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Studies on Hemicelluloses from Pine (*Pinus silvestris* L.)

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A number of glucomannan fractions and an araboxylan fraction have been isolated from pine (*Pinus silvestris* L.) holocellulose. The ratio glucose:mannose varied from 1:3.1 to 1:3.7 in the different glucomannans. All fractions contained a few per cent of galactose residues and the possible presence of galactoglucomannans cannot be excluded. The ratio arabinose:xylose in the isolated araboxylan was 1:7.4 and the uronic acid content 18.6 %.

The average degree of polymerisation ($\overline{DP}_n$) increased from about 70 for the most easily extractable glucomannan fractions up to about 115 for the most resistant. The $\overline{DP}_n$ value for the araboxylan was 121.

It seems probable that the glucomannans in a holocellulose are partly bound to the remaining lignin even when the holocellulose in question has a very low Klason-lignin content.

Studies were earlier reported [1] on the physical properties of glucomannans from Norwegian spruce (*Picea Abies* Karst.). Similar investigations have now been carried out on glucomannans and on an araboxylan from pine (*Pinus silvestris* L.).

ISOLATION OF THE CRUDE HEMICELLULOSES

The crude hemicelluloses were isolated by successive treatments of a chlorite-holocellulose with dimethyl sulphoxide, hot water, potassium hydroxide and finally with potassium hydroxide and boric acid. The xylans were practically completely extracted, but about 25 % of the glucomannans present in the holocellulose were resistant to those treatments. They could, however, be extracted, in part at least, after having destroyed the cellular structure of the residue by dissolving it in cupri-ethylene diamine and regenerating it by reprecipitation with acetic acid.

Table 1. Carbohydrate analyses of the crude hemicellulose extracts and of the residues.

Sample		Yield g	Galactose/Glucose %		Mannose %	Arabinose %	Xylose %
K_2	Holocellulose (yield with respect to acetone-treated wood: 69 %. Klason-lignin: 1 %)	147.1	1.6	71.8	15.6	1.7	9.3
$K_2GM_1X_1$	$(CH_3)_2SO$-extract	1.1	26.7		16.4	6.8	50.0
$K_2GM_2X_2$	H_2O-extract	2.9	24.4		72.6	1.2	1.8
$K_2GM_3X_3$	14 % KOH-extract	22.1	15.5		28.0	6.8	49.8
$K_2GM_4X_4$	(14 % KOH+3 % H_3BO_3)-extract	3.5	23.0		63.5	2.5	11.0
$K_2GM_5X_5$	(24 % KOH+3 % H_3BO_3)-extract	2.3	24.7		70.3	2.3	2.7
K_3	Residue after the previous extractions		92.9		7.1	trace	trace
$K_3GM_6X_6$	(24 % KOH+3 % H_3BO_3)-extract from the cupri-ethylene diamine-regenerate of K_3	2.5	29.0		65.3	2.1	3.6
K_4	Residue after the extraction of the cupri-ethylene diamine-regenerate of K_3		95.1		4.9	trace	trace

The composition of the different fractions is shown in Table 1. The dimethyl sulphoxide extracted principally xylans but only in small amounts. Hot water extracts contained an almost pure glucomannan having only 1.8 % of xylose residues while two samples of spruce holocellulose under similar conditions contained 16.5 and 11 % of xylose residues [1]. About a quarter of the material extracted by 14 % potassium hydroxide consisted of mannose residues while the rest of the fraction contained most of the xylans that could be extracted from the holocellulose. Relatively pure glucomannans were isolated in later extractions by a solution of potassium hydroxide and boric acid.

