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Structure of small lignin fragment retained in water-soluble polysaccharide extracted from sugi MWL isolation residue

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Abstract In order to analyze the structural features of small lignin fragments that are closely associated to polysaccharides, lignin carbohydrate complex (LCC) with low lignin content was extracted with water from the residual wood meal (sugi, *Cryptomeria japonica*) of milled wood lignin (MWL) isolation. This LCC exhibited almost the same neutral sugar composition as those extracted by other LCC solvents (dimethylformamide, dimethylsulfoxide, and others) but the lignin content was only 5.3%, which was much lower than others. Gel filtration chromatography demonstrated that lignin in this LCC was found together with carbohydrates at the higher molecular weight region, but after the treatment with carbohydrate-degrading enzymes the apparent molecular weight of both lignin and carbohydrates decreased significantly. Using a mild alkaline treatment, the apparent molecular weight of lignin also decreased while that of polysaccharide was not affected. These data indicated that lignin in this LCC is present as small fragments attached to high molecular weight polysaccharide at least partly by alkali-unstable linkages. Structural analysis by ozonation method revealed that the lignin in this LCC was lower in *erythro*/*threo* ratio of β-O-4 structure and relatively richer in the *threo* type structure carrying C-aryl linkages at β-position (β-5 and/or β-1) than other lignin fractions present in MWL, LCCs extracted by other solvents, and their extraction residues. It was suggested that the chemical structure of lignin closely associated to carbohydrates was different from that of the main part of lignin.

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Key words Lignin · Lignin-carbohydrate complexes · Cell wall · Driselase

Introduction

There are many factors that presumably affect the frequency of each linkage type of lignin. Those factors create differences in the lignin structure even in the same tree. Examples of such factors are the types of cells,[1] location of the lignin in the cell wall,[2] the maturity of the cell (period after the cell wall formation started),[3–5] proportion of each precursor (syringyl/guaiacyl ratio),[3] and the type of polymerization (endwise or bulk type).[1,6]

It is also an unresolved question whether the frequency of each linkage type is different from portion to portion even within one molecule. Some researchers have suggested that carbohydrates provide an initiation point for lignin polymerization during cell wall formation.[7] If this is the case, small lignin fragments retained in polysaccharides could represent lignin at the early stage of its polymerization. It has also been tried for many years to obtain evidence for the α-ether type lignin–carbohydrate complex (LCC) in which carbohydrate is linked to the α-position of a β-O-4 structure.[8] In such a case, the small lignin fragment retained in the cell wall could possess a structure preferable for the formation of this type of LCC.

There has been no concrete evidence obtained for this type of LCC, but it is possible to assume that the structure of lignin closely associated to carbohydrates could be different from that of lignin that is present apart from carbohydrates. From this point of view, we have tried to isolate a 'small lignin fragment' tightly retained in the cell wall. Such a lignin fragment could represent a special part of lignin that was separated from the main body of lignin or that could not grow to a larger lignin polymer for some reason. In the present study, in order to obtain such a lignin fragment, residual wood meals after the extraction of milled wood lignin (MWL) was subjected to extraction with various LCC solvent systems. Among these solvent systems,

Table 1. Abbreviations of fractions extracted with various solvent systems at room temperature and 60°C

Solvent systems	Abbreviations
100% Water	WS
Dioxane – water (96:4)	DW96
100% Dimethylformamide	DMF
Dimethylsulfoxide – water (1:1)	DMSO50
Dioxane – water (1:1)	DW50
100% Dimethylsulfoxide	DMSO
1.2 M Sodium *p*-ethylbenzenesulfonate aqueous solution	HT

water extraction gave a unique LCC in terms of lignin content and the gel filtration behavior. Based on the structural analysis after ozonation and alkaline nitrobenzene oxidation methods, it was ditermined whether lignin in this fraction was different from lignins present in MWL and LCCs extracted by other solvent systems.

