

Frequency and alkali resistance of lignin-carbohydrate bonds in wood

ABSTRACT

Milled-wood enzyme lignin, the residue obtained by polysaccharidase digestion of vibratory ball-milled wood, which contains nearly all of the lignin-carbohydrate bonds in wood, was used to investigate the nature of these bonds. A lignin-carbohydrate bond frequency of 0.028 per phenyl propane unit was experimentally determined. Mild alkaline treatment cleaved 10 to 20% of the lignin-carbohydrate bonds, and these alkali labile bonds were proposed to be mainly uronic acid ester linkages to lignin. Most of the remaining lignin-carbohydrate bonds in milled wood enzyme lignin were resistant to cleavage under the conditions of soda pulping.

KEYWORDS

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The results of research on lignin-carbohydrate (L-C) bonds have been reviewed (1-5), and the accumulated evidence strongly supports the existence of covalent links between polysaccharides and lignin. However, the nature and frequency of these bonds is not clearly understood. Many lignin-carbohydrate complexes (LCC's) have been isolated, but in low yield, and the actual L-C bonds therein represented only a fraction of the total L-C bonds in wood. Many LCC's can be cleaved by acids, alkali, or both. Some LCC's resist vigorous alkaline treatment and even survive pulping (6).

The likeliest bond types that would reflect these properties (3, 4) are benzyl ether or benzyl ester bonds of the type formed by addition of nucleophiles to quinone methide intermediates of lignification, as proposed by Freudenberg and Harkin (7). Guaiacylglycerol- β -carbohydrate ether bonds could be formed by free-radical exchange and subsequent radical pairing during lignification (7). Although the evidence for simple acetal L-C bonds is not very convincing, glycosides are more stable than simple acetals and have properties similar to those of many LCC's. Perhaps some of the mechanisms known to be operating in plants in the formation of phenol or alcohol glycosides of low molecular weight (8, 9) may also occur with oligosaccharides or oligolignols which can then be incorporated into the larger structures of polysaccharides and lignin. The chemical nature and frequency of L-C bonds is of considerable interest for developing specific methods of L-C bond cleavage and improved pulping and bleaching processes.

Results and discussion

Mild alkali labile L-C bonds

Currently, the isolated lignin most representative of the lignin in wood is that obtained by polysaccharidase hydrolysis of vibratory ball-milled wood (10, 11). This residue, milled-wood enzyme lignin (MWEL), contains not only almost all of the original lignin in the wood but also 10 to 15% of residual carbohydrates that resist further enzymatic hydrolysis. Since these residual carbohydrates cannot be removed by remilling and redigestion, it is most likely chemically bound to the lignin. Other treatments, except acid hydrolysis, have also been unsuccessful in significantly reducing these residual carbohydrates (10). All L-C bonds should be present in this lignin residue except probably glycosidic L-C bonds, since the polysaccharidase enzyme mixture used for the preparation of MWEL also contains phenyl glycosidases. It was experimentally determined that phenyl- β -D-glucopyranoside was 70% hydrolyzed in 4 days under the conditions of enzyme hydrolysis used for MWEL preparation. No evidence has been reported to indicate the formation of L-C bonds during ball-milling, and mechanical cleavage of such bonds is probably insignificant.

Milled-wood enzyme lignin is insoluble in water but dissolves extensively in 0.1N sodium hydroxide. For loblolly pine (*Pinus taeda* L.) MWEL, it was observed that, after treatment with 0.1N sodium hydroxide at 20°C for 90 min, about 11% of the residual carbohydrates and about 10% of the lignin, as measured by ultra-violet absorption, did not precipitate upon neutralization

