GLUCAN ISOLATED FROM LEAVES OF *Althaea officinalis* L.

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A water-soluble low-molecular D-glucan was isolated from leaves of the medicinal plant marsh-mallow (*Althaea officinalis* L.). The results of methylation analysis, partial hydrolysis, periodate oxidation, and ^{13}C NMR data indicated a virtually linear structure with α-(1→6) glycosidic bonds.

Althaea officinalis L. as a medicinal plant has already been known in ancient time; used were its roots, leaves, and flowers. Aqueous extracts were applied when curing cough, catarrh of respiratory system, kidney pain, tonsilitis, and inflammations of nasal and oral cavities[1]. The principal active substance of this drug is considered to be the mucilage, the substantial part of which is formed by polysaccharides. In order to shed light on the relation between the mucilageneous material and its therapeutic effect, it is necessary to determine the chemical structure of its purified polysaccharide components. So far, only few papers[2,3] have dealt with detailed structure of these polymers. In our previous work[4] we described an L-arabinan isolated from the roots of marsh-mallow. This paper deals with isolation and characterization of D-glucan from leaves of this plant grown in Czechoslovakia.

EXPERIMENTAL

Material and Methods

Leaves of marsh-mallow were collected in 1979 in the Centre for Medicinal Plant Cultivation, Faculty of Medicine, J. E. Purkyně University, Brno. The uronic acid content in polysaccharides was determined by the carbazole method[5]. The periodate absorption was measured at 223 nm using a Beckman DB-GT spectrophotometer.

All solutions were evaporated under reduced pressure at temperature not exceeding 40°C. For descending paper chromatography on paper Whatman No 1 the following systems were used (v/v): A ethyl acetate–acetic acid–water (18 : 7 : 8), B ethyl acetate–pyridine–water (8 : 2 : 1). Chromatograms were detected with anilinium hydrogen phthalate[6]. For preparative paper chromatography paper Whatman 3 MM and system B were employed. The mobility of the adsaccharide is expressed relative to that of D-glucose. Free electrophoresis with Zeiss 35 (Jena) piparatus proceeded in 50 mmol l^{-1} borate buffer of pH 9·3 at 10 V/cm and 6 mA per 30 min

at a 10 mg/ml polysaccharide concentration. Optical rotation was measured with a Perkin–Elmer, model 141 polarimeter in water at 20°C. Infrared spectrum of the methylated polysaccharide (*c* 5, $CHCl_3$) was recorded with a Perkin–Elmer, model 457 spectrophotometer. For gas chromatography Hewlett–Packard, model 5711 A, chromatograph was employed; column A (200 × 0·3 cm), stationary phase 3% OV-225 on Chromosorb W (AW-DMCS, 80–100 mesh), temperature gradient 120°C (4 min) up to 170°C (2°C/min); column B (200 × 0·3 cm), stationary phase 20% SF-96 at 110°C (2 min) up to 210°C (4°C/min). Column A was employed for quantitative determination of sugars in the form of their alditol trifluoroacetates[7]. Spectrometer JMS-D 100 (Jeol, Japan) was used for gas chromatography-mass spectrometry of alditol acetates of methylated sugars[8]; column (200 × 0·3 cm), stationary phase 3% SP 2340 on Supelcoport (100–120 mesh). The inlet pressure of helium was 101 kPa, temperature 160–240°C (6°C/min); the ionizing electron energy 23 eV.

FT ^{13}C NMR spectrum of the polysaccharide (4%-solution in ^2H_2O) was taken with a Bruker WM-250 instrument at total proton decoupling and 30°C. The spectral width was 15 kHz, acquisition time 0·5 s, data points 8 k, puls width 6 μs (45°C). Chemical shifts refer to dimethyl sulfoxide as an internal reference (40·00 ppm downfield shift from tetramethylsilane). The number average molecular weight of D-glucan ($\overline{M}_n$) was determined osmometrically in water at 35°C using Knauer Vapour-pressure osmometer.

Isolation of D-Glucan

Dried leaves (600 g) were macerated in cold water (25 l) for 2 days, the extract was filtered, evaporated to 6 l and poured into ethanol (96%, 36 l) containing acetic acid (1%). The precipitate was decanted, washed with dilute aqueous ethanol (60%), suspended in water and dialyzed against water for 3 days. The water-insoluble portion was separated by centrifugation and the supernatant was freeze-dried (the mixture of polysaccharides I, Table I). The remaining water-ethanolic solution was concentrated to 1 l and poured into ethanol (96%, 6 l) acidified with acetic acid (1%). By a procedure similar to that employed in the preceding case the mixture II was obtained; the third precipitation of the supernatant furnished fraction III. The second maceration of the debris in cold water followed by precipitation of the mixture of polysaccharides IV was analogous with the first one.