Experimental

Preparation of MWL and MWLR

Alcohol–benzene preextracted sugi (*Cryptomeria japonica*) wood meal (80 mesh passed) was ball-milled in dry toluene for 120 h and the milled wood extracted with dioxane–water 96:4 (v/v) for 24 h. Extraction was repeated five times and MWL was isolated according to Björkman's method.[9] The yield of MWL was 1.7% of wood meal. The residues after the extraction of MWL were suspended in dioxane solution and freeze dried, then dried under reduced pressure over P_2O_5. The MWL extraction residue (MWLR) was subjected to extraction with various solvent systems.

Extraction of LCCs with various solvent systems

Three-gram portions of MWLR were extracted with 100 ml of each solvent system listed in Table 1 for 24 h at room temperature or 60°C with stirring. Residues were removed by filtration and filtrates were subjected to the measurement of lignin content and neutral sugar analysis. Lignin content was determined by ultra violet (UV) absorbance at 290 nm. Gram extinction coefficient was obtained from MWL prepared in this experiment. Neutral sugars were determined by the alditol–acetate method.

Preparation of fractions from water and dimethylformamide extraction on a large scale

Twenty-four gram portions of MWLR were extracted with 800 ml of water or dimethylformamide for 24 h at room temperature with shaking. Residues were removed by filtration and fraction WS and fraction DMF were obtained from the filtrate by lyophilization or concentration, respectively. The yields of fractions WS and DMF were 3.7% and 3.0% on MWLR, respectively. Dimethylformamide extraction residue (DMFR) was washed three times with chloroform and dried successively in air and then under vacuum over P_2O_5.

Gel filtration chromatography

Gel filtration chromatography was performed using a column of Sepharose CL-6B or Sepadex G-50 (medium). As the eluent, 25 mM sodium acetate buffer (pH 5.0) was used for the analytical experiment and water was used for the preparative one. The bed height was 31.5 cm and the column diameter was 1.0 cm for the analytical experiment. The column dimensions for the preparative chromatography 46.0 cm high and 5.0 cm in diameter. The molecular weight distributions of carbohydrate and lignin were followed by the phenol-sulfuric acid method (482 nm) and UV absorbance at 280 nm, respectively.

Enzymatic treatment

Driselase (Kyowa Hakko) purified according to the method of Fry[10] except the freeze drying at the last stage was used as aqueous solution in this experiment. The WS fraction (78.2 mg or 51.6 mg) was treated with 25 mM sodium acetate buffer (pH 5.0, 5 ml) containing purified Driselase solution (0.03 ml) at 40°C with stirring. After 16 h and 88 h, parts of solution were taken and subjected to gel filtration chromatography. During and after the enzymatic treatment, no precipitate was observed. The presence of Driselase at the concentration used in the present experiment was confirmed to not interfere with the chromatographic analysis.

Mild alkaline treatment

WS fraction (95.0 mg) was treated with 0.25 M NaOH aqueous solution (3 ml) for 24 h at room temperature with stirring. After the treatment, the pH of the solution was adjusted to 5.0 with dilute acetic acid solution, and then a part of the solution was subjected to gel filtration chromatography. During and after the alkaline treatment, no precipitate was observed.

Lignin contents and neutral sugar analysis of preparative samples

Lignin contents of the high molecular weight portion (WSH) and low molecular weight portion (WSL) of the WS fraction were determined by UV absorbance at 280 nm and those of other fractions were determined by the Klason method. Neutral sugars were determined by the alditol–acetate method.

Alkaline nitrobenzene oxidation and ozonation

Alkaline nitrobenzene oxidation was conducted according to the procedure of Chen.[11] Ozonation analysis was con-

