ISOLATION AND STRUCTURAL CHARACTERISTICS OF NEUTRAL POLYSACCHARIDES FROM WHITE WILLOW (*Salix alba* L.)

Š.KARÁCSONYI and M.PAŠTEKA

Institute of Chemistry,
Slovak Academy of Sciences, 809 33 Bratislava

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Neutral polysaccharides from white willow (*Salix alba* L.) were fractionated and two electrophoretically homogeneous polysaccharides were isolated and structurally characterised. Glucomannan, $[\alpha]_D$ −16·4°, with the D-glucose to D-mannose ratio of 1 : 1·4, consists of a linear chain of about 20 β-D-glucopyranose and β-D-mannopyranose units mutually joined by the 1 → 4 glycosidic linkage. α-Glucan, $[\alpha]_D$ +157°, of the phytoglycogen type, exhibits the average length of polysaccharide chains equal to 9·1 and the average length of internal chains equal to 5·1 glucose units.

The structure of main components of wood hemicelluloses has been determined except for some details[1]. In addition to the hitherto identified polysaccharides, trace amounts of some further polysaccharides have been detected in some woods. Thus, from suspension-cultured sycamore cells[2] (*Acer pseudoplatanus* L.) there have been isolated the extracellular polysaccharides fucoxyloglucan and arabinogalactan (originally assumed to occur in coniferous woods only) and subjected to the structural characterization. From bark of the Engelmann spruce (*Picea engelmanni* PARRY)[3], xyloglucan has been isolated while galactoxyloglucan has been found in compression wood of the red spruce (*P. rubens* SARG.)[4]. The detailed structure of these polysaccharides has so far not been reported. In the present paper, we describe the fractionation process and structural characterization of polysaccharides occurring in wood of the white willow (*Salix alba* L.).

The alkaline extraction of hemicelluloses from the holocellulose and isolation of a part of 4-O-methylglucuronoxylans was performed by the reported procedure[5]. The non-precipitated portion of hemicelluloses, the polysaccharides *I* (3% of the wood weight), $[\alpha]_D$ −42·8°, consisted of D-galactose, D-glucose, D-mannose, L-arabinose, and D-xylose in the molar ratio 1·8 : 3·6 : 0·9 : 1·0 : 26·0, uronic acids, and a trace of L-rhamnose. The polysaccharides *I* were dissolved in water and precipitated with saturated aqueous barium hydroxide[6].

The polysaccharides *II* (yield, 1%), $[\alpha]_D$ +48·8°, were fractionated by means of preparative chromatography on a DEAE-Sephadex A-50 column (Table I). The thus-ob-

tained polysaccharide A was then subjected to an additional chromatography on a column of DEAE-cellulose in the borate form[7] (Fig. 1). Fractions 1 and 2 contained pure α-glucan, $\bar{P}n$ 25. Hydrolysates of fractions 3, 4, and 5 contained D-glucose, D-xylose, D-mannose, and traces of D-galactose. To remove a the α-glucan, fractions 3–5 were pooled and treated with crystalline α-amylase. The thus-obtained polysaccharides, $[\alpha]_D$ +7·7°, were dissolved in water and the aqueous solution was precipitated with saturated aqueous barium hydroxide to afford glucomannan consisting of D-glucose and D-mannose in the molar ratio 1 : 1·4, $\bar{P}n$ 19.

In the partial hydrolysate of α-glucan there was identified by gas liquid chromatography D-glucose, maltose, and isomaltose. The D-glucose O-methyl ethers obtained by hydrolysis of the methylated polysaccharide, were identified as 2,3,4,6-tetra-O-methyl-D-glucose (10·9 mol%), 2,3,6-tri-O-methyl-D-glucose (77·0%), and 2,3-di-O-methyl-D-glucose (12·1%). The average length of polysaccharidic chains equal to 9·1 glucose units as calculated from number-average degree of polymerization and the periodate oxidation data, was in accordance with results of the methylation analysis. By the action of crystalline β-amylase, 10·5% of α-glucan was degraded to maltose. This value in combination with results of the periodate oxidation corresponds to the average length of internal chains equal to 5·1 and to the average length of external chains equal to 3·0 glucose units. The thus-determined α-glucan branching relating to every fifth glucose unit is different from branching of amylopectins or phytoglycogens of higher plants[8,9].

