POLYSACCHARIDES FROM THE FLOWERS OF *Malva silvestris* L., ssp. *mauritiana* (L.) THELL.: THE STRUCTURE OF D-GLUCAN

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The neutral polysaccharide α-D-glucan was isolated from the flowers of *Malva silvestris* L., ssp. *mauritiana* (L.) THELL. using a combination of ion exchange and gel chromatography. It was homogeneous under the conditions of free electrophoresis of average molecular weight $\overline{M}_n$ 25 260. The chemical and spectroscopic investigations indicated a linear structure of the polysaccharide in which the α-D-glucopyranose units were linked predominantly by 1 → 6 glycosidic bonds, while some saccharides were the place of branching in position C-3.

Malva silvestris L., ssp. *mauritiana* (L.) THELL. (*Malvaceae*) has been known since ancient times as a medicinal plant, owing to its emollient and antiinflammatory effect. The main indication area in which the curative effect of this drug (flower, leaf) is used are inflammatory processes in diseases of the respiratory tract, the oral cavity, and catarrhs of the digestive tract[1]. It is assumed that its active component is mucilage. Its chemical composition is, however, little known so far. Till now the mucilage of this plant has been characterized merely from the point of view of the composition of monosaccharides or amino acids[2,3]. The subject of this paper is the isolation of a mixture of polysaccharides from the flowers of *M. silvestris*, ssp. *mauritiana*, its fractionation to an acid and a neutral component and a detailed structural characterization of one of the polymers of the neutral mixture of α-D-glucan.

EXPERIMENTAL

Material and Methods

The flowers of *Malva silvestris* L., ssp. *mauritiana* (L.) THELL. are from Slovakofarma (the factory Medicinal plants, Malacky), collected in 1989. Polysaccharides were hydrolyzed with 2M trifluoroacetic acid at 120 °C for 1 h. After evaporation of the acid the monosaccharides were reduced with $NaBH_4$, acetylated (pyridine–acetic anhydride 1 : 1) and their molar ratios were determined by gas chromatography on column A. Optical rotation of 1% aqueous polysaccharide solution was measured on a Perkin–Elmer Model 141 polarimeter at 20 °C. Free electrophoresis of 1% solutions of polysaccharides in 0.05M borate buffer of pH 9.25 was carried out on a Zeiss 35 instrument at 10 V/cm and 6 mA for 30 min. The content of uronic acids was determined spectrophotometrically with a 3-hydroxydiphenyl reagent[4], and potentiometrically.

