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ISOLATION AND CHARACTERISATION OF LIGNIN–CARBOHYDRATE COMPLEXES FROM THE MILLED-WOOD LIGNIN FRACTION OF *Pinus densiflora* SIEB. ET ZUCC.

JUN-ICHI AZUMA, NOBUYOSHI TAKAHASHI, AND TETSUO KOSHIJIMA
Research Section of Wood Chemistry, Wood Research Institute, Kyoto University, Gokasho, Uji, Kyoto 611 (Japan)
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ABSTRACT

The lignin–carbohydrate complex (LCC-W), isolated from the milled-wood, lignin fraction of *Pinus densiflora* Sieb. et Zucc., comprised three fractions (W-1,2,3) by gel filtration on Sepharose 4B. W-1 was eluted at the void volume, whereas W-2 and W-3 were included in the gel and had apparent weight-average molecular weights of 5.0×10^5 and 5.0×10^3, respectively. W-2 and W-3 were homogeneous in ultracentrifugal and electrophoretic analyses. The sedimentation coefficients of W-2 and W-3 were 25.7 and 0.4S, respectively. The chemical composition of W-2 was 38.0% of neutral sugar, 6.2% of uronic acid, 51.5% of lignin, and the corresponding values for W-3 were 73.1, 11.0, and 22.2%. The neutral carbohydrate residues of W-2 and W-3 were L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose in the ratios 15.8:16.2:37.3:16.7:14.0 and 27.6:16.5:26.1:19.3:10.5, respectively. Based on the results of methylation and Smith-degradation analyses, the carbohydrate moiety of the LCC-W fractions was found to be multiply branched. The major backbone structure was composed of (1→4)-linked D-mannopyranosyl residues. By hydrophobic-interaction chromatography on Phenyl- and Octyl-Sepharose CL-4B gels, it is concluded that the LCC-W fractions have a hydrophobic property that is exclusively ascribed to the lignin moiety.

INTRODUCTION

Milled-wood lignin (MWL), isolated by the method of Björkman, contains a few percent of carbohydrates[1,2]. Various procedures for removal of the carbohydrate moiety from MWL have been attempted[3,4], but complete removal has not been achieved. Although the importance of the carbohydrate in MWL has been stressed in the association between carbohydrate and lignin[5], the nature of the carbohydrate moiety has not been fully investigated. Our interest has been in the establishment of the existence of lignin–carbohydrate linkages in MWL and in the characterisation of the carbohydrate moiety.

After extraction of MWL from finely divided wood (*Pinus densiflora*), extraction

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with *N,N*-dimethylformamide gave a partially acetylated glucomannan and acidic polysaccharides having lignin contents of 4.17 and 13.5%, respectively[6-8]. We now report a new method for the isolation of lignin–carbohydrate complexes from MWL of *Pinus densiflora* and some chemical and physical properties of the complexes.

EXPERIMENTAL

Materials. — Phenyl- and Octyl-Sepharose CL-4B (ligand concentration, ~40 μmol/ml[9]), Sepharose 4B and Sephadex G-100, Dextran T fractions (10, 20, 40, 70, and 500), and FITC Dextran (FITC-3) were supplied by Pharmacia Fine Chemicals. Extra-pure propionic acid and acetic acid for g.l.c. were obtained from Gaskuro Kogyo Co., Ltd. *Pinus densiflora* Sieb. et Zucc. was obtained at the Kamigamo Experimental Forest, Kamigamo, Kita-ku, Kyoto. The tree was ~30 years old (total height, ~14 m). Acetyl-galactoglucomannan $\{[\alpha]_D^{20}$ −28° (*c* 0.5, water); weight-average molecular weight of 1.2×10^4; galactose, glucose, and mannose molar ratios of 6.6:21.8:71.6; and 5.9% of acetyl$\}$ was prepared[6-8] from Björkman LCC. Acetyl-arabinoglucuronoxylan $\{[\alpha]_D^{20}$ −13° (*c* 0.21, methyl sulfoxide); weight-average molecular weight of 2.23×10^4; arabinose and xylose molar ratio of 12.9:87.1; 5.6% of uronic acid and 3.0% of acetyl$\}$ was prepared[10] from Coir Fiber of *Cocos nucifera* L.

General. — Solvents were removed under diminished pressure below 45°. Optical rotations were determined with a JASCO DIP-181 digital polarimeter at 20°. T.l.c. was carried out on Silica gel 60 (Merck, 5715) with *A*, chloroform–methanol–acetic acid (6:4:1); *B*, ethyl acetate–pyridine–water (8:2:1); *C*, ethyl acetate–2-propanol–water (16:6:3), and *D*, 1-butanol–pyridine–water (6:4:3). P.c. was performed by the ascending method on Whatman 3MM paper with solvent *D*. Sugars were detected with alkaline silver nitrate[11], *p*-anisidine hydrochloride[12], and 10% ethylenediamine sulfate[13]. Total carbohydrate was determined by the phenol–sulfuric acid method[14], uronic acid by the modified carbazole method[15], and lignin by the standard TAPPI methods[16]. The lignin content was also determined by the acetyl bromide method[17].

