Phytochemistry, Vol 28, No 10, pp 2609–2611, 1989
Printed in Great Britain

0031-9422/89 $3.00+0.00

A RETICULOENDOTHELIAL SYSTEM-ACTIVATING GLYCAN FROM THE SEEDS OF *MALVA VERTICILLATA**

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(*Received* 3 *January* 1989)

Key Word Index—*Malva verticillata*; Malvaceae; seed, polysaccharide, structure analysis; phagocytic activity.

Abstract—From the hot water extract of the seeds of *Malva verticillata*, a polysaccharide (MVS-IIIA) has been isolated by fractionation on DEAE-Sephadex A-25 followed by chromatography on Sephacryl S-500 and Con A-Sepharose columns. Chemical and spectroscopic studies established that the polysaccharide possesses mainly an α-1,5-linked L-arabino-3,6-β-D-galactan structure α-1,3-Linked L-arabinopyranose, β-1,4-linked D-xylose and α-1,4-linked D-galacturonic acid residues were also identified as the component units. The polysaccharide showed remarkable reticuloendothelial system potentiating activity in the carbon clearance test.

INTRODUCTION

Recently, we have obtained and elucidated the structural features of three neutral polysaccharides (MVS-I, MVS-IIA and MVS-IIG) [1, 2], an acidic polysaccharide (MVS-IVA) [3] and the major peptidoglycan (MVS-V) [4] from the seed of *Malva verticillata* L. The present paper describes the isolation, structure analysis and phagocytic activity of a new acidic polysaccharide from this material, which is an Oriental crude drug.

RESULTS AND DISCUSSION

The crude polysaccharide fraction was isolated from the seeds of *M. verticillata* by hot water extraction followed by precipitation with ethanol. The aqueous solution was applied to a DEAE-Sephadex A-25 column (carbonate form). After elution with water, the eluate with 0.2 M ammonium carbonate was dialysed, concentrated and applied to Sephacryl S-500 column. The eluate containing the first peak fraction was subjected to affinity chromatography on Con A-Sepharose After elution with a buffer, followed by dialysis and gel chromatography with Sephadex G-25, a pure polysaccharide designated as MVS-IIIA was obtained.

The polysaccharide gave a single band on PAGE, after staining with periodate–Schiff and Coomassie Blue reagents. Further, it gave a single peak on gel chromatography with Toyopearl HW-75F. The polysaccharide showed a negative specific rotation ($[\alpha]_D^{24} -40.4°$). Gel chromatography using standard dextrans gave a value of about 8.5×10^6 for its M_r.

Quantitative analyses showed that the polysaccharide was composed of L-arabinose (52.9%). D-xylose (3.3%), D-galactose (33.0%), D-galacturonic acid (11.6%) and a peptide moiety (1.7%). The molar ratio of these component sugars was 16:1:8·3.

The carboxyl groups of the hexuronic acids in the polysaccharide were reduced with a carbodiimide reagent and sodium borohydride to give the corresponding neutral sugar residues [5]. Both the original polysaccharide and the carboxyl-reduced derivative were methylated with methylsulphinyl carbanion and methyl iodide in dimethyl sulphoxide [6]. The methylated products were hydrolysed, then converted into the partially methylated alditol acetates. Methyl ethers of hexuronic acids were removed from the hydrolysis products of the methylated native polysaccharide by treatment with anion-exchange resin. GC-MS [7] showed the presence of 2,3,5-tri-*O*-methyl arabinose, 2,4-di-*O*-methyl arabinose, 2,3-di-*O*-methyl arabinose, 2,3-di-*O*-methyl xylose, 2,4,6-tri-*O*-methyl galactose and 2,4-di-*O*-methyl galactose in a molar ratio of 4:2:10:1:4:4. The carboxyl-reduced derivative gave 2,3,5-tri-*O*-methyl arabinose, 2,4-di-*O*-methyl arabinose, 2,3-di-*O*-methyl arabinose, 2,3-di-*O*-methyl xylose, 2,4,6-tri-*O*-methyl galactose, 2,3,6-tri-*O*-methyl galactose and 2,4-di-*O*-methyl galactose in a molar ratio of 4:2:10:1:4:3:4.

The polysaccharide was subjected to periodate oxidation followed by reduction with sodium borohydride. The component sugar analysis of the product showed that all the galactose units and one-eighth of the arabinose units remained after periodate oxidation No xylose or galacturonic acid was found in the product.

The ^{13}C NMR spectrum of the polysaccharide showed six anomeric carbon signals at δ112.024, 110.162, 107.087, 105.818, 104.442 and 100.771. Among these, it was evident that those at δ112.024 and 110.162 were attributable to anomeric carbons of α-L-arabinofuranose [8], those at δ107.087 and 105.818 to anomeric carbons of α-L-arabinopyranose [3, 8] and β-D-galactopyranose [9], and those at δ104.442 and 100.771 to anomeric carbons of β-D-xylopyranose [9] and α-D-galactopyranosyluronic acid [10]. These results suggested that the minimal repeating unit of the polysaccharide is composed of seven sugar units as shown in Fig. 1.

