POLYSACCHARIDES FROM THE BARK OF THE WHITE WILLOW (*Salix alba* L.): STRUCTURE OF A GALACTAN

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(Received March 24th, 1972; accepted for publication in revised form, May 16th, 1972)

ABSTRACT

A water-soluble galactan, $[\alpha]_D$ +49.5°, has been isolated from the pectic material of the bark of the white willow (*Salix alba* L). The polysaccharide has a linear backbone of 33 β-D-galactopyranose residues linked in the main through C-1 and C-4, with approximately 4% of the residues linked through C-1 and C-6.

INTRODUCTION

Structural investigations of polysaccharides from wood and bark have been generally restricted to the hemicelluloses, and only a few studies of pectic substances have been reported. Knowledge about the composition and structural features of the neutral and acidic components of these complex polysaccharides is still meagre, probably because of their complex structure and their occurrence with the structurally related, plant gums and mucilages[1]. Both homopolymers, such as polygalacturonic acids[2–4], and heteropolymers of acidic galactans[5,6] differing in their composition and structural features have been isolated from wood and bark materials.

We now describe structural studies of a polysaccharide isolated from the bark of young twigs of the white willow (*Salix alba* L.).

RESULTS AND DISCUSSION

Sawdust was extracted with benzene–ethanol and then delignified[7]. Treatment of the resulting holocellulose with cold water gave crude pectic material which had $[\alpha]_D$ +168° and contained D-galacturonic acid (75%, degree of esterification 27). Acid hydrolysis of the polymer yielded D-galactose and L-arabinose in the molar ratio 1:0.8, together with small amounts of D-glucose, D-xylose, and L-rhamnose. Fractionation of the polysaccharide on microcrystalline DEAE-cellulose[8] gave a D-galactan which appeared to be homogeneous on free-boundary electrophoresis. This fractionation procedure, however, was not suitable for large-scale preparation of the polysaccharide. Treatment of the de-esterified pectic material with an endopolygalacturonase[9] and subsequent fractionation on Sephadex G-25 afforded the following fractions: (*I*) a macromolecular part (*MM*), $[\alpha]_D$ +108°, which on acid hydrolysis gave

TABLE I

FRACTIONATION OF THE POLYSACCHARIDES FROM THE BARK OF *Salix alba* L. ON DEAE-SEPHADEX A-50

Fraction	*Eluant*	*Yield* (%)	$[\alpha]_D$ (*degrees*)	*Molar ratios of monosaccharides*					*Uronic acid*
				Gal	*Glc*	*Ara*	*Xyl*	*Rha*	
MM	—	100	+108	10	tr[a]	2.5	0.7	1.5	+
A	2.5mM sodium formate	9.5	+52.6	10	—	tr	tr	—	—
B	0.1M sodium formate	6.0	+48.3	10	—	tr	tr	—	—
C	0.25M sodium formate	4.7	+76.6	10	—	4.7	0.8	0.8	+
D	0.5M sodium formate	75.2	+118	10	tr	3.2	0.7	1.7	+
E	0.3M sodium hydroxide	1.5	+84	10	tr	2.4	2.7	1.1	+

[a]tr = trace.

D-galactose, L-arabinose, D-xylose, and L-rhamnose in the molar proportions 10.0: 2.5:0.7:1.5; the D-galacturonic acid content was 26.6%. (*2*) Acidic fragments composed of D-galacturonic acid, D-galactose, L-arabinose, and traces of D-xylose. (*3*) A homologous series of D-galacturonic acid-containing oligosaccharides.

When the polysaccharide mixture *MM* was fractionated on DEAE-Sephadex A-50 (Table I), fractions *A* and *B* were obtained having slightly different molecular properties and composition. Mainly D-galactose and only traces of L-arabinose and D-xylose were detected on acid hydrolysis of *A* and *B*. Further attempts at fractionation failed. Combination of fractions *A* and *B* gave material having $[\alpha]_D$ +49.5° and $\overline{M}_n$ 5350 which corresponded to an average d.p. of 33.

