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Structure of small lignin fragments retained in water-soluble polysaccharides extracted from birch MWL isolation residue

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Abstract To analyze the structural features of lignin in the vicinity of lignin–carbohydrate linkages, water-soluble lignin–carbohydrate complex (LCC) with low lignin content was prepared from residual birch wood meal after the extraction of milled wood lignin (MWL). The molecular weight distribution of lignin in this LCC appeared together with carbohydrate in the relatively high molecular weight region of the gel permeation chromatogram. This result was consistent with our previous results obtained for the same fraction of Japanese cedar (sugi); however, after treatment with polysaccharide-degrading enzyme, the molecular weight distribution of carbohydrate and that of lignin shifted significantly to the lower region. These results demonstrated that molecular size of this LCC is determined by carbohydrates while lignin is present as a minor fragment in this fraction. The syringyl/guaiacyl (S/V) ratio of this LCC was higher than other lignin fractions. Ozonation analysis implied that this LCC has a relatively high number of β-1 structures. It is likely that lignin that exists near lignin–carbohydrate linkages has more endwise-type features than other lignin fractions.

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

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Introduction

Although there are many reports dealing with lignin–carbohydrate complexes (LCCs), almost all are focused on the carbohydrate part. The characteristics of the lignin part have not been seriously investigated. It seems that the interest in the lignin part has been limited to the types of linkage that connect the carbohydrate and lignin.[1–3] One of the reasons for this is that there are no enzymes or chemicals that can cleave specific parts of lignin. If such a chemical or enzyme were available, lignin that participates in the lignin–carbohydrate linkages (L–C linkages) could be concentrated in the LCC sample and therefore more easily characterized.

Because the formation mechanism of L–C linkages seems to be limited, characteristic features of lignin in the proximity of the L–C linkage are expected to be different from those of lignin in other parts. Nucleophilic addition of carbohydrate to β-*O*-4 type quinone methide has been widely accepted as the formation mechanism of L–C linkages. This mechanism was investigated by the use of low molecular weight model compounds and the occurrence of this type of L–C linkages in actual wood materials seems to be well proven,[1–3] even though direct evidence for this has not yet been obtained. In this case, an endwise-type lignin rich in β-*O*-4 linkages must be favorable for the formation of LCCs.

Some researchers have proposed that a different formation mechanism of L–C linkages may exist with hydroxycinnamic acids functioning as an "anchor" for lignification to occur.[4] This proposal is based on the isolation of a fragment from enzymatic hydrolyzates of several plant materials in which several feruloyl and coumaroyl residues are attached to hemicellulosic oligosaccharides.[5] This finding also suggested that hemicellulose may play a role as a template for formation of the lignin macromolecule.[6,7] Ralph and Helm[6] prepared the CA/FA-Ara DHP from coniferyl alcohol and methyl 5-*O*-feruloyl-α-L-arabinofuranoside and suggested that various linkage types can be formed from these precursors. These studies imply that lignification may start from

some specific point on the carbohydrate. According to this hypothesis, the lignin near the L–C linkages could represent lignin at the early stage of polymerization, which may have the nature of bulk-type lignin.

In our previous report,[8] it was suggested that water-soluble LCC (fraction WS) obtained from residual Japanese cedar wood meal after the extraction of milled wood lignin (MWL) contained lignin as relatively small fragments attached to high molecular weight polysaccharide chains. If this is the case, all the lignin in fraction WS must be in the vicinity of L–C linkages.

In this study, residual birch wood meal was extracted with water after the extraction of MWL in the same manner as the previous report.[8] It was examined whether the fraction obtained possessed the same characteristics as the water-soluble LCC of Japanese cedar. In addition to this, the water-soluble fraction was also prepared from crude MWL to investigate the entire water-soluble fraction of milled wood. The characteristic features of the lignin were investigated by alkaline nitrobenzene oxidation and ozonation analysis and compared with other fractions.