FRACTIONATION AND PURIFICATION OF THE CRUDE HEMICELLULOSES

The hemicelluloses extracted by hot water and by potassium hydroxide and boric acid contained primarily glucose and mannose residues. The contaminating araboxylans could to a large extent be removed by fractionation of the samples with Fehling's solution and then with barium hydroxide. Barium hydroxide has been shown [2] to be a good reagent for the selective precipitation of polysaccharides containing mannose residues. Xylans do not precipitate under the conditions employed, but in certain cases do so at higher concentrations of barium hydroxide. As will be seen in Table 2, glucomannans were obtained having only 1.2 to 2.8 % of xylose residues. Unlike the glucomannans isolated from spruce [1] they contained 2.8 to 4.7 % of galactose residues and normally also a low proportion of arabinose residues.

Table 2. Carbohydrate analyses and degree of polymerisation of the glucomannans and of an araboxylan obtained by fractionation using Fehling's solution and barium hydroxide.

Sample	Origin (*cf.* Table 1)	Galactose %	Glucose %	Mannose %	Arabinose %	Xylose %	Glucose: Mannose	$[\alpha]_D^{20}$ [a]	$\overline{DP}_n$	$\overline{DP}_w$	Nitrogen[b] %
K_2GM_2	from $K_2GM_2X_2$	4.7	19.9	73.8	trace	1.6	1:3.7	—37	70.8	40.0	12.65
K_2GM_3		3.9	22.6	63.1	1.8	8.6	1:2.8	—31	89.9	50.1	12.33
K_2GM_3*	from $K_2GM_3X_3$	5.2	22.4	70.0	trace	2.4	1:3.1	—33	—	—	—
K_2X_3		2.7	3.6	1.2	11.0	81.5	—	—63	121.0	—	—
K_2GM_4	from $K_2GM_4X_4$	2.8	20.0	73.5	0.9	2.8	1:3.7	—33	90.6	59.3	12.17
K_2GM_5	from $K_2GM_5X_5$	2.8	21.7	71.6	2.0	1.9	1:3.3	—27	92.5	61.2	12.25
K_3GM_6	from $K_4GM_6X_6$	3.1	22.4	72.1	1.2	1.2	1:3.2	—34	115.8	78.3	11.75

[a] In 2.5 N NaOH.
[b] Nitrogen content of the nitrated samples used in DP-determinations.

The material extracted by 14 % potassium hydroxide was sub-fractionated from an aqueous alkaline solution by the addition of Fehling's solution and then yielded a precipitate (A) enriched in glucomannan, and a supernatant (B) enriched in xylan. No significant reduction in the proportion of xylose residues in the glucomannan-rich fraction (A) was obtained when the material was again treated with Fehling's solution, but the xylose residues were reduced from 19.6 to 8.3 % in the precipitate obtained when barium hydroxide was added to a solution of the material (K_2GM_3, Table 2). The xylan-rich supernatant (B) contained 10.8 % of mannose residues but this was reduced to 1.2 % by the preferential precipitation with barium hydroxide solution of a fraction enriched in mannose residues. In this way an araboxylan (K_2X_3, Table 2) was obtained having a uronic acid content of 18.6 % and a ratio of arabinose to xylose residues of 1:7.4. (This ratio refers only to those xylose residues in the xylan which have no uronic acid residues bound to them since the latter, under the hydrolytic conditions used, are present in aldobiuronic acids.)

Although cetyltrimethylammonium bromide (CTA-Br) has been used [3] to separate acidic and neutral polysaccharides, its use in the present work did not result in any significant reduction in the proportion of xylose residues in fraction K_2GM_3.

Table 3. Carbohydrate- and lignin-analyses of two fractions from K_2GM_3 precipitated with cetavlon hydroxide in the presence of boric acid. (Carbohydrate content is assumed to be 100 %.)

Sample	Lignin %	Galactose %	Glucose %	Mannose %	Arabinose %	Xylose %
F_2	5.8	4.2	18.9	64.0	2.3	10.6
F_4	trace	2.5	18.5	77.0	trace	2.0

Finally K_2GM_3 was divided into five fractions (F_{1-5}) by adding boric acid and then making successive additons of cetyltrimethylammonium hydroxide (CTA-OH). This method has been used at this laboratory [4] to fractionate arabogalactans. The first three fractions (F_{1-3}), which accounted for about 80 % of the recovered material, were similar to one another but were still heterogeneous (*cf.* Table 3). The next two fractions ($F_{4,5}$), on the other hand, contained glucose and mannose residues almost exclusively.