TABLE I

Polysaccharide fractions from the leaves

Fraction	Yield[a] %	Molar ratios							
		D-Glc	D-Gal	D-Man	D-Xyl	L-Ara	L-Rha	L-Fuc	Uronic acid
I	4·33	1·00	0·16	0·12	0·12	0·08	1·12	—	0·11
II	0·33	1·00	0·36	0·36	0·22	0·54	traces	—	—
III	1·13	1·00	0·03	—	0·25	0·03	traces	—	—
IV	1·00	1·00	0·79	0·15	0·06	0·43	2·42	0·17	—

[a] With regard to the total weight of dry leaves.

A portion of fraction I (10 g) was suspended in aqueous ethanol (70%, 500 ml) and stirred at 40°C for 6 h. The insoluble residue was centrifuged off. This process was repeated 4 times. The insoluble portion (IP) was further separated: its aqueous solution (200 mg in 5 ml) was chromatographed on a Sephadex G-75 column (100 × 3 cm) using water as the eluent. Two fractions containing besides other saccharides different amounts of D-glucose were obtained. The fraction obtained at greater volume and enriched by D-glucose was further purified by successive elutions from Sephadex G-100 and G-75 columns (100 × 3 cm) with water. The polysaccharide obtained in this way afforded exlusively D-glucose upon total hydrolysis.

Hydrolysis

The polysaccharide was totally hydrolyzed with 0·5M-H_2SO_4 for 20 h, or with 2M-trifluoroacetic acid for 6 h at 100°C in a sealed tube. The glucan (200 mg) was partially hydrolyzed with 0·25M-H_2SO_4 (90 ml) on a steam bath under reflux for 4 h. The filtrate was neutralized with $BaCO_3$, deionized on a Dowex 50 WX4 (H^+ form) column, concentrated, and analyzed in system B. In addition to D-glucose and higher oligosaccharides, isomaltose was the only disaccharide found in the hydrolysate.

Methylation Analysis of D-Glucan

A solution of sodium dimethylsulfinylmethanide in dimethyl sulfoxide[9] (4 ml) was added to the polysaccharide (50 mg) dissolved in dimethyl sulfoxide (5 ml) and the suspension was stirred at room temperature for 6 h in nitrogen atmosphere. Methyl iodide (5 ml) was added dropwise during 30 min and further methyl iodide (2 ml) was added after stirring the mixture overnight. The solution was then poured into cold water (40 ml), dialyzed against water for 48 h and concentrated. The syrupy product was dissolved in methyl iodide (5 ml), silver oxide (100 mg) was added and the mixture was refluxed with stirring for 24 h (ref.[10]). The procedure was repeated three times. The methylated glucan (40 mg) was hydrolyzed with 2M-trifluoroacetic acid (5 ml) at 105°C for 3 h and the partially methylated D-glucose derivatives were transformed into the corresponding alditol acetates[8] and quantitatively analyzed by gas chromatography-mass spectrometry. The identified products are listed in Table II.

Periodate Oxidation of D-Glucan

Glucan (20 mg) dissolved in aqueous sodium periodate (15 mM, 20 ml) was allowed to stay at 4°C in the dark. The periodate consumption was spectrophotometrically[11] monitored during oxidation in aliquot portions of the reaction mixture. The reaction was complete after 48 h.

RESULTS AND DISCUSSION

Four fractions of polysaccharides, differing in quantitative representation of constituent saccharides, were obtained by re-precipitation of water-extracts with ethanol (Table I). Since D-glucose was found to be the dominant saccharide in all fractions, systematic investigation of polysaccharides started by removing the glucan from the main fraction I. The polymeric material containing 37% of glucose was suspended in 70% aqueous ethanol. The ethanol-insoluble portion was loaded onto a Sephadex G-75 column; elution with water gave two polysaccharide fractions. The fraction obtained at greater elution volume contained enhanced amount of D-glucose. Suc-

cessive chromatography of this fraction on Sephadex G-100 and G-75 packed columns afforded D-glucan homogeneous upon electrophoresis. It was well soluble in water, its specific optical rotation was +150·6°, number average molecular weight $\bar{M}_n$ 6 950, and electrophoretic mobility 6·37 × 10^{-5} cm^2 V^{-1} s^{-1}.

Partial hydrolysis of the polysaccharide afforded a mixture of D-glucose and one disaccharide of R_{Glc} 0·16 in system B. The disaccharide was separated from the hydrolysate by preparative paper chromatography. Chromatographic mobility of this disaccharide was found to be identical with that of the isomaltose standard and its specific optical rotation was +100·5° (ref.[12] gives +98° for isomaltose). The disaccharide was methylated and the product of methanolysis were identified by combined gas chromatography-mass spectrometry as methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside and methyl 2,3,4-tri-O-methyl-D-glucopyranoside thus evidencing the presence of (1→6) glycosidic bond between the D-glucose units. The high positive value of specific optical rotation both of the D-glucan (+150·6°) and the disaccharide (+100·5°) as well as the ^{13}C NMR spectrum of D-glucan indicated the α-D type of the saccharide units involved.

