A Neutral Heteropolysaccharide from the Flowers of *Malva mauritiana* L.

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A neutral heteropolysaccharide consisting of D-galactose (23.4 %), L-arabinose (34.3 %), and L-rhamnose (42.2 %) has been isolated from the mucilage of the flowers of *Malva mauritiana* L. Its relative molecular mass determined by the light-scattering method was unusually high, $M_r = 3.71 \times 10^6$. This finding was supported by gel-permeation chromatography whereby the polysaccharide was totally excluded from several gels with exclusion limits 0.05—1.5 × 10^6. The results of chemical and spectroscopic investigations indicated a branched structure of the polysaccharide with 3,6-linked D-galactopyranose, 5-linked L-arabinofuranose as well as 4-linked and terminal L-rhamnopyranose residues as the main building units.

The medicinal plants containing mucilage are still the subject of investigation because of the therapeutical effects of the drug. The flowers and leaves of *Malva mauritiana* L. have been used in treatment of catarrhs of the respiratory system and various inflammations of the nasal and oral cavities. Recently, we have found that the mucilage isolated from the flowers of this herb and especially its acidic heteropolysaccharide component exhibited cough-suppressing activity [1]. We isolated from this mucilage three polysaccharide species, a linear 1,6-α-D-glucan [2], a highly branched 1,5-α-L-arabino-3,6-β-D-galactan [3], and a branched rhamnogalacturonan [4]. To characterize the polysaccharide component of the mucilage in more detail, we continued in its fractionation and now we report on another neutral polysaccharide, an arabinogalactorhamnoglycan, which is remarkable for high proportion of the rhamnose component as well as for its unusually high relative molecular mass.

EXPERIMENTAL

The flowers of *Malva mauritiana* L. were purchased from Slovakofarma, Medicinal plants, Malacky (Slovak Republic).

Solutions were concentrated under reduced pressure below 40 °C. Free-boundary electrophoresis of 1 % solution of polysaccharide was effected with a Zeiss 35 apparatus, using 0.05 M sodium tetraborate buffer (pH 9.2) at 150 V cm^{-1} and 6 mA for 30 min.

Values of relative molecular mass (M_r), of second virial coefficient (A_2), and of radius of gyration (R_g) were obtained with BI-200SM goniometer equipped with BI-2030 correlator (Brookhaven Instruments Co., Holtsville, USA). The light employed was a vertically polarized 514 nm green line from Lexel argon ion laser. Static light scattering data were analyzed according to the Berry plot. The refractive index increment of the sample in distilled water was determined by the Brice-Phoenix, BP-2000V differential refractometer and the value 0.137 $cm^3\ g^{-1}$ was obtained. Diffusion coefficients were obtained with the same goniometer and the experimental autocorrelation curves were analyzed using CONTIN and cumulant methods. From these values the hydrodynamic radii R_h were calculated using the Stokes—Einstein equation.

Viscosity measurements were made in distilled water at (25 ± 0.05) °C using an Ubbelohde dilution viscometer. Intrinsic viscosity [η] and Huggins constant k_H were obtained from the intercept and slope of solution viscosity dependence on concentration according to the Huggins equation.

High-performance gel permeation chromatography (HPGPC) was performed using a commercial instrument (Laboratorní přístroje, Prague, Czech Republic) equipped with two Tessek Separon HEMA-BIO 1000 columns (8 × 250 mm) with a 0.1 cm^3 sample loop at room temperature. The mobile phase used was 0.1 M-$NaNO_3$ at a flow rate 0.4 $cm^3\ min^{-1}$ and the carbohydrate content was monitored by a differential refractometer (RIDK-101).

