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Studies on Glucomannans from Norwegian Spruce

II. Structural Investigations

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The structures of two glucomannan fractions from *Picea abies* (Norwegian Spruce) have been investigated by methylation and periodate oxidation techniques. It is concluded that both polysaccharides (degree of polymerization, DP_n, 68 and 100, respectively) are composed essentially of α-1,4 linked sugar residues. Both polysaccharides had the same general structure. Each molecule had 3—4 branch points, which were attached to the 3-position of glucose residues. The nonreducing end groups consist principally of mannose residues.

In Part I[1] of this series the isolation of a number of glucomannan fractions from Norwegian Spruce *(Picea abies)* was reported. One fraction was isolated by hot water treatment of the holocellulose (previously swollen with dimethyl sulphoxide [2]). Most of the xylan was then removed from the holocellulose by extraction with 14 % potassium hydroxide. Subsequently, fractions of glucomannan were obtained by treatment of the residual holocellulose with potassium hydroxide-borate solutions by the method of Jones *et al.*[3] This holocellulose after dissolution in cuprammonium solution was regenerated by the addition of acetic acid. From this material another fraction was extracted by treatment with alkali borate. After purification of the fractions by way of their copper complexes they had a nearly constant proportion of glucose to mannose (1:3.5-4). The degree of polymerization of the fractions, determined osmometrically on their nitrates, increased from about 70 for the most easily extractable to 140 for the most difficultly extractable fraction. It appears to be unlikely that the very great differences in the readiness with which the various glucomannan fractions may be extracted can be due only to differences in molecular weight. Variations in the microarrangement of the molecules and in their localisation in the fibre wall could explain the differences as could also variations in their chemical structures, since a highly branched polysaccharide is more soluble than is one which is either less branched or linear.

The present paper reports studies on the structures of the glucomannans in two fractions from *Picea abies*. One was extracted with hot water and the

other with alkali-borate. The glucomannan fractions were investigated by electrophoresis on glass paper, using borate buffer and α-naphthol-sulphuric acid in butanol as spraying reagent. They proved to be electrophoretically homogeneous. No differences in mobilities between the fractions were observed. These fractions were investigated by methylation and periodate oxidation techniques. The properties of the two fractions and the results of the structural studies are given in Table 1.

The methylations were performed in each case by treating the polysaccharide once with methyl sulphate in sodium hydroxide and three times with methyl iodide and silver oxide in dimethylformamide. The latter technique, successfully used by Kuhn *et al.*[4] for the methylation of low molecular weight carbohydrates, has proved also to be very useful for the methylation of polysaccharides such as arabogalactans [5] and glucomannans. The fractionation of the products of the two hydrolyses were performed by carbon column chromatography using the gradient elution technique. In each case the only tetramethyl hexose isolated was 2,3,4,6-tetra-*O*-methyl-D-mannose, identified as its crystalline aniline derivative. On demethylation of the fraction, a trace of glucose was observed in addition to the mannose. One hydrolysate yielded two, the other three, trimethyl-hexoses. Two of those were common to both hydrolysates and were shown to be 2,3,6-tri-*O*-methyl-D-glucose and 2,3,6-tri-*O*-methyl-D-mannose. These substances are very difficult to separate by ordinary paper chromatography but were readily separated on the carbon column. The former crystallised spontaneously when the solvent was removed. The latter, which on demethylation gave mannose only, gave a crystalline aniline derivative in good yield. The fraction containing the 2,3,6-tri-*O*-methyl-D-mannose was converted into its α-acetate and chromatographed on dimethylsulphoxide treated paper using *iso*propyl ether as solvent. The fraction was found to be chromatographically homogeneous. This method was devised by Wickberg [7] and has proved most valuable for the separation of acetylated and methylated sugars. The third trimethyl-hexose (that found in only one hydrolysate) on demethylation yielded glucose. The compound was chromatographically distinguishable from both the 2,3,4- and the 2,3,6-derivatives and may have been present in the other hydrolysate but have escaped detection there due to the larger amount of material added to the carbon column; such a condition would not favour the separation of compounds travelling at nearly the same rate. A number of dimethyl-hexoses was obtained from each hydrolysate as a mixed fraction and they were separated by paper electrophoresis in borate buffer [8]. The principal component was 2,6-di-*O*-methyl-D-glucose, identified by its chromatographic and electrophoretic behaviour. Three di-*O*-methyl-D-hexoses were also observed in each hydrolysate, which were shown by demethylation studies to be mannose derivatives.