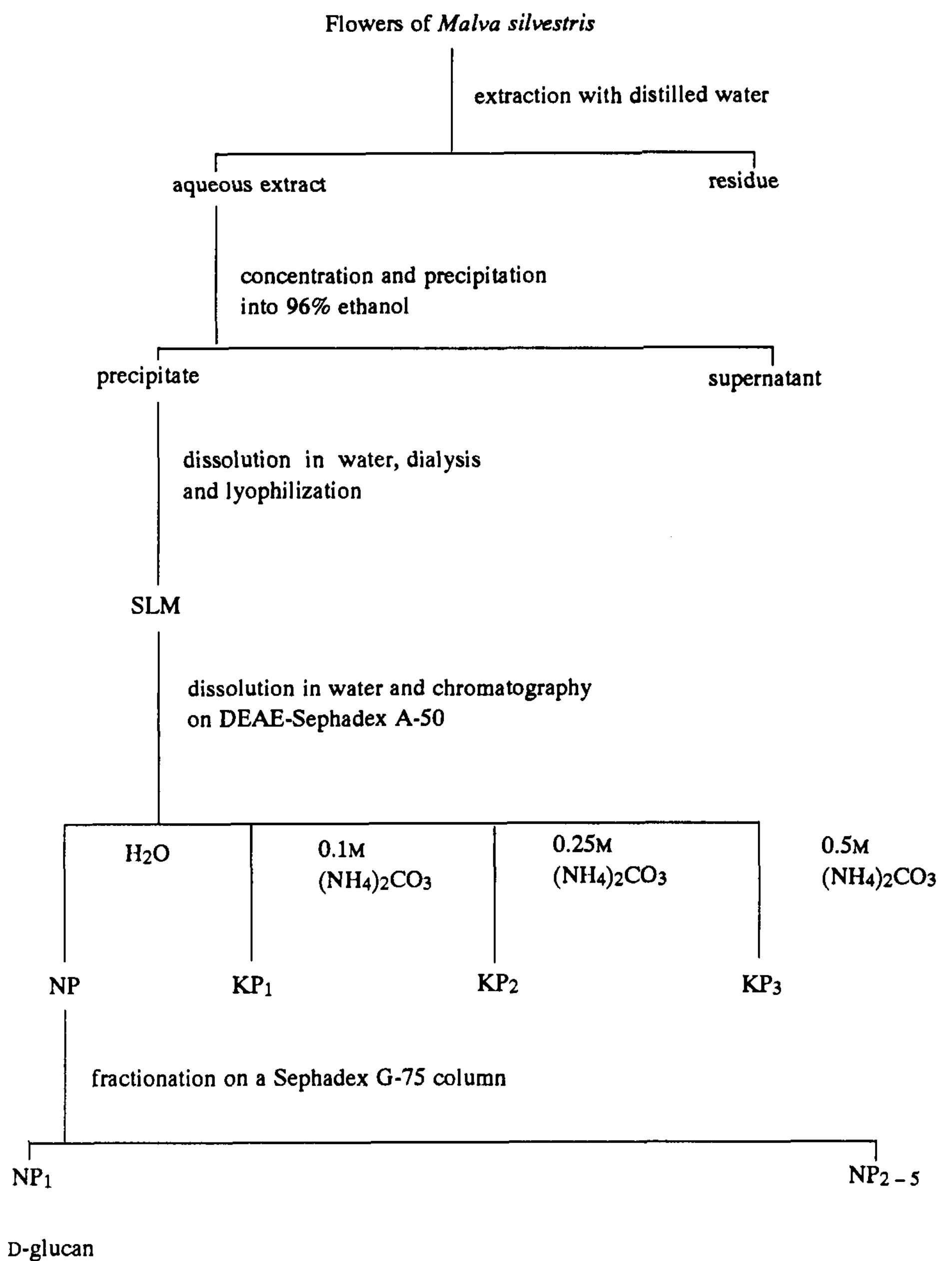
The number average molecular mass ($\overline{M}_n$) was determined osmometrically on a Knauer Vapour-pressure osmometer. The limit viscosity number was measured on Ubbelohde's flow-through viscosimeter. The content of proteins was determined according to Lowry et al.[5]. The content of amino acids was determined on an automatic amino acid analyzer, type 6020 (Mikrotechna Praha) after acid hydrolysis of the sample with 6M-HCl at 105 °C for 20 h. Methoxy groups were determined according to Viebock and Brecher[6]. The nitrogen content was determined on a Perkin–Elmer 240 elemental analyzer. Paper chromatography was performed using the descending mode, on Whatman No. 1 paper in the following systems: ethyl acetate–pyridine–water (8 : 2 : 1), ethyl acetate–acetic acid–formic acid–water (5 : 5 : 1 : 3) and ethyl acetate–acetic acid–water (18 : 7 : 8). The saccharides were detected with anilinium hydrogen phthalate[7]. Gas chromatography (GLC) was carried out on Hewlett-Packard Model 5711 A on column A (200 × 0.3 cm) with stationary phase 3% OV-225 on chromosorb W (AW-DMCS, 80 – 100 mesh), and 120 °C (4 min) to 170 °C (2 °C/min) temperature interval, and column B (200 × 0.3 cm) with the stationary phase 3% SP-2340 on chromosorb W (AW-DMCS, 80 – 100 mesh) at 180 – 220 °C (4 °C/min). Gas chromatography–mass spectrometry (GLC–MS) of partially methylated alditol acetates of saccharides[8] was carried out using a Hewlett–Packard 5711 A instrument, and a column (200 × 0.3 cm) with 3% SP-2340 on Supelcoporte (100 – 120 mesh) as stationary phase. The injection pressure of helium was 101.3 kPa, the temperature 160 – 240 °C (6 °C/min) and the spectra were measured at 23 eV ionization energy. The infrared spectra of the samples were measured on a Perkin–Elmer, model 9836 instrument. FT ^{13}C NMR spectra of polysaccharides were measured on a Jeol FX-100 apparatus with complete proton decoupling in D_2O, with methanol as internal reference (50.15 ppm with respect to tetramethylsilane) at 30 °C.

