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STUDIES ON THE DISTRIBUTION OF THE *O*-ACETYL GROUPS IN PINE GLUCOMANNAN

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ABSTRACT

The distribution of the *O*-acetyl groups in pine glucomannan has been determined. The method used involved acetalation of the glucomannan with methyl vinyl ether, removal of the *O*-acetyl groups by treatment with base, and cleavage of the glycosidic linkages of the D-mannose residues carrying the original *O*-acetyl groups by a technique involving oxidation, β-elimination, and mild hydrolysis with acid. After removal of the acetal functions, the composition and molecular-weight distribution of the resulting oligosaccharide mixture indicated that the *O*-acetyl groups are irregularly distributed in the pine glucomannan.

INTRODUCTION

Pine glucomannan is essentially a linear polysaccharide composed of β-(1→4)-linked D-mannose and β-(1→4)-linked D-glucose residues. The polysaccharide contains some terminal D-galactose residues linked to the 6-positions of the D-mannose and D-glucose residues[1]. Previous analyses[2] using partial hydrolysis techniques indicated that the D-mannose and D-glucose residues are randomly distributed in the chain. About half of the D-mannose residues are substituted with *O*-acetyl groups equally distributed[1] between positions 2 and 3.

The present study describes an analysis of the distribution of the *O*-acetyl groups in the glucomannan.

RESULTS AND DISCUSSION

Pine glucomannan was isolated by extraction of pine-wood meal with hot water, and further purified by removal of acidic polysaccharides using Cetavlon precipitation and DEAE-cellulose chromatography[3]. Sugar analysis showed D-mannose, D-galactose, and D-glucose to be present in the molar proportions 72:8:20, in accordance

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with previous studies[1-3]. Methylation analysis showed that the glucomannan had an essentially linear structure with (1→4)-linked D-glucose and D-mannose residues. Only a small amount of branching was observed. The distribution of the *O*-acetyl groups at positions 2 and 3 of the D-mannose residues was in agreement with previous results[1]. Molecular-weight determination gave $\bar{M}_w$ 26,300 and $\bar{M}_n$ 16,500, corresponding to $\bar{P}_n$ 102.

Previous studies[2], using partial hydrolysis techniques, indicated that the β-(1→4)-linked D-mannose and the β-(1→4)-linked D-glucose residues in pine glucomannan are arranged randomly. The distribution of the D-galactose side-chains and the *O*-acetyl groups along the backbone of the polysaccharide is not known. Recently developed techniques for sequential analysis of polysaccharides, using a new, specific method of degradation[7,8,11], can also be used to study the distribution of substituents[12]. In the present study of the distribution of *O*-acetyl groups, the native, acetylated glucomannan was converted into a derivative having all free hydroxyl groups protected as part of an acetal function. Deacetylation then created free hydroxyl groups at the positions originally substituted by *O*-acetyl groups.

TABLE I

SUGAR ANALYSES OF ORIGINAL, MODIFIED, AND DEGRADED PINE GLUCOMANNAN

Polysaccharide material		*Relative molar proportions*				
		2-Man[a] (T *4.8*[b])	*3-Man* (T *5.9*)	*Man* (T *6.3*)	*Gal* (T *6.9*)	*Glc* (T *7.7*)
A	Original			72	8	20
B	Acetalated, methylated	16	17	40	8	19
C	Acetalated, deacetylated, methylated	16	17	41	8	18
D	Acetalated, deacetylated, oxidised			60	12	28
				(43	9	20)[c]
E	Degraded			60	13	27

