Constituents of the Seed of *Malva verticillata*. VIII.[1] Smith Degradation of MVS-VI, the Major Acidic Polysaccharide, and Anti-complementary Activity of Products

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The controlled Smith degradation of MVS-VI, the major acidic polysaccharide having remarkable anti-complementary activity isolated from the seeds of *Malva verticillata* L., was performed. Methylation analysis of both the primary and the secondary Smith degradation products indicated that the core structural features of MVS-VI include a backbone chain composed of β-1,3-linked D-galactose residues. The majority of galactose units in the backbone carry side chains composed of β-1,3- and β-1,6-linked D-galactosyl residues at position 6. The controlled Smith degradation products showed considerable anti-complementary activity.

Keywords *Malva verticillata*; seed; MVS-VI; polysaccharide structure; Smith degradation; partial hydrolysis; anti-complementary activity

MVS-VI is the major acidic polysaccharide isolated from the seed of *Malva verticillata* L. (Malvaceae).[2] As the other glycans of this material, the isolation and structural features of three neutral polysaccharides (MVS-I, -IIA and -IIG),[1,3] two acidic polysaccharides (MVS-IIIA and-IVA)[4,5] and a peptidoglycan (MVS-V)[6] were reported in previous papers. Among them, especially remarkable anti-complementary activity was observed for MVS-VI[2] in addition to MVS-I and -IIA.[7]

The present paper described the controlled Smith degradation and limited hydrolysis of MVS-VI, and presents the core structural features. This paper also describes the anti-complementary activity of the Smith degradation products.

Materials and Methods

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

Deacetylation Followed by Controlled Smith Degradation MVS-VI (250 mg) was dissolved in 0.05 N sodium hydroxide (50 ml), and after standing at room temperature for 30 min, the solution was neutralized with 5 M acetic acid. The solution was adjusted to 62.5 ml with water, then 0.1 M sodium metaperiodate (62.5 ml) was added and kept at 4 °C in the dark. The periodate consumption was measured by a spectrophotometric method.[8] Oxidation was completed after 72 h. The reaction mixture was successively treated with ethylene glycol (1.25 ml) at 4 °C for 1 h and with sodium borohydride (1.25 g) at 4 °C for 16 h, then adjusted to pH 5.0 by the addition of acetic acid. The solution was concentrated and applied to a column (5 × 86 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.[9] The eluates obtained from tubes 32 to 36 were combined, concentrated and lyophilized. Yield, 202.7 mg.

The product was dissolved in 0.5 N sulfuric acid (20 ml). After standing at 24 °C for 16 h, the solution was neutralized with barium carbonate. The filtrate was concentrated and 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 30 to 34 were combined, concentrated and lyophilized. The yield of the Smith degradation product (SDP) was 35.4 mg.

Separation of SDP into Three Fractions SDP (28.4 mg) was dissolved in a 0.1 M Tris–HCl buffer (pH 7.0) and applied to a column (2.6 × 95 cm) of Sephacryl S-200, pre-equilibrated and developed with the same buffer. Fractions of 5 ml were collected and analyzed by the phenol-sulfuric acid method. The eluates obtained from tubes 47 to 55 were combined, concentrated and applied to a column (2.6 × 86 cm) of Sephadex G-25. The column was eluted with water and fractions of 10 ml were collected. The eluates obtained from tubes 20 to 22 were combined, concentrated and lyophilized. The yield of this fraction (SDP-a) was 9.4 mg. The eluates obtained from tubes 58 to 68 of the Sephacryl S-200 column chromatography were applied to the same column of Sephadex G-25, and the second fraction (SDP-b) was obtained. Yield, 8.5 mg. The eluates obtained from tubes 69 to 87 of the same column of Sephacryl S-200 gave the third fraction (SDP-c). This fraction was rechromatographed using a column (2.6 × 88 cm) of Sephacryl S-200 and with the same buffer. The eluates obtained from tubes 62 to 77 were combined, concentrated and applied to the same column of Sephadex G-25, and the purified fraction (SDP-c1) was obtained. Yield, 3.9 mg. Gel chromatography on a column of Sephacryl S-200 with the same buffer as described above was used for estimation of molecular masses. Standard pullulans (Shōwa Denkō Co.) having known molecular masses were run on the column to obtain a calibration curve.