Isolation of the Mixture of Polysaccharides

Dry flowers (5 kg) of *Malva silvestris* L., ssp. *mauritiana* (L.) THELL. were macerated in distilled water (125 l) at room temperature for 24 h. The aqueous extract was concentrated to 10 l and precipitated by pouring it into 96% ethanol (50 l) acidified with acetic acid (1%). The precipitated polysaccharide was washed with ethanol, dissolved in distilled water, dialyzed for 4 days and lyophilized. A dark brown powdered material (SLM) was obtained, weighing 175 g (3.5% per weight of dry flowers) and containing 3.8% of nitrogen and 2.0% of methoxy groups (Scheme 1). Amino acid composition of the protein part of the glycoprotein complexes after acid hydrolysis is given in Table I. After acid hydrolysis the sample contained D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose, L-fucose, L-rhamnose and uronic acids (Table II).

TABLE I
Amino acid composition of the protein moiety of SLM

Amino acid	Mole %	Amino acid	Mole %
Aspartic acid	11.70	Isoleucine	5.58
Threonine	5.46	Leucine	9.09
Serine	6.21	Tyrosine	2.78
Glutamic acid	11.75	Phenylalanine	3.30
Proline	6.77	Histidine	2.41
Glycine	9.95	Lysine	5.30
Alanine	9.96	Arginine	3.98
Valine	5.77		



SCHEME 1
Isolation of the neutral polysaccharide

Isolation of α-D-Glucan

A polysaccharide mixture (SLM; 2.5 g) was dissolved in distilled water (100 ml) and separated on a column (5 × 100 cm) of DEAE-Sephadex A-50 in carbonate form. The column was eluted gradually with water, 0.1M, 0.25M and 0.5M ammonium carbonate solution. Elution with water gave a mixture of neutral polysaccharides (NP) containing D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose, L-fucose and L-rhamnose (Table II). Elution with the ammonium carbonate solutions gave acid fractions of polysaccharides (KP_{1-3}) containing various molar ratios of constitutive saccharides (Table II). The mixture of neutral polysaccharides (1 g), composed of D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose, L-fucose and L-rhamnose was dissolved in distilled water (10 ml) and chromatographed on a Sephadex G-75 column (4 × 100 cm). Elution with water gave, as the first fraction, D-glucan in 12% yield (120 mg), homogeneous under the conditions of free electrophoresis, with $[\alpha]_D$ +128° and limiting viscosity number η 0.25. Its average molecular weight ($\overline{M}_n$) was 15 260, determined osmometrically. The fraction NP_2 contained in addition to D-galactose, D-glucose and L-arabinose also traces of D-mannose, D-xylose and L-rhamnose. The fractions of NP_{3-5} polysaccharides differed by various contents of constitutive saccharides, D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose, L-fucose and L-rhamnose.