U.v. spectra were recorded for solutions in 50% aqueous 1,4-dioxane with a JASCO UVDEC-1 spectrophotometer. I.r. spectra were determined for KBr discs, using a JASCO IR-S spectrophotometer. G.l.c. was conducted on a JEOL JGC 1100 gas chromatograph equipped with flame-ionisation detectors. Separations were performed on *A*, 3% of ECNSS-M on Gas Chrom Q; *B*, 3% of OV-225 on Gas Chrom Q; and *C*, 20% of tetramethylcyclobutanediol adipate–4% phosphoric acid on Chromosorb W in glass columns (2 m × 0.3 cm); and *D*, an SP-1000 S.C.O.T. glass column (25 m × 0.28 mm). Analytical ultracentrifugation was performed at 42,040 r.p.m. and 20° in a Spinco model E ultracentrifuge equipped with Schlieren optics. G.l.c.–m.s. was conducted with a Shimadzu LKB-9000 system, using column *B* at 170 and 150°. The ionisation potential was 70 eV, the ionization current was 30 μA, and the temperature of the ion-source was 210°.

Electrophoretic studies. — The lignin–carbohydrate complex (LCC) was pre-coloured with Procion Blue MX-R and subjected to electrophoresis[18] on Toyo GB-60 glass-fiber filter paper (30 × 20 cm) in 0.05M borate buffer at 50 mA for 20 min and 0.1M borax–sodium chloride buffer at 100 mA for 20 min.

Determination of O-acetyl groups. — The sample (9 mg) was hydrolysed with M HCl (0.3 ml) in a sealed tube at 100° for 2 h. Propionic acid was used as the internal standard. The hydrolysate was directly applied[19] in g.l.c. on column *C* at 120°.

Determination of component sugars. — The sample (10 mg) was hydrolysed with 5 ml of 0.5M sulfuric acid at 100° for 6 h, and 1 mg of methyl β-D-glucopyranoside was added as the internal standard[20]. The hydrolysate was neutralised with barium carbonate, deionised with Dowex 50W-X8 (H^+) and 1-X8 (AcO^-) resins, and concentrated to dryness. The neutral sugars were converted[21] into the corresponding alditol acetates, and separated by g.l.c. on column *A* at 180°. Identification of the monosaccharides was also carried out by t.l.c. (solvents *A*–*C*).

Determination of the configurations of the sugars. — The LCC-W was hydrolysed[22] with 72% sulfuric acid followed by 3% sulfuric acid. The hydrolysate was neutralised and deionised as described above. The neutral monosaccharides were separated by p.c. and t.l.c. (solvents *D* and *B*, respectively), to give arabinose, xylose, mannose, galactose, and glucose, the configurations of which were determined[23]. Each monosaccharide (1 mg) was treated with 0.5 ml of D-(+)-2-octanol and one drop of trifluoroacetic acid overnight at 130° in a sealed tube. After evaporation, D-(+)-2-octyl glycosides were acetylated, and analysed by g.l.c. on column *D* at 200°. The split ratio was 1/50 and the carrier-gas flow was 2 ml/min.

Isolation of lignin–carbohydrate complex. — The sap wood of *Pinus densiflora* (24–80 mesh), extracted previously with ethanol–benzene (1:2) for 48 h, was depectinated[6] with 0.25% aqueous potassium acetate at 60° for 24 h. The wood meal was then Vibromilled for 48 h under nitrogen with external cooling by tap water. More than 80% of particles were accumulated in the range of 12–36 μm. Dried, milled wood (500 g) was extracted with 80% aqueous 1,4-dioxane (5.0 litres) for 48 h at room temperature. The residue was washed with 80% aqueous 1,4-dioxane and re-extracted with the same solvent. The combined filtrates and washings were concentrated to a small volume, to give milled-wood lignin (MWL). In order to obtain water-soluble lignin–carbohydrate complex (LCC), this solution was dialysed against distilled water. The precipitate formed during dialysis was removed by centrifugation at 8,000*g* for 15 min at 10°. The aqueous layer was lyophilised to give LCC-S fraction. The carbohydrate-less lignin fraction was removed[4] from the fraction LCC-S; LCC-S (1 g) was solubilised in 56 ml of pyridine–acetic acid–water (9:1:4) and extracted with chloroform (6 vol.). After addition of 40 ml of distilled water, the aqueous layer was recovered by centrifugation at 8,000*g* for 15 min at 10°. The chloroform layer was washed with distilled water (4 × 40 ml). The combined aqueous layers were thoroughly dialysed against distilled water and lyophilised, to give the LCC-W fraction. The chloroform layer was concentrated to dryness, to give carbohydrate-less lignin fraction.

Fractionation of the lignin–carbohydrate complex. — Fractionation of LCC-W was performed by gel filtration on a column (115 × 2.4 cm) of Sepharose 4B equilibrated with 25mM sodium phosphate buffer (pH 6.8). LCC-W (~50 mg) was solubilised in the equilibrating buffer (5 ml), applied to the column, and eluted with the same buffer. Fractions (6 ml) were collected at room temperature, using a Toyo model SF-160K fraction collector at 40 ml/h. A 0.5-ml sample of each fraction was analysed for carbohydrate content. The lignin content of each fraction was determined by measuring the absorbance at 280 nm.

Determination of molecular weight. — The molecular weight distribution of LCC-W was analysed by gel filtration on columns of Sephadex G-100 (60 × 1.0 cm) and Sepharose 4B (55 × 1.0 cm), using 25mM sodium phosphate buffer (pH 6.8) as eluent. Each 1-ml fraction was collected using a Toyo model SF-100G mini fraction-collector equipped with a drop counter. The column was calibrated against the dextrans having known molecular weight.