Determination of Components Neutral sugars were analyzed by gas chromatography (GC) after conversion of the hydrolyzate into alditol acetates as described previously.[10] Galacturonic acid was determined by the *m*-hydroxybiphenyl method.[11]

Methylation Analysis Methylation was performed with powdered sodium hydroxide and methyl iodide in dimethyl sulfoxide as previously described.[12] The products were hydrolyzed with dilute sulfuric acid in acetic acid, then reduced and acetylated as described in a previous report.[13] The partially methylated alditol acetates obtained were analyzed by gas chromatography–mass spectometry (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.

Secondary Smith Degradation SDP (20.2 mg) was oxidized with 0.05 M sodium metaperiodate (10.6 ml) at 4 °C for 48 h in the dark. The reaction mixture was successively treated with ethylene glycol and sodium borohydride as described above. After addition of acetic acid, the solution was applied to a column (2.6 × 90 cm) of Sephadex G-25. The column was eluted with water, and fractions of 10 ml were collected. The eluates obtained from tubes 21 to 23 were combined and lyophilized. Yield, 20.0 mg. This product was treated with 0.5 N sulfuric acid at 24 °C for 16 h, and after neutralization, the solution was applied to a column (2.6 × 90 cm) of Sephadex G-25. The secondary Smith degradation product was obtained from the eluates in tubes 21 and 22. Yield, 9.0 mg.

Limited Acid Hydrolysis MVS-VI (50 mg) was dissolved in 0.05 M trifluoroacetic acid (5 ml), and the solution was heated at 100 °C for 2 h. The acid was removed by evaporation, then 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. The eluates obtained from tubes 29 to 31 were combined, concentrated and lyophilized. Yield, 15.4 mg.

Anti-complementary Activity The activity was measured as described previously.[14] The sample solutions were incubated with normal human serum and gelatin–veronal–buffered saline containing Mg^{2+} and Ca^{2+}, and the residual total hemolytic complement (TCH_{50}) was determined by a method using immunoglobulin M-hemolysin-sensitized sheep erythrocytes. The activities of the samples were expressed as the percentage inhibition of the TCH_{50} of the control. Plantago-mucilage A[15] was used as a positive control.

Results

The crude glycan fraction obtained from the seeds of

Malva verticillata by hot water extraction, followed by the addition of ethanol, was applied to a column chromatography of diethylaminoethyl (DEAE)-Sephadex A-25. After elution with water, the eluate with 0.2 M ammonium carbonate was subjected to successive gel chromatographies on Sephacryl S-500, Toyopearl HW-60F and Sephadex G-25. The major acidic polysaccharide, MVS-VI, thus obtained is composed of terminal α-L-arabinose, α-1,3-linked L-arabinose, α-1,5-linked L-arabinose, α-2,5-branched L-arabinose, β-1,3-linked D-xylose, β-1,4-linked D-xylose, terminal β-D-galactose, β-1,3-linked D-galactose, β-1,4-linked D-galactose, β-1,6-linked D-galactose, β-3,6-branched D-galactose, α-1,4-linked D-glucose, α-1,2-linked L-rhamnose, α-2,4-branched L-rhamnose and α-1,4-linked D-galacturonic acid residues. The minimal repeating unit of MVS-VI possesses these component units in the ratio of 10:1:16:3:6:9:1:8:1:3:7:3:1:1:10.[2]

The controlled Smith degradation[16] of MVS-VI by periodate oxidation and reduction followed by mild hydrolysis gave a polymer (SDP) composed of L-arabinose and D-galactose. SDP was divided into three products (SDP-a, SDP-b and SDP-c1) by gel chromatography on Sephacryl S-200. Each of them gave a single peak on gel chromatography, and values for the molecular mass were 37,000 in SDP-a, 17,000 in SDP-b, and 7,000 in SDP-c1. The value of 56000 was obtained for the molecular mass of MVS-VI under the same conditions. The difference of molecular mass of MVS-VI between this value and that in a previous report[2] must result from the difference in gel used. Sephacryl S-400 previously used might not be suitable for this polysaccharide. Quantitative analysis showed that both SDP-a and SDP-b are composed of L-arabinose and D-galactose in the molar ration of 1:19, and that SDP-c1 is composed of the same component sugars in the molar ratio of 1:9.

