Constituents of the Seed of *Malva verticillata*. VII.¹⁾ Structural Features and Reticuloendothelial System-Potentiating Activity of MVS-I, the Major Neutral Polysaccharide

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The structural features of MVS-I, the major neutral polysaccharide isolated from the seeds of *Malva verticillata* L., were elucidated by controlled Smith degradation, methylation analysis, partial acid hydrolysis and enzymic degradation studies. It has a backbone chain composed of β -1,3-linked D-glucose and D-galactose residues having branches composed of α -1,5-linked L-arabinosyl β -1,4-linked D-galactose and of β -1,4-linked D-galactosyl β -1,3-linked D-glucose residues at position 6 of a part of D-galactose units as side chains. MVS-I showed remarkable reticuloendothelial system-potentiating activity in a carbon clearance test.

Keywords *Malva verticillata*; seed; MVS-I; polysaccharide structure; immunological activity; reticuloendothelial system; Smith degradation; partial hydrolysis; enzymic degradation; methylation analysis

MVS-I is the major neutral polysaccharide obtained from the seed of *Malva verticillata* L. (Malvaceae).²⁾ The seed of this plant is an Oriental crude drug used as a diuretic, laxative and galactopoietic. As the other glycans of this material, the isolation and structural features of two neutral polysaccharides (MVS-IIA and MVS-IIG),³⁾ three acidic polysaccharides (MVS-IIIA, MVS-IVA and MVS-VI)^{1,4,5)} and the major peptidoglycan (MVS-V)⁶⁾ were reported in previous papers. These substances were examined for anti-complementary and hypoglycemic activities.⁷⁾ Among them, MVS-I showed especially remarkable activities. The polysaccharide is composed of L-arabinose: D-galactose: D-glucose in the molar ratio of 3:6:7.²⁾ The present paper describes controlled Smith degradation, partial acid hydrolysis, enzymic degradation and methylation analysis of the products, and presents its structural features and reticuloendothelial system (RES)-potentiating activity.

Materials and Methods

Isolation of Polysaccharide This was performed as described in a previous report.²⁾

Periodate Oxidation The polysaccharide (100 mg) was oxidized with 0.05 M sodium metaperiodate (50 ml) at 4°C in the dark. The periodate consumption was measured by a spectrophotometric method,⁸⁾ and the oxidation was completed after 5 d. The reaction mixture was successively treated with ethylene glycol (0.5 ml) at 4°C for 1 h and sodium borohydride (0.5 g) at 4°C for 16 h, then adjusted to pH 5.0 by addition of acetic acid. The solution was concentrated and applied to a column (5×85 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 31 to 34 were combined, concentrated and lyophilized. Yield, 86 mg.

Controlled Smith Degradation The product (300 mg) obtained by periodate oxidation was dissolved in 0.5 N sulfuric acid (25 ml). After standing at 26°C for 16 h, the solution was neutralized with barium carbonate. The filtrate was concentrated and applied to a column (5×85 cm) of Sephadex G-25. The column was eluted with water, and fractions of 20 ml were collected. The eluates obtained from tubes 31 to 34 were combined, concentrated and lyophilized. Yield, 89 mg.

Methylation The sample (5 mg) was dissolved in dimethyl sulfoxide (0.5 ml), then finely powdered sodium hydroxide (20 mg) and methyl iodide (0.1 ml) were added to the solution. The whole was stirred at room temperature for 1 h. All procedures were carried out under nitrogen. Chloroform and water (2 ml each) were then added to the reaction mixture, and the whole was extracted three times with chloroform (2 ml each). The combined extract was washed three times with water (6 ml each), then dried over sodium sulfate. The filtrate was concentrated to dryness, and the residue was dissolved in a 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, 3 mg.

Analysis of Methylated Products The products were hydrolyzed with diluted sulfuric acid in acetic acid, then reduced and acetylated as previously described.⁹⁾ 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.) with a programmed temperature increase of 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-GX303 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.

Partial Acid Hydrolysis and Isolation of Products The controlled Smith degradation product (192 mg) was dissolved in 0.5 M trifluoroacetic acid and heated at 80°C for 8 h. After evaporation for the removal of acid, the residue was dissolved in water and applied to a column (5×80 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.¹⁰⁾ Fraction 1 was obtained from tubes 51 to 56, fr. 2 from tubes 57 to 59, fr. 3 from tubes 60 to 62, and fr. 4 from tubes 63 to 70. The yields were 24.9, 9.7, 38.0 and 110.0 mg, respectively.