with acid. These water-soluble carbohydrates were completely digestible by polysaccharidases (Table I), while the residual carbohydrates of the precipitated lignin remained indigestible. No monosaccharides were detected in the neutralized saponification liquor but, after acid hydrolysis, all five common wood sugars were found (Table I), with xylose being the major component. The enzyme-digestible carbohydrates found in the soluble fraction most likely resulted from L-C bond cleavage. When the reaction time of the mild alkaline treatment was increased to 60 hr, 19% of the carbohydrates became water soluble, but only 13% of the total was enzyme-digestible. Although most of the mild-alkali labile L-C bonds can be cleaved rapidly, an increase of almost 20% cleavage occurred with the longer reaction time. The indigestible carbohydrates solubilized after 60 hr were probably water-soluble LCC's formed by alkaline hydrolysis of the MWEL. For this reason, the 90-min saponification was used for characterizing the carbohydrates cleaved from the lignin under mild conditions. Assuming that the frequency of L-C linkages in the mild alkali-cleaved oligosaccharide is the same as the frequency in the whole residual oligosaccharide of the MWEL, then 11-13% of the L-C bonds are mild-alkali labile.

As mentioned before, MWEL dissolves extensively in 0.1N sodium hydroxide. When a suspension of MWEL in alkali was centrifuged, only 10% was recovered as a residue. The alkali-insoluble residue contained 21% of the original carbohydrates in MWEL. Its composition suggested a lignin-glucan bond since it contained 48% of the total

I. Carbohydrate composition of loblolly pine MWEL and of soluble and insoluble fractions after mild alkaline treatment^a and neutralization

	Total sugar anhydride, mg/g MWEL		Sugar composition, %					Uronic anhydride, mg/g MWEL
	Enzymatic hydrolysis	Acid hydrolysis	Glucose	Mannose	Xylose	Galactose	Arabinose	
MWEL	0	107.9	27.1	22.4	19.1	23.3	8.1	7.4
Soluble fraction	11.6	11.6	8.6	7.8	58.6	11.2	13.8	n.d ^b
Residue	0	91.8	28.5	23.3	14.7	27.2	6.2	5.0

^a0.1N NaOH at 20°C for 90 min. Neutralized to pH 6.5 with acetic acid. ^bNot determined.

glucose and only 16 to 18% of the other sugars.

L-Cbond frequency

The water-soluble fractions obtained from the alkaline treatment of MWEL and MWEL itself were treated with sodium borohydride to reduce oligosaccharide aldehydic end groups. Reducing-sugar analysis after acid hydrolysis and comparison with the carbohydrate content and composition of the unreduced samples showed a 25% loss of reducing sugar for the alkali-labile carbohydrates and a 17% loss for the MWEL residual carbohydrates (Table 11). Assuming one reducing end group for each polysaccharide residue, the average oligosaccharide composition was calculated as 4 and 5.9 sugar units, respectively. If one L-C linkage per oligosaccharide is assumed, the average frequency of L-C bonds in MWEL is 0.028 per lignin C₉ unit or one L-C bond for every 36 phenyl propane units. Mild-alkali labile L-C bonds are much less frequent, approximately one for every 200 phenyl propane units. The presence of acid-resistant L-C bonds, for example, carbon-carbon linkages or stable ethers, would result in monosaccharides remaining attached to the lignin after carbohydrate analysis. Because the frequency of occurrence of acid-resistant bonds is unknown, calculated L-Cbond frequency is uncorrected for this type of bond. The experimentally determined L-C bond frequency in loblolly pine MWEL should be very similar to that of lignin in wood and is about one-half that predicted on the basis of dehydrogenation studies (12).

