POLYSACCHARIDES OF THE WHITE WILLOW BARK (*Salix alba* L.). THE STRUCTURE OF GLUCOMANNAN I

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Received September 20th, 1971

From the bark of young twigs of white willow (*Salix alba* L.) glucomannan was isolated, $[\alpha]_D$ −6·6°, $\overline{M}n$ 5280, in which the ratio of D-glucose and D-mannose was 1 : 1·4. It is supposed that the isolated glucomannan is composed of a linear chain of approximately 32 units of β-D-glucopyranoses and β-D-mannopyranoses linked by 1 → 4 glycosidic bonds.

In spite of extended investigations carried out in the field of the chemistry of wood hemicelluloses[1] the papers dealing with the structural characteristics of polysaccharides of wood bark are scarce. The wood and the bark have differing morphological structure and chemical composition what follows from their different function in the tree[2]. For this reason differences between the polysaccharides occuring as components of their cellular membranes[3,4] may be supposed. In the course of the investigation of the polysaccharides of the wood and the bark of the white willow (*Salix alba* L.) we want to describe in this paper the isolation and the structural characteristics of water soluble glucomannan of the mentioned bark.

The extracted saw-dust was delignified by a modification of Wise's method and the holocellulose obtained (89% per bark weight) was submitted to gradual extraction[5]. The mixture of polysaccharides extracted from holocellulose with 8% NaOH additioned with 4% of boric acid (4·8% per holocellulose weight) had $[\alpha]_D$ +3·4°. After hydrolysis it gave D-galactose, D-glucose, D-mannose, L-arabinose, and D-xylose in a 1·0 : 4·1 : 4·2 : 0·1 : 0·6 molar ratio, and traces of uronic acids. Fractionation of the mixture of polysaccharides was carried out by preparative chromatography on microcrystalline DEAE-cellulose[6]. The results of the fractionation are summarised in Table I. After dissolution in water polysaccharide A was fractionated by precipitation with a saturated barium hydroxide solution[7]. The precipitated polysaccharide, $[\alpha]_D$ −6·6° (1·57% of the holocellulose weight) was homogeneous under the conditions of free-boundary electrophoresis, and its mobility in the electrostatic field was $u = 4{\cdot}25 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}. It consisted of D-glucose and D-mannose in a 1 : 1·4 molar ratio. The molecular weight of the polysaccharide was $\overline{M}n$ 5280, which corresponds to an average polymerization degree $\overline{P}n$ 32.

On partial hydrolysis of glucomannan a series of disaccharides was obtained: 4-O-β-D-mannopyranosyl-D-mannose, 4-O-β-D-glucopyranosyl-D-glucose, 4-O-β-D-mannopyranosyl-D-glucose, and 4-O-β-D-glucopyranosyl-D-mannose. Identification was carried out on the basis of their mobility in paper or gas chromatography (comparison with standards[8]) and from the products of enzymatic hydrolysis with emulsin. A part of glucomannan was methylated with methyl iodide

in the presence of methylsulfinylmethyl carbanion[9]. Hydrolysis of the methylated polysaccharide gave a mixture of methylated sugars the qualitative and quantitative evaluation of which is given in Table II. Glucomannan was treated with sodium metaperiodate and 1·08 mol of IO_4^- was consumed per one hexose unit. The products of Smith's degradation[10], glycerol and erythritol, were identified and quantitatively estimated by gas chromatography. The molar ratio of glycerol and erythritol was 1 : 27. D-glucitol and D-mannitol were not identified.

The overwhelming amount of 2,3,6-tri-O-methyl-D-glucopyranose and 2,3,6-tri-O-methyl-D-mannopyranose in the hydrolysate of the methylated polysaccharide indicates that D-glucose and D-mannose units are bound in the polysaccharide chain by 1→4 glycosidic linkages. The effect of emulsin on isolated disaccharides shows that

TABLE I

Fractionation of the Mixture of Polysaccharides on DEAE-Cellulose Column

Fraction	Eluent	Yield, %	$[\alpha]_D$	Molar ratios of monosaccharides				
				gal.	gluc.	mann.	arab.	xyl.
Mixture	—	100	+ 3·4°	1·0	4·1	4·2	0·1	0·6
A	water	58·6	+ 7·7°	1·0	5·3	5·4	—	—
B	0·05M–CH_3COOK	3·8	+15·5°	1·0	2·5	2·1	—	tr
C	0·5M–CH_3COOK	1·8	+ 8·2°	1·0	2·8	2·3	tr	tr
D	0·1M–NaOH	32·3	− 2·6°[a]	1·0	2·3	2·4	0·4	2·0
E	0·4M–NaOH	2·4	+ 4·6°[a]	1·0	3·4	2·1	tr	0·2

[a] Specific rotations were measured in 5% NaOH.