SDP-a, SDP-b and SDP-c1 were respectively methylated with sodium hydroxide and methyl iodide in dimethyl sulfoxide.[17] The methylated products were hydrolyzed, then converted into the partially methylated alditol acetates. Analysis by GC-MS[18] showed the presence of the same methylation products from these three materials. They are 2,3,5-tri-*O*-methyl-L-arabinose, 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose. Their molar ratios were 1:10:10:5:11 in the products from SDP-a, 0.5:10:10:5:11 in the products from SDP-b, and 0.4:10:10:5:11 in the products from SDP-c1. The decrease of arabinosyl units in the methylated products was probably caused by their partial elimination in the reaction process.

In order to elucidate the structural features of SDP, secondary Smith degradation was performed under the same conditions as the isolation of SDP from MVS-VI. The product thus obtained was composed of only D-galactose. Methylation analysis revealed derivatives of 2,3,4,6-tetra-*O*-methyl-D-galactose, 2,4,6-tri-*O*-methyl-D-galactose, 2,3,4-tri-*O*-methyl-D-galactose and 2,4-di-*O*-methyl-D-galactose as the products in a molar ratio of 3:14:1:3.

From these results of methylation analysis of the primary and secondary Smith degradation products, it can be presumed that SDP possesses a backbone chain composed of β-1,3-linked D-galactose units. About ten-seventeenths of the galactose residues in the backbone chain must carry four kinds of side chains at position 6. Terminal and β-1,6-linked D-galactosyl side chains were lost by the secondary degradation. Thus, the possible structural features of the primary and secondary Smith degradation products are shown in Chart 1.

Limited acid hydrolysis of MVS-VI with very dilute trifluoroacetic acid resulted in the nearly complete removal of arabinose and glucose moieties. The product obtained was composed of D-xylose, D-galactose, L-rhamnose and D-galacturonic acid in the molar ratio of 3:20:2:10. Methylation analysis of this product showed the presence of 2,3-di-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-

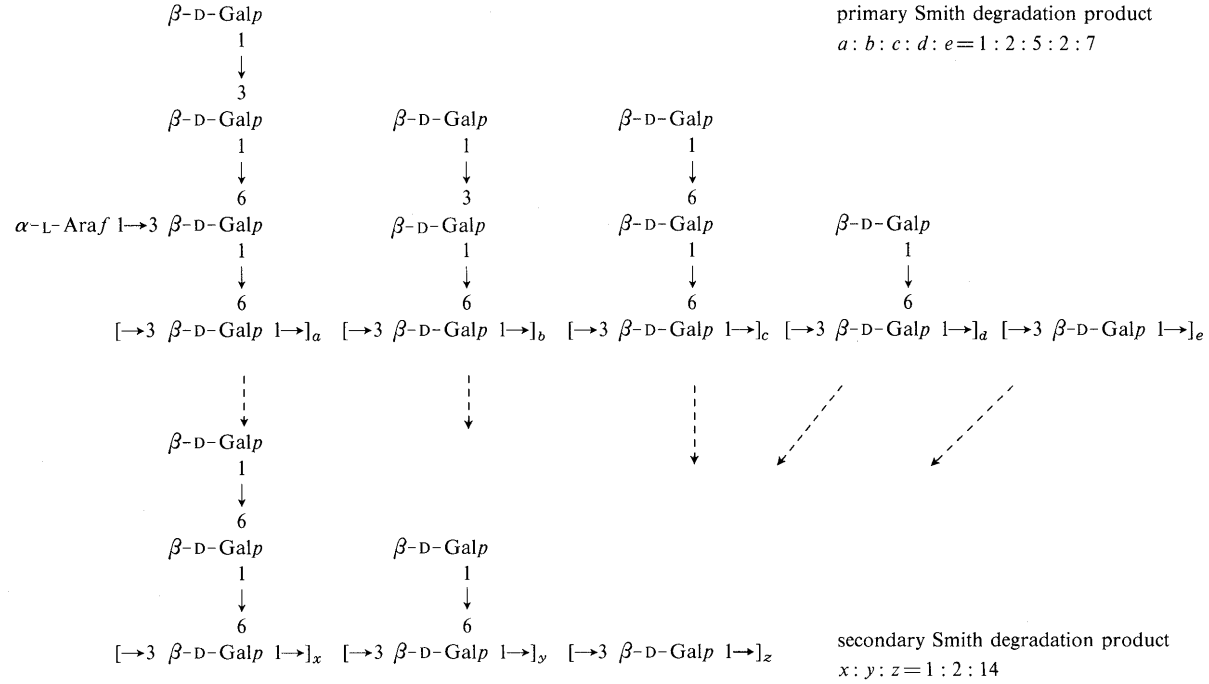

Chart 1. Possible Structural Units of the Primary and the Secondary Smith Degradation Products of MVS-VI