Experimental

Preparation of MWL fractions

Birch (*Betula maximowicziana*) wood meal (190 g, passing 40 mesh) previously extracted with ethanol–benzene (1:2, v/v) was ball-milled in a 3-l jar in dry toluene for 120 h. The milled wood was then extracted with dioxane–water (96:4, v/v) for 24 h. The extraction was repeated five times and crude MWL (CMWL) was obtained from these extracts by concentration. The yield of CMWL was 2.1% on wood meal. The residue that remained after the CMWL extraction was designated as MWLR. The CMWL was dissolved in 90% acetic acid and the solution was added dropwise to a large volume of water with stirring. After centrifugation, the precipitate was subjected to further purification steps described by Björkman[9] to give purified MWL. The supernatant was concentrated and lyophilized to give the water-soluble fraction (MWLW). The yields of MWL and MWLW were 0.9% and 0.6%, respectively, on wood meal.

Preparation of fractions from residual wood meal

Residual birch wood meal (MWLR) was fractionated after the extraction of MWL according to the procedure described in our previous report[8] to give the water-soluble fraction (WS) and its high molecular weight (WSH) and low molecular weight (WSL) components. The dimethylformamide-soluble fraction (DMF) and its residue (DMFR) were also prepared by extraction of MWLR with dimethylformamide (Fig. 1). The yield of fraction WS was 3.3% and those of fractions DMF and DMFR were 2.8% and 95.4% on MWLR, respectively. The yields of fractions WSH and WSL on fraction WS were 84.3% and 7.1%, respectively.

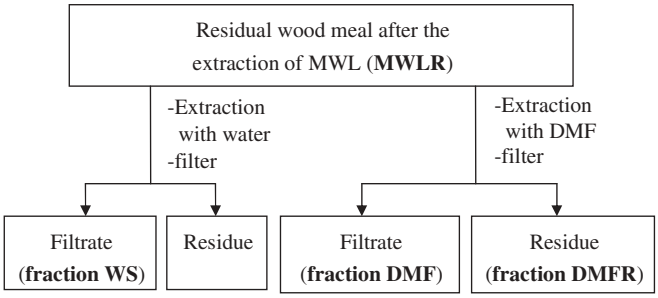

Fig. 1. Preparation of fractions from milled wood lignin residue (*MWLR*). *WS*, water-soluble fraction of MWLR; *DMF*, dimethylformamide-soluble fraction of MWLR; *DMFR*, residue after dimethylformamide extraction of MWLR

Enzymatic treatment and gel filtration chromatography

Gel filtration chromatography was conducted according to the method described in our previous report.[8] In the case of enzymatic treatment, fraction WS (49.9 mg or 50.2 mg) was treated with 25 mM sodium acetate buffer (pH 5.0, 5 ml) containing purified Driselase (Kyowa Hakko) solution (0.03 ml), which was prepared in our previous study,[8] at 40°C with stirring. After 16 and 88 h, part of the solution was taken and subjected to gel filtration chromatography on a column of Sepharose CL-6B or Sephadex G-50 (medium). Because extension of the enzyme treatment from 16 to 88 h did not create any significant change in the chromatograms with the two different gels, the amount of enzyme applied was believed to be sufficient. The similarity between the chromatograms for 16 and 88 h also suggests that the scission of the polysaccharide chain by the enzyme was already complete within the first 16 h. During and after the enzymatic treatment, no precipitate was observed.

Lignin content and neutral sugar analysis

Lignin contents of fractions WSH, WSL, and CMWL were determined by ultraviolet (UV) absorbance at 280 nm. The lignin contents of other fractions were determined by the Klason method. Neutral sugars were analyzed by the alditol-acetate method.[10]

Alkaline nitrobenzene oxidation and ozonation

Alkaline nitrobenzene oxidation was conducted according to the procedure of Chen.[11] Ozonation analysis was conducted according to the modified procedure of Akiyama et al.[12] For ozonation of WS and WSH, treatment was conducted for 0.5, 1, 2, 3, and 6 h.