Recent studies by Lindgren[5] strongly indicate that in coniferous woods the hemicelluloses are in part chemically bound to lignin. It is, therefore, possible that the major component, F_{1-3}, is a hemicellulose-lignin complex with one or more types of hemicelluloses bound to lignin. By spectral analysis the lignin content in F_{1-3} was determined to be about 6 %; F_4 and F_5, on the other hand, contained no lignin (the Klason-lignin in the starting holocellulose was less than 1 %). On electrophoresis of a sample of F_2 on glass-fibre sheets wet with 0.05 N sodium hydroxide, it gave a single spot with a migration distinct from that of the glucomannan sample F_5. This indicated that in F_2 there was no glucomannan similar to that in F_5 and that the mannose residues present in the former fraction must either have been present in a different type of molecule, or be in a glucomannan chemically bound to some other species. There were two items indicating that a lignin-hemicellulose complex was present. Firstly, Lindgren [5] has noted that when lignin-free hemicelluloses are chromatographed on glass-fibre paper, using 0.05 N sodium hydroxide as irrigant, then the hemicelluloses migrate with the solvent front; lignin-hemicellulose complexes, on the other hand, have R_F values below 1. F_2 and F_5 had R_F 0.3—0.4 and 1, respectively, indicating that the former was a lignin-hemicellulose complex. Secondly, a sample of K_2GM_3 (lignin content 5 %) was delignified by chlorination and by extraction with ethanolic ethanolamine, and the product, in solution, was afterwards treated with barium hydroxide. The precipitate then obtained (K_2GM_3*) contained a much smaller amount of xylose residues than did K_2GM_3 (see Table 2). This indicated that, prior to delignification, the xylose residues had been present in some molecular species other than the glucomannan but that both species were probably bound to lignin molecules. The mobility of K_2GM_3* on an electrophoretogram was similar to that of the glucomannan F_5.

The samples K_2X_3, K_2GM_4 and K_2GM_6 contained, respectively, 1.1, 3.6 and 5 % lignin. By chromatographic examination on glass-fibre paper a sample of K_2X_3 was found to contain two components; one having an R_F of 0.3, the other of 1, indicative, it is believed, of their having been produced by a hemicellulose-lignin complex and a hemicellulose, respectively. The spot produced by the former was very weak, and that by the latter strong, while K_2GM_4 and K_2GM_6 gave two spots each of about equal intensity. It seems, therefore, that in the latter two samples also a considerable part of the glucomannan was bound to the lignin.

DP-DETERMINATIONS

The degrees of polymerisation based on number-average molecular weights ($\overline{DP}_n$), were determined osmometrically on the nitrates in butyl acetate [1]. The results are shown in Table 2. The $\overline{DP}_n$ value for the glucomannan extracted by water was *ca.* 70 and was practically identical to that of the corresponding fraction from spruce holocellulose [1]. The value for K_2GM_3 was relatively high (89.9) probably due to the presence, as a contaminant, of an araboxylan of higher molecular weight. The fractions that had been extracted with potassium hydroxide/boric acid differed but little from one another. The fraction extracted from the regenerated cellulose (K_2GM_6) had a $\overline{DP}_n$ of *ca.* 115, a value rather lower than that given by the corresponding fraction from spruce [1].

The $\overline{DP}_n$ value of the sodium salt of the acidic araboxylan (K_2X_3) was determined in 0.1 N sodium chloride (*cf.* Ref.[7]) to be *ca.* 120.

The problem had to be considered of whether these $\overline{DP}_n$ values related to hemicelluloses or to lignin-hemicellulose complexes. Lignin was, however, found to be absent from the nitrated samples, and this indicated that delignification had taken place during nitration and that, in consequence, the $\overline{DP}_n$ values were those of hemicelluloses and not of lignin-hemicellulose complexes.