TABLE II

Methylated Sugars in the Hydrolysate of Methylated Glucomannan

Sugars	Molar ratio	*B*[a]		*C*[a]	
Di-O-methylhexoses	1·6	—	—	2·96	3·27 s
2,3,6-Me_3-Gluc[b]	11·1	3·23	4·30 s	1·96	2·07 w
				2·24 s	
2,3,6-Me_3-Mann	15·5	3·96	—	2·34	—
2,3,4,6-Me_4-Gluc	1·0	1·00	1·36 s	1·00	1·27 s

[a] Retention values of methyl glycosides on columns, with respect to methyl 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside; the peaks correspond to α and β anomers if separated under the given conditions; Relative intensities of peaks: s strong, w weak; [b] 2,3,6-Tri-O-methyl-D-glucose.

the sugar residues are linked by β-glycosidic bonds. The presence of 2,3,4,6-tetra-O-methyl-D-glucopyranose indicates that non-reducing terminal units of the polysaccharide chain are composed of D-glucose exclusively. The identified linkages in the polysaccharide, the absence of D-glucitol or D-mannitol in the products of Smith's degradation, and also the molar ratio of glycerol and erythritol 1 : 27 corroborate the linearity of the isolated glucomannan. The occurrence of di-O-methylhexoses in a 5·4 molar percent concentration may result from incomplete methylation of the polysaccharide, and therefore we do not attach any structural importance to it.

On the basis of the mentioned results it may be supposed that the isolated glucomannan forms a linear chain composed of β-D-glucopyranose and β-D-mannopyranose units in a 1 : 1·4 ratio, mutually bound by 1→4 glycosidic linkages. Similar, though not identical, structural features occur in glucomannans isolated from the wood of the beech (*Fagus grandifolia*), birch (*Betula papyrifera* and *B. lutea*)[11], and willow-tree (*Salix alba* L.)[8], which differ mutually in the ratio of D-glucose to D-mannose, or also in the length and the branching of the polysaccharide chain.

EXPERIMENTAL

Optical rotations were determined at room temperature in aqueous solutions. unless stated otherwise. The solutions were evaporated under reduced pressure at 40°C.

Apparatus and methods. Chromatographic separations were carried out on Whatman No 1 and No 3 MM papers (the latter were used for preparative purposes) using descending technique in the following solvent mixtures: S_1 ethyl acetate–pyridine–water 8 : 2 : 1, S_2 n-butanol–pyridine–water 10 : 3 : 3, S_3 butanone–water–ammonia 90 : 8 : 2, S_4 n-butanol–ethanol–water 4 : 1 : 5. Anilinium hydrogen phthalate[12] was used for detection. R_G and R_{Cel} represent relative mobilities related to 2,3,4,6-tetra-O-methyl-D-glucose and cellobiose. For quantitative evaluation of sugars gas chromatography of trifluoroacetyl derivatives of their alditols[13] on column A was applied. The hydrolyses of polysaccharides were carried out with 72% H_2SO_4. Free-boundary electrophoresis was carried out in a solution of 0·05M borate buffer (pH 9·2), at a polysaccharide concentration of 10 mg/ml at 150 V and 8 mA for 30 minutes (apparatus Zeiss 35). Gas chromatography was carried out on a Hewlett-Packard 5750 G model, the following columns were employed: *A*) 1% XE-60 on Gas-Chrom. Z. (80—100 mesh) on a 305 × 0·3 cm column, flow rate 13 ml of He/min, temperature programme 130—150°C/1°C/min; *B*) 5% BDS on Gas-Chrom. (80—100 mesh) on a 183 . 0·3 cm column, 20 ml N_2/min, 170°C; *C*) 3% ECNSS-M on Chromaton-NAW DMCS (80—100 mesh) on a 183 . 0·3 cm column, 18 ml N_2/min, 130—210°C/2°C/min; *D*) 10% UC-W-98 on Chromosorb WAW (80—100 mesh) on a 183 × 0·3 cm column, 35 ml N_2/min, 120—280°C/4°C/min; *E*) 5% SE-30 on Chromosorb WAW (70—80 mesh) on a 122 × 0·25 cm column, 30 ml N_2/min, 210°C. The molecular weight determination was carried out with a Knauer osmometer at 128 sensitivity and 37°C, using a universal probe (25—60°C). The calibration curve was constructed for Dextran T 10 (Uppsala) $\overline{Mn}$ 5700 as standard.