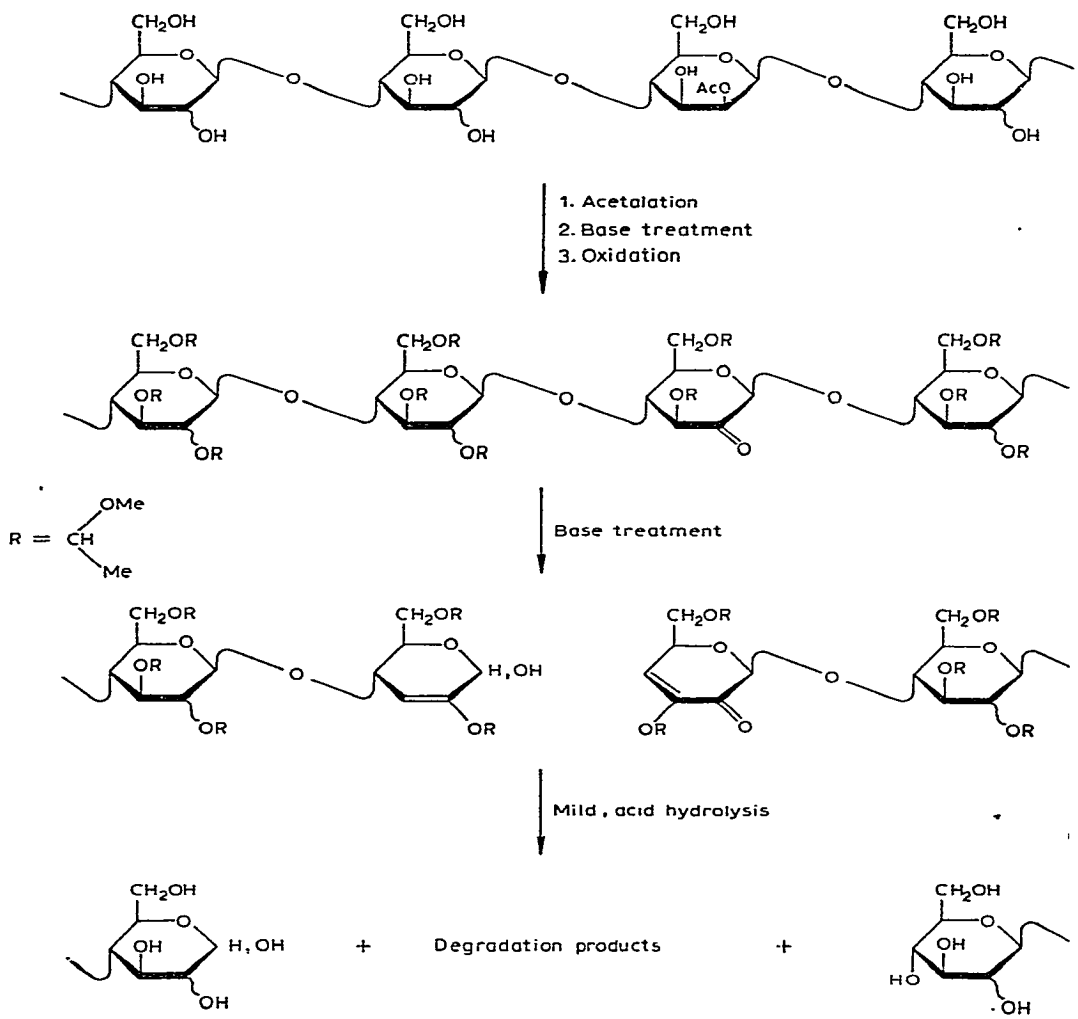
[a]2-Man = 2-*O*-methyl-D-mannose, etc. [b]Retention times of the corresponding alditol acetate relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an OV-225 column at 190°. [c]Adjusted to the D-glucose ratio of the original polysaccharide.

Methylation analysis demonstrated that the steps involved in this modification of the original glucomannan were quantitative (Table I, *C*). Oxidation of the acetalated, deacetylated glucomannan with a chlorine—methyl sulphoxide complex[6] completely converted the free hydroxyl groups into keto functions, as shown by hydrolysis and analysis of the resulting, partially methylated ethers (Table I, *D*). The oxidised D-mannose residues, with keto groups at either position 2 or 3, are degraded by base, as has previously been shown by using model compounds of analogous, alkylated pento- and hexo-siduloses[7,8]. The degradations are depicted in Schemes 1 and 2.