The R_{cel} and R_T values (paper chromatography and gas liquid chromatography) of oligosaccharides obtained by the partial hydrolysis of glucomannan were identical with those of 4-O-β-D-glucopyranosyl-D-mannose, 4-O-β-D-glucopyranosyl-D-glucose, and

TABLE I
Fractionation of the Polysaccharides on a DEAE-Sephadex A-50 Column

Fraction	Eluant	$[\alpha]_D$	mol %					Uronic acids
			Gal	Glc	Man	Ara	Xyl	
Polysaccharides *II*	—	+48·8°	9·5	38·9	14·3	1·7	35·6	+
A	0·02M-CH_3COONa	+98·7°	[a]	68·8	20·0	—	8·2	—
B	0·05M-CH_3COONa	+51·1°	1·9	49·7	16·0	—	32·5	+
C	0·1M-CH_3COONa	−21·9°	6·3	20·4	10·2	—	63·1	+
D	0·3M-CH_3COONa	+13·2°	22·5	17·7	11·6	4·4	43·8	+
E	0·5M-CH_3COONa	+ 7·9°	29·0	22·7	13·6	5·5	29·2	+
F	0·1M-NaOH	− 6·6°	12·5	24·0	7·7	3·7	52·1	+

[a] Trace.

TABLE II

Molar Ratio of Components in the Methylated Glucomannan Hydrolysate

Methyl ether	Mol %	Retention times (R_T) on columns[b]			
		B		C	
2,3,4,6-Me_4-Glucose[a]	3·5	1·00	1·36 s	1·00	1·27 s
2,3,4,6-Me_4-Mannose	1·4	1·19	—	1·12	—
2,3,6-Me_3-Glucose	39·0	2·23	4·21 s	1·95	2·07 w
				2·24 s	
2,3,6-Me_3-Mannose	56·1	4·23	—	2·34	—

[a] 2,3,4,6-Tetra-O-methyl-D-glucose. [b] Referred to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside. Peaks correspond to α and β anomers of methyl glycosides and their separation under experimental conditions. Relative peak intensities; s, strong; w, weak.

4-O-β-D-mannopyranosyl-D-glucose[10]. The hydrolysate of the methylated polysaccharide contained a mixture of methylated sugars (for the evaluation see Table II). The products of the Smith degradation, glycerol and erythritol (molar ratio 1 : 17), were identified on the basis of authentic specimens; glucitol and mannitol were not identified. The predominating amount of 2,3,6-tri-O-methyl-D-glucose and 2,3,6-tri-O-methyl-D-mannose in the hydrolysate of the methylated glucomannan and the products of the Smith degradation of the polysaccharide confirm the 1 → 4 glycosidic linkage between the hexose units; the identified disaccharides prove the β-configuration of this linkage. The linearity of polysaccharide chains may be inferred from the molar ratio of products of the methylation analysis and the Smith degradation. As shown by comparison of structures of this polysaccharide and that isolated from the same wood under different conditions[10], the glucomannans of white willow are heterogeneous with respect to composition, size, and shape of the macromolecules.