Partial hydrolysis of the galactan gave a homologous series of D-galactose oligomers, which was resolved on cross-linked starch. The galactose disaccharide was characterised by enzymic hydrolysis and, after permethylation, by mass spectrometry. From these results, the disaccharide was shown to have a β-(1→4)-linkage[10,11]. The polysaccharide consumed 1.06 moles of periodate per anhydrohexose unit, and Smith degradation[12] gave glycerol and threitol in the molar ratio 1:17 (g.l.c.); D-galactitol could not be detected.

After hydrolysis of the methylated galactan, the partially methylated sugars were separated by t.l.c., converted into the glycosides, and identified by mass spectrometry[13–16] (see Table II); the molar ratios are summarized in Table III.

TABLE II

MASS SPECTRA OF THE PARTIALLY METHYLATED METHYL D-GALACTOSIDES

m/e	*Relative intensities*[a]			m/e	*Relative intensities*[a]		
	2,3,4[b]	*2,3,6*	*2,6*		*2,3,4*	*2,3,6*	*2,6*
205	0.7	0.3	—	111	2.8	1.5	—
191	—	0.3	0.4	102	5.0	6.9	0.2
177	—	—	0.7	101	67.9	100.0	5.3
176	2.8	—	—	99	1.0	8.7	5.7
173	1.8	1.1	0.6	89	7.6	4.2	6.4
161	1.3	8.5	—	88	100.0	33.3	17.8
159	—	2.2	0.9	87	5.8	10.1	100.0
155	—	0.6	—	85	6.2	9.8	16.6
149	1.3	0.3	0.3	75	52.4	45.8	10.8
147	—	—	3.8	74	3.3	8.4	63.2
145	2.8	1.4	3.1	73	22.7	13.7	10.0
144	1.5	0.5	1.9	71	10.7	21.9	17.8
141	1.0	0.9	0.7	69	6.2	2.9	4.6
131	2.4	4.0	0.3	61	2.2	2.6	6.3
130	0.6	2.1	3.4	60	—	—	2.6
129	1.6	2.0	2.2	59	7.1	6.9	13.4
127	1.5	2.4	0.4	57	9.8	7.2	14.5
117	1.8	1.2	3.7	55	9.8	5.6	4.5
115	2.1	1.9	5.8	45	32.0	80.1	57.2
113	2.2	2.0	0.4				

[a]Percentage of the base peak. [b]The numbers indicate the positions of the methoxyl groups.

TABLE III

METHYLATED SUGARS FROM THE HYDROLYSATE OF THE METHYLATED GALACTAN

Sugar	*Molar ratio*	*Retention time* (T)[b]	R_{GAL}[c]
2,3,4,6-Me_4-Gal[a]	1.0	1.00	1.00
2,3,6-Me_3-Gal	31.2	1.40s 1.75w 1.81m	0.66
2,3,4-Me_3-Gal	1.1	2.95s	0.44
2,6-Me_2-Gal	1.4	2.83s 3.13w 3.32m	0.27

[a]2,3,4,6-Tetra-*O*-methyl-D-galactose, etc. [b]G.l.c., column (*b*): s, strong; m, moderate; w, weak. [c]T.l.c.

The identification of the linkage in the galactose disaccharide, the abundance of 2,3,6-tri-*O*-methyl-D-galactose in the hydrolysate of the methylated polysaccharide, and the identity of the products of Smith degradation suggest that the galactan is mainly built up of (1→4)-linked D-galactopyranose residues. Small amounts of 2,3,4-tri-*O*-methyl-D-galactopyranose (Table III) indicate the presence of some (1→6)-linkages. The low, positive optical rotation (+49.5°) of the polysaccharide, the i.r. band at 890 cm^{-1}, and the stability of the disaccharide to α-D-galactosidase, indicate the presence of β-D linkages. The molar ratio of the methylated sugars and the absence of D-galactose residues after periodate oxidation prove the linearity of the polysaccharide backbone. The formation of 2,6-di-*O*-methyl-D-galactose is probably structurally insignificant and may be due to incomplete methylation of the polymer.

In general, the results obtained indicate that the galactan from the bark of *Salix alba* L. is different both in composition and structural features from the bark galactan of white spruce (*Picea glauca*)[17], as well as from the compression and tension wood-galactans of red spruce (*Picea rubens* Sarg.)[5] and American beech (*Fagus grandifolia* Ehrl.)[6]. The structure is essentially the same as that proposed for the galactan from *Lupinus albus*[18].