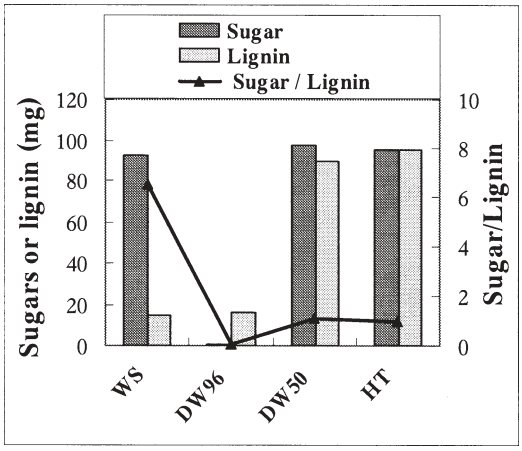

Fig. 1. Amount of neutral sugars and lignin extracted from residual wood meal of MWL isolation (MWLR) with various solvent systems at 60°C. Three grams of MWLR was used as starting material

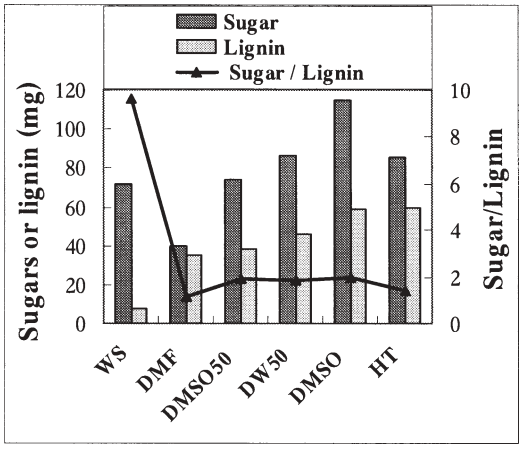

Fig. 2. Amount of neutral sugars and lignin extracted from MWLR with various solvent systems at room temperature. Three grams of MWLR was used as starting material

ducted according to the modified procedure of Akiyama et al.[12]

Results and discussion

Investigation for suitable extraction solvents

To obtain a fraction containing small lignin fragments, the residual wood meal (sugi, *Cryptomeria japonica*) after MWL extraction (MWLR) was further extracted with various solvent systems for 24 h at either room temperature or 60°C with stirring. The solvent systems used and the abbreviations of fractions obtained by the extractions are listed in Table 1 in order of low to high extractability of lignin. The solvent systems used in this experiment, except for dioxane–water (96:4) (DW96), have been used for the extraction of LCC,[13,14] or could be expected to have the ability to extract it. Sodium *p*-ethylbenzenesulfonate is known as a hydrotropic agent, and is known to be an excellent solvent for lignin under the high concentration of this agent.[15] The lignin content in each fraction was determined by its UV absorbance at 290 nm instead of 280 nm in order to avoid the interference from the strong absorbance of sodium *p*-ethylbenzenesulfonate.

Figure 1 shows the results of extraction with various solvent systems at 60°C. Only a small amount of lignin and carbohydrate were extracted from MWLR with 96% aqueous dioxane, indicating that extraction by this solvent system had been almost complete at the stage of MWL extraction. Therefore, major parts of the fractions obtained from MWLR were not the remains of the MWL extraction stage but became extractable by changing the extraction solvent. Among these fractions, fraction WS seemed to be unique because of its quite high sugar/lignin ratio compared with other fractions.

The results of extraction at room temperature are shown in Fig. 2. Similarly as above, fraction WS had quite high sugar/lignin ratio compared with other fractions, but the ratio was higher than that observed at 60°C (Fig. 1). As shown in Table 2, it is interesting that the neutral sugar compositions of fraction WS were almost identical with the others except DW96 and MWL, although the sugar/lignin ratio was quite different (Figs. 1 and 2). Björkman[14] reported that the neutral sugar compositions in the LCCs extracted by dimethylformamide, dimethylsulfoxide, and aqueous acetic acid were almost the same. The result that fraction WS also had a similar neutral sugar composition suggests that the major factor distinguishing the fraction WS from other fractions is lignin content. Because the high sugar/lignin ratio of the WS fraction suggested that lignin in this fraction could be present as small fragments attached to carbohydrate chains, this fraction was further analyzed.