Analysis of Products Thin-layer chromatography (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. High-performance liquid chromatography (HPLC) of *p*-aminobenzoyl derivatives purified using a column (6×22 mm) of PBA-60 (Amicon Co.)¹¹⁾ was performed with a column (4.6×250 mm) of TSK-gel Amide-80 (Tosoh Co.) and detected by absorption at 304 nm. The HPLC system used consisted of a Hitachi L-6200 intelligent pump, a JASCO Uvidec-100 spectrophotometer and a Hitachi D-2000 chromatointegrator. Elution was done at a flow rate of 1 ml per min at room temperature using a linear gradient of acetonitrile-

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

	Relative retention time ^{a)}	Main fragments (<i>m/z</i>)
1,4-Ac ₂ -2,3,5-Me ₃ -L-arabinitol	0.69	43, 45, 71, 87, 101, 117, 129, 161
1,4,5-Ac ₃ -2,3-Me ₂ -L-arabinitol	1.13	43, 87, 101, 117, 129, 189
1,5-Ac ₂ -2,3,4,6-Me ₄ -D-glucitol	1.00	43, 45, 71, 87, 101, 117, 129, 145, 161, 205
1,3,5-Ac ₃ -2,4,6-Me ₃ -D-glucitol	1.39	43, 45, 87, 101, 117, 129, 161
1,3,5-Ac ₃ -2,4,6-Me ₃ -D-galactitol	1.34	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
1,3,5,6-Ac ₄ -2,4-Me ₂ -D-galactitol	2.01	43, 87, 117, 129, 189

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-Ac₂-2,3,5-Me₃=1,4-di-O-acetyl-2,3,5-tri-O-methyl-).

water (75:25 to 50:50) for 20 min after sample injection. Retention times of glucose, laminaribiose, laminaritriose and laminaritetraose were 3.3, 4.4, 6.0 and 8.1 min.

Enzymic Degradation and Isolation of Products The polysaccharide (200 mg) was dissolved in 0.05 M acetate buffer (pH 6.0, 10 ml) and β -glucosidase preparation from almonds (10 mg of Emulsin, 900 units per mg, Tokyo Kasei Co.) was added. The solution was incubated with a few drops of toluene at 37 °C for 24 h, then applied to a column (5 × 15 mm) of Dowex 50W-X8 (H⁺). The eluate with water was applied to a column (5 × 80 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. Fraction a-1 was obtained from tubes 33 to 38 and fr. b-1 from tubes 62 to 66. Their yields were 165.0 and 10.9 mg. Fraction a-1 was treated with the same enzyme preparation once again, and the resulting solution was applied to the two columns successively as described above. Fraction a-2 was obtained from tubes 31 to 35 and fr. b-2 from tubes 61 to 65. The yields were 135.6 and 19.0 mg, respectively.

Analysis of Degradation Products Molecular masses were estimated by 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 Toyopearl HW-55F, 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 masses were run on the column to obtain a calibration curve.

Component sugars were analyzed by GC after conversion of the hydrolyzate into alditol acetates as described previously.¹²⁾

Phagocytic Activity This was measured as described in a previous report.¹²⁾ The sample and a positive control, zymosan (Tokyo Kasei Co.), were each dissolved and suspended in physiological saline and dosed i.p. (20 or 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). The comparison of results was performed by means of Student's *t*-test.

Results

The hot water extract obtained from the seed of *Malva verticillata* was applied to a column chromatography of diethylaminoethyl (DEAE)-Sephadex A-25. The eluate with water was subjected to affinity chromatography on concanavalin A (Con A)-Sephadex. The passed-through fraction was dialyzed and purified by gel chromatography with Sephadex G-25, then the major neutral polysaccharide, MVS-I, was obtained after lyophilization.²⁾ The polysaccharide was homogeneous on electrophoresis and gel chromatography, and it was composed of L-arabinose, D-galactose and D-glucose in the molar ratio of 3:6:7.²⁾ Its molecular mass was estimated to be 7.7×10^4 . The minimal repeating unit of MVS-I is composed of terminal α -L-arabinofuranose, α -1,5-linked L-arabinofuranose, β -1,3-linked D-galactopyranose, β -1,4-linked D-galactopyranose, β -3,6-branched D-galactopyranose, terminal β -D-glucopyranose and β -1,3-linked D-glucopyranose residues in the ratio of 3:3:2:6:4:1:13.²⁾

The controlled Smith degradation¹³⁾ of MVS-I by periodate oxidation and reduction followed by mild hydrolysis yielded a polymer composed of D-galactose and D-glucose in a molar ratio of 6:13. Its molecular mass was 2.4×10^4 . The controlled Smith degradation product was methylated with solid sodium hydroxide and methyl iodide in dimethyl sulfoxide.¹⁴⁾ The methylated product was hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS revealed derivatives of 2,3,4,6-tetra-*O*-methyl D-glucose, 2,4,6-tri-*O*-methyl D-glucose, 2,4,6-tri-*O*-methyl D-galactose and 2,4-di-*O*-methyl

D-galactose in a molar ratio of 1:12:5:1.