Methylation analysis. — The sample (100 mg) was methylated by the method of Hakomori[24] followed by the method of Kuhn[25]. The methylated product, extracted with chloroform, showed no i.r. absorption for hydroxyl groups. The fully methylated LCC was treated with 2 ml of 90% formic acid for 2 h at 100°. Formic acid was then evaporated, and the residue was hydrolysed with 3 ml of 0.25M sulfuric acid for 12 h at 100°. The partially methylated monosaccharides were analysed by g.l.c. on columns *A* and *B*, and/or by g.l.c.–m.s. on column *B*.

Smith-degradation analysis of the lignin–carbohydrate complex. — The deacetylated LCC fractions were prepared by treating the native LCC fractions with 25mM NaOH for 4 h at 56°, followed by neutralisation, dialysis, and lyophilisation. The deacetylated LCC fraction had no i.r. absorption for acetyl carbonyl groups. Samples (50 mg) of the native and the deacetylated LCC fractions were separately treated with 50 ml of 0.05M sodium metaperiodate in the dark for 20 days at 5°. Each oxidation product was reduced with sodium borohydride, exhaustively dialysed against distilled water, and lyophilised. The product (5 mg) was hydrolysed with 2 ml of 2M trifluoroacetic acid. The hydrolysate was converted into the corresponding alditol acetates and analysed by g.l.c. on column *A*. After injection of the sample at 90°, the temperature was increased linearly at 6°/min to 190°.

Hydrophobic-interaction chromatography of the lignin–carbohydrate complex. — Hydrophobic-interaction chromatography was performed on columns of Phenyl-Sepharose CL-4B (14 × 1.8 cm) and Octyl-Sepharose CL-4B (15 × 1.8 cm) gels at ambient temperature. The columns were equilibrated with 25mM sodium phosphate buffer (pH 6.8) containing 0.8M ammonium sulfate. The LCC fractions (50 mg) were solubilised in ~20 ml of the equilibrating buffer and applied to the column. After the unadsorbed material had been eluted with the equilibration buffer, the adsorbed material was eluted with 50% Ethyl Cellosolve in 25mM sodium phosphate buffer (pH 6.8). The column was checked for its saturation with LCC by re-chromatography of the unadsorbed fraction on a new column.

RESULTS

Isolation and fractionation of the lignin–carbohydrate complex. — The water-soluble, lignin–carbohydrate complex (LCC-S) was isolated from the milled-wood lignin fraction of *Pinus densiflora*. Purification of LCC was effected by treatment of LCC-S in a pyridine–acetic acid–water–chloroform system. The carbohydrate-less lignin fraction recovered from the chloroform layer contained only 0.92% of carbohydrate. The yields of the LCC-S and LCC-W fractions were 1.55–2.91 and 0.75–1.10% of the depectinated wood-meal, respectively. A typical elution profile of LCC-W on an analytical column of Sephadex G-100 is shown in Fig. 1. LCC-W was separated into two fractions, one of which was eluted at the void volume (W-G-1); the other was included in the gel matrices, showing a very broad, symmetrical peak (W-G-2). However, gel filtration of LCC-W on Sepharose 4B gave (Fig. 2) three fractions (W-1,2,3), corresponding to 4.1, 34.8, and 37.7%, respectively, of the material applied on the column. W-1, the fraction of highest molecular weight, was eluted at the void volume, whereas fractions W-2 and W-3 were included in the gel. Re-chromatography of each fraction on the same Sepharose 4B column did not reveal reversible formation of the other fraction. On an analytical column of Sephadex G-100, fractions W-2 and W-3 were recovered at the same positions as fractions W-G-1 and W-G-2, respectively. The distribution curve for carbohydrate overlapped completely with that of lignin, as shown in Figs. 1 and 2. The presence of lignin and carbohydrate in the LCC fractions was also proved

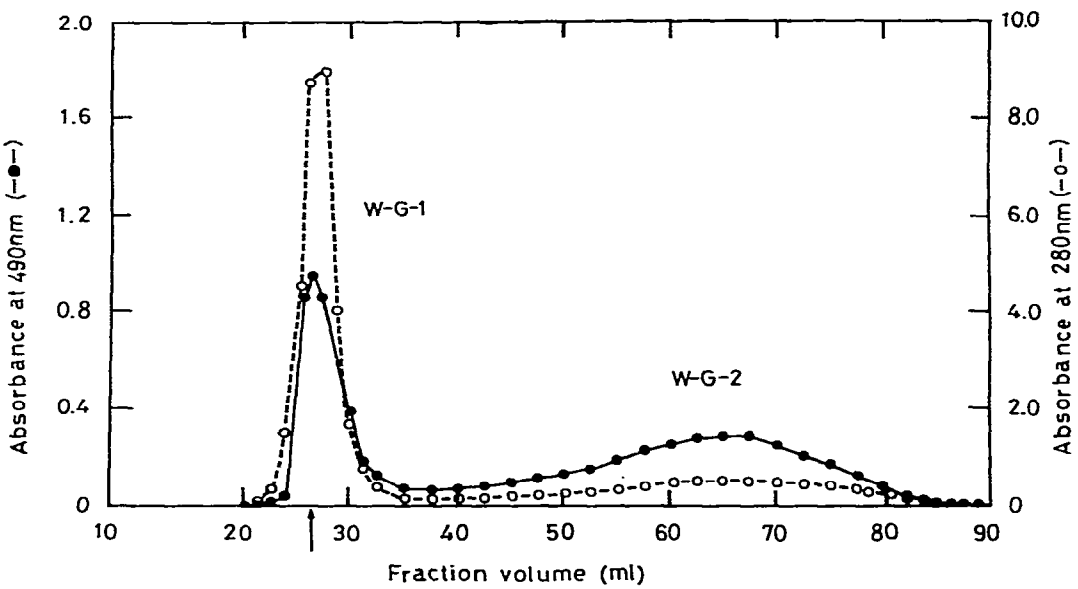

Fig. 1. Gel filtration of the LCC-W fraction on Sephadex G-100 (See Experimental); the arrow represents the void volume determined with Blue Dextran. Each fraction was analysed for carbohydrate (——) and lignin (– – –).