The results of the methylation studies indicate that both glucomannans contain chains of 1,4-linked glycopyranosidic glucose and mannose residues. From the value of the optical rotation those residues appear to be linked by β-glycosidic bonds. The presence of a low percentage of other linkages, for example of glucose or of mannose residues linked to the 3-positions of glucose residues, is not excluded. A part of the dimethyl hexoses isolated is certainly derived from incomplete methylation of the polysaccharide. The fairly high

Table 1. Norwegian spruce glucomannan: Properties and results of periodate oxidation and methylation studies.

	Glucomannan A	Glucomannan B
Gal:Gluc:Man:Xyl	(+):21.8:76.9:1.3	(+):19.4:79.9:0.7
DP_n	68	100
$[\alpha]_D^{20}$ (c = 1, in 2 N NaOH)	−37°	−39°
Periodate oxidation		
Consumption of periodate (moles per $C_6H_{10}O_5$)	—	0.97
Formic acid released, extrap. to zero time (moles per $C_6H_{10}O_5$)	—	0.09
Molar % methylated hexoses		
2,6-Di-*O*-methyl-D-glucose	6.4	4.4
Di-*O*-methyl-D-mannoses	2.0	0.8
2,3,6-Tri-*O*-methyl-D-mannose	66.6	72.2
2,3,6-Tri-*O*-methyl-D-glucose	17.7	18.6
? -Tri-*O*-methyl-D-glucose	1.4	—
2,3,4,6-Tetra-*O*-methyl-D-mannose	5.8	3.6
% OCH_3 in the methylated polymers	44.0	44.6

yields of 2,6-di-*O*-methyl-D-glucose and of 2,3,4,6-tetra-*O*-methyl-D-mannose, however, strongly indicate that the polysaccharides are slightly branched and that the branches are on the 3-positions of glucose residues. From the analytical figures (Table 1) the number of branches per macromolecule in the glucomannan of DP 68 is calculated as 3—4. As the corresponding calculated value for the glucomannan of DP 100 is the same it must have a relatively lower degree of branching than the other.

Those conclusions are supported by the results of the periodate oxidation studies. The consumption of about 1 mole of periodate per hexose residue accords with such a 1,4-linked structure. The glucomannan of DP 100 on oxidation released 9 moles of formic acid per 100 hexose residues. This value is somewhat higher than that to be expected from a glucomannan having only 3—4 branch-points. The reducing group, unless modified by oxidation during delignification, would yield 2 moles and the non-reducing groups 4—5 moles of formic acid. The total yield then would be 6—7 moles but if the former group were modified it would be less. The oxidised polysaccharide after reduction with borohydride was hydrolysed and the hydrolysate examined chromatographically. Glucose and a trace of mannose were detected as was expected from the methylation studies, since those glucose residues which yield 2,6-di-*O*-methyl-D-glucose should not consume periodate.

These studies do not give any other details of the glucomannan structure. Points remaining undetermined are the distribution of glucose and mannose residues and of branching points and whether the branches are of uniform length or not. The rather small differences observed between the DP_n and

DP_w values, might suggest that the polysaccharide has a main chain with rather short branches (the DP_w was calculated from viscosity measurements making the assumption that the molecules are linear). The polysaccharide is non-crystalline but yields a crystalline product on mild acid hydrolysis [1]. The obvious explanation of this is that it is hydrolysed to shorter and linear fragments which, in contradistinction to the native, branched polysaccharide, have a strong tendency to crystallise. The X-ray diagram of this degraded glucomannan is identical to that given both by ivory nut mannan A and by a hemicellulose fraction from spruce sulphite pulp. During the hydrolysis the ratio of mannose (M) to glucose (G) residues in the polymeric material dropped from 3.6:1 to 2.9:1. This is to be expected as mannosidic linkages are more susceptible to acid hydrolysis than are glucosidic linkages.