The infrared spectrum of the methylated polysaccharide was recorded with a Nicolet Magna 750 spectrometer. Carbohydrates were determined by the

Table 1. Sugar Composition of Sephadex G-75 Fractions of the Neutral Polysaccharide Mixture (NP)

Fraction	Monosaccharide composition (x_i/mole %)						
	D-Gal	D-Glc	D-Man	L-Ara	D-Xyl	L-Fuc	L-Rha
NP[a]	23.7	33.2	9.5	19.3	5.8	2.1	6.4
NP-1[b]	19.2	28.2	5.8	29.4	*c*	*c*	17.3
NP-2	*c*	99.8	*c*	*c*	–	–	–
NP-3	36.1	12.3	6.1	45.3	*c*	*c*	*c*
NP-4	20.8	31.5	14.3	12.4	18.3	2.6	*c*

a) Neutral polysaccharide mixture. *b*) Sephadex G-75 fractions of NP. *c*) Traces.

phenol—sulfuric acid assay [5]. Polysaccharides were hydrolyzed with 2 M-TFA for 1 h at 120 °C. Paper chromatography was performed by the descending method on Whatman No. 1 paper in the system S, ethyl acetate—pyridine—water (φ_r = 8 : 2 : 1). The saccharides were detected with anilinium hydrogen phthalate. Quantitative determination of the neutral sugars was carried out in the form of their trifluoroacetates [6] by gas chromatography on a Hewlett—Packard Model 5890 Series II chromatograph equipped with a PAS-1701 column (0.32 mm × 25 m) at the temperature program of 110—125 (2 °C min^{-1})—165 °C (20 °C min^{-1}) and flow rate of hydrogen 20 cm^3 min^{-1}. Gas chromatography—mass spectrometry of partially methylated alditol acetates [7] was effected on a FINNIGAN MAT SSQ 710 spectrometer equipped with an SP 2330 column (0.25 mm × 30 m) at 80—240 °C (6 °C min^{-1}), 70 eV, 200 μA, and ion-source temperature 150 °C.

The one-dimensional ^1H and ^{13}C, and two-dimensional ^{13}C-^1H heteronuclear correlated NMR spectra were measured in deuterated water (at 25 °C or 60 °C) on an FT NMR Bruker AVANCE DPX 300 spectrometer equipped with gradient-enhanced spectroscopy kit (GRASP) for generation of *z*-gradients up to 5 × 10^{-3} T cm^{-1} in 5 mm inverse probe kit. Chemical shifts were referred to external acetone (δ = 2.225 and 31.07 for ^1H and ^{13}C, respectively).

Isolation of Arabinogalactorhamnoglycan

The dry flowers of *M. mauritiana* (500 g) were macerated in distilled water (1.2 dm^3) for 24 h at room temperature. The aqueous extract was centrifuged, concentrated to 1 dm^3 and poured into 96 % ethanol (4 dm^3) containing 1 vol. % of acetic acid. The precipitate was washed with 70 % aqueous ethanol, suspended in water, dialyzed, and freeze-dried. The dark brown material (17.5 g), containing 22.8 % of protein, yielded on hydrolysis D-galactose (30.8 %), D-glucose (6.1 %), D-mannose (6.1 %), L-arabinose (15.0 %), D-xylose (7.5 %), L-rhamnose (11.1 %), uronic acids (23.4 %), and trace amount of L-fucose. The crude product (4.3 g) was dissolved in water (100 cm^3) and loaded on a column (4 × 40 cm) of DEAE-Sephadex A-50 in carbonate form and eluted with water. The retained material was washed with ammonium carbonate solutions. Fractions of 10 cm^3 were collected and analyzed for sugar content with the phenol—sulfuric acid assay. The water eluate (1 g in 10 cm^3 of water) composed of D-galactose (23.7 %), D-glucose (33.2 %), D-mannose (9.5 %), L-arabinose (19.3 %), D-xylose (5.8 %), L-fucose (2.1 %), and L-rhamnose (6.4 %) was further separated on a column (4 × 100 cm) of Sephadex G-75 with water irrigation. Fractions of 4 cm^3 were collected and analyzed for sugar content. Four distinct fractions differing in proportions of sugar components were obtained (Table 1). The first fraction (NP-1, ≈ 250 mg) eluted in the void volume was rich in D-galactose, D-glucose, L-arabinose, and L-rhamnose. Rechromatography of NP-1 (200 mg in 2 cm^3) on a column (2.5 × 120 cm) of Sephadex G-100 gave a polysaccharide (in the void volume) that appeared homogeneous upon free-boundary electrophoresis. The elution profile of this polymer was identical on Sephadex G-150 and G-200, Sepharose 6B, and Bio-Gel A-1.5m, *i.e.* it was eluted in the void volume and its monosaccharide composition was constant. The polymer was composed of D-galactose (23.4 %), L-arabinose (34.3 %), L-rhamnose (42.2 %), and trace amounts of D-mannose, D-xylose, and L-fucose.