The extent of the loss of reducing end groups for each sugar after borohydride reduction of the MWEL was not uniform (Table II). This may reflect polysaccharide structural differences yielding different degrees of hydrolysis by the polysaccharidases. With the exception of the xylan, borohydride reduction indicated that the carbohydrate liberated by mild alkaline treatment was composed of fewer sugar units than the oligosaccharide of the whole MWEL. Although the liberated sugars represent only a small fraction of the total residual carbohydrates in MWEL, there appears to be a correlation of polysaccharide

II. Reducing sugar loss by sodium borohydride reduction of loblolly pine MWEL and of the soluble fraction from mild alkaline treatment

	Loss of total sugar anhydride, %	Loss of individual sugars, %				
		Glucose	Mannose	Xylose	Galactose	Arabinose
MWEL	17.1	21.8	12.8	15.0	20.3	9.1
Soluble fraction	25.0	50.0	44.4	17.6	39.6	16.5

III. Carbohydrate composition of MWEL from sodium hydroxide pretreated loblolly pine wood, and of its soluble and insoluble fractions after mild alkaline treatment^a

	Total sugar anhydride, mg/g MWEL		Sugar composition, %				
	Enzymatic hydrolysis	Acid hydrolysis	Glucose	Mannose	Xylose	Galactose	Arabinose
p-MWEL	0	75.3	29.9	23.9	9.3	28.4	8.5
Soluble fraction	2.6	4.9	20.4	16.3	34.6	14.3	14.3
Residue	0	65.7	29.8	25.6	11.1	25.4	8.1

^a0.1N NaOH at 20°C for 90 min Neutralized to pH 6.5 with acetic acid

fragment size and the type of L-C chemical bond.

Although arabinose occurs as non-reducing side chains in hemicellulose (13), some arabinose was reduced by borohydride treatment (Table II). A possible source of reducible arabinose could result from an L-C linkage to a 3-O-β-L-arabinopyranosyl-L-arabinose side chain of an arabinogalactan (13) after enzymatic hydrolysis of the galactan backbone. Young and Sarkanen (14) have indicated that arabinose may occur as a backbone unit of the arabinogalactan of *Larix*. If arabinose similarly occurred in the backbone of pine hemicellulose, reducible arabinose would be expected in the MWEL residual carbohydrate.

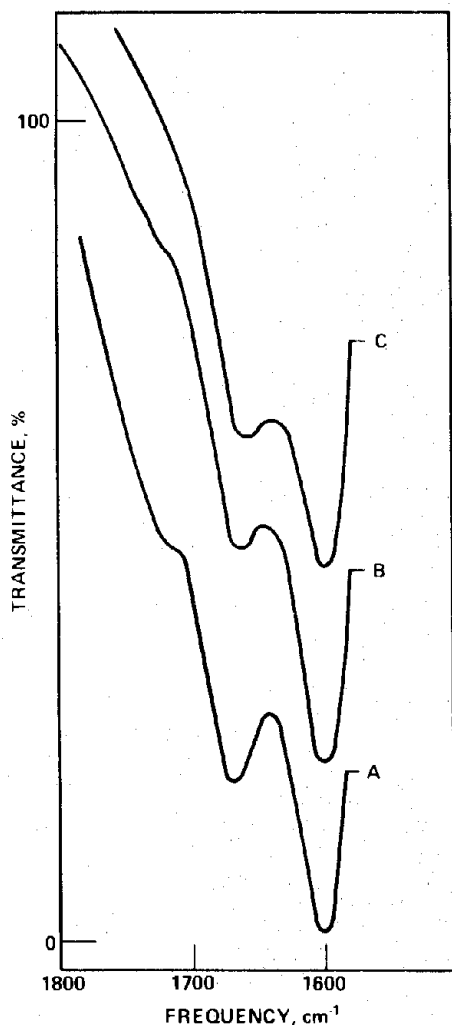
Alkali prehydrolysis of labile L-Cbonds

The question arises whether the mild alkali labile L-C bonds just described in MWEL can be cleaved in wood before lignin isolation. An attempt to hydrolyze these bonds was made by treating Wiley-milled loblolly pine with 1.0N sodium hydroxide for 18 days at 20°C. After neutralization, washing and drying the prehydrolyzed wood, a milled-wood enzyme lignin, p-MWEL, was prepared in the usual way. The p-MWEL had a lower residual carbohy-

drate content and contained less xylose than the MWEL from the untreated wood (Table III). Mild alkaline treatment of p-MWEL gave a soluble fraction after neutralization which contained about 7% of the total carbohydrate. However, only half of these soluble carbohydrates were enzyme-digestible. The alkaline pretreatment was effective in reducing the residual carbohydrates of the p-MWEL by cleaving most of the mild alkali labile L-C bonds. The alkaline pretreatment also promoted formation of water-soluble LCC's, the enzyme-indigestible carbohydrates of the soluble fraction.