The degrees of polymerisation based on weight-average molecular weight ($\overline{DP}_w$), were determined viscometrically on the nitrates in butyl acetate. $K\eta$ in the equation of Schulz and Blaschke

$$Z\eta = \frac{\eta_{sp}}{c(1 + K_\eta \cdot \eta_{sp})}$$

was determined to be 0.29 and the $\overline{DP}_w$ values were then obtained by application of the equation derived by Marx [8]:

$$Z_{\eta \text{ acetone}} = 1.5 \times 10^{-3} \overline{DP}_w{}^{0.9}$$

In a separate experiment $Z_{\eta \text{ butyl acetate}}$ was determined to be $1.13 \times Z_{\eta \text{ acetone}}$.

The derivation of Marx' equation was based on measurements carried out on cellulose nitrates which contained *ca.* 13.6 % nitrogen. The calculation of the $\overline{DP}_w$ values of the nitrated glucomannans was based on the average weight of the residues, allowance being made for their incomplete nitration. It must, however, be stressed that the $\overline{DP}_w$ values in Table 2 are of necessity highly uncertain as it is very doubtful if the formula of Marx can legitimately be applied to nitrated glucomannans of low molecular weight. The $\overline{DP}_n$ and $\overline{DP}_w$ values of the various fractions both increase in the same order; the lowest values being given by the most readily extracted glucomannans and the highest values by those most difficult to extract. This indicates that all of the molecular species probably possess a similar degree of branching.

DISCUSSION

The mannose residues in the holocellulose of *Pinus silverstris* are present in glucomannans as was also earlier found in work on *Picea Abies* [1]. The ratio of glucose to mannose appears, however, to be rather higher in the former (*ca.* 1:3.5 as compared to *ca.* 1:4 in *Picea Abies*).

From the various fractions glucomannans were precipitated by the addition of Fehling's solution and those precipitates were dissolved in water and fractionated by the addition of barium hydroxide. The glucomannan then precipitated generally contained about 2 % xylose, 2 % arabinose, and up to 4.2 % galactose residues. The material extracted by the 14 % potassium hydroxide ($K_2GM_3X_3$) did not yield a similar polysaccharide when treated in like manner but from its mannose-rich sub-fraction, K_2GM_3, by fractional precipitation with cetavlon hydroxide and boric acid there was obtained a small amount of a polysaccharide containing principally glucose and mannose residues. The major part of the K_2GM_3 sub-fraction contained xylose and galactose residues in addition to glucose and mannose residues. As the xylose residues could largely be removed after delignification of the material, it seems probable that they and the glucomannan were bound independently to the lignin. It is uncertain whether or not the galactose residues were present as part of the glucomannan. The galactose residues may be present in contaminating polysaccharides as arabogalactans or pectic galactans.

The various glucomannans had optical rotations differing relatively widely from one another. This variation may have been due to varying amounts of lignin and to differences in the proportions of the different sugar residues.

The $\overline{DP}_n$ values of the different purified glucomannans were similar to those given by the corresponding fractions from spruce. The araboxylan K_2X_3 had a $\overline{DP}_n$ of 121, that is a value even higher than that given by the least readily extracted glucomannan (K_2GM_6).

EXPERIMENTAL

Quantitative paper chromatography. The hydrolyses and the quantitative sugar analyses of the hemicelluloses were carried out by the method of Saeman *et al.*[9] using the chromatographic irrigants ethyl acetate — acetic acid — water (3:1:3), and, for the separation of glucose and galactose, ethyl acetate — pyridine — water (2:1:2).

Electrophoresis. The electrophoresis was carried out on glass-fibre sheets [5]. The spray-reagent was a mixture of *a*-naphthol (1 g), conc. sulfuric acid (5 ml) and *n*-butanol (100 ml).