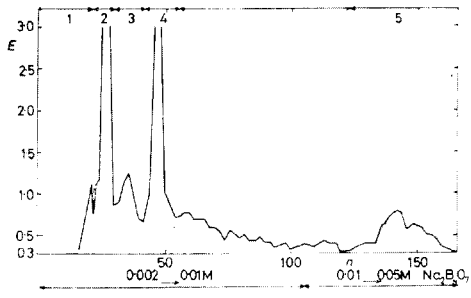

FIG. 1

Fractionation of the Polysaccharide A on DEAE-Cellulose in the Borate Form

EXPERIMENTAL

General Methods

Solutions were evaporated under diminished pressure at temperatures bellow 40°C. Paper chromatography was performed by the descending technique on papers Whatman No 1 and 3 MM in solvent systems S_1, ethyl acetate–pyridine–water (8 : 2 : 1); S_2, 1-butanol–ethanol–water (10 : 3 : 3); S_3, 1-butanol–ethanol–water (4 : 1 : 5); S_4, 1-butanol–water–aqueous ammonia (90 : 8 : 2); and S_5, acetone–water–1-butanol (7 : 2 : 1). Aniline hydrogen phthalate and ammoniacal silver nitrate were used as detecting agents. Sugars were determined by gas chromatography as trifluoroacetyl derivatives of their alditols[11] on column A (see bellow). Free-boundary electrophoresis was performed on a Zeiss 35 apparatus at 150 V, 8·2 mA, 30 min, and the concentration of polysaccharides 10 mg/ml in E_1, 0·05M borate buffer (pH 9·2), and E_2, 0·2M acetate buffer (pH 6·8). Gas chromatography was carried out on a Hewlett-Packard Model 5750 G apparatus equipped with a two-column FID system, on the following columns: A 1% XE-60 on Gas-Chrom. Z (80–100 mesh), 305 × 0·3 cm column, 13 ml He per min, 130–150°C/1°C / / min; B 5% BDS on Gas-Chrom. Z (80–100 mesh), 183 × 0·3 cm column, 20 ml N_2 per min, 170°C; C 3% ECNSS-M on Chromaton-N AW-HMDS (80–100 mesh), 183 × 0·3 cm column, 18 ml N_2 per min, 130–210°C/7°C/min; D 10% UC-W-98 on Chromosorb WAW (80–100 mesh) 183 × 0·3 cm column, 35 ml N_2 per min, 120–280°C/4°C/min; E 5% SE-30 on Chromosorb WAW (70–80 mesh), 122 × 0·25 cm column, 30 ml N_2/min, 210°C. The R_G and R_{Cel} values refer to the mobility of 2,3,4,6-tetra-O-methyl-D-glucose and cellobiose. The R_T retention times of methylated methyl glycosides refer to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside.

Molecular weight determinations were performed on a Knauer osmometer (sensitivity, 128) at 37°C. The calibration curve was made for Dextran T 10 (Pharmacia, Uppsala, Sweden) $\overline{M}n$ 5700 as the standard. Optical rotations were measured in aqueous solutions at room temperature (20–22°C).

Extraction and Fractionation of Polysaccharides

The alkaline extraction of hemicelluloses from the holocellulose of *S. alba* L. and the partial precipitation of 4-O-methylglucuronoxylans with cetyltrimethylammonium bromide was performed by the reported procedure[5]. The non-precipitated portion of polysaccharides was processed as usual to afford the polysaccharides *I* (yield, 3% by weight), $[\alpha]_D$ −42·8° (*c* 1·0). Analysis: OCH_3 2·5%, $\overline{M}n$ 12800. As shown by paper chromatography in the solvent system S_1, the hydrolysate consisted (in mol%) of D-galactose (5·4), D-glucose (10·7), D-mannose (2·8), L-arabinose (3·0), D-xylose (78·2), and traces of L-rhamnose and uronic acids.

The polysaccharides *I* (60·0 g) were dissolved in water (1 000 ml), the aqueous solutions precipitated with saturated aqueous barium hydroxide, the precipitate collected by centrifugation, and washed by a dilute solution of the precipitating agent[6]. The precipitate was dissolved in 2M acetic acid (300 ml), the solution deionized with Ionenaustauscher V (E. Merck, Darmstadt, Federal Republic Germany) mixed ion exchange resin, and freeze-dried to yield 15·1 g of polysaccharides *II*, $[\alpha]_D$ +48·8° (*c* 1·0). Analysis: OCH_3 1·6%; $\overline{M}n$ 7250. In the hydrolysate, the following compounds were identified by paper chromatography in S_1: D-galactose (9·5 mol%), D-glucose (38·9), D-mannose (14·3), L-arabinose (1·7), D-xylose (34·8), and traces of L-rhamnose and uronic acids. The free-boundary electrophoresis in E_1 and E_2 indicated heterogeneity and gel chromatography on Sephadex G-75 showed polymolecularity in the range $\overline{M}n$ 3 . 10^3 to 1·5 . 10^4.