Methylation Analysis of α-D-Glucan

The polysaccharide (20 mg) was vacuum dried at 50 °C for 5 h and then dissolved in anhydrous dimethyl sulfoxide (3 ml). A solution of methylsulfinyl carbanion[9] (2 ml) was added and the reaction mixture was stirred at room temperature for 6 h. After cooling of the solution in an ice bath, methyl iodide (2 ml) was added and the mixture was stirred overnight. The reaction mixture was dialyzed for 2 days, the methylated polysaccharide was extracted with chloroform and the solution was dried over anhydrous sodium sulfate and evaporated to dryness. The syrupy product was dissolved in methyl iodide (2 ml) and refluxed for 1 day in the presence[10] of silver(I) oxide (100 mg). The permethylated polysaccharide was hydrolyzed with 2M trifluoroacetic acid at 120 °C for 1 h and after evaporation of the acid the partially methylated saccharides were reduced and acetylated to corresponding alditol acetates and analysed by GLC–MS (ref.[8]). The results of the methylation analysis are given in Table III.

TABLE II
Fractionation of the mixture of polysaccharides (SLM) on DEAE-Sephadex A-50

Fraction	Eluent	Molar ratios of monosaccharides							
		D-Gal	D-Glc	D-Man	L-Ara	D-Xyl	L-Fuc	L-Rha	Uronic acids
SLM	–	1.00	0.22	2.22	0.62	0.30	0.03	0.42	0.68
NP	H_2O	1.00	1.41	0.40	1.03	0.34	0.10	0.29	–
KP_1	0.1M $(NH_4)_2CO_3$	1.00	0.57	0.30	1.61	0.25	0.03	0.34	0.39
KP_2	0.25M $(NH_4)_2CO_3$	1.00	0.15	0.04	0.31	0.11	0.01	0.53	0.58
KP_3	0.5M $(NH_4)_2CO_3$	1.00	0.28	0.10	0.33	0.34	0.01	1.10	1.60

RESULTS AND DISCUSSION

Dry leaves of *M. silvestris*, ssp. *mauritiana* were macerated in distilled water. Precipitation of the aqueous extract with ethanol gave a mixture of glycans or proteoglycans which gave, after dialysis and lyophilization, a dark brown powdery material (SLM) in a 3.5% yield (per weight of the dry flowers, Scheme 1). The content of the methoxy groups was 2.07% and of nitrogen 3.86%. Since the presence of amino acids could not be detected (negative reaction of saccharides with ninhydrin, after paper chromatography), the content of nitrogen indicates a high proportion of proteins in the sample. They can occur either in the form of free peptides or proteins, or bound in complexes with polysaccharides. Their amino acid composition is given in Table I.

In order to separate neutral polysaccharides from the acid ones the crude mixture of polysaccharides (SLM), composed of D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose, L-fucose, L-rhamnose and uronic acids (Table II) was chromatographed on a DEAE-Sephadex A-50 column. The column was gradually eluted with water and ammonium carbonate solution of increasing ionic strength. Elution with water gave a neutral fraction composed of D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose, L-fucose and L-rhamnose. Elution with ammonium carbonate solutions gave acid polysaccharide fractions (PK_{1-3}) containing various molar ratios of constitutive sugars. The content of uronic acids in individual fractions (Table II) increased with increasing ionic strength of the eluents. Since NP was not homogeneous under the conditions of free electrophoresis it was further purified on a Sephadex G-75 column. The first fraction (NP_1) contained D-glucan, homogeneous under the conditions of free electrophoresis, of average molecular weight $\overline{M}_n$ 25 260, optical rotation $[\alpha]_D$ +128° and limiting viscosity number η 0.25. Fraction NP_2 contained, in addition to D-galactose, D-glucose and L-arabinose, also trace amounts of D-mannose, D-xylose, and L-rhamnose. The poly-

TABLE III
GLC–MS Analysis of methylated saccharides of α-D-glucan

Methylated alditol acetate	Relative ratio	Bond	Relative content (%) of glycosidic bonds	
2,3,4,6-Me_4-D-Glc[a]	5.81	Glc 1 →		
2,3,4-Me_3-D-Glc[b]	85.84	→ 6 Glc 1 →	90.01	1 → 6
2,4-Me_2-D-Glc[c]	8.34	→ 6 Glc 1 → 3 ↑	4.17	1 → 3

[a] 1,5-Di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol; [b] 1,5,6-tri-O-acetyl-2,3,4-tri-O-methyl-D-glucitol; [c] 1,3,5,6-tetra-O-acetyl-2,4-di-O-methyl-D-glucitol.

saccharide fraction NP_{3-5} differed in varying molar proportion of D-galactose, D-glucose, D-mannose, L-arabinose, D-xylose, L-fucose and L-rhamnose.

