A POLYSACCHARIDE FROM THE PITH OF THE STEMS OF Althea flavovirens

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It has been established that a polysaccharide from the pith of the stems of Althea flavovirens Boiss et Buhse growing in Azerbaidzhan (environs of the village of Dzhul'fy) has a branched molecule consisting of residues of ribose, rhamnose, glucose, and glucuronic and galacturonic acids (3 : 6 : 3 : 7 : 2). Among the products of the methylation of the polysaccharide reduced at the carboxy groups the following sugar derivatives have been identified: 2,3,4-tri-O-methyl-L-rhamnose, 3,4-di-O-methyl-L-rhamnose, 2,3,4,6-tetra-O-methyl-D-glucose, and 2,3,6-tri-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-galactose (unresolved mixture), and also 2,6-di-O-methyl-D-glucose (2 : 10 : 24 : 7 : 2).

Previously, one of us [1] isolated and characterized a polysaccharide (yield 10.5%) from the pith of the stems of Althea flavovirens Boiss et Buhse (yellowish-green hollyhock) growing in Azerbaidzhan (environs of the village of Dzhul'fy). The plants were collected in the mass-flowering phase.

The homogeneity of the polysaccharide with respect to molecular weight was determined with the aid of ultracentrifugation and with respect to chemical composition by electrophoresis. The polysaccharide contained 45.3% of uronic acids.

When the products of the hydrolysis of the polysaccharide were separated by paper electrophoresis in a 1% solution of acetic acid, glucuronic and galacturonic acids were found, and glucose and rhamnose were identified by paper chromatography in system 1 (1 : 2.4, densitometrically).

Continuing a study of the polysaccharide, with the aid of solvent system 2 we have detected ribose in the hydrolysate, in addition to glucose and rhamnose; in system 1 the ribose was not separated from the rhamnose.

The IR spectrum of the initial polysaccharide (Fig. 1) showed strong bands at (cm^{-1}) 1600 (COO^- group), 1245, 1375, and 1720 (acetyl group), and 3300-3400 (the broad absorption band of OH groups), and also bands at 850 and 890 cm^{-1} (α- and β-glycosidic bonds, respectively).

The relative viscosity of a 0.6% solution of the polysaccharide in borate buffer (pH 9.21) $\eta_{rel} = 1.5$.

Since the initial polysaccharide was not soluble in dimethyl sulfoxide (DMSO), which is a necessary condition for successful methylation by the Hakomori method [2], we decided to obtain a neutral polysaccharide from it and for a start to determine the types of bonds in the molecules of the latter. For this purpose the initial polysaccharide was deionized on KU-2 resin [3], esterified with ethylene oxide [4], and reduced with sodium tetrahydroborate. The gas–liquid chromatography (GLC) analysis of the acetates of the polyols obtained after acetylation of the hydrolysis products of the neutral polysaccharide that had been reduced with sodium tetrahydroborate showed that the ratio of ribose, rhamnose, glucose, and galactose in them was 3 : 6 : 10 : 2. Thus, in the initial polysaccharide from the pith of the stems of the yellowish-green hollyhock the ratio of ribose, rhamnose, glucose, and glucuronic and galacturonic acids was 3 : 6 : 3 : 7 : 2.

The completely methylated neutral polysaccharide was subjected to methanolysis. Part of the methanolysis products was separated by the GLC method on columns I and II, and another part was hydrolyzed with a solution of sulfuric acid [5]. A small amount of the hydrolysis products was separated by paper chromatography in systems 3, 4, and 5, and the bulk of the hydrolysate was reduced with sodium tetrahydroborate, acetylated, and analyzed by the GLC method on columns III, IV, and V. The acetates of the partially methylated polyols were identified by mass spectrometry [6]. The GL-chromatogram on column IV is shown in Fig. 2A, and the results of a determination of the types of bonds in the molecule of the reduced polysaccharide are given below:

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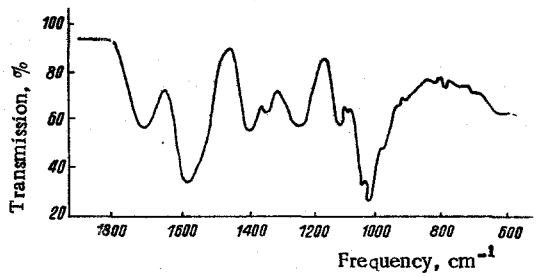