Isolation of Glucomannan

Holocellulose of the bark of young twigs of willow (*Salix alba* L.) was extracted according to the procedure devised by Pavlovová and coworkers[5]. The mixture of polysaccharides obtained by extraction with 8% NaOH and 4% boric acid (4·8% per holocellulose weight) had $[\alpha]_D$ +3·4°

(*c* 1·0) and after hydrolysis it gave D-galactose, D-glucose, D-mannose, L-arabinose, and D-xylose in molar ratio 1·0 : 4·1 : 4·2 : 0·1 : 0·6 and traces of uronic acids. The polysaccharide (1·53 g) was dissolved in water (15 ml) and put on a DEAE-cellulose column (70 . 2·5 cm) having an exchange capacity of 1·76 meq/g in phosphate form[6]. Elution was carried out gradually by water, 0·05M and 0·5M potassium acetate solution, 0·1M and 0·4M-NaOH (Table I). The fractions were 15 ml each, and flow rate was 1·5 ml/min. The presence and the concentration of polysaccharides were determined by phenol-test[14]. Fractions were recovered by freeze-drying in the case of B,C,D,E after deionisation (Ionenaustaucher I E. Merck in H^+ form, Dowex 1 × 4 in OH^- form). Polysaccharide A (0·88 g), $[\alpha]_D$ +7·7° (*c* 1·0) was dissolved in 40 ml of water and a saturated barium hydroxide solution was added dropwise until precipitation stopped[7]. The precipitate was centrifuged, washed several times with 3% barium hydroxide and dissolved in 10 ml of 2M acetic acid. After deionisation with a mixed bed ion exchanger (Ionenaustauscher V, Merck) the polysaccharide was obtained by freeze-drying. This procedure was repeated three times. The polysaccharide obtained (0·5 g, 1·57% of the holocellulose weight) had $[\alpha]_D$ −6·6° (*c* 1·0) and contained D-glucose and D-mannose in a 1 : 1·4 molar ratio. Free boundary electrophoresis indicated that the polysaccharide of the mobility $u = 4{\cdot}25 \,.\, 10^{-5}$ cm^2 V^{-1} s^{-1} in the electrostatic field was homogeneous. Its degree of polymerization was $\bar{P}n$ 32.

Partial Hydrolysis of Glucomannan

Glucomannan (150 mg) was dissolved in 5 ml of 98% formic acid and after dilution to a 45% concentration the solution was heated at 100°C for 2 h. The hydrolysate was evaporated to dryness then hydrolysed in 0·5N-H_2SO_4 at 100°C for 10 minutes. The solution was neutralised with a weakly basic ion exchanger (Ionenaustauscher II, Merck, in OH^- form). Paper chromatography in S_2 demonstrated the presence of D-glucose, D-mannose, and a series of disaccharides which were isolated preparatively.

4-O-β-D-*Glucopyranosyl*-D-*mannose*. The disaccharide (1 mg) having R_{Cel} 1·50 in solvent mixture S_2 was dissolved in phosphate buffer (2 ml, pH 7), and after the addition of emulsin, allowed to stand at room temperature for 10 h. After deionisation with mixed bed ion exchanger (Ionenaustauscher, Merck) paper chromatography in S_1 indicated the presence of D-glucose and D-mannose.

4-O-β-D-*Glucopyranosyl*-D-*glucose and* 4-O-β-D-*mannopyranosyl*-D-*mannose*. A mixture of disaccarides with R_{Cel} 1·0 in S_2 was submitted to enzymatic hydrolysis with emulsin under the same conditions as in the preceding case. Using paper chromatography in S_2 in addition to D-glucose, disaccharide with R_{Cel} 1·05 could also be identified, which remained unchanged by emulsin (of which it is known that it has only α-mannosidase activity)[15] and which after acid hydrolysis gave only D-mannose.