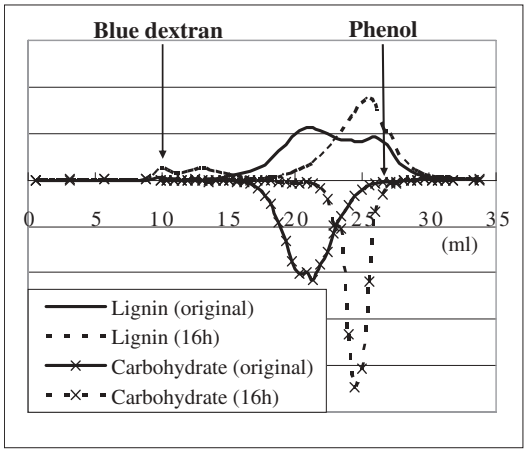

Fig. 2. Molecular weight distributions of lignin and carbohydrate of fraction WS before and after treatment with Driselase (Sepharose CL-6B). Eluent: 25 mM sodium acetate buffer (pH 5.0); fractionation range (dextrans): 10 000–1 000 000

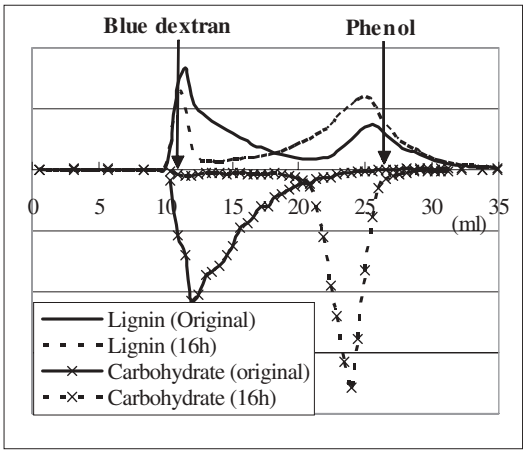

Fig. 3. Molecular weight distributions of lignin and carbohydrate of fraction WS before and after treatment with Driselase (Sephadex G-50). Eluent: 25 mM sodium acetate buffer (pH 5.0); fractionation range (dextrans): 500–10 000

Results and discussion

Changes in gel filtration chromatogram of fraction WS by enzymatic treatment

Fraction WS was prepared from residual birch wood meal after the extraction of MWL. Lignin content of this fraction was only 8.5%, which was comparable with the value of 5.3% obtained in our previous study using Japanese cedar.[8]

To examine the interaction between lignin and carbohydrate, fraction WS was treated with the polysaccharide-degrading enzyme Driselase. Untreated and treated samples were subjected to gel filtration chromatography using two different gels (Sepharose CL-6B and Sephadex G-50) to evaluate the changes in molecular weight distributions of both lignin and carbohydrate. As shown in Fig. 2 (Sepharose CL-6B), the molecular weight distribution of the major part of lignin in the untreated WS fraction appeared together with carbohydrate at relatively high molecular weight. However, after the treatment by Driselase, the molecular weight distribution of carbohydrate and lignin shifted to the lower molecular weight. The use of Sephadex G-50 as gel permeation bed resulted in a similar distribution pattern, as shown in Fig. 3. This behavior and the low lignin content in this fraction can be explained by assuming that the molecular size of this LCC is determined by carbohydrates, and lignin is present as small fragments in this fraction. The results obtained here and in the previous report[8] indicate that water extraction of residual wood meal after MWL isolation could be a good method to prepare a fraction containing small lignin fragments that are closely associated to the polysaccharides, from both angiosperm and gymnosperm.

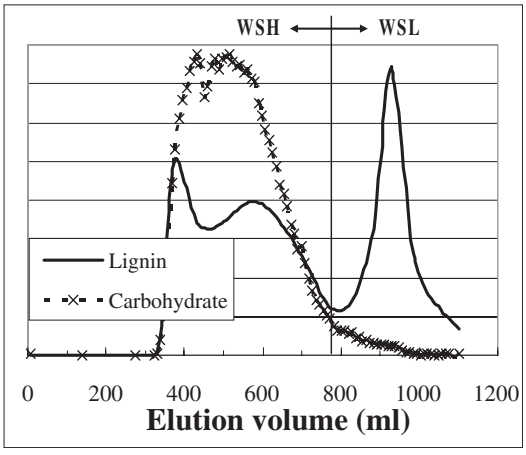

Fig. 4. Chromatogram of preparative gel filtration of fraction WS. Eluent: water; gel: Sephadex G-50