The carbohydrates of the soluble fraction were rich in xylose and arabinose, but not nearly as high in xylose as those from the untreated wood MWEL. A simple comparison of the composition of the carbohydrates solubilized by mild alkaline treatment of MWEL to those from p-MWEL is not valid because the contribution of the indigestible portion from p-MWEL is now known. However, the major alkali-labile L-C bond in MWEL was lignin-xylan bond, and the alkali pretreatment effectively reduced the number of these linkages.

Since benzyl ethers and esters are thought to be likely types of L-C linkages (7), a likely lignin hemicellulose



1. Infrared ester absorption of loblolly pine. MWEL (A), MWEL from loblolly wood meal pretreated with sodium hydroxide (B), and their saponification residues (C).

linkage would be a lignin-xylan (4-*O*-methylglucurono) ester bond. This prediction is supported by the high uronic anhydride content of the MWEL and of its saponification residue (Table I) and by the high xylose content of the alkali-cleaved carbohydrates. Comparison of the infrared spectra of loblolly MWEL, *p*-MWEL, and their saponification residues (Fig. 1) showed removal of ester

absorption (1730 cm^{-1}) by alkaline treatment. The alkaline pretreatment removed a considerable amount of ester absorbance in agreement with the lower amount of residual carbohydrates in *p*-MWEL. The remaining ester absorption was completely removed by subsequent mild alkaline treatment which is supportive of lignin-uronic acid ester bonds.

A test of the type of mild alkali labile L-C bond was diazomethane methylation of loblolly MWEL free phenolic hydroxyls before mild alkaline treatment. L-C ester bonds should remain saponifiable, but α -ethers of free phenolic units would be stabilized in the methylated MWEL. Since the methylated MWEL was not soluble in aqueous alkali and swelling was limited, the reaction time was extended to 4 hr. About 4% of the original carbohydrates were recovered in the soluble fraction after neutralization. Only half were enzyme-digestible while none of the carbohydrates in the saponification residue were digestible. Although this suggests that α -ethers of free phenolic units are the major mild alkali labile bond type, the infrared spectrum of the methylated MWEL saponification residue showed considerable ester absorption. The stabilization of esters may be chemical—for example, α -esters of free phenolic units are more readily cleaved than those of etherified units—ophysical, since methylated MWEL does not dissolve or appreciably swell in alkali. Thus, under the conditions employed, methylation of free phenolic hydroxyls in the MWEL probably stabilized both α -ester and α -ether L-C bonds, and no distinction may be made between these types. However, the persistence of the infrared ester absorption after alkaline treatment of the methylated MWEL is indicative of L-C ester linkages.

L-C bonds in other woods

Milled-wood enzyme lignins were also prepared from Sitka spruce (*Picea sitchensis* [Bong] Carr.), aspen (*Populus tremuloides* Michx.), and red pine (*Pinus resinosa* Ait.) compression wood and normal wood. The softwood lignins,