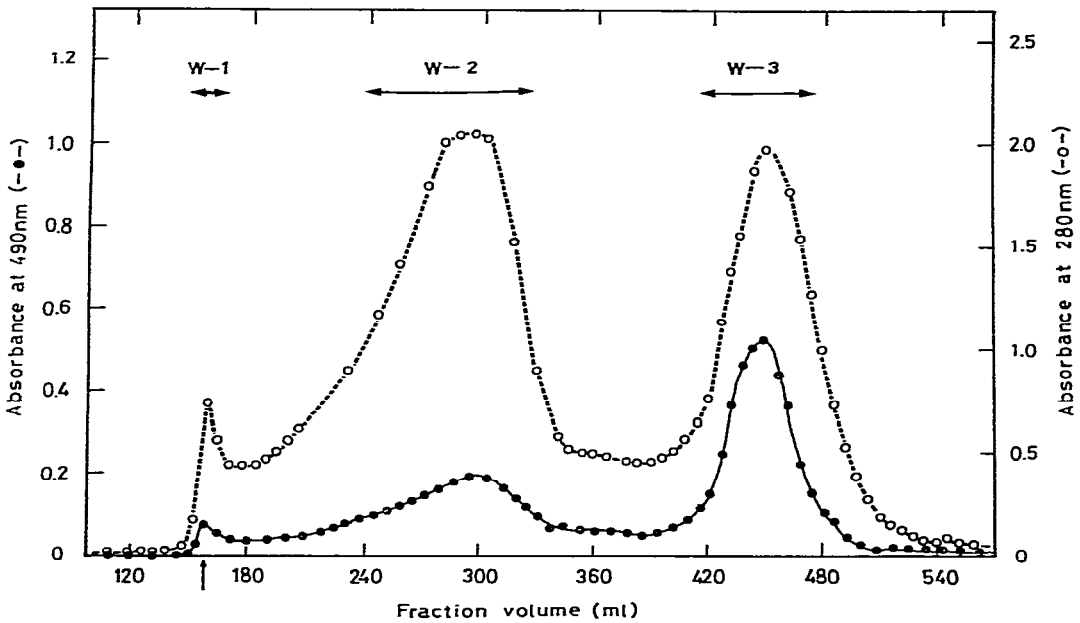

Fig. 2. Gel filtration of the LCC-W fraction on Sepharose 4B (See Experimental); the arrow represents the void volume determined with Blue Dextran. Each fraction was analysed for carbohydrate (——) and lignin (– – –).

by chromatography on Phenyl-Sepharose and Octyl-Sepharose (Fig. 3). The unadsorbed and adsorbed fractions were designated as fractions I and II, respectively. In each LCC fraction, there was overlapping of the two peaks due to the lignin and carbohydrate moieties. The affinity of each LCC for Phenyl-Sepharose was higher than that for Octyl-Sepharose. The amounts of the adsorbed materials of the LCC-W, W-2, and W-3 fractions were 83.1–89.5, 85.8–93.8 and 73.1–77.0% for Phenyl-Sepharose, and 80.4–87.0, 84.4–92.6, and 56.5–65.7% for Octyl-Sepharose, respectively. In velocity sedimentation, fractions W-2 and W-3 showed single, symmetrical boundaries, indicative of general homogeneity of molecular size. Extrapolation of sedimentation coefficients of W-2 and W-3 to infinite dilution gave values of 25.7 and 0.4S, respectively. In paper electrophoresis in borate buffer, fractions W-1, W-2, and W-3 each showed a single spot. LCC-W which contained these three fractions showed a very broad spot.

U.v. and i.r. spectra of the lignin-carbohydrate complex. — The u.v. spectra of the LCC-W, W-1, W-2, and W-3 fractions are shown in Fig. 4. All LCC fractions showed a maximum at 281 nm and minimum at 261 nm. The absorption coefficient at 281 nm, a_{281} ($l.g^{-1}.cm^{-1}$), and the ratio of the absorbance at 280 nm to that at 260 nm, a_{280}/a_{260}, are listed in Table I. The typical i.r. spectra of LCC-W, W-1, W-2, and W-3 are shown in Fig. 5. Together with those of the carbohydrate-less lignin fraction prepared from LCC-S, acetyl-galactoglucomannan and acetyl-arabino-