The result of methylation analysis revealed that it was mainly composed of β -1,3-linked D-galactose and D-glucose units and that a part of the D-galactose residues in the backbone carries side chains having terminal D-glucose at position 6.

On the other hand, the controlled Smith degradation product was partially hydrolyzed with dilute trifluoroacetic acid under the condition providing the maximum yield of total oligosaccharides. After removal of the acid, the products were isolated by chromatography with Sephadex G-25. In addition to galactose and glucose in an approximate ratio of 2:3 (fr. 4), three oligosaccharide fractions (frs. 1 to 3) were obtained. Laminaritetraose, laminaritriose and laminaribiose were identified in these fractions by HPLC of *p*-aminobenzoyl derivatives and by TLC. No galactose-containing oligosaccharide was obtained under this condition. This result suggested the presence of linear oligosaccharide chains composed of β -1,3-linked D-glucose units in MVS-I.

MVS-I was treated with β -glucosidase preparation from almonds followed by chromatography with Sephadex G-25. A high molecular mass fraction (fr. a-1) was treated once more with the same enzyme preparation. The final high molecular mass fraction (fr. a-2) is tentatively designated as EDP. The yields of the monosaccharide fraction (frs. b-1 and b-2) of the primary and secondary enzymic degradations were 5.4% and 11.5%, respectively. The yields of fr. a-1 and EDP were 82.5% and 82.2%, respectively. Fraction b-1 contained glucose, galactose and arabinose in an approximate ratio of 5:1:1, and fr. b-2 had glucose, galactose and arabinose in an approximate ratio of 12:1:1.

EDP was homogeneous on gel chromatography, and its molecular mass was estimated to be 2.4×10^4 . It was composed of L-arabinose, D-galactose and D-glucose in the molar ratio of 6:9:4. The methylation analysis of EDP was performed in the similar manner to those described above, and GC-MS revealed derivatives of 2,3,5-tri-*O*-methyl L-arabinose, 2,3-di-*O*-methyl L-arabinose, 2,3,6-tri-*O*-methyl D-galactose, 2,4,6-tri-*O*-methyl D-galactose, 2,4-di-*O*-methyl D-galactose and 2,4,6-tri-*O*-methyl D-glucose in a molar ratio of 3:3:3:3:3:4. These results suggested that EDP was appeared from MVS-I by the loss of side chains composed of both β -1,4-linked D-galactose and β -1,3-linked D-glucose. Glycosidase activities of the enzyme preparation on these types of glycosidic linkage were ascertained using lactitol and laminaribitol as substrates. Weak α -L-arabinofuranosidase activity of the enzyme preparation used was also confirmed using a larch wood arabinogalactan (Sigma Co.) as the substrate. It caused about a 6% liberation of terminal arabinose residues in the polysaccharide. In the side chains, β -1,4-linked D-galactosyl units must be located on the outer side of β -1,3-linked D-glucosyl units because of the loss of the former group by the controlled Smith degradation.

Mild treatment of MVS-I with 0.05 M trifluoroacetic acid at 100 °C for 3 h caused partial liberation of some component sugars. The liberation rates of arabinose, galactose and glucose were 90.6%, 16.4% and 1.8%, respectively. This result showed the presence of arabinosyl arabinose units in the terminals of side chains in MVS-I.

Based on the accumulated evidence described above, it

Chart 1. Possible Structural Units of MVS-I

(a + c + e) : b : d : f = 4 : 3 : 2 : 1.

Fig. 1. Effects of MVS-I and Its Degradation Products on Phagocytosis
Significantly different from the control, a) $p < 0.01$, b) $p < 0.001$.

can be concluded that MVS-I has the structural features shown in Chart 1.

The effects of MVS-I, and its periodate oxidation and reduction product (POP), the controlled Smith degradation product (SDP) and EDP on the RES were demonstrated by a modification¹²⁾ of the *in vivo* carbon clearance test¹⁵⁾ using zymosan as a positive control. As shown in Fig. 1, MVS-I showed remarkable activity. POP showed significant activity, however, neither SDP nor EDP showed RES activity.

Discussion

We have obtained three neutral polysaccharides, MVS-I,²⁾ MVS-IIA and MVS-IIG,³⁾ and three acidic polysaccharides, MVS-IIIa,⁴⁾ MVS-IVa⁵⁾ and MVS-VI,¹⁾ from the seed of *Malva verticillata*. As one of their immunological activities, especially remarkable anti-complementary activity was found in MVS-I, MVS-IIA and MVS-VI.^{1,7)} In addition, both MVS-IIIa and MVS-IVa also showed considerable anti-complementary activity.⁷⁾ Further, these two acidic polysaccharides showed remarkable RES-potentiating activity in the carbon clearance test, while this activity of MVS-VI was relatively low.¹⁾ The phagocytic index was remarkably increased, suggesting the marked activation of RES by i.p. injection of MVS-I.