Analyses of lignin chemical structures

In order to analyze structural features of lignin in fraction WS, several samples were prepared. Fraction WS was fractionated into the high molecular weight part (WSH) and low molecular weight part (WSL) by gel filtration chromatography on a preparative scale (Fig. 4). Fraction DMF was prepared by dimethylformamide extraction of MWLR as a reference sample of "normal" LCC and its residue was used as fraction DMFR. To investigate the entire water-soluble fraction from milled wood, the water-soluble fraction obtained during the purification procedure of crude MWL (CMWL) was also analyzed as fraction MWLW. Because this fraction was water soluble, it was interesting to examine whether it had properties similar to fraction WS. As a fraction similar to this, Azuma et al.[13,14] reported the preparation of the water soluble fraction of crude MWL.

Lignin contents and neutral sugar compositions of these samples are listed in Table 1. In the case of Japanese cedar

Table 1. Lignin content and neutral sugar compositions

Sample	Lignin (%)	Neutral sugars (%)	Rha	Ara	Xyl	Man	Glc	Gal
Wood meal	24.0[a]	–	–	–	–	–	–	–
MWLR	23.3[a]	56.7	0.5	0.6	32.1	2.2	63.9	0.8
WS	8.5[a]	55.1	1.3	2.7	74.3	10.4	7.7	3.6
WSH	5.7[b]	59.2	1.3	2.7	75.5	9.8	7.0	3.6
WSL	41.6[b]	–	–	–	–	–	–	–
DMF	19.9[a]	41.2	1.0	2.7	87.4	2.4	3.6	2.8
DMFR	23.0[a]	–	–	–	–	–	–	–
MWL	–	2.7	2.9	1.8	86.4	0.5	6.2	2.2
CMWL	81.9[b]	7.5	2.8	2.0	77.6	0.4	15.1	2.2
MWLW	54.1[a]	17.8	3.0	2.2	74.1	0.5	18.1	2.1

Makeup of individual sugars are expressed as molar ratios
Rha, rhamnose; Ara, arabinose; Xyl, xylose; Man, mannose; Glc, glucose; Gal, galactose; MWLR, milled wood lignin residue; WS, water-soluble fraction from MWLR; WSH, high molecular weight fraction of WS; WSL, low molecular weight fraction of WS; DMF, dimethylformamide-soluble fraction from MWLR; DMFR, residue after dimethylformamide extraction of MWLR; MWL, milled wood lignin; CMWL, crude milled wood lignin; MWLW, water-soluble fraction of CMWL
[a] Lignin content was determined by the Klason method
[b] Lignin content was determined by UV absorption (280 nm)

Table 2. Results of alkaline nitrobenzene oxidation

Sample	S/V	S + V yield (mol/200 g lignin)[a]
Wood meal	2.66	0.45
MWLR	2.77	0.50
WS	3.03	0.40
WSH	3.31	0.52
WSL	2.36	0.19
DMF	2.17	0.43
DMFR	2.82	0.51
MWL	1.40	0.39
CMWL	1.48	0.40
MWLW	1.61	0.39

V, vanillin; S, syringaldehyde; S/V, molar ratio of syringaldehyde/vanillin
[a] One unit of hardwood lignin was calculated as 200 g

