysis of the Aldobiouronic Acid——Analysis of component sugars, TLC, and GLC of the trimethylsilylated derivative were performed as described in previous reports.[10–12]

TABLE III. *Rf* Values and Retention Times of Low-Molecular-Weight Fractions and Derivatives

	TLC (*Rf*)	HPLC (t_R) (min)	GLC of TMS-derivatives (t_R) (min)
Fr. 2	0.25	19.4	—
Fr. 3	0.36	12.3	44.4
Fr. 4, 5	0.48, 0.59	5.7, 7.8	8.4, 25.4
Fr. 6	0.51	6.2	11.0
EDP of fr. 2[a]	0.29, 0.59	5.7, 16.1	8.4, 32.7
EDP of fr. 3[a]	0.40, 0.59	5.7, 10.0	8.4, 29.3
Fructose	0.59	5.7	8.4
Glucitol	0.51	6.2	11.0
Sucrose	0.48	7.8	25.4
Melibiose	0.40	10.0	29.3
Manninotriose	0.29	16.1	32.7
Raffinose	0.36	12.3	44.4
Stachyose	0.25	19.4	—

a) EDP = enzymatic degradation products. See Experimental for the solvents and conditions.

Isolation of Oligosaccharides——The hot water extract from the material (16.7 g) was poured into two volumes of ethanol. After centrifugation, the supernatant was evaporated to dryness. The residue was dissolved in water, and one-tenth of the solution was applied to a column (5 × 73 cm) of Sephadex G-15. The column was eluted with water, and fractions of 20 ml were collected and analyzed by the phenol–sulfuric acid method. The eluates obtained from the column were divided into six groups: fr. 1, tubes 27 to 28; fr. 2, tubes 37 to 38; fr. 3, tues 40 to 42; fr. 4, tubes 44 to 45; fr. 5, tubes 46 to 47; fr. 6, tubes 49 to 50. The yields were 11.4 mg in fr. 1, 12.0 mg in fr. 2, 51.1 mg in fr. 3, 23.3 mg in fr. 4, 10.1 mg in fr. 5, and 10.5 mg in fr. 6. Fraction 1 contained high-molecular-weight components. Fractions 2 and 3 were stachyose and raffinose, respectively. Both sucrose and D-fructose were found in frs. 4 and 5, and they were separated by HPLC. Fraction 6 gave D-glucitol.

Analysis by TLC——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.

Analysis by HPLC——The HPLC system used was operated at room temperature with a Waters μBondapak CH column (4 mm i.d. × 30 cm). The solvent was acetonitrile–water (3 : 1, v/v) and the flow rate was 1 ml per min. The peaks were monitored with an RI detector. The results are also listed in Table III.

Analysis by GLC——Trimethylsilyl (TMS) derivatives were prepared in the usual way. GLC of the derivatives was performed with a column (3 mm × 2 m long spiral glass) packed with 2% OV 101 on Uniport HP (80 to 100 mesh) and with a flow of 50 ml per min of helium. The programmed temperature was increased at 4 °C per min from 180 to 300 °C. The results are also listed in Table III.

Enzymatic Degradation——A sample (3 mg) was dissolved in water (0.06 ml) and β-fructofuranosidase (0.3 mg, Boehringer Co.) was added. The solution was incubated at 40 °C for 17 h, and the hydrolyzate was analyzed by TLC and HPLC. The hydrolysis rates were 100% for both raffinose and stachyose.

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