MVS-IIIa, -IVa and -VI possess acidic arabino-3,6-galactan structure as their major parts. We have already isolated and characterized saposhnikovian A¹²⁾ from the root and rhizome of *Saposhnikovia divaricata*, ukonans A¹⁶⁾ and B¹⁷⁾ from the rhizome of *Curcuma longa*, glycyrrhizan UA¹⁸⁾ from the root of *Glycyrrhiza uralensis*, and

glycyrrhizan GA¹⁹⁾ from the stolon of *G. glabra* var. *glandulifera* as the other RES active polysaccharides belonging to this structural group.

MVS-I possesses both α -1,5-linked L-arabinofuranosyl and β -3,6-branched D-galactosyl units. However, unlike typical arabino-3,6-galactan, the major part of this polysaccharide is occupied by β -1,3-linked D-glucose residues with dispersed β -1,3-linked D-galactose units. In addition, it has considerable β -1,4-linked D-galactose residues and no hexuronic acid. Thus MVS-I is a novel RES active neutral polysaccharide having a new structural type. Two kinds of side chains were found in it. They are composed of β -1,4-linked D-galactosyl β -1,3-linked D-glucosyl units and α -1,5-linked L-arabinosyl β -1,4-linked D-galactosyl units. The removal of one of each caused the disappearance of the RES-potentiating activity.

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References

- 1) Part VI: R. Gonda, M. Tomoda, M. Kanari, N. Shimizu and H. Yamada, *Chem. Pharm. Bull.*, **38**, 2771 (1990).
- 2) N. Shimizu and M. Tomoda, *Chem. Pharm. Bull.*, **35**, 4981 (1987).
- 3) N. Shimizu and M. Tomoda, *Chem. Pharm. Bull.*, **36**, 2778 (1988).
- 4) M. Tomoda, M. Kanari, R. Gonda and N. Shimizu, *Phytochemistry*, **28**, 2609 (1989).
- 5) R. Gonda, M. Tomoda, N. Shimizu and M. Kanari, *Planta Med.*, **56**, 73 (1990).
- 6) R. Gonda, M. Tomoda, M. Kanari, N. Yoneda, T. Shimizu, K. Shigeshiro and T. Yazawa, *Chem. Pharm. Bull.*, **36**, 2790 (1988).
- 7) M. Tomoda, N. Shimizu, R. Gonda, M. Kanari, H. Yamada and H. Hikino, *Planta Med.*, **56**, 168 (1990).
- 8) G. O. Aspinall and R. J. Ferrier, *Chem. Ind.* (London), **1957**, 1216.
- 9) M. Tomoda, K. Shimada, Y. Saito and M. Sugi, *Chem. Pharm. Bull.*, **28**, 2933 (1980).
- 10) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
- 11) H. Higashi, M. Ito, N. Fukaya, S. Yamagata and T. Yamagata, *Anal. Biochem.*, **186**, 355 (1990).
- 12) N. Shimizu, M. Tomoda, R. Gonda, M. Kanari, M. Takanashi and N. Takahashi, *Chem. Pharm. Bull.*, **37**, 1329 (1989).
- 13) I. J. Goldstein, G. W. Hay, B. A. Lewis and F. Smith, "Methods in Carbohydrate Chemistry," Vol. 5, ed. by R. L. Whistler, Academic Press, New York and London, 1965, pp. 361–370.
- 14) I. Ciucanu and F. Kerek, *Carbohydr. Res.*, **131**, 209 (1984).
- 15) G. Biozzi, B. Benacerraf and B. N. Halpern, *Br. J. Exp. Pathol.*, **34**, 441 (1953).
- 16) M. Tomoda, R. Gonda, N. Shimizu, M. Kanari and M. Kimura, *Phytochemistry*, **29**, 1083 (1990).
- 17) R. Gonda, M. Tomoda, N. Shimizu and M. Kanari, *Chem. Pharm. Bull.*, **38**, 482 (1990).
- 18) M. Tomoda, N. Shimizu, M. Kanari, R. Gonda, S. Arai and Y. Okuda, *Chem. Pharm. Bull.*, **38**, 1667 (1990).
- 19) N. Shimizu, M. Tomoda, M. Satoh, R. Gonda and N. Ōhara, *Chem. Pharm. Bull.*, **39**, 2082 (1991).