Methylation Analysis

The dry polysaccharide (≈ 5 mg) was solubilized in dry dimethyl sulfoxide (1 cm^3) and methylated by the Hakomori method [8]. The solution was then poured into distilled water (2 cm^3), dialyzed for 48 h, and evaporated. The residue was dried and methylated according to the Purdie method [9] to give a fully methylated product. The permethylated polysaccharide was hydrolyzed first with 90 % HCOOH (1 h, 100 °C) and then with 2 M-TFA (1 h, 120 °C). The partially methylated saccharides were reduced with sodium borodeuteride, acetylated and analyzed [7] by GLC-MS (Table 2).

RESULTS AND DISCUSSION

The mucilage isolated from the flowers of *Malva mauritiana* L. was resolved by ion-exchange chromatography to neutral and acid portions. The mix-

Table 2. Methylation Analysis Data of the Neutral Heteropolysaccharide

Sugar derivative	x_i/mole %		Mode of linkage
2,3,4-Me_3-Rha-ol[a]	16.3	(38.7)[b]	Rha*p*-(1→
3,4-Me_2-Rha-ol	0.8	(1.9)	→2)-Rha*p*-(1→
2,3-Me_2-Rha-ol	19.5	(46.3)	→4)-Rha*p*-(1→
2,4-Me_2-Rha-ol	4.8	(11.4)	→3)-Rha*p*-(1→
2-Me-Rha-ol	0.2	(0.5)	→3,4)-Rha*p*-(1→
4-Me-Rha-ol	0.5	(1.2)	→2,3)-Rha*p*-(1→
Total:	42.1		
2,3,4-Me_3-Fuc-ol	0.2	(100)	Fuc*p*-(1→
2,3,5-Me_3-Ara-ol	3.2	(9.8)	Ara*f*-(1→
2,3,4-Me_3-Ara-ol	0.7	(2.1)	Ara*p*-(1→
3,5-Me_2-Ara-ol	0.8	(2.4)	→2)-Ara*f*-(1→
2,5-Me_2-Ara-ol	1.3	(4.0)	→3)-Ara*f*-(1→
2,3-Me_2-Ara-ol	24.3	(74.1)	→5)-Ara*f*-(1→ or→4)-Ara*p*-(1→
Ara-ol	2.5	(7.6)	→2,3,5)-Ara*f*-(1→
Total:	32.8		
2,3,4-Me_3-Xyl-ol	0.1	(3.3)	Xyl*p*-(1→
2,4-Me_2-Xyl-ol	1.9	(63.3)	→3)-Xyl*p*-(1→
2,3- + 3,4-Me_2-Xyl-ol	0.6	(20.0)	→4)-Xyl*p*-(1→ or →2)-Xyl*p*-(1→
2-Me-Xyl-ol	0.4	(13.3)	→3,4)-Xyl*p*-(1→
Total:	3.0		
2,3,5,6-Me_4-Gal-ol	0.3	(1.4)	Gal*f*-(1→
2,3,4,6-Me_4-Gal-ol	1.5	(7.0)	Gal*p*-(1→
2,4,6-Me_3-Gal-ol	3.0	(14.0)	→3)-Gal*p*-(1→
2,3,6-Me_3-Gal-ol	3.6	(16.7)	→4)-Gal*p*-(1→
2,3,4-Me_3-Gal-ol	2.7	(12.5)	→6)-Gal*p*-(1→
2,6-Me_2-Gal-ol	0.6	(2.8)	→3,4)-Gal*p*-(1→
2,4-Me_2-Gal-ol	9.8	(45.6)	→3,6)-Gal*p*-(1→
Total:	21.5		
2,4-Me_2-Man-ol	0.4	(100)	→3,6)-Man*p*-(1→

a) 2,3,4-Me_3-Rha-ol = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol. *b*) Numbers in brackets indicate fraction/% of each linkage with the respective sugar residue.