The effect of the polysaccharide MVS-IIIA on a reticuloendothelial system (RES) was demonstrated by the *in*

*Part 5 in the series 'Constituents of the seed of *Malva verticillata*'. For Part 4, see ref [3].

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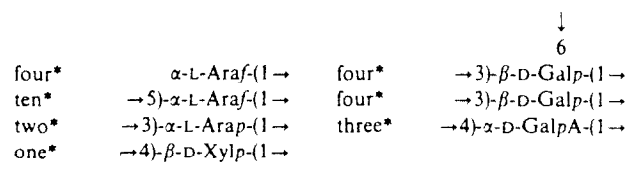

Fig 1 Component sugar residues in the minimal repeating units in the structure of MVS-IIIA. *Number of residues

vivo carbon clearance test [11] using zymosan as a positive control. When administered i.p (50 mg/kg), the phagocytic indices of MVS-IIIA, zymosan and control (blank) were 0.4492 ± 0.1048, 0.1713 ± 0.0361 and 0.0778 ± 0.0108. Thus the value was remarkably increased, suggesting powerful activation of RES by i.p injection of MVS-IIIA

As examples of plant polysaccharides having a phagocytosis-enhancing effect, several acidic and branched heteroglycans have been obtained from plants belonging to *Echinacea*, *Eupatorium*, *Chamomilla*, *Calendula*, *Baptisia*, *Achyrocline*, *Arnica*, *Sabal* and *Eleutherococcus* genera [12]. Two 4-methylglucuronoxylans have been obtained from the herbal part of *Eupatorium cannabium* and *E perfoliatum* [13] An active fucogalactoxyloglucan has been isolated from *Echinacea purpurea* cell cultures [14]. Further, an acidic arabinogalactan has been isolated from *Viscum album* berries [15].

As the RES activating polysaccharides from Oriental crude drugs, sanchinan A from the root of *Panax notoginseng* [16], saposhnikovan A from the root and rhizome of *Saposhnikovia divaricata* [17] and MVS-IVA from the seed of *Malva verticillata* [3] have been reported so far. These polysaccharides contain arabino-3,6-branched galactan units in common as their major parts, though they have different types of backbone chain The structural characterization of MVS-IIIA is similar to that of MVS-IVA in regard to both their high arabinose content and the presence of α-1,3-linked L-arabinopyranose residues Further investigations of the relationship between biological activities and structural features are in progress

EXPERIMENTAL

Plant material The seed of *M verticillata* was imported from China, and its plant of origin was identified by cultivation in the botanical garden of this College

Isolation of the polysaccharide The seeds (200 g) were homogenized and extracted with hot H_2O (2000 ml) with stirring for 1 hr After suction filtration, the filtrate was poured into two vols of EtOH The ppt was dissolved in H_2O (200 ml) and applied to a 5 (i d) × 78 cm DEAE-Sephadex A-25 column (Pharmacia Co) DEAE-Sephadex was pretreated as described in a previous report [18] After elution with water (1760 ml), the column was eluted with 0 2 M $(NH_4)_2CO_3$ Fractions of 20 ml were collected and analysed by the PhOH–H_2SO_4 method [19] The eluates obtained from tubes 135 to 161 were combined, dialysed, concd and applied to a 5 (i d) × 74 cm Sephacryl S-500 column (Pharmacia Co) 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 the eluates obtained from tubes 26 to 34 were combined, dialysed and concd The soln was applied to a 1 5 (i d) × 40 cm Con A-Sepharose column (Pharmacia Co) The column was pre-equilibrated with 0 067 M Pi buffer (pH 7 0) containing 0 15 M NaCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and kept at 4° and eluted with the same buffer Fractions of 10 ml were collected, and the eluates obtained from tubes 4 to 10 were combined, dialysed and concd The soln was applied to a 5 (i d) × 78 cm Sephadex G-25 column The column was eluted with H_2O, and fractions of 20 ml were collected The eluates obtained from tubes 27 to 40 were combined, concd and lyophilysed MVS-IIIA (13 5 mg) was obtained as a white powder

Determination of M_r The sample (3 mg) was dissolved in 0 1 M Tris–HCl buffer (pH 7 0) and applied to a 2 6 (i d) × 95 cm Toyopearl HW-75F column (Tosoh Co), pre-equilibrated and developed with the same buffer Fractions of 5 ml were collected and analysed by the PhOH–H_2SO_4 method Standard dextrans having known M_rs were run on the column to obtain a calibration curve Fraction numbers of the peaks of dextrans 2 0 × 10^6, 2 7 × 10^5, 1 5 × 10^5, MVS-IIIA and native dextran (void, Tokyo Kasei Co) were 53, 63, 65, 46 and 41