Fig. 1

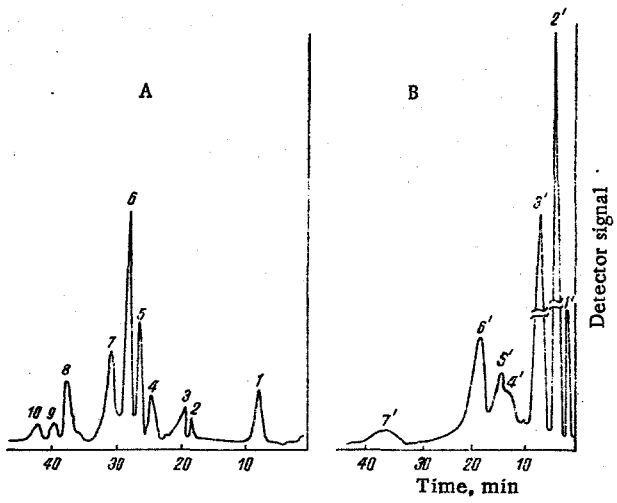

Fig. 2

Fig. 1. IR spectrum of the initial (undeionized) polysaccharide from the pith of the stems of yellowish-green hollyhock (in a film).

Fig. 2. GL chromatogram of the acetates of partially methylated polyols (conditions of separation IV, A) and of the methyl glycosides of partially methylated carbohydrates (conditions of separation II, B): 1) presumably a tetra-O-acetyl-O-methyl-6-deoxyhexitol; 2) a 1,5-di-O-acetyl-2,3,4-tri-O-methyl-6-deoxyhexitol; 3) unidentified; 4, 7) these apparently relate to derivatives of pentitols obtained from ribose; 5) a 1,2,5-tri-O-acetyl-3,4-di-O-methyl-6-deoxyhexitol; 6) a 1,5-di-O-acetyl-2,3,4,6-tetra-O-methylhexitol; 8) a 1,4,5-tri-O-acetyl-2,3,6-tri-O-methylhexitol; 9) unidentified; 10) a 1,3,4,5-tetra-O-acetyl-2,6-di-O-methylhexitol; 1') methyl 2,3,4-tri-O-methyl-L-rhamnoside; 2') methyl 3,4-di-O-methyl-L-rhamnoside; 3') methyl 2,3,4,6-tetra-O-methyl-D-glucoside; 4', 7') unidentified; 5', 6') methyl 2,3,6-tri-O-methyl-D-glucoside + methyl 2,3,6-tri-O-methyl-D-galactoside.

Sugar residue	Positions of the O-methyl groups	Glycosidic bonds	Quantitative ratio of the sugar residues*	
			by weight	molar
L-rhamnose	2, 3, 4	terminal	1	2
	3, 4	2	5,5	10
D-glucose	2, 3, 4, 6	terminal	13,2	24
	2, 3, 6†	4	4,2	7
	2, 6	3,4	1,3	2

*Mean of eight chromatograms

†Apparently containing 2,3,6-tri-O-methyl-D-galactose as impurity.

Unfortunately, it was impossible to identify the ribose derivatives reliably. Peaks 4 and 7 in Fig. 2A apparently correspond to them. The presence of peak 1 can probably be explained by the existence of a 1-O-acetyl-2,3,4,5-tetra-O-methyl-6-deoxyhexitol in the mixture.

The results of the GLC analysis of the methyl glycosides of the partially methylated carbohydrates (Fig. 2,B) and the PC analysis of the partially methylated monosaccharides did not contradict the conclusions drawn on the basis of a mass-spectrometric investigation of the polyols. The chromatographic mobilities of the partially methylated monosaccharides were as follows:

Partially methylated monosaccharide	R_g^* in the following systems		
	3	4	5
2,3,4-Tri-O-methyl-L-rhamnose	1,01	1,03	1,00
2,3,4,6-Tetra-O-methyl-D-glucose	1,00	1,00	1,00
3,4-Di-O-methyl-L-rhamnose	0,90	0,89	0,92
2,3,6-Tri-O-methyl-D-glucose + 2,3,6-tri-O-methyl-D-galactose	0,80	0,79	0,85
2,6-Di-O-methyl-D-glucose	0,62	0,65	0,69

*Relative to 2,3,4,6-tetra-O-methyl-D-glucose.