Table 2. Molar ratios of neutral sugars extracted with various solvent systems

Fraction extracted	Neutral sugars composition (%)					
	Rha	Ara	Xyl	Man	Glc	Gal
Room temperature						
WS	1.1	4.5	19.7	49.5	18.9	6.3
DMSO	0.8	4.2	17.5	44.4	28.0	5.1
DMF	1.4	6.9	20.0	41.2	20.5	10.1
HT	1.1	5.3	18.1	50.3	18.6	6.7
DW50	1.7	6.9	18.7	49.5	15.9	7.3
DMSO50	2.8	5.5	19.3	48.0	16.2	8.1
MWL	4.5	13.7	20.3	16.2	28.5	16.8
60°C						
WS	1.2	5.4	18.8	51.2	16.9	6.5
DW96	6.5	21.2	22.0	16.0	20.6	13.7
DW50	1.2	4.7	19.0	56.4	13.0	5.9
HT	1.3	5.7	18.4	50.9	17.2	6.4

Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Glc, glucose; Gal, galactose; MWL, milled wood lignin

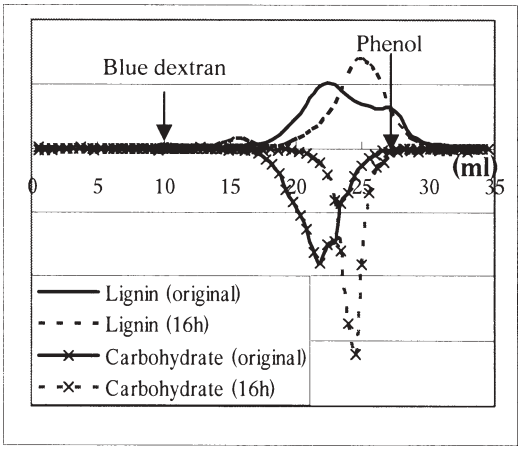

Fig. 3. Molecular weight distributions of lignin and carbohydrate in fraction WS before (original) and after (16h) treatment with Driselase (Sepharose CL-6B)

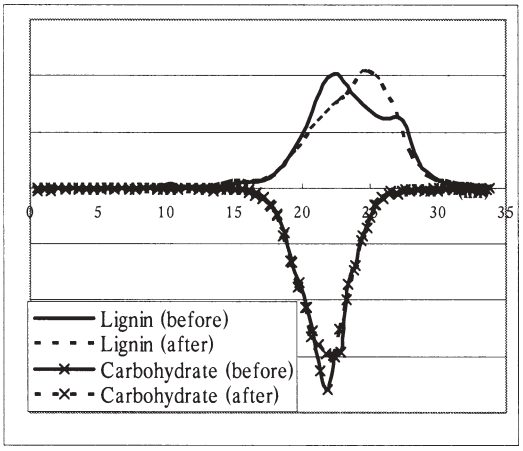

Fig. 5. Molecular weight distributions of lignin and carbohydrate in fraction WS before and after mild alkaline treatment (Sepharose CL-6B)

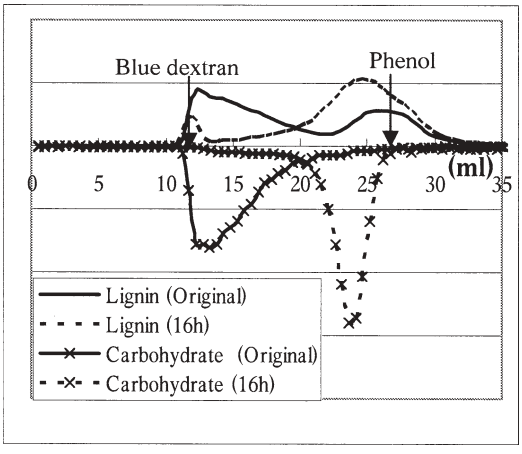

Fig. 4. Molecular weight distributions of lignin and carbohydrate in fraction WS before (original) and after (16h) treatment with Driselase (Sephadex G-50)

Changes in gel filtration chromatograms of WS fraction by enzymatic or alkaline treatment