PAGE This was carried out in an apparatus equipped with gel tubes (0 4 i d × 13 7 cm) and 0 005 M Tris–glycine buffer (pH 8 3) at 5 mA/tube for 40 min Gels were stained by the PAS procedure and with Coomassie Blue reagent MVS-IIIA gave a clear band at a distance of 5 7 cm from the origin

Analyses of the components Hydrolysis and cellulose TLC of component sugars were performed as described in a previous report [18] The configurations of component sugars were proved by GC of trimethylsilylated α-methylbenzylaminoalditol derivatives [20] The hydrolysate was reduced, acetylated and analysed for component sugars by GC [17] a fused silica capillary column (0 053 i d × 1500 cm) of SP2380 (Supelco Co), programmed temperature increase of 3° per min from 160 to 200°, He 10 ml/min Galacturonic acid was estimated by a modification of the carbazole method [21] Peptide determination was performed by the method of ref [22]

Reduction of carboxyl groups This was carried out with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulphonate and $NaBH_4$ as described in a previous report [23] The reaction was repeated × 3 under the same conditions Yield was 20 mg from 50 mg of the sample

Methylation analysis Methylation was performed with methylsulphinyl carbanion and MeI in dimethyl sulphoxide as described in a previous report [24] The products were hydrolysed with dilute H_2SO_4 in HOAc, then reduced and acetylated as described in [25] The partially methylated alditol acetates obtained were analysed by GC/MS using a fused capillary column (0 032 i d × 3000 cm) of SP2330 (Supelco Co) and with programmed temperature increase of 4° per min from 160 to 220°, He 1 ml/min The RR_ts of the products with respect to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol and their main fragments in the MS are shown in Table 1

Periodate oxidation The polysaccharide (5 0 mg) was oxidized with 0 05 M Na metaperiodate (4 ml) at 5° in the dark The oxidation was completed after 4 days The reaction mixture was

Table 1 RR_1s on GC and main fragments in the mass spectra of partially methylated alditol acetates

Acetate	RR_1*	Main fragments (*m/z*)
1,4-Ac-2,3,5-Me-L-Arabinitol	0 69	43, 45, 71, 87, 101, 117, 129, 161
1,3,5-Ac-2,4-Me-L-Arabinitol	1 04	43, 87, 117
1,4,5-Ac-2,3,-Me-L-Arabinitol	1.12	43, 87, 101, 117, 129, 189
1,4,5-Ac-2,3-Me-D-Xylitol	1 21	43, 87, 101, 117, 129, 189
1,3,5-Ac-2,4,6-Me-D-Galactitol	1.29	43, 45, 87, 101, 117, 129, 161
1,4,5-Ac-2,3,6-Me-D-Galactitol	1 43	43, 45, 87, 99, 101, 113, 117, 233
1,3,5,6-Ac-2,4-Me-D-Galactitol	2 01	43, 87, 117, 129, 189

*Relative to 1,5-Ac-2,3,4,6-Me-D-glucitol

successively treated with ethylene glycol (0 02 ml) at 5° for 1 hr and $NaBH_4$ (15 mg) at 5° for 16 hr, and was adjusted to pH 5 0 by addition of HOAc The soln was concd and applied to a 2 6 (i d.) × 96 cm Sephadex G-25 column The column was eluted with water, and fractions of 10 ml were collected The eluates obtained from tubes 21 to 26 were combined, concd and lyophilysed Yield, 3.3 mg Determination of the components was carried out as described above.

Phagocytic activity Male mice (ICR-SPF, 25-30 g) were used in groups of five The sample was dissolved in physiological saline and administered i p (50 μg/g body weight) once a day for 5 days 48 hr after this treatment, the mice were injected via the tail vein with colloidal carbon (Pelican AG, F R G) The ink was diluted (× 8) with Pi buffered saline containing 1% gelatine before use, and the amount of resulting soln used was 10 μl/g body weight Blood samples were drawn from the orbital vein at 0, 3, 6, 9, 12 and 15 min The blood (25 μl) was dissolved in 0 1% Na_2CO_3 (2 ml) and the absorbance at 660 nm was determined The phagocytic index, K, was calculated by means of the following equation $K=(\ln A_1-\ln A_2)/(t_2-t_1)$, where A_1 and A_2 are the absorbancies at times t_1 and t_2, respectively Results were expressed as the arithmetic mean ± s d of five mice

Acknowledgement—We are grateful to Prof K Nagasawa (Kitasato University) for the gift of standard dextrans

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