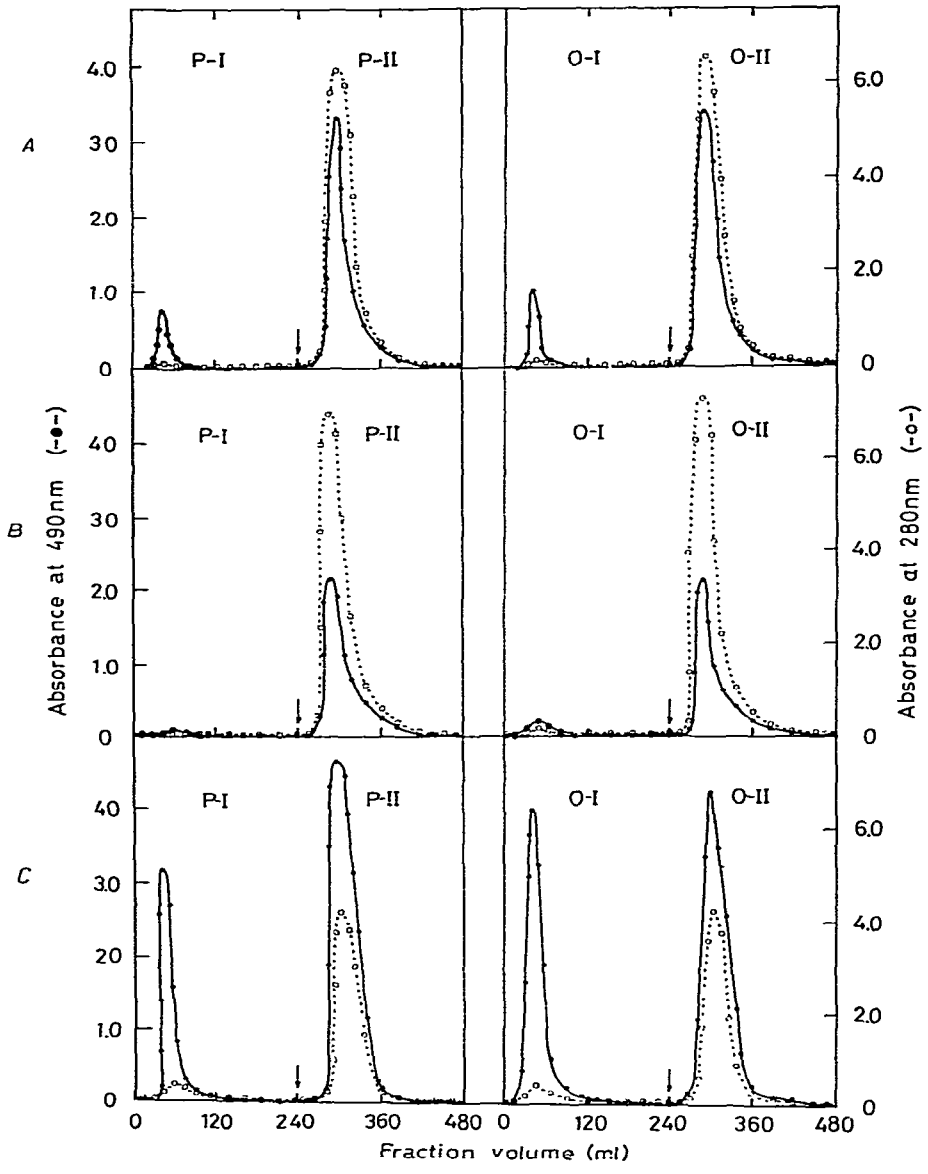

Fig. 3. Hydrophobic-interaction chromatography on Phenyl-Sepharose CL-4B (P) and Octyl-Sepharose CL-4B (O) (See Experimental): *A*, LCC-W; *B*, W-2; and *C*, W-3; fractions I and II represent the unadsorbed and adsorbed fractions, respectively. The arrows indicate the change of elution conditions from 25mM sodium phosphate buffer (pH 6.8) containing 0.8M ammonium sulfate to 50% Ethyl Cellosolve in the same buffer. Each fraction was analysed for carbohydrate (——) and lignin (– – –).

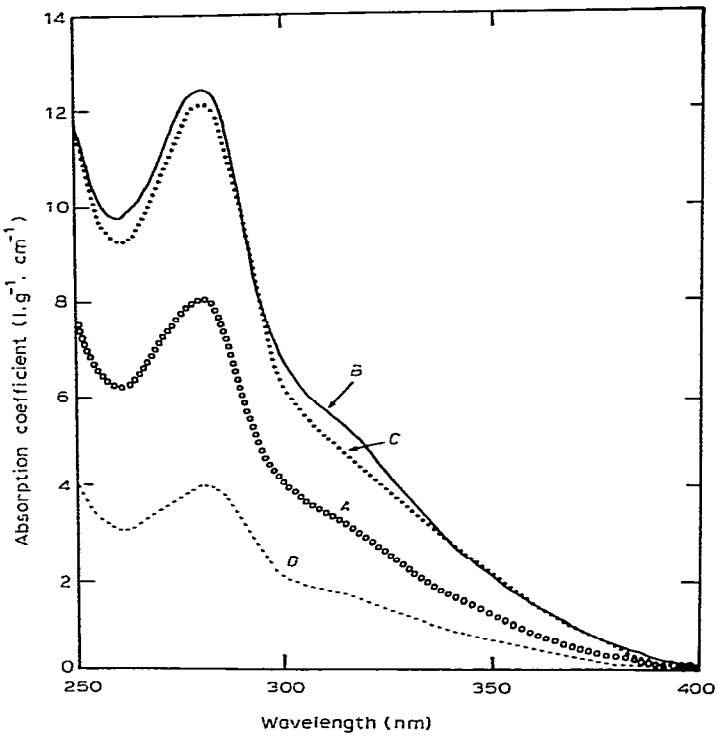

Fig. 4. U.v. spectra: *A*, LCC-W; *B*, W-1; *C*, W-2; and *D*, W-3 (See Experimental).

glucuronoxylan were included in Fig. 5. All four LCC fractions showed absorptions typical of carbohydrate and lignin moieties and of ester carbonyl groups.