ture of neutral polysaccharides (NP) containing D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose, L-fucose, and L-rhamnose was further separated by gel-filtration to four fractions (NP *1*—*4*) differing in molecular mass as well as in composition of the constitutive saccharides (Table 1). Of these, we have previously dealt with the fractions NP-*2* and NP-*3* and described the structures of their dominant polysaccharide components, a linear 1,6-α-D-glucan [2] and a highly branched 1,5-α-L-arabino-3,6-β-D-galactan [3]. The first fraction (NP-*1*), eluted in the void volume from the Sephadex G-75 column, was rich in D-galactose, D-glucose, L-arabinose, and L-rhamnose. This fraction, showing molecular heterogeneity upon free-boundary electrophoresis, was further purified on a gel with higher exclusion limit to give a homogeneous polysaccharide, composed of L-rhamnose (42.2 %), L-arabinose (34.3 %), D-galactose (23.4 %), and trace amounts of D-mannose, D-xylose, and L-fucose. Its optical rotation was −12° and relative molecular mass (M_r) 3.71 × 10^6 (in water) and 3.37 × 10^6 (in DMSO).

As the relative molecular mass seemed to be unusually high for a polysaccharide of such an origin, we performed chromatographic separations on several gels with exclusion limits above 0.05—1.5 × 10^6. All experiments confirmed the large molecules of the polysaccharide that were excluded from the gels in the void volume of the column. From combination of static and dynamic light scattering, the ratio of $R_g/R_h = \rho$ can be derived. This parameter is sensitive to the structure of macromolecules in solution and thus is a useful parameter for their characterization [10]. The values of ρ lower than 1.8—2.0 predict polydisperse flexible branched chains or densely packed clusters. On the other hand, higher values indicate linear chains of increasing stiffness. Table 3 shows lower values of this parameter suggesting the presence of branched aggregated structures. The same conclusion can be derived from high value of the Huggins constant (k_H) obtained by viscosimetry. The k_H values for linear high-molecular-mass polymers in the thermodynamically good solvents [11] are in the range 0.2—0.4. Higher values (in our case 0.72) are characteristic of branched or aggregated structures of polymers in solution. However, the molecular mass measured in dimethyl sulfoxide, which is supposed to be a hydrogen-bonding destructor, was also very high, M_r = 3.37 × 10^6 and,

Table 3. Molecular Characteristics of the Polysaccharide in Two Solvents

Solvent	$M_r \times 10^{-6}$	R_g	$A_2 \times 10^6$	$[\eta]$	k_H	R_h	$\rho = R_g/R_h$
		nm	$cm^3\ mol\ g^{-2}$	$cm^3\ g^{-1}$		nm	
Water	3.71	73.0	8.96	72.3	0.72	57.5	1.27
DMSO	3.37	81.7	−8.55	–	–	62.0	1.30

thus, has not confirmed the presence of aggregated structures of macromolecules in solution.

The results of methylation analysis are given in Table 2. The great number of sugar derivatives indicated a wide range of glycosidic linkages and demonstrated the occurrence of rhamnose in the pyranose form and arabinose and galactose in both pyranose and furanose forms.

The dominant derivatives of the rhamnose residue were 2,3,4-tri-*O*-methyl- (38.7 %) and 2,3-di-*O*-methylrhamnose (46.3 %), representing 1- and 1,4-linked units. Though rhamnose was found to occupy terminal position in many plant arabinogalactans [12—15], the high portion in this polysaccharide is remarkable. Uncommon is also the high proportion of 1,4-linked rhamnose units in neutral polysaccharides isolated from plant sources. In addition to the abovementioned linkages, detected were also small proportions of 3-, 2-, 3,4-, and 2,3-linked residues.

Arabinose was found to be involved in 6 types of linkages. The main derivative 2,3-di-*O*-methylarabinose (74.1 %) indicated the presence of the 5-linked arabinofuranose units. This type of the arabinose linkages is common in plant arabinogalactans [12—17]. The other two methylated products, *i.e.* 3,5-di-*O*-methyl- and 2,5-di-*O*-methylarabinose found in small proportions proved the 2- and 3-linked position of arabinofuranose units. Some terminal arabinose residues are present both in pyranose (2.1 %) and in furanose (9.8 %) forms, as indicated by the 2,3,5-tri-*O*-methyl- and the 2,3,4-tri-*O*-methylarabinose derivatives. Some units (7.6 %) were found to be fully substituted.