WS fraction was prepared from MWLR on a large scale. The contents of lignin and the neutral sugar in the fraction were 5.3% and 58.6%, respectively, which were almost the same as those prepared on a small scale. To examine the interaction between lignin and carbohydrate, WS fraction was treated with the polysaccharide-degrading enzyme Driselase (Kyowa Hakko), which is a mixture of pectolytic, hemicellulolytic, and cellulolytic enzymes but lacks esterase and α-xylosidase activity, and has no peroxidase or laccase activity.[10,16] WS fraction, before and after the enzymatic treatment, was subjected to two kinds of gel filtration chromatography (Sepharose CL-6B and Sephadex G-50) and the changes in molecular weight distribution of the both lignin and carbohydrate were measured.

As shown in Fig. 3, the molecular weight distribution of the major part of the lignin appeared together with carbohydrate in the relatively high molecular weight region before Driselase treatment. However, after 16h of Driselase treatment, the molecular weight distribution of carbohydrate and that of lignin shifted to the lower molecular weight region although the scission of molecules must have occurred only on carbohydrate. When Sephadex G-50, which has smaller fractionation range compared to Sepharose CL-6B, was used for gel filtration chromatography, the same tendency was observed (Fig. 4). These results indicate that the molecular weight distribution of lignin obtained for the untreated WS fraction is an apparent one and the real molecular weight of lignin in WS fraction is rather small. In this context, the extension of enzyme treatment from 16h to 88h did not create any significant change in the chromatograms for both gels, suggesting that the scission of polysaccharide chain by this enzyme preparation was already completed within the first 16h.

Interestingly, a mild alkaline treatment of WS fraction resulted in a lowering of the molecular weight distribution of lignin while that of carbohydrate was not affected (Fig. 5), suggesting that at least some part of the linkage between lignin and carbohydrate in WS fraction is alkali-labile. This finding seems to coincide with the report by Lundquist et al.[17] which found that mild alkaline treatment of birch MWL could remove the major part of carbohydrate, whereas it was considerably stable toward acid treatment. Joniak et al.[18] reported that alkaline treatment (1M NaOH, 24h, room temperature), which was stronger than the conditions employed in the present study, could not cleave the LCC linkage of the phenolic benzyl ether type. Therefore, it appears a candidate for the alkali-labile linkage found in this experiment could be an ester.

All the results obtained in this section seem to suggest an image of LCC composing of carbohydrate polymer chains to which small lignin fragments are linked at least partly by alkali-labile linkages. It is interesting to compare the present results with those reported by Takahashi and Koshijima[19] who observed the decrease in molecular weight distribution, not only for lignin but also for carbohydrate,

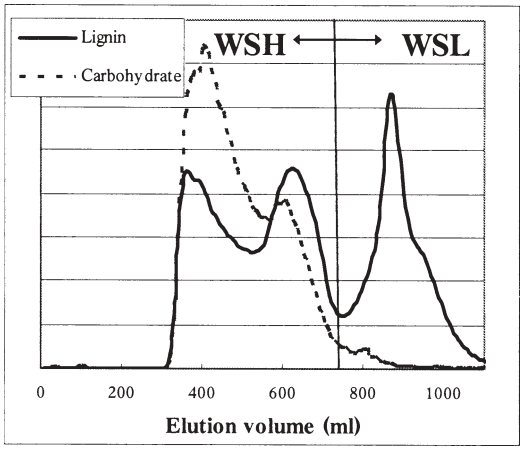

Fig. 6. Preparative gel filtration of WS (Sephadex G-50). *WSH*, high molecular weight fraction; *WSL*, low molecular weight fraction