Chemical properties of the lignin–carbohydrate complex. — The chemical properties of the LCC fractions are shown in Tables I and II. As expected from the gel-filtration profile (Fig. 2), in contrast to the lignin content, the neutral and uronic acid content gradually became higher from fraction W-1 to W-3. The $[\alpha]_D$ value and the acetyl content became larger in accordance with the increase of the carbohydrate content. Each LCC contained L-arabinose, D-xylose, D-mannose, D-galactose, and D-glucose. The configurations were determined by g.l.c. of the acetylated D-(+)-2-octyl glycosides. The mannose content of W-2 was higher than those of W-1 and W-3, whereas the arabinose content of W-3 was higher than those of W-1 and W-3. The glucose content gradually became lower from W-1 to W-3.

The apparent weight-average molecular weights ($\overline{M}_w$) of W-2 and W-3 were estimated to be 5.0×10^5 and 5.0×10^3, respectively, from the plot of $\overline{V}_e$ *vs.* log $\overline{M}_w$. The apparent number-average molecular weights ($\overline{M}_n$) of W-2 and W-3 were estimated to be 1.8×10^5 and 3.4×10^3, respectively, from the plot of $\overline{V}_e$ *vs.* log $\overline{M}_n$. It was not possible to estimate the molecular weight of W-1, since this fraction did not penetrate into the gel matrices.

Methylation analysis of the lignin–carbohydrate complexes. — The LCC fractions

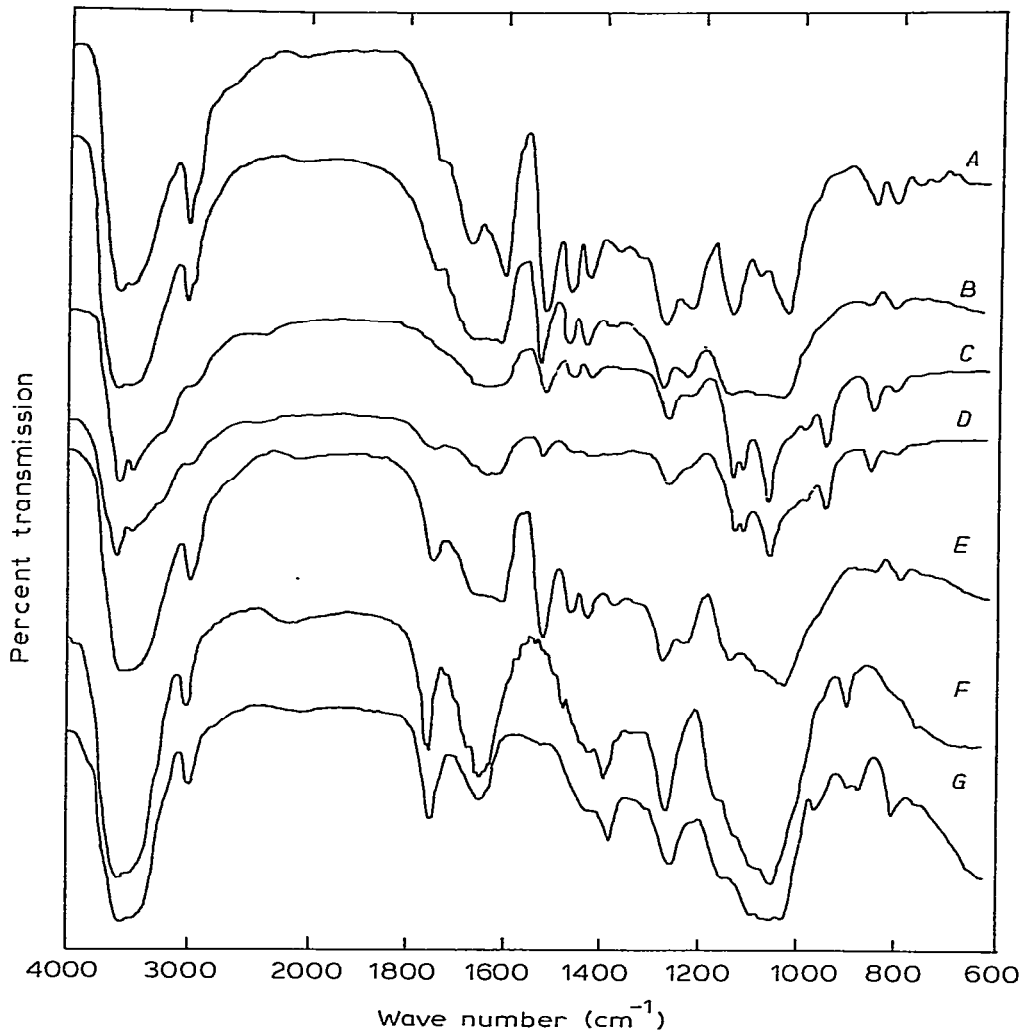

Fig. 5. I.r. spectra: *A*, carbohydrate-less lignin; *B*, W-1; *C*, W-2; *D*, W-3; *E*, LCC-W; *F*, acetyl-arabinoglucuronoxylan; and *G*, acetyl-galactoglucomannan (See Experimental).