4-O-β-D-*Mannopyranosyl*-D-*glucose*. The disaccharide with R_{Cel} 0·65 in S_2 was stable against emulsin and after acid hydrolysis it gave D-glucose and D-mannose. The identity of the isolated disaccharides mentioned above was also corroborated on the basis of their mobility on paper chromatograms, using cellobiose as standard, or by gas chromatographic analysis of their trimethylsilyl derivatives[16] on column E, using authentic samples as standards[8].

Methylation of Glucomannan

Glucomannan (36 mg) in 5 ml of dimethyl sulfoxide was added dropwise to a solution of sodium hydride (300 mg) in 5 ml of dimethyl sulfoxide[9]. The mixture was stirred at room temperature for 8 h. Methyl iodide (4 ml) was then added at a temperature below 30°C. The reaction mixture

was allowed to stand for 4 h and then poured into 50 ml of water and dialysed in distilled water for 2 days. Eventually it was evaporated to dryness. Hydrolysis of methylated glucomannan was carried out in 72% H_2SO_4. Paper chromatography of the hydrolysate of the methylated glucomannan in S_4 indicated the presence of di-, tri-, and tetramethylhexoses with R_G 0·63, 0·80, and 1·0, which were analysed qualitatively and quantitatively by a combination of gas and paper chromatography. After transformation to methyl glycosids (3% methanolic hydrogen chloride, 100°C, 8 h) they gave on gas chromatography, columns B and C, R_T values identical to corresponding anomers of methyl glycosides of 2,3,4,6-tetra-O-methyl-D-glucopyranose, 2,3,6-tri-O-methyl-D-glucopyranose, and 2,3,6-tri-O-methyl-D-mannopyranose. Quantitative evaluation indicated the molar ratio of di-, tri-, and tetramethylhexoses to be 1·6 : 26·6 : 1. According to paper chromatography in S_3 the molar proportion of 2,3,6-tri-O-methyl-D-glucopyranose (R_G 0·72) and 2,3,6-tri-O-methyl-D-mannopyranose (R_G 0·66) was 1 : 1·4. Of the dimethylhexoses present only 2,3-di-O-methyl-D-glucopyranose (R_T 2·96–3·27 on column C) was identified. After demethylation with boron trichloride[17] D-glucose and D-mannose were obtained in a 1 : 2 molar ratio. The results of methylation analysis are summarised in Table II.

Degradation of Glucomannan

Glucomannan (9 mg) was oxidised in 10 ml of 0·015M sodium metaperiodate in the dark at 5°C. Aliquots (0·1 ml) were withdrawn at defined intervals and the consumption of IO_4^- was determined spectrophotometrically at 225 nm[18]. After 168 hours the constant consumption was 1·08 mol per one hexose unit. The excess periodate was precipitated with 2·5 ml of 0·03M lead acetate. The precipitate was filtered off, the solution evaporated to 5 ml and 15 mg of sodium borohydride was added. After 4 h Na^+ ions were eliminated with a cation exchanger (Ionenaustauscher I, Merck, in H^+ form), and the boric acid formed was removed, after transformation to its methyl ester, with methanol by repeated evaporation to dryness. The dry residue was hydrolysed at room temperature with 4 ml of 0·5N-H_2SO_4 for 8 h. After neutralisation with a weakly basic anion exchanger (Ionenaustauscher II, Merck, in OH^- form) 5 ml of the solution were repeatedly reduced with 15 mg of sodium borohydride for 4 h, in order to reduce the glycol aldehyde obtained by hydrolysis to ethylene glycol (in view of equal R_T values of glycolaldehyde and ethylene glycol under the conditions of the analysis)[19]. After deionisation the products of Smith degradation[10] were identified and determined quantitatively by gas chromatography in the form of their trimethylsilyl derivatives[16] on column D, in comparison with authentic samples of glycerol and erythritol. The molar ratio of glycerol and erythritol determined was 1 : 27 (average of two determinations). D-glucitol or D-mannitol were not identified.

The authors thank Mr G. Košický for electrophoretic measurements as well as for the determination of molecular weights.

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Translated by Ž. Procházka.