For the sake of structural studies the polysaccharide was methylated using a combination of the methods by Hakomori[9] and Purdie[10]. After hydrolysis of the permethylated polymer the partially methylated derivatives of D-glucose were reduced and acetylated to corresponding alditol acetates and identified by GLC–MS combination[8]. The results of the methylation analysis are shown in Table III. The main methylation product was 2,3,4-tri-O-methyl-D-glucose, which indicates the linear character of the polysaccharide chains in which D-glucose units are linked by a 1 → 6 glycosidic bond. The presence of 2,4-di-O-methyl-D-glucose shows that some units are the sites of branching in position C-3. Approximately 4% of the sugar units are linked in polysaccharide chains by a 1 → 3 glycosidic bond.

The results of the methylation analysis of D-glucan were correlated with the results of the ^{13}C NMR measurements. Its data were in good agreement with the information obtained from the ^{13}C spectrum of D-glucan. Chemical shifts of individual atoms are listed in Table IV. The signals at the lowest magnetic field at 100.46 and 98.81 ppm

TABLE IV
^{13}C NMR parameters (δ, ppm; D_2O) of α-D-glucan, methanol as internal reference (50.15 ppm) at 30 °C

Chemical shift	Position of the carbon	Type of link of α-D-glucopyranose
100.46[a]	C-1	→ 6 Glcp 1 → 3 ↑
98.81	C-1	→ 6 Glcp 1 →
81.03[a]	C-3	→ 6 Glcp 1 → 3 ↑
74.65	C-3	→ 6 Glcp 1 →
72.63	C-2	
71.34	C-4	
70.76	C-5	
67.50[a]	C-6	→ 6 Glcp 1 → 3 ↑
66.69	C-6	→ 6 Glcp 1 →
61.71[a]	C-6	6 Glcp 1 →

[a] Signal of low intensity.

were assigned on the basis of analogies with the literature data[11,12] to anomeric carbon atoms of 1,3,6-tri-O-substituted and 1,6-di-O-substituted D-glucose. Their values, as well as the high positive value of the specific rotation, indicated the α-D-type of the sugar units. The relative representation of the 1 → 3 bonds (3.3%), obtained from the integrated intensities of the anomeric signals, indicates a low degree of branching of the polymer, which is in agreement with the results of chemical analysis. In the bond region the signal with low intensity at 81.03 ppm was assigned to the atom C-3 in 1,3,6-substituted sugar unit, which represents a downfield shift of about 7 ppm with respect to the unsubstituted carbon. Further signals in the 75 – 70 ppm region belong to the carbon atoms C-2 to C-5 in 1,6-di-O-substituted D-glucopyranose. As is evident from the Table IV two signals with low intensity at 67.50 and 61.71 ppm occur within the 68 – 61 ppm interval, which permits their being assigned to the C-6 atom in 1,3,6-substituted glucopyranose and the non-bonding atom C-6 of the non-reducing terminal units, and one signal at 66.69 ppm, typical of C-6 in 1,6-substituted glucopyranose.

The results of the methylation analysis and the ^{13}C measurement of D-glucan indicated a linear character of the polysaccharide chains in which α-D-glucopyranose units are linked by 1 → 6 glycosidic bonds, while some saccharides are the place of branching in position C-3. D-Glucan of the given structure is similar to a certain type of extracellular bacterial dextrans[11,13], but it occurs in other sources of plant origin as well[14 - 17].

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