POLYSACCHARIDES FROM THE ROOTS OF *Althaea officinalis* L.: STRUCTURAL FEATURES OF D-GLUCANS

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From the roots of the medicinal plant *Althaea officinalis* L., three D-glucans were isolated by gel chromatography which differed in molecular weights. The results of methylation analyses and ^{13}C NMR measurements indicated predominantly linear character of the polysaccharide chains composed of α-D-glucopyranose units linked by 1 → 6 glycosidic bonds almost exclusively. The polymers had essentially the same structural features as D-glucan isolated from the leaves of this plant.

In our preceding paper[1] we described the isolation of polysaccharides from the mucilage of the roots of *Althaea officinalis* L., and the structural characteristics of the neutral polysaccharide L-arabinan. In this paper, we present the basic structural features of three D-glucans from the same plant source.

EXPERIMENTAL

Material and Methods

The roots of *Althaea officinalis* L., variety *rhobusta*, were collected in the autumn of 1979 and 1981 in the Botanical Garden of the Medical faculty, Purkyně University, Brno.

The isolated polysaccharides were hydrolysed with 72% sulfuric acid[2] or with 2M trifluoroacetic acid at 100°C for 2 h. The content of uronic acids was determined using the carbazole method. Optical rotation of 1% aqueous solutions of polysaccharides was measured on a Perkin–Elmer polarimeter, model 141, at 20°C. Free electrophoresis of 1% solutions of polysaccharides in 0·05M tetraborate buffer (pH 9·2) was carried out and measured with a Zeiss 35 (Jena) instrument, at 10 V/cm and 6 mA, for 30 min.

Paper chromatography was carried out using the descending method on No 1 paper Whatman in the solvent systems ((v/v) ethyl acetate–pyridine–water (8 : 2 : 1) and ethyl acetate–acetic acid–water (18 : 7 : 8). Saccharides were detected with anilinium hydrogen phthalate[4].

Gas chromatography (GLC) was carried out on a Hewlett–Packard instrument, model 5 711 A on a column A (200 × 0·3 cm) with 3% of OV-225 on Chromosorb W (AW-DMCS, 80—100 mesh) as stationary phase, temperature range 120°C (4° min) to 170°C (2°/min), and column B (200 × 0·3 cm) with 3% of SP-2340 on Chromosorb W (AW-DMCS, 80—100 mesh) as stationary phase, at 190°—220°C (2°/min). Column A was used for quantitative determination of sugars

in the form of their alditol trifluoroacetates[5]. Gas chromatography-mass spectrometry (GLC-MS) of alditol acetates of methylated saccharides[6] was carried out on a JMS-D 100 (JEOL) instrument, using a column (200 × 0·3 cm) with 3% of SP-2340 on Supelcoport (100—120 mesh) as stationary phase. The pressure of helium at inlet was 101·3 kPa, temperature 160—240°C (6°/min), and the spectra were measured at 23 eV ionization energy.

The infrared spectra (IR) of methylated polysaccharides (5% in $CHCl_3$) were measured on a Perkin–Elmer spectrometer, model 457. The FT-^{13}C NMR spectra of polysaccharides (3% solutions in D_2O) were recorded on a JEOL FX-100 spectrometer at complete proton decoupling at 30°C. The spectral width was 15 kHz, acquisition time 1 s, data points 8 k and pulse width 6 μs (45° flip angle). Chemical shifts were measured relative to dimethyl sulfoxide as internal standard (39·45 ppm from tetramethylsilane in the direction to lower magnetic field).

For the determination of the degree of polymerization of D-glucans the polysaccharides (10 mg) were dissolved in water (2 ml) and reduced with $NaBH_4$ (30 mg) at room temperature for 24 h. Deionized products were hydrolysed with 72% sulfuric acid. After neutralization with $BaCO_3$ and deionization the mixture of D-glucitol and D-glucose was identified by means of GLC in the form of aldononitrile acetate[7] (column B).

The number average molecular weight ($\overline{M}_n$) of D-glucans *I* and *III* was also determined osmometrically in water at 35°C, on a Knauer Vapour Pressure Osmometer.

Isolation of D-Glucans

Ground roots (collected in 1979) were macerated in cold water at room temperature for 48 h. The aqueous extract was concentrated to a small volume and then fractionally precipitated with 96% ethanol. Four water-soluble (A—D) and four water-insoluble fractions of polysaccharides were obtained[1]. After acid hydrolysis, fraction A contained D-galactose, D-glucose, L-arabinose, L-rhamnose, uronic acids in molar ratio 1 : 1·3 : 0·3 : 0·1 : 0·3, and traces of D-mannose, D-xylose, and L-fucose. The mixture of polysaccharides of this fraction (1 g) was dissolved in water (80 ml) and separated on a column of DEAE-Sephadex A-50 (95 × 4 cm) in carbonate form. Elution with water gave D-glucan *I* (373 mg, 37%), homogeneous under the conditions of free boundary electrophoresis ($\mu = 3{\cdot}61 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}), $[\alpha]_D + 61°$. After reduction with $NaBH_4$ and hydrolysis the molar ratio of D-glucitol and D-glucose was determined by GLC to be 1 : 64, from which the number average molecular weight was calculated: $\overline{M}_n = 10\,548$. Osmometrically, the value $\overline{M}_n = 10\,040$ was determined.