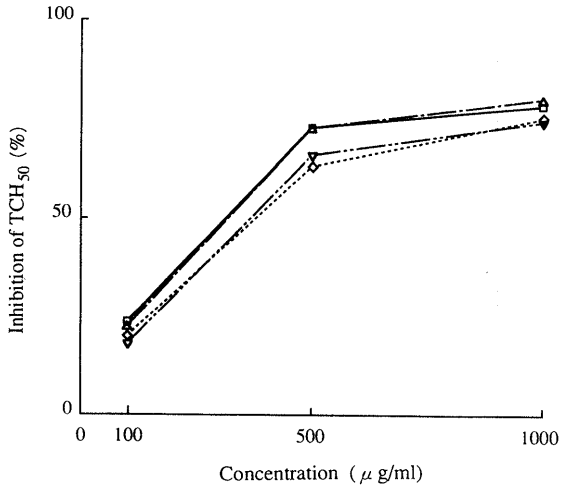

Fig. 1. Anti-complementary Activity of SDP-a, SDP-b and SDP-c1
SDP-a, □——; SDP-b, △—·—; SDP-c1, ▽—··—; Plantago-mucilage A, ◇------.

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, 3,4-di-*O*-methyl-L-rhamnose and 3-*O*-methyl-L-rhamnose as the products in the molar ratio of 2:5:4:1:8:5:1:1. In this case, galacturonic acid methyl ether was removed from the products by treatment with anion-exchange resin. Thus, the methylation analysis revealed a pronounced increase in terminal and 1,6-linked D-galactose units, and a distinct decrease in 1,3-linked and 3,6-branched D-galactose units. These results indicate that the arabinosyl units are mainly connected to terminal and intermediate galactose residues *via* position 3 in the side chains of MVS-VI.

MVS-VI showed especially remarkable anti-complementary activity. The activities of SDP-a, SDP-b and SDP-c1 are shown in Fig. 1. These Smith degradation products showed significant anti-complementary activity, though their values were lower than that of the original polysaccharide. These activities were at almost the same level as that of the positive control (Plantago mucilage A, the polysaccharide from the seed of *Plantago asiatica*[15,19]).

Discussion

MVS-VI is an acidic polysaccharide having a complicated structure. It is composed of fifteen kinds of component units. About 60 percent of the polysaccharide is occupied by L-arabinose and D-galactose moieties. In addition, D-xylose is found in approximately one sixth of the component sugars. The distinct liberation of xylose by limited acid hydrolysis suggests the presence of many xylose moieties in the side chains of MVS-VI.

The controlled Smith degradation of the polysaccharide revealed the presence of a backbone chain composed of β-1,3-linked D-galactose residues in the core of the polysaccharide. The majority of these galactose units carry galactosyl side chains at position 6. The result of methylation analysis of the de-arabinosylated product obtained by the limited hydrolysis indicated that many arabinosyl units must be linked to the galactosyl residues via position 3 in these side chains of MVS-VI.

Most of the polysaccharides, MVS-I, -IIA, -IIIA, -IVA and -VI, obtained from the seeds of *Malva verticillata* had potent anti-complementary activity.[2,7] Those of MVS-IIIA and -IVA were at almost the same level as that of the positive control. The values of MVS-I and -IIA were superior to those of these two acidic polysaccharides. Among them, MVS-VI showed the highest value on the activity. The controlled Smith degradation of MVS-VI afforded three peoducts: SDP-a, SDP-b and SDP-c1. Methylation analysis of each product gave similar results, though different values of molecular mass were observed in them. They showed significant anti-complementary activity. This fact suggests that the core structural features shown in these products must contribute to the activity. It is interesting that SDP-a and SDP-b showed almost the same value on the activity despite a marked difference in molecular mass between the two products.

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