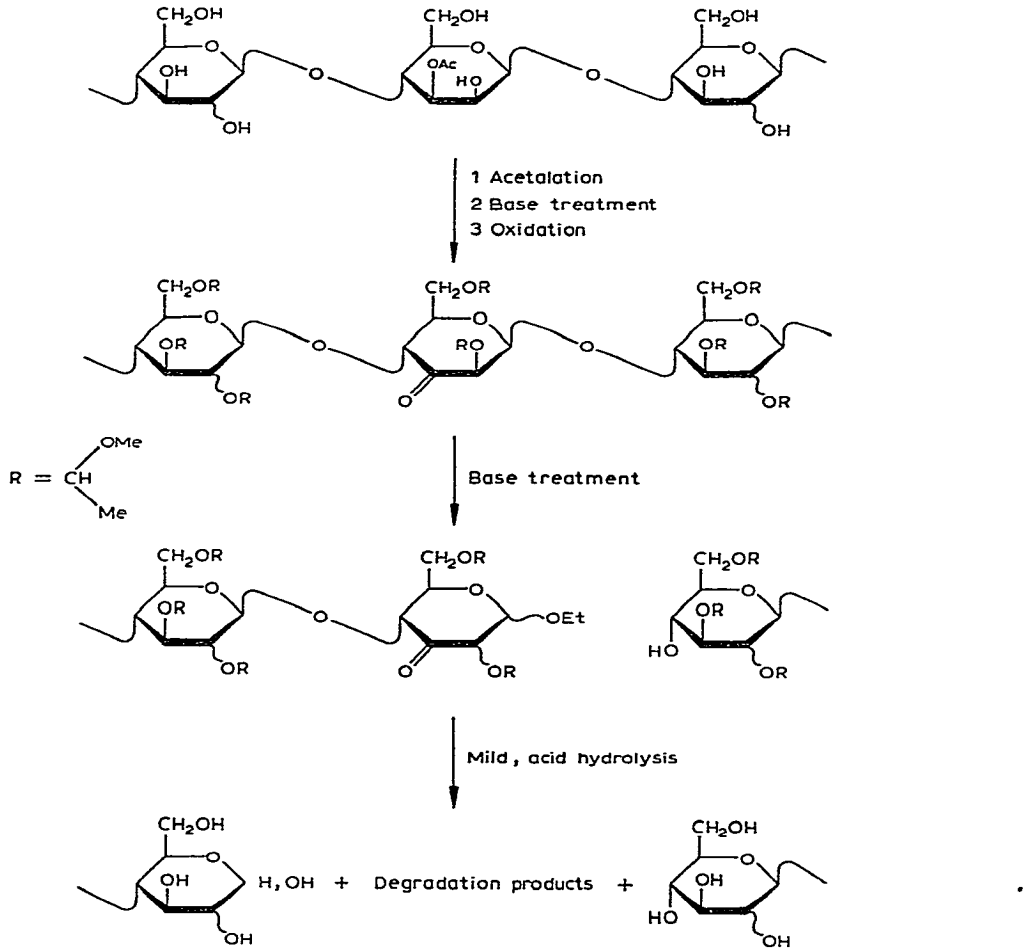
Alkaline treatment of the D-mannose residues containing a keto group at

position 2 resulted in β-elimination and formation of reducing end-groups. The reducing, terminal residues were further degraded under the alkaline conditions, with the formation of 2,3-ene derivatives[9,13] (Scheme 1).

The α,β-unsaturated keto sugars formed by treatment with base, together with the acetal functions, were then cleaved by mild hydrolysis[9,10] with acid, and the resulting oligosaccharide mixture was subjected to sugar and methylation analysis (Table I, *E*; and Table II, *B*). The molecular-weight distribution of the oligosaccharide mixture (Fig. 1) ($\overline{M}_w$ 900; $\overline{M}_n$ 440) was determined by gel filtration[4] with a calibrated column of Sephadex. The main part of the material (70% by weight) had $n \leqslant 5$. Less than 1% of the mixture had $n \geqslant 48$, and less than 10% had $n \geqslant 12$.



Scheme 1



Scheme 2

TABLE II

METHYLATION ANALYSES OF NATIVE (*A*) AND DEGRADED (*B*) PINE GLUCOMANNAN

Sugars[a]	T[b]	*Mole %*	
		A	B
2,3,4,6-Man + Glc	1.00	1	29
2,3,4,6-Gal	1.19	6	7
2,3,6-Man	2.01	69	34
2,3,6-Glc	2.32	17	21
2,3-Man	3.91	5	6
2,3-Glc	4.50	2	3

[a]2,3,4,6-Man + -Glc = 2,3,4,6-tetra-*O*-methyl-D-mannose + 2,3,4,6-tetra-*O*-methyl-D-glucose.
[b]Retention times of the corresponding alditol acetates on an OV-225 S.C.O.T. column relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