were subjected to conventional methylation analysis[26–28]. The results are shown in Table III. 2,3,6-Tri-*O*-methyl-D-mannose was the most prominent, partially methylated monosaccharide in each LCC fraction. 2,3,4,6-Tetra-*O*-methyl-D-mannose was contaminated with 2,3,4,6-tetra-*O*-methyl-D-glucose. Mannose and glucose residues in each LCC fraction were also recovered as tri- and di-*O*-methylated hexoses. Galactose was recovered only as 2,3,4,6-tetra-*O*-methyl-D-galactose, and arabinose as 2,3,5-tri- and 2,5-di-*O*-methyl-L-arabinose. Tetra-, tri-, di- and mono-*O*-methyl-D-xylose residues were also formed.

Smith degradation of the lignin–carbohydrate complexes. — The native and deacetylated LCC fractions were subjected to complete Smith degradation. The result

TABLE I

CHEMICAL COMPOSITIONS AND PROPERTIES OF THE LCC FRACTIONS OF *Pinus densiflora*[a]

Components	*Lignin–carbohydrate complexes*			
	LCC-W	*W-1*	*W-2*	*W-3*
Carbohydrate content[b]				
Neutral sugar	51.6	20.4	38.0	73.1
Uronic acid	8.9	1.5	6.2	11.0
Lignin content[b]				
Klason	38.7	66.0	51.0	21.3
Acid-soluble	1.2	0.7	0.5	0.9
Acetyl bromide	40.5	67.2	46.6	25.5
Acetyl content[b]	1.9	0.7	1.1	2.6
$[\alpha]_D^{20}$ (50% 1,4-dioxane, degrees)	−13.5	−4.5	−6.0	−17.0
$S_{20}°$ (S)	n.d.[c]	n.d.	25.7	0.4
$\bar{M}_w$	n.d.	n.d.	5.0×10^5	5.0×10^3
$\bar{M}_n$	n.d.	n.d.	1.8×10^5	3.4×10^3
λ_{max} (50% 1,4-dioxane, nm)	281	281	281	281
λ_{min} (50% 1,4-dioxane, nm)	261	261	261	261
a_{281} ($l.g^{-1}.cm^{-1}$)	8.2	12.5	12.4	4.0
a_{280}/a_{260}	1.29	1.26	1.30	1.29

[a]Details of the procedure are given in the Experimental section. [b]Values are expressed as a percentage of the dry weight of each lignin–carbohydrate complex. [c]Not determined.

TABLE II

NEUTRAL-SUGAR COMPOSITION OF THE LCC FRACTIONS OF *Pinus densiflora*[a]

Monosaccharides	*Lignin–carbohydrate complexes*[b]			
	LCC-W	*W-1*	*W-2*	*W-3*
L-Arabinose	22.5	14.4	15.8	27.6
D-Xylose	18.2	10.0	16.2	16.5
D-Mannose	29.2	33.7	37.3	26.1
D-Galactose	19.1	21.8	16.7	19.3
D-Glucose	11.0	20.1	14.0	10.5

[a]Details of the procedure are given in the Experimental section. [b]Values are expressed as a percentage of the total, neutral sugar.

is shown in Table IV. The solution became turbid during periodate oxidation, and insoluble material was formed which could be solubilised by reduction with sodium borohydride. There are no substantial differences in the compositions of the native LCC fractions. In the native LCC fractions, D-galactose and D-glucose were almost completely degraded, whereas some L-arabinose and D-xylose and much D-mannose remained. However, after de-acetylation, only a small proportion of D-mannose

TABLE III

METHYL ETHERS FROM THE HYDROLYSATE OF THE LCC FRACTIONS OF *Pinus densiflora*[a]

Methylated sugars[b]	T[c]	*Lignin–carbohydrate complexes*[d]		
		LCC-W	*W-2*	*W-3*
2,3,4,6-Glc or Man	1.00	3.6	4.0	3.1
2,3,4,6-Gal	1.24	10.6	16.5	10.4
2,3,5-Ara	0.44	3.8	6.5	12.3
2,3,4-Xyl	0.64	10.5	4.8	12.5
2,3,6-Man	2.16	27.6	21.9	15.4
2,3,6-Glc	2.46	6.9	5.1	5.1
2,5-Ara	1.16	9.3	3.2	11.3
2,3-Xyl	1.45	5.6	11.1	4.0
2,3-Man	4.63	3.9	3.7	4.0
2,3-Glc	5.22	3.9	6.4	3.2
2 or 3 or 4-Xyl	3.04	2.5	2.1	2.4
Others[e]		11.8	14.7	16.3

[a]Details of the procedure are given in the Experimental section. [b]2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, *etc.* [c]Retention times are relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol on an ECNSS-M column. [d]Values are expressed as a relative molar percentage of the total partially methylated sugars. [e]Unidentified, partially methylated sugars.