EXPERIMENTAL

General. — Free-boundary electrophoresis was effected with a Zeiss 35 apparatus, using 0.05M sodium tetraborate buffer (pH 9.2), at 175 volts and 6 mamp for 40 min, and a polysaccharide concentration of 10 mg/ml. Optical rotations were measured with a Thorn–Bendix Model 143A polarimeter at 21 ±3°. Molecular weights were determined on Knauer's vapor pressure osmometer; readings were taken at sensitivity 128 and 37°. The calibration curve was constructed using Dextran T-10 (Uppsala), $\overline{M}_n$ 5700, as standard.

G.l.c. was performed on a Hewlett–Packard Model 5750G instrument, using (*a*) a column (305 × 0.3 cm) of 1% w/w of XE-60 on 80–100 mesh Gas Chrom Z, at a programmed temperature-range of 130–150° at 1°/min, with helium as carrier gas at a flow rate of 14 ml/min. (*b*) a column (183 × 0.3 cm) of 3% w/w of ECNSS-M on

80–100 mesh, acid-washed Chromaton N-DMCS, at 120–220° and 2°/min, (*c*) a cclumn (183 × 0.3 cm) of 10% w/w UC-W-98 on 80–100 mesh, acid-washed Chromosorb W, at 120–280° and 4°/min. For (*b*) and (*c*), nitrogen was the carrier gas at a flow rate of 35 ml/min. Mass spectra were obtained with a MCh Model 1306 spectrometer (U.S.S.R.) at an ionizing potential of 70 eV. The inlet temperature was 25–35° and that of the ionizing chamber 120–130°. Paper chromatography was performed by the descending method on Whatman No. 1 paper with (*d*) 8:2:1 ethyl acetate–pyridine–water, (*e*) 10:3:3 butyl alcohol–pyridine–water, (*f*) 90:8:2 butanone–water–conc. ammonia, (*g*) 18:7:8 ethyl acetate–acetic acid–water, (*h*) 4:1:5 butyl alcohol–ethanol–water. Reducing sugars were detected with aniline hydrogen phthalate[19]. Column (*a*) was used for quantitative analysis of the sugars as the alditol trifluoroacetates[20]. T.l.c. was carried out on silica gel (Merck) with solvent (*f*). The mobility (R_{Gal}) of the methylated sugars is expressed relative to that of 2,3,4,6-tetra-*O*-methyl-D-galactose, and the rate of movement (R_{Ara}) of oligouronic acids is related to that of L-arabinose. The retention times (*T*) of the methylated methyl glycosides are relative to that of methyl 2,3,4,6-tetra-*O*-methyl-αβ-D-galactopyranoside. The polysaccharides were hydrolyzed with 72% sulphuric acid[21]. The uronic acid content was determined by the carbazole method[22].

Isolation of the galactan. — Sawdust prepared from the bark of twigs of young white-willow (*Salix alba* L.) was pre-extracted with benzene–ethanol (1:3) for 30 h, and then delignified according to Wise's procedure[7] using 80% ethanol. The holocellulose obtained (85% of the bark) was twice extracted at room temperature with water to give a crude, pectic material (57 g, 11.2%) which had $[\alpha]_D$ +168° (*c* 1.0, water) (Found: uronic acid, 75%; degree of esterification, 27). On hydrolysis, the sample yielded D-galactose and L-arabinose (molar ratio 1:0.87), together with traces of D-glucose, D-xylose, and L-rhamnose (solvent *d*).