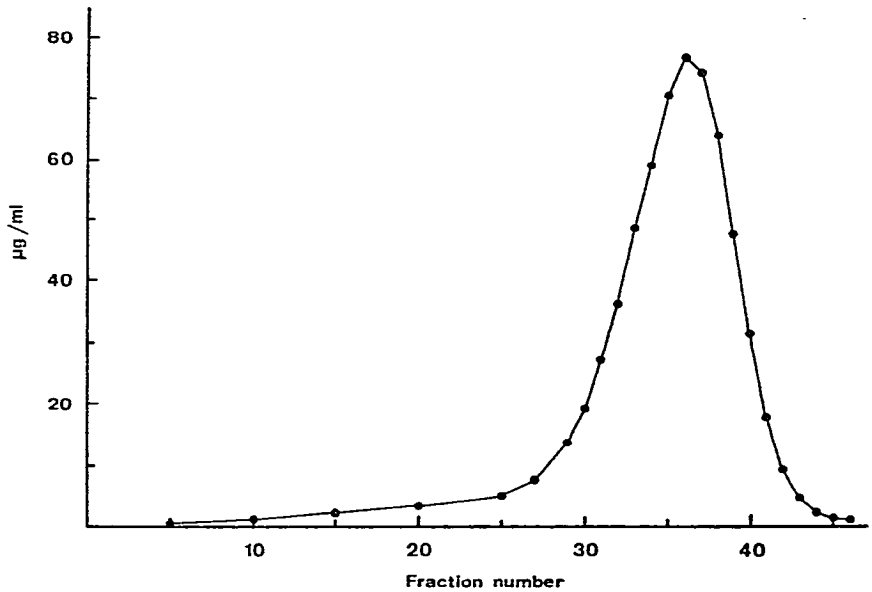


Fig. 1. Elution curve for the oligosaccharide mixture from a Sephadex column (see Experimental section) calibrated with oligosaccharides from the isomaltose series. The eluant was analysed colorimetrically ($\lambda = 620$ nm) using anthrone. Fraction 5 corresponds to n = 98, 10 to n = 58, 15 to n = 37, 20 to n = 24, 25 to n = 15, 30 to n = 8, 35 to n = 3, and 40 to n = 1.

Methylation analysis of the oligosaccharide mixture (Table II, *B*) showed that ~30% of new non-reducing terminals had been formed. This analysis, together with the analysis of the size of the oligosaccharides formed, indicated that the *O*-acetyl groups are irregularly distributed in pine glucomannan and that only a small proportion of glucomannan contains large *O*-acetyl-free regions.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at bath temperatures not exceeding 40°. G.l.c. was performed on a Perkin–Elmer 990 instrument, fitted with flame-ionisation detectors, and glass columns (190 × 0.15 cm) containing 3% of OV-225 on Gas Chrom Q (100–120 mesh) at 190° or OV-225 S.C.O.T. columns (15 m × 0.5 mm) at 190°. For quantitative evaluation of the g.l.c. results, a Hewlett Packard 3370 B integrator was used. For mass spectrometry, a solution of the mixture of alditol acetates in chloroform was injected into a Perkin–Elmer 270 combined gas chromatograph–mass spectrometer. Optical rotations were recorded with a Perkin–Elmer 141 instrument, and i.r. spectra with a Perkin–Elmer 257 instrument.

Preparation of the polysaccharide. — Acetone-extracted pine wood (*Pinus silvestris*) of particle size 0.5–2.0 mm was delignified by the chlorite method[14]. The

pine holocellulose was obtained in 78% yield. The holocellulose (500 g) was extracted twice with methyl sulphoxide (7.5 l) at room temperature overnight. The extracted holocellulose was collected by filtration, washed with cold water, and extracted twice with water (7.5 l) at 100° for 30 min. The combined aqueous extracts were concentrated under diminished pressure to 1 litre, and adjusted to pH 4 with hydrochloric acid, and polysaccharides were precipitated by addition to 4 vol. of ethanol with vigorous stirring. The polysaccharides were recovered by centrifugation and washed first with ethanol and then with acetone. The product (11.9 g) was dissolved in water (200 ml), and a small, insoluble fraction was removed by centrifugation. A 5% aqueous solution of cetyltrimethylammonium bromide was added until no further precipitate was formed. The precipitate was removed by centrifugation, the clear solution was dialysed against water for 72 h and then adjusted to pH 4, and polysaccharide (7.8 g) was precipitated with ethanol as described above. A small part of the product (5 mg) was subjected to sugar analysis. The presence of xylose necessitated further purification.

The material (7 g) was dissolved in water (30 ml) and applied to the top of a column (50 × 5 cm), of DEAE-cellulose, previously equilibrated with 0.5M phosphate buffer (pH 5.5) and then washed with water. The column was eluted first with water (2 l) and then with 0.05M phosphate buffer (pH 5.5; 2 l). The separation was monitored by sugar analysis, and the fractions containing the glucomannan were concentrated to small volumes, dialysed overnight, and lyophilized, yielding 3.1 g of product, $[\alpha]_{578}^{22}$ −28° (*c* 0.5, water). The molecular weight was determined on a calibrated Sephadex G-75 column[4], giving $\overline{M}_w$ 26,300 and $\overline{M}_n$ 16,500.

Sugar analysis. — The polysaccharide (5 mg) and D-arabinose (as internal standard) were hydrolysed in 0.25M sulphuric acid (3 ml) at 100° for 16 h. The hydrolysate was neutralised with barium carbonate, filtered, and concentrated to dryness. The resulting sugars were converted into alditol acetates, and analysed by g.l.c.–m.s.[15,16] (Table I, *A*). Reducing sugars in the hydrolysate accounted for 90% of the product.