EXPERIMENTAL

For paper chromatographic analysis we used FN-2, FN-4, and FN-12 papers (GDR) and the following solvent systems: 1) ethyl acetate—pyridine—water (8 : 2 : 1); 2) butan-1-ol—pyridine—water (6 : 4 : 3; 2-3 times); 3) water-saturated butan-1-ol; 4) butan-1-ol—ethanol—water (40 : 11 : 19); and 5) butan-1-ol—acetic acid—water

(4 : 1 : 5; upper layer). Aniline hydrogen phthalate and dimethylaniline were used as chromogenic agents.

The IR spectra were recorded in paraffin oil on a UR-10 instrument and in the form of films on a Perkin-Elmer 577 instrument.

The gas–liquid chromatography (GLC) of the methyl glycosides of the methylated carbohydrates was performed on a Pye-Unicam series 104 instrument using glass columns: I) 15% of NPGA, 1.5 m × 4 mm, temperature 150 °C, V_{ar} = 30 ml/min; II) 15% of BDS, 1 m × 4 mm, temperature 175°C, V_{Ar} = 40 ml/min. Acetate of partially methylated polyols were separated on 1.5 m × 4 mm columns; III) 3% of QF-1 with programming of the temperature from 110 to 220 °C at 4°C/min; V_{Ar} = 60 ml/min; IV) 3% of OV-225, temperature from 130 to 220°C at the rate of 2°C/min; V_{Ar} = 30 ml/min; V) 15% of NPGS, temperature from 125 to 225°C at the rate of 6°C/min, V_{Ar} = 60 ml/min.

The complete acetates of polyols were analyzed on column VI – 3% of OV-225, 1.5 m × 4 mm, temperature 190°C, V_{Ar} = 40 ml/min. Chromaton (0.16–0.20 mm) was used as the inert support.

The weight ratios of the individual compounds were calculated from the areas of the peaks on the GL-chromatograms and were recalculated to molar ratios on the basis of the molecular weights of the corresponding compounds.

The mass spectra of acetates of partially methylated polyols were recorded in chloroform on LKB-2091 and LKB-90003 instruments (with separation of the mixtures on column IV).

The isolation and purification of the polysaccharide from the pith of the stems of the yellowish-green hollyhock were performed as described previously [1].

The relative viscosity of a 0.6% solution of the polysaccharide was determined after the heating of an aqueous solution of it (in a sealed tube at 105°C for 5 h) to which borate buffer had then been added (pH of the solution used 9.21).

The deionization of the polysaccharide was carried out by passing a 0.3–0.5% aqueous solution of the polysaccharide through a column of KU-2 resin (H^+ form) in a proportion of 200 ml of KU-2 per 1 g of polysaccharide. The column was carefully washed with water.

The polysaccharide was esterified with ethylene oxide, which was added in 15-ml portions every three days to a 1–2% aqueous solution of the deionized polysaccharide (50–60 ml per 0.1 g of polysaccharide). The mixture was kept for four days in the refrigerator and then at room temperature until the pH of the solution had become equal to the pH of distilled water (~4 weeks). The mixture was evaporated in a rotary evaporator to small volume and the reaction product was isolated by the addition of acetone (1 : 5, v%v).

The polysaccharide was reduced with $NaBH_4$ in borate buffer (pH 7.6). Dry $NaBH_4$ was added in small portions (15 mg) with vigorous stirring of the solution until it ceased to foam. The mixture was left at room temperature for 3 h and was then dialyzed against running water. The reduction operation was repeated twice more. The completeness of the reaction was judged from the absence of absorption bands at 1600–1640 and 1720–1740 cm^{-1} in the IR spectrum.

The neutral polysaccharide (0.2 g) was methylated by Hakomori's method [2] three times, after which the absorption band of OH groups in the IR spectrum of the reaction product had disappeared.