In another purification procedure fraction A (0·5 g in 20 ml of water) was separated on a Sephadex G-75 column (100 × 3 cm) and that part of the polysaccharide material (151 mg, 30%) was further investigated which was eluted from the column first and which had — in comparison with the original mixture — an increased content of D-glucose by about 29%. Its subsequent chromatography on a column of DEAE-Sephadex A-50 (95 × 4 cm) in carbonate form, with water as eluent, gave D-glucan II (30 mg, 6%). The molar ratio of D-glucitol to D-glucose in the polymer was 1 : 123, which corresponded to $\overline{M}_n$ 20 106.

During the isolation of the new polysaccharide material (fraction A) from the roots collected in 1981 the above-mentioned procedure was followed. Acid hydrolysis of fraction A obtained in this way showed that it differed from the preceding one by an increased content of D-glucose (about 15%). The polysaccharide material (1·65 g) was dissolved in water (125 ml) and also purified on the DEAE-Sephadex A-50 column (95 × 4 cm) in carbonate form. Elution with water gave an electrophoretically homogeneous D-glucan *III* ($\mu = 4{\cdot}79 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}) (310 mg, 19%), $[\alpha]_D + 101°$. The molar ratio of D-glucitol to D-glucose was 1 : 48, which represents an $\overline{M}_n = 7\,956$. The osmometrically determined $\overline{M}_n$ was 8 235.

Methylation Analysis of D-Glucans

The polysaccharide (50 mg) was dissolved in 30% aqueous NaOH solution (3 ml) and dimethyl sulfate (1 ml) was added[8]. The suspension was stirred at room temperature for 24 h, under simultaneous addition of 30% NaOH (15 ml) and dimethyl sulfate (7 ml) in small portions. After termination of methylation, the mixture was heated at 60°C for 30 min, then cooled and neutralized with sulfuric acid. The methylated polymer was extracted with chloroform (3 × 60 ml), the solution was dried over sodium sulfate, filtered and evaporated. The syrupy residue was dissolved in methyl iodide (10 ml) and refluxed after addition of 200 mg of Ag_2O for 24 h (ref.[9]).

This procedure was repeated until the band at 3 500 cm^{-1} in the IR spectrum (characteristic of OH groups) was no longer observable. The permethylated polysaccharide was hydrolysed with 72% sulfuric acid and the partially methylated derivatives of D-glucose were converted in to corresponding glucitol acetates in a conventional manner and identified by GLC-MS. The results of methylation analyses of D-glucans *I*—*III* are given in Tables I and II.

RESULTS AND DISCUSSION

From the aqueous extract of the roots collected in 1979 four water-soluble (A—D) and four water-insoluble polysaccharide fractions were obtained by fractional precipitation with ethanol[1]. The water-soluble fraction A was used in this study for the isolation of D-glucans. Purification on DEAE-Sephadex A-50 (elution with water) gave D-glucan *I* which was homogeneous under the conditions of free-boundary electrophoresis and had a average molecular weight number $\overline{M}_n$ 10 548 (GLC) or 10 040 (osmometrically).

In order to determine possible differences in the structural features of D-glucans in dependence on their molecular mass fraction A was first separated on a Sephadex G-75 column. In the first stage of our further studies we concentrated on the polysaccharide material, which was eluted from the column first and which represented up to 30% of the original polysaccharide mixture. It was also remarkable for the fact that it contained about 29% of D-glucose more than fraction A. After chromatography on DEAE-Sephadex A-50, D-glucan *II* was isolated, which had molecular mass $\overline{M}_n$ 20 106 (GLC), and which was only partly soluble in water, aqueous alkali, dimethyl sulfoxide and in corresponding buffer solutions, in contrast to the preceding one. For this reason, it was impossible to check its homogeneity by free-boundary electrophoresis and to determine $\overline{M}_n$ reliably by osmometry, or to measure optical rotation. To a certain extent, this fact was surprising for us and so far we do not have an explanation for it. We assume that the relatively high molecular mass of the polysaccharide might be one of the sources of this phenomenon.