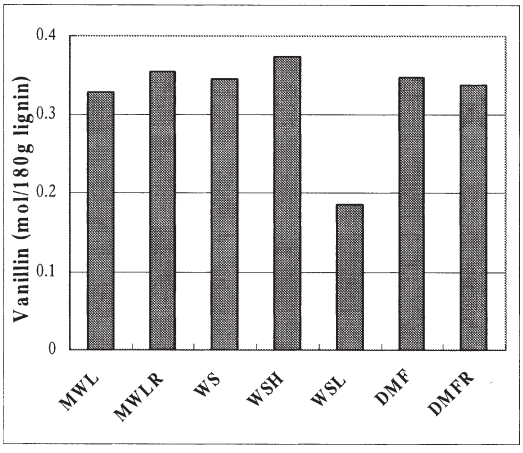

Fig. 7. Vanillin yields obtained by alkaline nitrobenzene oxidation of extracts and residues

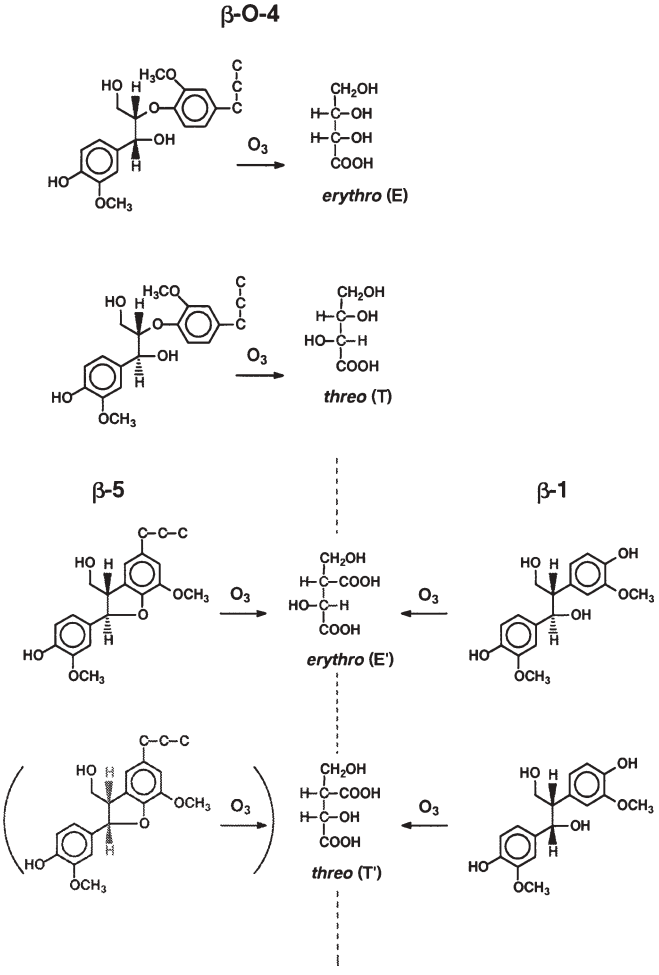

Fig. 8. Reaction products by ozonation from β-O-4, β-5, and β-1 structures

by mild alkali treatment of LCC. The difference could suggest that the molecular size of LCC in the present study is mainly determined by carbohydrate, while both carbohydrate and lignin contributed to the molecular size of LCC examined in the study of Takahashi and Koshijima.

Analyses of lignin chemical structure

To analyze the structural features of lignin in the WS fraction, the fraction was fractionated into the high molecular weight part (WSH) and low molecular weight part (WSL) by gel filtration chromatography on a preparative scale (Fig. 6). As a reference sample to represent normal LCC, the DMF fraction was also prepared on a large scale. MWL, and fractions WS, WSH, WSL, and DMF, and their extraction residues from the same wood meal (MWLR, DMFR) were subjected to alkaline nitrobenzene oxidation and ozonation analyses.

Figure 7 shows the yields of vanillin from the alkaline nitrobenzene oxidation. No significant difference was observed except for the WSL fraction, suggesting that the proportion of noncondensed type guaiacyl unit was almost the same for those fractions. The extremely low vanillin yield from the WSL fraction could be due to the uncertainty of lignin determination for this fraction, because the yields of two ozonation products, erythronic and threonic acids, from this fraction were also very low.