TABLE IV

COMPOSITION OF THE HYDROLYSATES OF THE LCC FRACTIONS OF *Pinus densiflora* AFTER PERIODATE OXIDATION[a]

Components	*Lignin–carbohydrate complexes*[b]					
	LCC-W		*W-2*		*W-3*	
	A	*B*	*A*	*B*	*A*	*B*
Glycerol	31.0	46.1	35.9	48.6	40.6	52.5
Erythritol	27.7	41.8	26.3	23.0	24.8	38.5
L-Arabinose	5.1	2.1	4.5	4.0	4.1	3.2
D-Xylose	4.9	4.0	4.0	7.5	4.5	5.8
D-Mannose	31.3	6.0	29.3	16.9	26.0	trace

[a]Details of the procedure are given in the Experimental section. [b]Lignin–carbohydrate complex (for details, see text): A, original; and B, de-acetylated. Values are expressed as a relative molar percentage of the component sugars.

remained in fractions LCC-W and W-2, and essentially all D-mannose was degraded in fraction W-3. A small proportion of L-arabinose and D-xylose remained in the de-acetylated LCC fractions.

DISCUSSION

The lignin–carbohydrate complex (LCC-W) can be easily isolated from the

milled-wood lignin (MWL) fraction of *Pinus densiflora*. Separation of lignin from carbohydrate cannot be achieved by gel filtration, electrophoresis, ultracentrifugation, and hydrophobic-interaction chromatography, strongly indicating the existence of chemical bonds between lignin and carbohydrates. Lundquist and Simmonson[4] prepared lignin having a very low content of carbohydrate after treatment of MWL with a pyridine–acetic acid–water–chloroform system and suggested the presence in the aqueous layer of LCC, a considerable part of which had a low molecular weight. Fraction W-3 corresponds to this material. Each LCC fraction has a complex neutral-carbohydrate composition which cannot be deduced from the structure of hemicellulose reported so far[29,30]. However, methylation analysis and Smith degradation of W-2 and W-3 suggest the presence of a hemicellulose closely similar to the acetylated galactoglucomannan isolated[6–8] from Björkman LCC. Also, the methylation analysis indicates the existence of a chain of (1→4)-linked xylopyranosyl residues. These results suggest a cross-linked network of lignin and several kinds of hemicelluloses, the structure of which is already known, or the possibility of new, complex polysaccharides specific to LCC. The mannose content of the lignin-rich fractions (W-1 and W-2) is relatively higher than that of fraction W-3 (Table I), suggesting that some of the mannose residues may be linked to lignin. The resistance of the mannose residues of the native LCC fractions to periodate oxidation is ascribed mainly to the acetyl groups located[31] at positions 2 and 3. It is not clear whether the carbohydrates remaining after periodate oxidation of the deacetylated LCC fractions are involved in the chemical linkage with lignin or survived because of steric hindrance to periodate oxidation. The optical activity of each LCC signified the carbohydrate moiety, since the lignin moiety is optically inactive. The negative optical rotation is suggestive of β-D-glycosidic linkages. The u.v. and i.r. data revealed that each LCC fraction contained principally guaiacyl lignins. The u.v. spectra of all the LCC fractions are similar to those of MWLs obtained from *Pinus densiflora*[32] and the other soft woods[2,33–35]. However, there were marked differences in the strengths of the absorptions (Fig. 4). Thus, a_{280} for guaiacyl lignin is[36] 15.6 $l.g^{-1}.cm^{-1}$ and that for syringyl lignin is[37] 4.5 $l.g^{-1}.cm^{-1}$. The a_{280} value of soft-wood MWL is[38] in the range of 16–20 $l.g^{-1}.cm^{-1}$. An a_{280} value for MWL of *Pinus densiflora* of 19.3 $l.g^{-1}.cm^{-1}$ has been reported[32]. The a_{281} values reported herein are considerably smaller than those reported so far. The a_{281} value of fraction W-3 is similar to that of the syringyl lignin. The low a_{281} values may simply reflect the carbohydrate content. Indeed, from the results of Fig. 4 and Table I, the a_{280} values of fractions LCC-W, W-1, W-2, and W-3 are calculated to be 20.4, 18.7, 24.0, and 17.8 $l.g^{-1}.cm^{-1}$, respectively, assuming that each LCC is exclusively composed of lignin. The ratio of the absorbance at 280 and 260 nm tends to confirm that the lignin moiety of the present LCCs are composed of guaiacyl lignin[37].

The i.r. spectra of the LCC fractions were quite similar to those of MWLs obtained from *Pinus densiflora*[32,39] and various other soft-woods[34,38,40,41]. However, MWL contains discrete amounts of carbohydrate, and the contribution of carbohydrate to the i.r. spectra of various lignin preparations has been discussed[41–45].

Acetyl-galactoglucomannan and acetyl-arabinoglucuronoxylan were used to assess the effect of carbohydrate on the i.r. spectra of the LCC fractions. The absorptions due to aliphatic hydroxyl groups, C-H, and carbonyl groups of these hemicelluloses overlapped with those of lignin, so that interpretation is difficult[43,45]. In addition, there is possible overlap of the glucomannan specific bands at 870 and 810 cm^{-1} with the guaiacyl-ring specific bands at 855 and 815 cm^{-1}, respectively[40].

The ability of LCC fractions to bind to Phenyl-Sepharose and Octyl-Sepharose relates to their hydrophobic properties. The affinity of the LCCs for Phenyl-Sepharose is higher than that for Octyl-Sepharose, and the amount of the materials adsorbed on to the gel increased with increase of lignin content (Fig. 3, *A–C*). These results indicate that the hydrophobicity is ascribed to the lignin moiety. Further studies of the elucidation of the hydrophobicity of LCC might assist in the clarification of the function of LCC in living plants.

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