A solution of the polysaccharide (0.65 g) in water (30 ml) was added to the top of a column (2 × 50 cm) of DEAE-cellulose (capacity, 1.75 mequiv. g^{-1}; phosphate form[8]). The column was eluted successively with water, sodium hydrogen phosphate (0.05–2.0M) and 0.3M sodium hydroxide. The fractions were deionized and then lyophilized. The water-eluted fraction (3 mg) gave only D-galactose on acid hydrolysis. The other fractions contained increasing amounts of material which, on hydrolysis, gave D-galacturonic acid, and L-rhamnose, D-galactose, and L-arabinose in different molar ratios, together with traces of D-glucose and D-xylose. The following procedure was used to obtain larger amounts of neutral polysaccharide. The pectic material (57 g) was de-esterified (pH 9.0, 2 h) and then dissolved in an acetate buffer (1.5 l, pH 4.6) and treated with endopolygalacturonase[9] (600 mg, Koch–Light) for 3 days at 35°. The solution of degraded polysaccharide was heated for 15 min at 60°, and then evaporated to small volume and centrifuged. The solution was added to the top of a column (6.5 × 100 cm) of Sephadex G-25 and eluted with water to give three fractions: (*I*) a macromolecular portion (*MM*, 8.2 g), $[\alpha]_D$ +108° (*c* 1.0, water) (Found: uronic acid, 26.6%), which, on hydrolysis, yielded D-galactose, L-arabinose, D-xylose, and L-rhamnose in molar proportions 10:2.5:0.7:1.5, together with traces

of D-glucose; (*2*) a mixture of acidic fragments (16.4 g) composed of D-galacturonic acid, D-galactose, L-arabinose, and traces of D-xylose; (*3*) a mixture containing mono- up to penta-galacturonic acids (the last two only in traces) with R_{Ara} 0.87, 0.55, 0.35, 0.18, and 0.08 (solvent *g*).

A solution of polysaccharide *MM* (4.0 g) in water (40 ml) was added to the top of a column (4 × 80 cm) of DEAE-Sephadex A-50 in the formate form. The column was eluted successively with 0.0025, 0.1, 0.25, and 0.5M solutions of sodium formate, and finally with 0.3M sodium hydroxide (Table I).

Polysaccharides *A* (0.38 g), $[\alpha]_D$ +52.6°, and *B* (0.24 g), $[\alpha]_D$ +48.3°, were obtained, having similar compositions and identical electrophoretic mobilities. The average d.p. of the combined fractions, $[\alpha]_D$ +49.5° (*c* 1.0, water), was 33.

Partial, acid hydrolysis of the galactan. — A sample of the galactan (100 mg) was hydrolyzed with 0.25M sulphuric acid (10 ml) for 1 h at 100°. The hydrolysate was neutralized (Ionenaustauscher II, free base) and concentrated to a syrup (80 mg). The oligomers were fractionated on a column (2.8 × 170 cm) of cross-linked starch[23], using water as eluant, to give D-galactose (38 mg, R_{Ara} 0.67), disaccharide (18 mg, R_{Ara} 0.28), trisaccharide (10 mg, R_{Ara} 0.11), and tetrasaccharide (3 mg, R_{Ara} 0.03). The R_{Ara} values were obtained for solvent (*e*). The higher oligosaccharides could not be separated. A sample of the disaccharide (1 mg) in a sodium acetate buffer (1.25 ml, pH 4.8) was treated with α-D-galactosidase for 10 h at 37°; toluene was used as a preservative. No release of D-galactose could be detected by paper chromatography, using solvent (*e*). The i.r. spectrum of the disaccharide contained bands at 760, 895, and 945 cm^{-1}.

Sodium hydride powder (10 mg) was added to a solution of the disaccharide (10 mg) in *N*,*N*-dimethylformamide (2 ml), and the suspension was stirred for 1 h before the addition of methyl iodide (0.5 ml). The reaction mixture was allowed to stand at room temperature for 2 h. Water (5 ml) was added and the methylated product was extracted with chloroform (3 × 6 ml). The organic extracts were washed with water and concentrated to dryness. Peaks in the mass spectrum of the permethylated disaccharide at *m/e* 380, 305, 219, and mainly at 161, proved[10,11] the glycosidic bond to be (1→4).

Degradation of the galactan. — A sample of galactan (9 mg) was oxidized with 15mM sodium metaperiodate (10 ml) in the dark at 5°. Aliquots (0.1 ml) were withdrawn at intervals and the periodate consumption was followed spectrometrically[24]. After 72 h, the consumption of oxidant was 1.06 mole per hexose unit. The excess of periodate was removed with 30mM lead acetate (2.5 ml), and the polyaldehyde was reduced with sodium borohydride (9 mg). The deionized product was hydrolyzed with 0.25M sulphuric acid (4 ml) for 8 h at room temperature. The solution was neutralized (Ionenaustauscher II, free base), and the glycolaldehyde obtained on acid hydrolysis was treated again with sodium borohydride (9 mg) because of the identical *T* values of glycolaldehyde and glycerol[25]. G.l.c. [column (*c*)] was used to determine the resulting alditols as their trimethylsilyl ethers[26]. The molar ratio of glycerol and threitol was 1:17 (average of two determinations).