Fractional extraction of the hemicellulose. Wood meal (18—35 mesh) of *Pinus silvestris* L. was treated in a Soxhlet-type extractor with acetone and the residue was then delignified by the chlorite method [10]. The product remaining after delignification was 68.9 % of the acetone-treated wood. The holocellulose (147 g) was thoroughly washed with water and was then mechanically shaken, at room temperature, with dimethyl sulphoxide (1.5 l) in an atmosphere of nitrogen. Three such treatments were carried out over a total period of 24 h. Then the residual material was washed with cold water. The combined extracts were slightly acidified by the addition of a little hydrochloric acid and the hemicelluloses were precipitated from the solution by the addition of twice its volume of ethanol. The precipitate was centrifuged down and, after washing with *ca.* 65 % aqueous ethanol, was suspended in water. The remaining ethanol was distilled off, and the solution then freezedried. The residual material from the dimethyl sulphoxide

treatment was then treated successively, and in a similar way, with boiling water (giving extract $K_2GM_2X_2$), 14 % potassium hydroxide (giving extract $K_2GM_3X_3$), 14 % potassium hydroxide and 3 % boric acid (giving extract $K_2GM_5X_5$), and 24 % potassium hydroxide and 3 % boric acid (giving extract $K_2GM_4X_4$). In all cases a total of 4.5 l of liquid was employed over three treatments. A sample of the final residue (K_3) was dissolved in cupriethylene diamine and precipitation was effected by the addition of acetic acid. The precipitate was treated once with 24 % potassium hydroxide + 3 % boric acid (giving extract $K_2GM_6X_6$). All extractions were carried out in an atmosphere of nitrogen. The extracts were sligthly acidified and the hemicelluloses precipitated, washed, and dried as described earlier. Those precipitates contaminated by borate were washed also with warm methanol to remove the boric salts.

Isolation and purification of the glucomannans and of an araboxylan. The various crude hemicellulose fractions were suspended in fifty times their weight of water and the mixture was homogenized in a Turmix blender. After boiling the mixture for a few minutes, then cooling, it remained turbid, but complete dissolution was effected on the addition of a little dilute sodium hydroxide. To these solutions Fehling's solution was added, and the resultant precipitate was centrifuged down and washed once with water. The centrifugate from the $K_2GM_3X_3$ fraction, after acidification with hydrochloric acid, was passed through a column of cation-exchange resin (IR 120). The hemicelluloses were then precipitated by the addition of ethanol and the precipitates, after washing, were dried. As this product still contained *ca.* 10 % mannose residues it was redissolved in water and was treated with barium hydroxide and the precipitate centrifuged down. The centrifugate, after acidification with acetic acid, was treated with ethanol and a relatively pure araboxylan (K_2X_3) was obtained.

Those precipitates that had been obtained from the crude hemicelluloses with Fehling's solution were dissolved in N hydrochloric acid and glucomannans were precipitated by the addition of ethanol. The precipitates were separated and washed until neutral. They were then suspended in water, and, after distilling off the remaining ethanol, were freeze-dried. As some samples were still obviously heterogeneous all were redissolved in water and were precipitated by the addition of saturated barium hydroxide solution. The precipitates were dissolved in either 2 N acetic acid or in N hydrochloric acid. The glucomannans were precipitated with ethanol, then washed and dried.

Fractionation of the sample K_2GM_3 *with cetyltrimethylammonium hydroxide* (CTA-OH). A sample (500 mg) of the K_2GM_3 fraction was dissolved in water (25 ml) and CTA-OH (0.3 mmole) was added. The precipitate was separated and dissolved in N sodium chloride. From this solution the CTA was precipitated by potassium iodide and was centrifuged down and washed with potassium iodide solution. The centrifugate and the washings were combined and acidified and the hemicelluloses, after precipitation with ethanol, were washed and dried (yield 175 mg). The material was hydrolysed and the hydrolysate examined chromatographically. The sugars were all present in amounts similar to those found in a hydrolysate of the starting material. The hemicelluloses, left in the supernatant after the cetavlon precipitation, were precipitated by the addition of ethanol. This material (180 mg) on hydrolysis was found to contain all of the sugars noted in the hydrolysate of the previous precipitate, but the galactose and xylose were present in somewhat lower amounts.