Table 3 shows the yields of ozonation products and the molar ratios among them. The E/T ratio is a ratio of erythronic acid and threonic acid obtained from the *erythro* and *threo* type β-O-4 structures, respectively, and is used as an indication of the *erythro*/*threo* ratio of this structure (Fig. 8). E/T ratio of hardwood species are known to range widely from 1.17 to 3.35 depending on wood species, cell types, and the portion within a cell wall, but that of softwood is normally 1.0 regardless of wood species, cell types, or other factors.[20] Therefore, it is not surprising to observe only small variation in the E/T ratio for different fractions obtained in this study.

What is interesting is the ratio between *erythro* and *threo* types of 3-hydroxy-2-hydroxymethyl butanedioic acids that are ozonation products of *erythro* and *threo* type structures

Table 3. Results of ozonation of various samples

Samples	E/T	E + T (mol/180 g lignin)	E′/T′[a]	(E′ + T′)/(E + T)[a]
MWL	0.93	0.14	3.35	0.14
MWLR	1.01	0.22	3.89	0.08
WS	0.88	0.21	0.98	0.20
WSH	0.88	0.25	1.11	0.16
WSL	0.91	0.06	1.34	0.29
DMF	0.92	0.14	3.52	0.14
DMFR	1.01	0.20	4.02	0.08

E, erythronic acid; T, threonic acid; E′ and T′, *erythro* and *threo* type of 3-hydroxy-2-hydroxymethyl butanedioic acids; WSH, high molecular weight fraction of water extract; WSL, low molecular weight fraction of water extract; DMFR, extraction residue from dimethylformamide extraction

[a] Calculated from the peak areas of each compound from the gas chromatograph

carrying a C-aryl linkage at the β-position, for which β-5 and β-1 structures are known to be present in native lignin. As is illustrated in Fig. 8, only the *trans* (*erythro*) configuration is known for the β-5 structure, but the β-1 structure may have both *erythro* and *threo* configurations. Therefore, if the ratio between *erythro* and *threo* types of this acid (E′/T′ ratio) is very high, it suggests that the β-5 structure predominantly contributes to the formation of this acid, because the number of β-1 structures is known to be very low.[6,21] Based on this speculation, Habu et al.[22] proposed that the β-1 structure was not a main structural unit in spruce lignin. In support of Habu's report, relatively high E′/T′ ratios were found for MWL, MWLR, and the DMF and DMFR fractions (Table 3). However, the ratio obtained for the WS fraction was extremely low. Although a clear explanation for this result cannot be provided at this moment, it could suggest either the relative importance of the β-1 structure or the presence of the *cis* (*threo*) type β-5 structure which has not been known to be present in lignin.

The area ratio of (E′ + T′)/(E + T) on the gas chromatogram was also interesting. This ratio indicates the frequency of the C-aryl linkage at the β-position relative to β-O-4 structures. This ratio for WS fraction was clearly higher than those of other samples (Table 3), suggesting the importance of β-5 and/or β-1 structures in this fraction.

As discussed above, ozonation analysis revealed that the structure of small lignin fragments closely associated with carbohydrate are different from lignin in other parts. It should be noted that although the analysis of the carbohydrate part of LCC has been studied by many researchers, the structural characteristics of the lignin part have been scarcely analyzed except for several trials to examine linkage types between lignin and carbohydrate.[8,23] We are now trying to examine whether the difference found in the present study can be attributed to any of the factors discussed in the introductory part. For this purpose, water extraction under the same conditions was applied to hardwood residual wood meal after MWL extraction and the obtained WS fraction was analyzed by the same method. The results will be described in our next report.

Summary

In order to analyze the structural features of small lignin fragments that are closely associated to polysaccharides, residual wood meal after the extraction of MWL was extracted with various solvent systems, and a unique LCC fraction was obtained by water extraction. Treatment by a carbohydrate-degrading enzyme and by weak alkali indicated that the molecular size of this LCC fraction was mainly dependent on the carbohydrate chain and lignin is present as a small fragment attached to the carbohydrate. Ozonation analysis suggested that the lignin structure of this fraction is different from those of other lignin fractions.

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