From literature[10] it is known that the content of polysaccharides and their composition in the plant is affected by several factors, as for example the locality where it was grown, the degree of the development stage, time of harvest *etc.* To our knowledge, however, the possible changes in their primary structures have not yet been investigated in this connection. Therefore, we were interested in whether D-glucan isolated from the polysaccharide material obtained from roots of the 1981 collection

would differ in its basic structural features from the above-mentioned D-glucans. The isolation of the new polysaccharide material (fraction A) was carried out in the same way as in the preceding case. The analysis of its sugar components showed that it differed from the preceding one by an increased content of D-glucose (by about 15%). Fractionation of DEAE-Sephadex A-50, using water as eluent, gave D-glucan *III*. The polymer was homogeneous on free-boundary electrophoresis and it had $\bar{M}_n$ 7 956 (GLC) or 8 235 (osmometrically).

For structural studies, the polysaccharides were first methylated in dimethyl sulfate and sodium hydroxide[8]. A complete methylation was achieved by the repeated Purdie[9] method. After hydrolysis of permethylated polymers the partially methylated derivatives of D-glucose were converted to corresponding glucitol acetates and identified[6] by GLC-MS.

The results of methylation analyses of D-glucans *I*–*III* are shown in Tables I and II. From them it is immediately evident that D-glucans *I*–*III* are bound almost exclusively by 1→6 glycosidic bonds. The presence of other types of bonds (Table II) indicates a very slight branching of these polysaccharides, differing in the position and number of branching sites.

The results of methylation analyses of water-soluble D-glucans *I* and *III* were correlated with the data obtained in their ^{13}C NMR measurements (Table III). The

TABLE I

Methylated saccharides in the hydrolysates of methylated D-glucans *I*–*III*

Saccharide	Relative ratios, %			Bond		
	I	*II*	*III*	*I*	*II*	*III*
2,3,4,6-Me_4-Glc[a]	22	19	26	—	—	—
2,4,6-Me_3-Glc	—	—	2	—	—	1→3
2,3,4-Me_3-Gl	70	74	59	1→6	1→6	1→6
2,4-Me_2-Glc	5	3	5	1→6 1→3	1→6 1→3	1→6 1→3
2,3-Me_2-Glc	1	4	8	1→6 1→4	1→6 1→4	1→6 1→4
3,4-Me_2-Glc			—	1→2	1→2	—
D-Glc	2	—	—	1→6 1→4 1→3 1→2	—	—

[a] 2,3,4,6-Me_4-Glc = 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol, *etc.*

signals were assigned on the basis of known rules[11,12] valid for ^{13}C-chemical shifts of O-alkylated saccharides and by comparison with the ^{13}C-spectral data of methyl 6-O-methyl-α-D-glucopyranoside, isomaltose, isomaltooligosaccharides, and α-(1→6) D-glucans[13,14]. The spectra of the mentioned polysaccharides are relatively simple and only a downfield shift of the signals $C_{(6)}$ by 5·27 and 5·30 ppm, resp., is evident in them, which confirms the presence of 1→6 glycosidic bonds. The signals of the anomeric carbon atoms at 98·12 and 98·94 ppm, resp., indicate[14,15] that the sugar units are of the α-D-type. The relative proportion of 1→6 bonds in D-glucan *I* (69%) and *III* (62%) were calculated[15] from the integrated intensities of the signals belonging to substituted $C'_{(6)}$ and unsubstituted $C_{(6)}$ atoms. The spectra did not afford any information concerning the branching sites in the polysaccharide chains, in consequence of the small number of 1→4, 1→3 and 1→2 bonds with respect to the main 1→6 bond.

The isolated D-glucans have essentially the same structural features as the D-glucan from the leaves of the investigated plant[15]. Certain differences are evident in the variability of the determined low degree of branching at $C_{(4)}$, $C_{(3)}$ and $C_{(2)}$, and especially in the molecular mass. The mentioned facts may be considered as a further proof for the polydispersity in individual types of polysaccharides occurring in plant

TABLE II

Relative content (%) of glycosidic bonds in D-glucans *I*—*III*

Bond	*I*	*II*	*III*
1→6	73·3	76·8	65·5
1→4	0·8	1·3	4·0
1→3	3·0	1·5	4·5
1→2	0·8	1·3	—

TABLE III

^{13}C NMR chemical shifts (δ, ppm) of D-glucans *I* and *III*

Glucan	$C_{(1)}$	$C_{(2)}$	$C_{(3)}$	$C_{(4)}$	$C_{(5)}$	$C'_{(6)}$	$C_{(6)}$
I	98·12	72·98	73·31	71·85	70·39	66·18	60·91
III	98·94	72·67	74·66	71·45	70·80	66·80	61·50

cell walls. This means that the proposed structural features for the polysaccharides investigated are only a certain average of the structural properties of the group of related polymers.

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