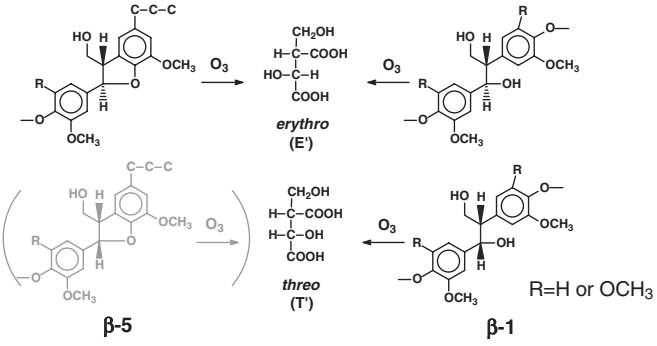

Fig. 5. Reaction products by ozonation of β-5 and β-1 structures

analyzed in the previous report,[8] the neutral sugar composition of the WS fraction was similar to those of other LCCs and was quite different from that of MWL. However, neutral sugar composition of the birch WS fraction was different from that of LCC (fraction DMF) by its high mannose content.

The results of alkaline nitrobenzene oxidation are listed in Table 2. Quite high syringaldehyde/vanillin (S/V) ratios were observed for fractions WS and WSH compared with the other extracted fractions DMF, MWL, CMWL, and MWLW. Fraction WSH showed the highest S/V ratio and highest S + V yield. These results indicate that lignin in the WSH fraction has a more non-condensed-type nature than the other fractions. The vanillin yield of WSH fraction obtained from Japanese cedar was also the highest among the different lignin fractions.[8] Comparison of the results for MWLW and WS fractions also proved interesting because both of them were water soluble but quite a low S/V ratio and low S + V yield were observed for fraction MWLW. This suggests that the structural characteristics of lignin in fraction MWLW are quite different from those in fractions WS and WSH, even though they are all water soluble. This result clearly demonstrates that WS fraction is not a "left over" from the MWL extraction. If water extraction is conducted without the preceding MWL extraction, both MWLW and WS will be extracted by water at the same time as a mixture and a unique fraction like WS could not be obtained. Although the clear difference between WSH and WSL also requires an explanation, only the WSH fraction is examined as a typical fraction of WS in this research.

Table 3 shows results of ozonation analysis. The WS fraction series (WS, WSH, WSL) showed quite low E′/T′ ratios, which is consistent with the results for the fraction WS series for Japanese cedar in our previous study.[8] E′ and T′ represent the *erythro* and *threo* forms of 3-hydroxy-2-hydroxymethyl butanedioic acid, respectively, as shown in Fig. 5. This acid is obtained by ozonation of structures carrying a C-aryl linkage at the β-position of the side chain.[8,15,16] Of such structures, β-5 and β-1 structures are known in lignin. Because the β-5 structure only has the *trans* form,[16–20] lower E′/T′ ratios could suggest the relative importance of the β-1 structure or the presence of the *cis* (*threo*) type β-5 structure, which is not known to be present in lignin.

Because authentic samples of E′ and T′ were not available, the relative yields of E′ and T′ from each fraction were estimated by using the following equation based on peak areas on the gas chromatogram and the weight of internal standard and lignin:

Table 3. Results of ozonation analysis

Sample	E / T	E + T (mol/200 g lignin)	E′/T′	E′ + T′[a]
Wood meal	2.70	27.24	3.42	0.37
MWLR	2.67	30.24	2.76	0.36
WS	1.92	23.83	1.30	0.47
WSH	2.07	31.35	1.65	0.56
WSL	1.44	9.77	0.71	0.37
DMF	2.18	17.81	2.10	0.35
DMFR	2.71	30.35	2.85	0.38
MWL	1.92	12.72	3.10	0.32
CMWL	1.89	13.32	2.45	0.35
MWLW	1.75	13.05	2.13	0.48

Ozonation treatment was conducted for 2 h according to the modified method of Akiyama et al.[12]
E, erythronic acid; T, threonic acid; E′, *erythro* type of 3-hydroxy-2-hydroxymethyl butanedioic acid; T′, *threo* type of 3-hydroxy-2-hydroxymethyl butanedioic acid
[a] Refer to text

$$E' + T' = \frac{\text{area of } E' \text{ and } T'}{\text{area of internal standard}} \times \frac{\text{weight of internal standard}}{\text{weight of lignin}}$$

It is possible to compare the total yield of E′ and T′ for each fraction by the calculation of E′ + T′ according to this formula. The estimated amounts of E′ and T′ in lignin samples are shown in Table 3. It is interesting to note that clearly high values of E′ + T′ are observed for fractions WS, WSH, and MWLW. Thus, lignin in fractions WS and WSH are characterized by a high E′ + T′ value and a low E′/T′ ratio.