Galactose was found to occur predominantly in pyranose form (98.6 %). The methylated derivatives pointed to seven types of glycosidic linkages of this saccharide component. The most abundant derivative, 2,4-di-*O*-methylgalactose (45.6 %), indicated branched units in 3,6-linked position. Less amounts of 2,4,6-tri-*O*-methyl- (14.0 %) and 2,3,4-tri-*O*-methylgalactose (12.5 %) demonstrated the presence of 3- and 6-linked units. Such glycosidic linkages together with the aforementioned type are common for 3,6-branched arabinogalactans [16] (Type II) widely distributed in plant sources. The other indicated linkages 1→4 (16.7 %); 1→3,4 (2.8 %) are characteristic of 3,4-branched plant arabinogalactans [16] (Type I). This sugar component was found also at nonreducing end position both in pyranose (7.0 %) and furanose (1.4 %) forms.

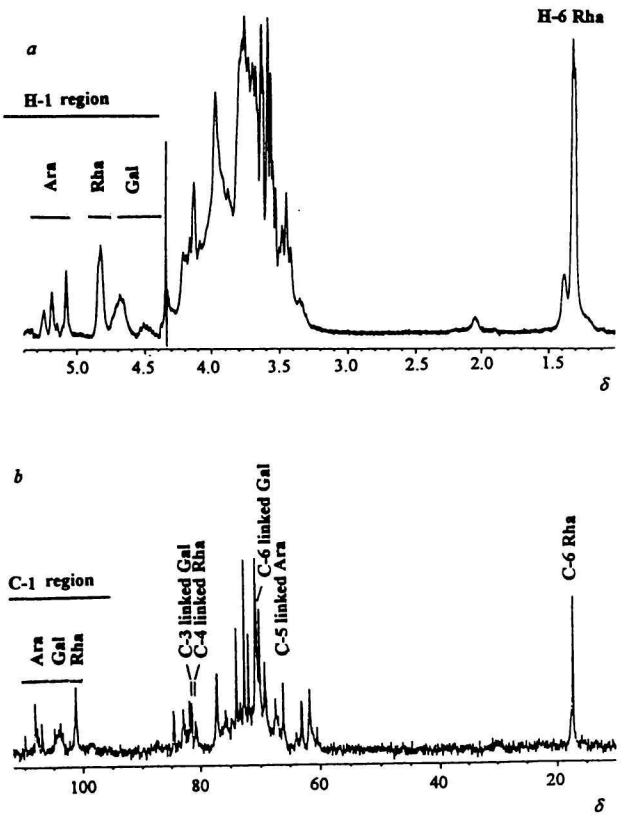

Fig. 1. ^1H (*a*) and ^{13}C (*b*) NMR spectra of arabinogalactorhamnoglycan.

The methylated derivatives of the trace saccharides pointed to terminal position of fucose, 3,6-linked mannose units as well as terminal, 2-, 3-, 4-, and 3,4-linked xylose residues.

The ^1H NMR spectrum of the polysaccharide (Fig. 1*a*) was recorded at 60 °C, assuring the resolution of anomeric proton signals of all sugar components. These appeared at δ = 5.25, 5.18, 5.08, 4.83, 4.69, and 4.49. The signal characteristic of the protons of the methyl group (H-6) of 6-deoxyhexose was observed at a higher magnetic field (δ = 1.19).

The ^{13}C NMR spectrum of the arabinogalactorhamnoglycan is complex (Fig. 1*b*). The signals in the anomeric region δ = 101.14—109.86 assigned to the anomeric carbons of rhamnose, galactose, and arabinose moieties indicated a branched structure of the polysaccharide. The five signals at the lowest magnetic field at δ = 107.03—109.86 reflected the reso-