In another experiment a sample (1 g) of K_2GM_3 was dissolved in water (100 ml) and boric acid (6.2 mmole) was added. To this solution there were added four successive portions of both sodium hydroxide and cetavlon hydroxide with intermediate separation of the precipitate formed on each addition (fractions: F_{1-4}). A total of 4 mmole of each reagent was added. The precipitates were dissolved in 6 % acetic acid and the hemicelluloses precipitated by the addition of ten times the volume of ethanol. After centrifugation the samples were washed and dried. The solution remaining after the final precipitation was passed successively through columns of cation-, and anion-exchange resins (IR 120 and IR 4B), then the solvent was distilled off from the eluate and a further fraction (F_5) was obtained. Samples of the first three fractions (F_{1-3}), after hydrolysis, were found to contain similar amounts of the same sugars as found in the hydrolysate of the non-fractionated material. F_4 and F_5 accounted for about 20 % of the weight of F_{1-5} and contained glucose and mannose residues almost exclusively.

Delignification of K_2GM_3. A 1 % aqueous solution of K_2GM_3 was treated with chlorine by passing the gas for 5 min through the solution which was kept at 6° [11]. The hemicellu-

loses were then precipitated by the addition of four times the volume of ethanol. After washing the precipitate, it was suspended in a 3 % solution of ethanolamine in ethanol and the mixture was boiled for 5 min. The residue was separated and, after treating it again in the same way with ethanolic ethanolamine, it was redissolved in water and the above procedure repeated twice from the chlorination stage. By those treatments the amount of lignin was reduced from *ca.* 5 % in K_2GM_3 to 0.4 % in K_2GM_3*.

Lignin analyses. Samples (0.5 g/l) of the hemicellulose fractions were dissolved in 0.5 N sodium hydroxide and the absorption curves from 250—320 mμ were determined with a Beckman spectrophotometer. A lignin-free ivory nut mannan was used as reference substance. Those samples which contained lignin had a sharp absorption maximum at 280—285 mμ (*cf.* Ref.[6]) whereas in the chlorinated sample the corresponding maximum was at *ca.* 295 mμ. From the height of the maximum the lignin content was calculated using the equation:

$$\log \frac{I_o}{I} = \varepsilon \cdot c \cdot d$$

in which $\log \frac{I_o}{I}$ is the determined absorption value. ε is the "molar" extinction coefficient, c is the "molar" concentration of lignin, and d is the thickness of the liquid layer in cm. The "molarity" of lignin solutions is arbitrarily based upon the methoxyl content of the lignin, which, is taken to be 15 %. A "molar" solution of lignin then contains 206 g (*i.e.* $\frac{1 \text{ mole } OCH_3}{15} \times 100$ g) of lignin per litre. The value of ε used, *viz.* 3 200, was that determined for native lignin by Aulin-Erdtman [6]. Our determinations are therefore only valid if we are justified in assuming that no changes took place in the lignin structure during the various treatments.

Osmotic determinations. The osmotic determinations were carried out on the nitrated glucomannans as described in an earlier paper [1].

An aqueous solution of the acidic araboxylan (K_2X_3) was passed through a column of the cation-exchange resin IR 120 (Na^+) and the osmotic pressure was then determined on the sodium salt of the araboxylan dissolved in 0.1 N sodium chloride (cf. Ref[7].). The semipermeable membranes used were "Ultracellafilter allerfeinst" supplied by "Membranfiltergesellschaft Göttingen".

Viscometric determinations. The relative viscosity was determined on the nitrated samples in butyl acetate in an Ostwald viscometer at 25°.

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