The results of this investigation are in good agreement with those reported from the studies of other wood hemicellulose glucomannans. Hamilton *et al.*[9] isolated [from *Tsuga heterophylla* (Western hemlock)] a glucomannan of specific rotation $-38°$ (in 8 % sodium hydroxide) and having M:G = 4:1. Periodate oxidation studies of this material gave results very similar to those obtained in the present investigation. Aspinall *et al.*[10] isolated [from *Picea sitchensis* (Sitka spruce)] a glucomannan having $[\alpha]_D$ $-33°$ (in 10 % sodium hydroxide) and M:G = 2.5:1. The mannose content is quite low, compared to that of other glucomannans from coniferous woods, and it is possible that the material was contaminated by a degraded cellulose of which a fraction was also, separately, isolated. A structural investigation of the glucomannan gave results fairly similar to those reported in the present paper. From aspen wood hemicellulose Jones *et al.*[11] obtained a low yield of a glucomannan fraction having M:G = 2:1. Methylation studies indicated that it had the same general structure. The presence of glucomannans in coniferous wood has also been demonstrated by other investigators [12,13] and it seems fairly certain that glucomannans are amongst the principal hemicellulose components of those woods. The presence of other mannose containing polysaccharides (galactomannans or glucogalactomannans) has also been indicated [14,15] but awaits confirmation.

EXPERIMENTAL

Paper chromatography was carried out on Whatman No. 1 and on 3 MM filter paper. Chromatographic solvents: (a) ethyl acetate-acetic acid-water (3:1:1);
(b) butan-2-one, saturated with water;
(c) *iso*propyl ether (separations on dimethylsulphoxide-impregnated paper).

Paper electrophoresis was carried out on Whatman No. 3 MM filter paper in borate buffer at pH 10.

All concentrations were performed under reduced pressure and at a bath temp. of about 40°C.

Isolation of glucomannan fractions. The procedure was described in a previous paper [1] and further work on one of the fractions (A: DP_n 68) is now described. The other fraction, on which work has been carried out, was removed from a pretreated chlorite holocellulose by 27.6 % potassium hydroxide + 4 % boric acid. The pretreatment involved treating the holocellulose successively with dimethyl sulphoxide, with hot water, with 14 % potassium hydroxide and finally with 5 % potassium hydroxide + 3 % boric acid. The fraction (B) was purified as previously described. From the results of previous studies [1] on similarly derived fractions the DP_n of this fraction was estimated to be about 100.

Methylation of the glucomannans. Material A (2.0 g) was dissolved in 22.5 % sodium hydroxide (50 ml), under nitrogen, and methyl sulphate (10 ml) was added during 1 h with vigorous stirring, the temperature being kept at 20°C by external cooling. After 6 h the solution was neutralised with 6 N sulphuric acid and the polysaccharide recovered by dialysis. Yield 2.13 g (OCH_3, 28.4 %).

The partially methylated product was dissolved in dimethyl formamide (30 ml) and methyl iodide (6 ml) was added in one portion. The solution was kept under nitrogen and silver oxide (3 g) was added during 1 h under vigorous stirring. After 20 h the insoluble material was filtered off and washed with dimethyl formamide (10 ml) and the combined filtrate and washings were poured into water (800 ml) and then concentrated. Most of the dimethyl formamide was removed by repeated co-distillation with water. 200 ml of water and potassium cyanide (2 g) was added and the methylated polysaccharide was then extracted with chloroform (3 × 150 ml). The combined chloroform phases were washed with water (3 × 50 ml) and after drying over anhydrous sodium sulphate were taken to dryness. A further quantity of methylated polysaccharide was recovered from the later aqueous washings which were dialysed against tap water and then lyophilised. The two fractions were combined and were further methylated by two treatments with methyl iodide and silver oxide in dimethyl formamide. The product (0.58 g) had OCH_3, 44.0 %.

The second glucomannan fraction (B) (4.0 g) was methylated in the same way and yielded a product (1.64 g) having OCH_3, 44.6 %.