The polysaccharides *II* (1·5 g) were dissolved in 0·02M sodium acetate (8 ml) and the solution was applied to a column of DEAE-Sephadex A-50 (42 × 3·5 cm) in the acetate form. The elu-

tion was performed successively with 0·02M, 0·05M, 0·1M, 0·3M, and 0·5M solutions of sodium acetate or with 0·1M aqueous sodium hydroxide. For the composition of particular fractions see Table I. This process was repeated several times and the identical fractions were combined.

The polysaccharide A (2·4 g), $[\alpha]_D$ +98·7° (*c* 1·0), proved to be (free electrophoresis in E_1 an E_2) a mixture of neutral polysaccharides consisting of D-glucose, D-mannose, D-xylose, and traces of D-galactose. The polysaccharide A (2·4 g) was applied to a column (42 × 3·5 cm) of DEAE-cellulose in the borate form[7]; the elution was performed with a gradient of sodium tetraborate solutions 0·002M → 0·01M and 0·01M → 0·05M (2·5 l) and finally with a solution of 0·05M hydrochloric acid and sodium chloride (0·5 l), 15-ml fractions being taken. The concentration of polysaccharides was determined by the phenol test[12] (Fig. 1). After hydrolysis, fractions 1 and 2 contained only D-glucose while D-glucose, D-mannose, D-xylose, and a lesser amount of D-galactose was present in fractions 3, 4, and 5. The obtained α-glucan (280 mg), $[\alpha]_D$ +157·4° (*c* 1·0), was homogeneous under conditions of free-boundary electrophoresis; $u = 1{\cdot}93 \cdot 10^{-5}$ . . $cm^2\ V^{-1}\ s^{-1}$. The observed $\bar{M}n$ 4100 corresponds to $\bar{P}n$ 25. Fractions 3, 4, and 5 were combined (2·05 g), dissolved in 240 ml of a 0·2M phosphate buffer solution (pH 6·9), and subjected to the action (20 h at 20°C) of α-amylase (*Bacillus subtilis*; 2 mg). The thus-obtained polysaccharides, $[\alpha]_D$ +7·7°, were dissolved in water (110 ml), and the solution was precipitated as above with saturated aqueous barium hydroxide to afford 250 mg of glucomannan, $[\alpha]_D$ −16·4°, containing D-glucose and D-mannose in the molar ratio 1 : 1·4. The free-boundary electrophoresis in E_1 indicated a homogeneous substance with the mobility $u = 4{\cdot}66 \cdot 10^{-5}\ cm^2\ V^{-1}\ s^{-1}$. The observed molecular weight $\bar{M}n$ 3100 corresponds to $\bar{P}n$ 19.

Glucomannan

Methylation. Glucomannan (42·6 mg) in dimethyl sulfoxide (3 ml) was treated with the reagent prepared from sodium hydride (200 mg) and dimethyl sulfoxide (4 ml), ref.[13]. The mixture was stirred under nitrogen at 20°C for 7 h, treated dropwise with methyl iodide (2 ml), stirred for additional 2 h, poured into water (30 ml), dialysed overnight, and evaporated. The residue was hydrolysed at 100°C with 0·25M-H_2SO_4 (5 ml), neutralised with Dowex 1X4 ion exchange resin, and the filtrate evaporated. Paper chromatography of the residue in S_3 indicated the presence of tetramethyl- and trimethylhexoses (R_G 1·00 and 0·86), the molar ratio (1 : 19·8) of which was determined by gas chromatography on the column B. The R_T values of methyl glycosides (3% methanolic hydrogen chloride, 100°C, 8 h) of methylated sugars were identical (columns B and C) with those of methyl glycosides of 2,3,4,6-tetra-O-methyl-D-glucose, 2,3,4,6-tetra-O-methyl-D-mannose, 2,3,6-tri-O-methyl-D-glucose, and 2,3,6-tri-O-methyl-D-mannose. In S_4, there was quantitatively determined the molar ratio of 2,3,6-tri-O-methyl-D-glucose to 2,3,6-tri-O-methyl-D-mannose as 1 : 1·4. Demethylation of tetramethylhexoses with borontrichloride[14] afforded a mixture of D-glucose and D-mannose in the molar ratio 2·5 : 1. For the detailed results see Table II.