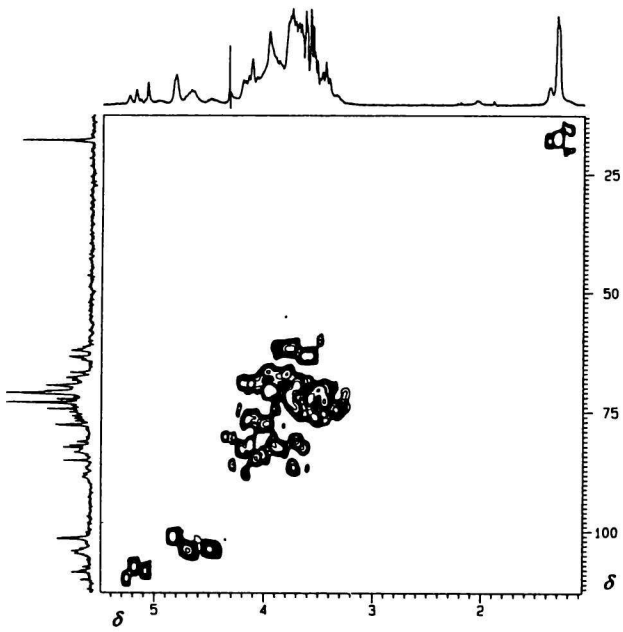

Fig. 2. 2D ^{13}C-^{1}H NMR spectrum (HSQC) of arabinogalactorhamnoglycan.

nances of C-1 of nonreducing terminal, internal, and branched α-arabinose residues [3, 14, 17—19]. The other not very well resolved C-1 signals in the region δ = 103.50—104.82 could be assigned to β-galactose units (terminal, internal, and branched) variously linked (as seen also from the results of methylation analysis) in the polymeric chain [3, 14, 18—20]. The last anomeric signal observed at δ = 101.14 represented the resonances of C-1 of terminal, internal as well as branched rhamnose residues [20, 21]. The resonances of the carbons involved in glycosidic linkages were observed at δ = 81.54 (C-3 of 3- and 3,6-linked Gal), 80.90 (C-4 of 4-linked Rha), 70.50 (C-6 of 6- and 3,6-linked Gal), and 66.1 (C-5 of 5- and 2,3,5-linked Ara). The other signals at δ = 63.1, 61.7, and 17.40 arose from resonances of C-5, C-6, and C-6 of the arabinose, galactose, and rhamnose residues, respectively.

The two-dimensional ^{13}C-^{1}H NMR (HSQC) spectrum of the polysaccharide is presented in Fig. 2. The ^{13}C NMR spectrum is displayed along the vertical axis and the ^{1}H NMR spectrum along the horizontal axis. The HSQC spectrum shows that each cross peak has coordinates corresponding to the respective chemical shift values of a ^{13}C atom and its directly bonded proton. The anomeric protons resonating at δ = 5.25, 5.18, and 5.08 correlated with carbon signals at δ = 109.25, 107.40, and 108.13, respectively. These chemical shifts are characteristic of anomeric carbons of arabinofuranose residues. The proton signal at δ = 4.83 corresponds to the signal at δ = 101.1, arising from the resonances of C-1 of rhamnose units. The two broad signals at δ = 4.69 and 4.49 correlated with the anomeric carbon shifts at δ = 103.95 and 103.69, assigned to C-1 atoms of galactose residues. The cross peak in the high magnetic field at δ = 1.19 (protons of CH_3 group) and at δ = 17.43 (carbon of CH_3 group) confirmed the presence of rhamnose units in the polysaccharide. Due to complexity of the heterocorrelated spectrum of the polymer, unambiguous assignment of all resonances was not possible.

The results of chemical and spectroscopic analyses pointed to a branched structure of the arabinogalactorhamnoglycan. The dominant rhamnopyranose component (42.1 %) of the polysaccharide was predominantly linked by α-(1→4)-glycosidic bonds (19.5 %) and high portion (16.3 %) of this sugar occupied nonreducing terminal position. Arabinose occurred almost exclusively in the furanose form, mainly 5-linked, and was found in α-configuration. On the contrary, galactose was in the pyranose form, β-configuration and mainly 3,6-linked. It is to be stressed that the occurrence of a neutral heteropolysaccharide with such a large proportion of the rhamnose component is unusual. It is characteristic rather of acidic polysaccharides. Anyway, we have not found any reference to a neutral heteropolysaccharide, isolated by water extraction from flowers of any medicinal plant, with such a high relative molecular mass and containing rhamnose as the dominant sugar component.

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