Methylation of the galactan. — A solution of the polysaccharide (200 mg) in dry methyl sulphoxide (5 ml) was added to a solution of methylsulphinyl sodium in methyl sulphoxide (5 ml). The base was prepared[27] by adding sodium hydride (300 mg) to methyl sulphoxide (5 ml). The suspension was kept at room temperature in a nitrogen atmosphere for 6 h. Methyl iodide (4 ml) was then added with cooling. The resulting solution was stirred for 4 h, poured into water (50 ml), dialyzed for 2 days against water, and concentrated to dryness.

Hydrolysis of the methylated galactan. — The methylated polysaccharide (180 mg) in 0.25M sulphuric acid (10 ml) was heated for 10 h at 100°. Paper chromatography of the neutralized hydrolysate indicated the presence of di-, tri-, and tetra-*O*-methylgalactoses with R_{Gal} 0.27, 0.42, 0.63, and 1.0 [solvent (*f*)]. The mixture of methylated sugars was fractionated by preparative t.l.c. The corresponding methyl glycosides, prepared with 3% methanolic hydrogen chloride for 8 h at 100°, were identified by mass spectrometry (see Table II).

Methyl 2,3,4,6-tetra-*O*-methyl-αβ-D-galactopyranoside: the mass spectrum of this substance was identical with that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-galactopyranoside[14], thereby establishing the substitution pattern.

Methyl 2,3,4-tri-*O*-methyl-αβ-D-galactopyranoside gave peaks of relatively high intensity at *m/e* 101, 88, and 75. The appearance of low-intensity peaks at *m/e* 87 and 74, in addition to the absence of peaks of ions $(M-45)^+$ and $(M-45-32)^+$ at *m/e* 191 and 159, proved[15] the location of methoxyl groups at C-2, C-3, and C-4.

Methyl 2,3,6-tri-*O*-methyl-αβ-D-galacto-pyranoside and -furanoside: the splitting off of the glycosidic radical gives rise to the ion $(M-31)^+$ with *m/e* 205, which further decomposes by eliminating methanol and produces ions with *m/e* 173 and 141, establishing the parent compound as a tri-*O*-methyl derivative. The peaks of ions $(M-45)^+$ and $(M-45-32)^+$ at *m/e* 191 and 159 confirmed the location of a methoxyl group at C-6. The low-intensity peaks at *m/e* 102, 87, 74, and 71, in addition to peaks at *m/e* 101, 88, and 75, proved the presence of methoxyl groups at C-2 and C-3. Although an ion with *m/e* 161 is also formed in the course of fragmentation of methyl 2,3,6-tri-*O*-methyl-αβ-D-glucopyranoside[15], the high-intensity peak of ions with *m/e* 101 proves that the furanoid form is also present[16].

Methyl 2,6-di-*O*-methyl-αβ-D-galacto-pyranoside and -furanoside: the ion $(M-31)^+$ with *m/e* 191 forms an ion with *m/e* 173 after dehydration. The ion $(M-45)^+$ with *m/e* 177 produces ions with *m/e* 159 and 145 after elimination of water or methanol, thereby establishing the presence of a methoxyl group at C-6. The peak at *m/e* 88, and the low-intensity peaks at *m/e* 102, 75, and 71, in addition to the intense peaks at *m/e* 87 and 74, prove the substitution at C-2. The fact that the peak at *m/e* 87 is the most intense in the spectrum and the appearance of an ion at *m/e* 147 prove that the furanoid form is also present.

For quantitative analysis, the methylated galactan (2 mg) was treated with 3% methanolic hydrogen chloride (0.1 ml) for 12 h at 100°, and the products were analyzed by g.l.c. [column (*b*)]. The results are given in Table III.

ACKNOWLEDGMENTS

The authors thank Prof. J. E. Courtois, Faculté de Pharmacie, Université de Paris, for the gift of α-D-galactosidase, and Dr. D. Anderle for the g.l.c. analysis.

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