Partial hydrolysis. Glucomannan (150 mg) was heated at 100°C with 45% aqueous formic acid (6 ml) for 3 h, the mixture evaporated, the formyl groups hydrolysed (10 min at 100°C) with 0·25M-H_2SO_4 (3 ml), the mixture neutralized with barium carbonate, and the barium sulfate removed by centrifugation. The filtrate was deionized with Dowex 50 W ion exchange resin and subjected to paper chromatography in S_2 to show the presence of D-glucose, D-mannose, and oligosaccharides with R_{cel} 1·50, 1·00, and 0·65. After the preparative separation, their trimethylsilyl derivatives[15] exhibited (column E) R_T values identical with those of the disaccharides 4-O-β-D-glucopyranosyl-D-mannose (0·70—1·10), 4-O-β-D-glucopyranosyl-D-glucose (1·00—1·35), and 4-O-β-D-mannopyranosyl-D-glucose (1·02—1·25)[10].

Periodate oxidation. Glucomannan (10·5 mg) was oxidized at 5°C in the dark with 0·015M sodium metaperiodate (10 ml). The periodate uptake was checked spectrophotometrically at 225 nm[16]. The constant uptake was 1·03 mol IO_4^- per hexose unit. When the oxidation was complete, the solution was deionized, and treated with sodium borohydride (20 mg). The solution was neutralized and the glycol aldehyde (obtained by hydrolysis) reduced with sodium borohydride (10 mg). After deionisation, the products of the Smith degradation, glycerol and erythritol, were identified and quantitatively evaluated (column D) as trimethylsilyl derivatives on comparison with standards. The molar ratio of glycerol to erythritol was 1 : 17 (average of 3 determinations). Glucitol or mannitol were not identified.

α-Glucan

Methylation. α-Glucan (30 mg) was methylated analogously to glucomannan. The hydrolysate of the methylated polysaccharide was analyzed by paper chromatography in S_3 while the products of methanolysis were determined by gas chromatography (columns B and C). On comparison with standards, the following compounds were identified: 2,3-di-O-methyl-D-glucose (R_T 2·96—3·27; C); 2,3,6-tri-O-methyl-D-glucose (3·36—4·55; B), (1·97—2·22; C); 2,3,4,6-tetra-O-methyl-D-glucose (1·00—1·45; B), (1·00—1·27; C). The molar ratio of di-, tri-, and tetramethylglucoses was determined as 1·1 : 7·0 : 1·0.

Partial hydrolysis. α-Glucan (150 mg) was hydrolyzed (60 min at 100°C) with 0·25M-H_2SO_4 (5 ml). After neutralisation with Ionenaustauscher II (E. Merck) ion exchange resin, the hydrolysate contained D-glucose and oligosaccharides which were separated by preparative paper chromatography in S_5. From disaccharides *I* (30 mg; R_{Gluc} 0·30) and *II* (3 mg; R_{Gluc} 0·17) there were prepared trimethylsilyl derivatives, the R_T values of which were identical (column E) with maltose and isomaltose.

Periodate oxidation. α-Glucan (13·0 mg) was oxidized with 0·015M sodium metaperiodate (15 ml). After 96 h, the constant uptake was 1·20 mol IO_4^- per mol of glucose. A portion of the solution (4 ml) was treated with ethylene glycol (0·2 ml) and the formic acid was titrated with 0·01M-NaOH (bromocresol Red). Found: 0·19 mol of formic acid per mol of glucose unit. After the oxidation and treatment with sodium borohydride, the acidic hydrolysate of the deionized residue contained glycerol and erythritol only.

Action of β-amylase. The experiment was performed at 30°C using a 4% concentration of α-glucan in 30 mM sodium acetate–acetic acid (pH 4·8), 0·05% serum albumin, and β-amylase in concentration of 1 mg/ml. The course of the degradation was checked by dinitrosalicylic acid[17]. The average length of external and internal chains was calculated according to Manners[18].

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