Treatment of D-glucan with sodium methylsulfinylmethanide in dimethyl sulfoxide with addition of methyl iodide[9] followed by methylation according to Purdie[10] (3 treatments) yielded a fully methylated products (as evidenced by the absence of —OH group absorption in the IR spectrum of the methylated product). This polymer was hydrolyzed and the resulting saccharides were reduced to alditols and

TABLE II

Methylated sugars from the hydrolysate of the methylated D-glucan

Sugar[a]	Relative proportion %	Bond	Relative proportion of bonds, %	
2,3,4,6-Me_4-D-Glc	11·8	—	—	—
2,3,4-Me_3-D-Glc	82·6	1→6	86·9	1→6
2,3,6-Me_3-D-Glc	1·3	1→4	2·3	1→4
2,4-Me_2-D-Glc	2·3	1→3 1→6	2·3	1→3
2,3-Me_2-D-Glc[b]	2·0	1→4 1→6	1·0	1→2
3,4-Me_2-D-Glc		1→2 1→6		

[a] 2,3,4,6-Me_4-D-Glc = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, *etc.*; [b] Identical retention times of the derivatives, distinguished by mass spectrometry.

acetylated. The partially methylated alditol acetates were identified[8] by combined gas chromatographic and mass spectrometric method (Table II). The main methylation product, 2,3,4-tri-O-methyl-D-glucose, indicated that this polymer consisted of glucose units linked virtually only by (1→6) glycosidic bonds; the latter together with (1→6) bonds in the branching points amounted totally 87%. The consumption of periodic acid within 48 h oxidation of the polysaccharide was 1·9 mol per glucose unit. This result also proved the presence of (1→6) bond in the polysaccharide. Theoretical consumption of the oxidation reagent by a unit bound in such a manner is 2 mol. The unit branched in the position 3 would be not oxidized at all whereas those branched in the positions 2 and 4, respectively, would consume only 1 mol reagent.

The ^{13}C NMR data of D-glucan are listed in Table III. The signals were assigned by analogy with ^{13}C NMR data of O-alkylated saccharides[13,14] and by comparison with those of methylated 6-O-methyl-α-D-glucopyranoside, isomaltose, isomaltooligosaccharides, and α-(1→6) glucans[15,16]. The $C_{(6)}$ signals of the glucan were ascribed considering their high-field position, too. Since the resonance signals of glucan carbons are sensitive towards the change of pD (the ability to participate in intramolecular hydrogen bonding[15]), the spectrum of glucan was measured in ^2H_2O at pD = 7 in order that the comparisons be authentic. Signals belonging to $C_{(6)}$ carbon atoms in the glycosodic bonds appeared at 66·8 ppm, *i.e.* they were shifted by 5·2 ppm downfield relative to their positions in hexapyranose. Signals belonging to anomeric carbon atoms were observed at 98·9 ppm in accordance with those reported by Friebolin[16] who measured the $C_{(1)}$ and $C_{(6)}$ signals of dextran and isomaltooligosaccharides up to 14 D-glucose units. This signal unambiguously indicates the α-anomeric character of glycosidic bonds in the D-glucan. Deviations due to the ring size or conformational differences of glycosidic bonds were not observed in the spectrum. The relative proportion of the (1→6) bonds (90%) was calculated from the integrated intensities of signals belonging to substituted $C'_{(6)}$ and unsubstituted $C_{(6)}$ carbon atoms. The legitimacy of this determination is based on the knowledge that the same-numbered carbons in glucan are in the same chemical environment and should experience identical nuclear Overhauser enhancement[17]. The spectrum

TABLE III
^{13}C NMR chemical shifts of D-glucan (ppm)

Atom	$C_{(1)}$	$C_{(2)}$	$C_{(3)}$	$C_{(4)}$	$C_{(5)}$	$C'_{(6)}$	$C_{(6)}$
Shift	98·9	72·7	74·7	71·4	70·8	66·8	61·8

did not offer information on branching points due to a restricted number of (1→4), (1→3), and (1→2) bonds relative to the main (1→6) bond.

The structural conclusions arrived at by ^{13}C NMR data are consistent with those based upon the results of methylation analysis. Both results revealed a practically linear structure of the D-glucan with α-(1→6) glycosidic bonds. The observations[18,19] that the solubility of glucan in water increases with the increase of (1→6) glycosidic bonds and decreases with the increase of (1→3) bonds were proved to be valid also in our case, since the D-glucan under investigation is readily soluble in water.

The described D-glucan resembles dextrans of bacterial origin. According to our knowledge, D-glucans of similar type have not been isolated from plant material yet.

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