Methanolysis of the methylated polysaccharide was carried out by boiling it with 5 ml of a mixture of methanol and acetyl chloride (50 : 3) for 36 h. After cooling, the solution was neutralized with dry Ag_2CO_3, and the precipitate was filtered off and was washed with methanol and a small amount of chloroform. The filtrate and the wash waters were passed through a column containing KU-2 resin (H^+ form, 15 ml), the column was washed with chloroform, and the solution was evaporated in vacuum.

The methyl glycosides of partially methylated monosaccharides were subjected to GLC analysis on columns I and II.

Hydrolysis of the methyl glycosides of the partially methylated monosaccharides was carried out with sulfuric acid, first with the 72% acid (2.5 ml, 0°C, 1 h) and then with the 8% acid by adding 20 ml of water to the mixture (100°C, 6 h). After cooling, the acid was neutralized with $BaCO_3$, and the precipitate was filtered off and washed with acetone. The combined solution was evaporated and was analyzed by PC in solvent systems 3, 4, and 5.

The reduction of the products of the hydrolysis of the methylated polysaccharide was carried out with $NaBH_4$, for which purpose ~500 mg of the latter was added in small portions (20 mg) to an aqueous solution of the partially methylated monosaccharides. The mixture was left at room temperature for 12 h and was then neutralized with acetic acid and passed through a column of KU-2 resin (H^+ form, 50 ml), which was subsequently washed with methanol. The solution was evaporated to dryness in the rotary evaporator with the addition of methanol repeatedly (six times). The residue was dried in a vacuum desiccator over $CaCl_2$.

The acetylation of the partially methylated polyols was carried out with a mixture of acetic anhydride (2.5 ml) and pyridine (2.5 ml). The mixture was heated at 100°C for 1 h and was left at room temperature for 12 h. Then it was poured into 50 ml of water containing ice and, after 3-4 h, the reaction products were exhaustively extracted with chloroform. The chloroform solution was dried over Na_2SO_4, filtered, and evaporated, and the residue was analyzed by GLC in columns III, IV, and V, the mass spectra of the individual compounds being recorded.

Determination of the Quantitative Ratio of the Neutral Monosaccharides in the Polysaccharide. The polysaccharide (50 mg) was hydrolyzed with 5 ml of 6 N CF_3COOH in a sealed tube at 120°C for 30 h. To eliminate the CF_3COOH, the hydrolysate was repeatedly evaporated in a rotary evaporator, with the addition of 2-5 ml of water each time. The residue was dried over NaOH and it was then dissolved in 5 ml of water, and 150 mg of NaBH was added in small portions (with stirring). The resulting mixture was left for 12 h and it was then neutralized with acetic acid and was evaporated to dryness several times, each time with the addition of 1.5-2 ml of methanol, and then 4-5 times with the addition of 2-3 ml of chloroform. The residue was dried in a vacuum desiccator over P_2O_5 and was dissolved in 5 ml of acetic anhydride; calcined CH_3COONa was added to this solution and it was kept in a sealed tube at 130°C for 5 h. The acetylation product was poured onto ice (50 mg) and, after 5 h, it was extracted with chloroform. The chloroform solution was washed with water to neutrality, dried with Na_2SO_4, filtered, evaporated to small volume, and subjected to GLC analysis on column VI.

The mass spectra of the acetates of the partially methylated polyols were taken by S. A. Zabelinskii and I. G. Zenkevich, while M. P. Filippov recorded and interpreted the IR spectra of the polysaccharide in the form of a film.

SUMMARY

1. It has been found that the acid polysaccharide from the pith of the stems of Althea flavovirens Boiss et Buhse growing in Azerbaidzhan consists of a branched polymer containing residues of ribose, rhamnose, glucose, and glucuronic and galacturonic acids (3 : 6 : 3 : 7 : 2).

2. The presence of the following derivatives in the products of the methylation of the polysaccharide reduced at the carboxy group was established: 2,3,4-tri-O-methyl-L-rhamnose, 3,4-di-O-methyl-L-rhamnose, 2,3,4,6-tetra-O-methyl-D-glucose, a mixture of 2,3,6-tri-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-galactose, and 2,6-di-O-methyl-D-glucose (2 : 10 : 24 : 7 : 2).

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