Akiyama et al.[21,22] reported that the ratio of erythronic and threonic acid (E/T ratio) was clearly correlated to the methoxyl group content in lignin. However, in this study, fractions WS and WSH gave almost the same E/T ratio as fractions DMF and MWLs, although quite high S/V ratios were observed for fractions WS and WSH. To investigate this abnormal behavior, fractions WS and WSH were subjected to ozonation treatment for prolonged treatment times (0.5, 1, 2, 3, 6 h).

According to Akiyama et al.,[12] the yields of erythronic and threonic acids from MWL and wood meal increase until 40–60 min of ozonation has been conducted, and after that only a moderate increase was observed and the E/T ratio became almost constant. However, in this study, the yields of these acids increased even at 6 h and the increasing trend was strong for threonic acid (Figs. 6 and 7).

One plausible explanation for this observation is the contribution of carbohydrate to produce these acids. Akiyama et al. (unpublished) confirmed that monosaccharides having the reducing end, especially pentose, create relatively large amounts of erythronic and threonic acids. Formation of these acids was largely suppressed by blocking the anomeric position of the monosaccharide as the methyl glycoside (Akiyama et al., unpublished) and $NaClO_2$-delignified wood meal created only a small amount of these acids.[12] Therefore, monosaccharides, especially pentose, and oligosaccharides that may create such monosaccharides during ozonation treatment may be the source of these acids.

As shown in Figs. 6 and 7, a more rapid increase of threonic acid was observed than for erythronic acid, which might support the above idea. This is because the major constituent of neutral sugar in fractions WS and WSH is xylose (Table 1) and xylose predominantly produces threonic acid during ozonation treatment (Akiyama et al.,

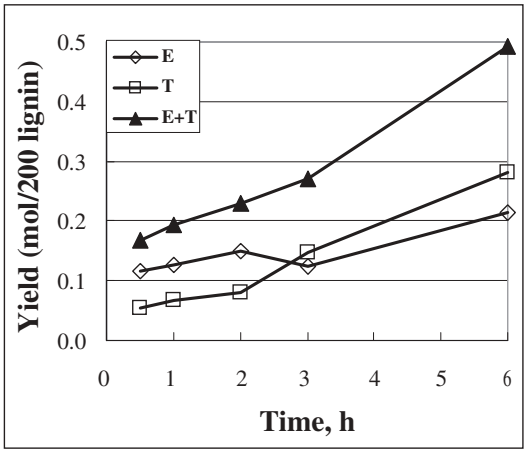

Fig. 6. Yields of ozonation products from fraction WS after prolonged reaction time

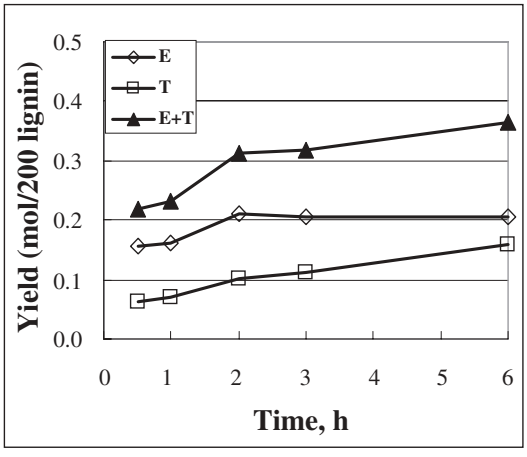

Fig. 7. Yields of ozonation products from high molecular weight fraction of WS after prolonged reaction time

unpublished). If carbohydrate is present as high molecular weight sugar, their contribution to the formation of these acids by ozonation will decrease. This should be the reason why the increase of threonic acid was moderate when WSH fraction was subjected to ozonation rather than in fraction WS (compare Figs. 6 and 7).

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