It is essential for the success of the methylations that all the chemicals are of high purity and perfectly dry. The silver oxide should be freshly prepared. The rather low yields of methylated products are certainly due to incomplete extraction of the aqueous solutions with chloroform and the procedure can probably be considerably improved.

Hydrolysis of the methylated glucomannans. The methylated glucomannans A and B (0.58 g and 1.64 g, respectively) were dissolved in anhydrous formic acid and kept at 100° for 6 h. After concentration of the solutions, the resulting syrup was dissolved in N sulphuric acid and was kept at 100° for 6 h. The solutions were then neutralised by passing them through a column of Amberlite IR-4B and on concentration they yielded syrups (0.44 g and 1.44 g, resp.). Chromatographic examination of the hydrolysates (Solvent a) revealed the presence of di-, tri- and tetra-*O*-methyl hexoses but no glucose, mannose or mono-*O*-methyl hexoses.

Examination of the hydrolysis products from methylated glucomannan A. The hydrolysate from methylated glucomannan A (444 mg) was added to the top of a carbon-Celite column (43 × 3.5 cm) which was then irrigated with the following solvents, using the gradient elution technique.

10 – 30 % aqueous ethanol	5 000 ml
30 – 60 » » »	3 000 »

The eluate was divided into fractions (30 ml) which were examined on paper chromatograms (Solvent a and b) and on paper electrophoretograms. Those fractions which were indistinguishable were combined and taken to dryness.

Fraction 1 (26.3 mg). By paper electrophoresis four components were isolated. The principal component on demethylation yielded glucose only and was chromatographically and electrophoretically indistinguishable from authentic 2,6-di-*O*-methyl-D-glucose. The components were fractionated, in two separate experiments, on paper chromatograms and on paper electrophoretograms and quantitative determinations of the principal component were made by hypoiodite oxidations [6]. The first experiment yielded a value of 82 % but in that case the separation of the components was not as good as when paper electrophoresis was used, then a value of 76 % was obtained. The analysis of mono-*O*-methyl-D-glucoses which had been separated by paper electrophoresis has previously been used [16].

Fraction 2 (219 mg). This fraction on demethylation yielded mannose only. The aniline derivative was prepared from it in a yield of 50 %. It had m. p. 125–127°, alone or in admixture with authentic 2,3,6-tri-*O*-methyl-*N*-phenyl-D-mannosylamine. The fraction was chromatographically homogeneous. Its α-diacetate had an R_F value of 0.26 (Solvent c).

Fraction 3 (58.1 mg). The material crystallised when the solvent was evaporated. One crystallisation from ethyl acetate yielded the pure substance, m. p. 117—119°C, undepressed on admixture with authentic 2,3,6-tri-*O*-methyl-α-D-glucose. The α-diacetate had an R_F value of 0.43 (Solvent c).

Fraction 4 (4.6 mg) on demethylation yielded glucose only. The substance was chromatographically distinguishable from 2,3,4- and 2,3,6-tri-*O*-methyl-D-glucose.

Fraction 5 (20.2 mg) on demethylation yielded mannose and traces of glucose. The aniline derivative had m. p. 142—144°, not depressed on admixture with that of 2,3,4,6-tetra-*O*-methyl-D-mannose.

Examination of hydrolysis products from methylated glucomannan B. The hydrolysate of methylated glucomannan B (1.440 mg) was fractionated as above on the same column. The results are summarised in Table 1.

Periodate oxidation studies. Glucomannan B (252 mg) was kept in water overnight in the refrigerator (2—4°). Then 0.4 M sodium metaperiodate solution (25 ml) was added and the volume was adjusted to 125 ml. The solution was kept in the refrigerator and at intervals samples (5 ml) were withdrawn and analysed for periodate consumption or for formation of acids. The results are summarised in Table 2.

Table 2. Periodate oxidation of glucomannan B.

Time, h	Moles of periodate consumed per $C_6H_{10}O_5$	Acid, equivalents per $C_6H_{10}O_5$
2	0.70	0.049
26	0.89	0.074
74	0.92	0.093
102	0.95	0.096
174	0.96	0.098

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