Methylation analysis. — The polysaccharide (5 mg) was dissolved in methyl sulphoxide (2 ml) in a flask sealed with a rubber cap. Nitrogen was flushed through the bottle, and 2M methylsulphinyl anion in methyl sulphoxide (2 ml) was added[17]. The gelatinous solution was agitated ultrasonically for 0.5 h and left at room temperature overnight. Methyl iodide (2 ml) was added dropwise with external cooling, and the resulting turbid solution was agitated ultrasonically for 0.5 h. The clear solution was diluted with water (10 ml) and dialysed against water overnight. After concentration to dryness, the product was treated with 90% formic acid (3 ml) at 100° for 2 h, the solution was concentrated to dryness, and the residue was hydrolysed with 0.25M sulphuric acid (3 ml) at 100° for 16 h. The hydrolysate was converted into alditol acetates, and analysed by g.l.c.–m.s.[18] (Table II, *A*).

Acetalation of the polysaccharide. — The native material (100 mg) and toluene-*p*-sulphonic acid (20 mg) were dissolved in methyl sulphoxide (20 ml) in a serum flask sealed with a rubber cap, and then cooled to 13°. Methyl vinyl ether (10 ml), which had

been condensed at −50°, was added and the solution was kept at 13–14° for 3.5 h. Excess of methyl vinyl ether was removed by evaporation and the clear solution was applied to a column (50 × 5 cm) of Sephadex LH-20, which was eluted with dry acetone. The separation was monitored by optical rotation. Only one fraction, which came with the void volume, was optically active, and was concentrated to dryness. Part of the product (5 mg) was subjected to methylation analysis, as described earlier. The results are summarized in Table I, *B*.

Deacetylation of the acetalated product. — A solution of the foregoing material (250 mg) in 0.25M methanolic sodium methoxide (20 ml) was heated under reflux for 20 h. The clear solution was concentrated to a small volume and applied to a column (50 × 5 cm) of Sephadex LH-20, which was eluted with dry acetone containing 1% of pyridine. The separation was monitored by optical rotation, and the acetalated, deacetylated product was eluted with the void volume. After concentration to dryness, part of the residue (5 mg) was subjected to methylation analysis (Table I, *C*).

Oxidation of the acetalated, deacetylated polysaccharide. — Methyl sulphoxide (10 ml) was added to a M solution of chlorine in anhydrous dichloromethane (25 ml) at −45°, with vigorous stirring. A white precipitate appeared during the addition. The modified glucomannan (200 mg) in dichloromethane (10 ml) was then added, and the mixture was stirred for 5 h at −45°. Triethylamine (8 ml) was added, and the mixture was kept at −45° for 10 min, allowed to reach room temperature, and then dialysed against water containing 0.1% of pyridine overnight. After concentration, the residue was applied to a column (40 × 4 cm) of Sephadex LH-20 and eluted with acetone containing 1% of pyridine. The void volume contained 165 mg of product, a part (10 mg) of which was hydrolysed and analysed as described above (Table I, *D*).

Alkaline treatment and acid hydrolysis of the acetalated, deacetylated, oxidised material. — The material (150 mg) was dissolved in dichloromethane (6 ml), and M ethanolic sodium ethoxide (3 ml) was added. The mixture was kept at room temperature for 1 h with continuous stirring, and then neutralised with acetic acid and concentrated to dryness. The residue was dissolved in methanol, and Dowex-50(H^+) resin was added to pH 4. The solution was filtered, and concentrated to dryness. The residue was treated with 50% aqueous acetic acid for 14 h at 100°, and the solution was evaporated to dryness. The residue was partitioned between chloroform–water, and the aqueous phase was freeze-dried. Part of the product (1/20), together with D-arabinose (as internal standard), was hydrolysed with 0.25M sulphuric acid for 16 h and 100°, and the sugars obtained were analysed as described above, showing that the sugar content was 40 mg (Table I, *E*).

Analysis of the degraded material. — Part of the product (1/10) was methylated as described above, and the reaction mixture was partitioned between chloroform and water. The organic phase was concentrated, hydrolysed, and analysed as described above (Table II, *B*). The molecular weight was determined on a calibrated Sephadex column[4], prepared from G-50 and G-75 (3:2; w/w) (Fig. 1), giving $\overline{M}_w$ 900 and $\overline{M}_n$ 400 ($\overline{M}_w/\overline{M}_n$ 2.013).

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