with the exception of compression wood MWEL, had residual carbohydrate contents and compositions similar to that from loblolly pine (Table IV). Reflecting the polysaccharide composition differences of hardwood and softwoods, aspen MWEL had higher glucose and xylose contents and lower mannose and galactose contents than did the softwood MWEL's. These MWEL's contained from 13 to 20% mild-alkali labile carbohydrates (Table IV). The composition of these cleaved carbohydrates was similar for all the MWEL's and xylose was the major component for each. Determination of reducing end groups by borohydride reduction indicated that polysaccharide fragments had an average composition of four to six units. Compression wood contains much more galactose and less mannose and xylose than normal wood (15). These differences are reflected in the very high galactose content of the red pine compression wood lignin (Table IV). The compression wood MWEL contained the highest amount of residual carbohydrates but had the least amount, about 4%, of carbohydrates solubilized by mild alkaline treatment. The small amount of alkali-labile carbohydrates may be a consequence of the low xylose content. Consistent with its low xylan content, an infrared spectrum of compression wood MWEL gave only a slight ester absorption compared to that of normal wood MWEL. Borohydride reduction of the compression wood MWEL yielded a 20% loss of reducing sugar after acid hydrolysis. Compression wood lignin apparently has more L-C bonds than normal wood, and most of these are lignin-galactan linkages.

Soda cooking of MWEL

The mild-alkali labile L-C bonds described comprise only 10–20% of the total L-C bonds and have little effect on the alkaline delignification of wood. It was, therefore, of theoretical and technical interest to determine the resistance of the majority of these bonds to cleavage under the conditions of alkaline pulping. When loblolly pine

IV. Carbohydrate composition of Sitka spruce, aspen, red pine compression and normal wood MWEL's and of their soluble fractions after mild alkaline treatment

	Total sugar anhydride, mg/g MWEL		Sugar composition, %				
	Enzymatic hydrolysis	Acid hydrolysis	Glucose	Mannose	Xylose	Galactose	Arabinose
Sitka spruce MWEL	0	100.00	33.8	29.6	14.7	16.9	5.1
Soluble fraction	12.9	13.1	10.2	14.0	59.2	10.4	6.3
Aspen MWEL	0	100.9	51.3	6.9	26.8	8.6	6.5
Soluble fraction	15.5	19.0	7.4	7.2	71.0	8.9	5.7
Red pine MWEL							
Normal wood	0	110.7	24.3	26.1	23.7	17.0	8.9
Soluble fraction	20.4	21.3	8.0	7.0	67.1	6.0	12.0
Compression wood	0	160.5	14.1	7.3	6.9	68.5	2.8
Soluble fraction	7.0	6.4	7.0	4.1	55.0	25.9	8.0

V. Loblolly pine borohydride-reduced MWEL treated under soda pulping conditions^d

	Total sugar anhydride mg/g MWEL ^b		Sugar composition, %				
	Enzymatic hydrolysis	Acid hydrolysis	Glucose	Man- nose	Xy- lose	Galac- tose	Arabi- nose
Reduced MWEL	0	88.1	26.3	25.8	12.7	27.2	8.1
Neutralized liquor	13.8	25.5	13.7	29.4	16.5	32.9	7.4
Residue	0	48.2	27.2	25.3	13.9	26.3	7.5

^aThree hours at 160°C in 0.5N sodium hydroxide ^bBased on reduced MWEL

MWEL was heated to 160°C in 0.5N sodium hydroxide for 3 hr, only 10% of the original reducing sugar could be accounted for after cooling and neutralization. This loss of sugar may be explained by the expected extensive alkaline degradation by end-group peeling. When the original carbohydrate was stabilized by borohydride reduction of aldehydic end groups and the reduced MWEL was treated under the same soda conditions, over 80% of the carbohydrate was recovered as reducing sugars.

Because the borohydride reduction was carried out in 0.1N sodium hydroxide, the low carbohydrate content of the reduced MWEL (Table V) resulted from loss of end groups and loss of the mild-alkali labile carbohydrate. About 30% of the original carbohydrates in the reduced MWEL were found in the neutralized liquor after cooking (Table V), but only half of this was enzyme digestible. In this case, partial enzyme digestibility of the soluble carbohydrates does not necessarily indicate L-C bond cleavage. These digestible carbohydrates may have been a result solely of the alkaline chain-splitting of the oligosaccharide residues. The carbohydrates of the MWEL precipitated after cooking were not enzyme-hydrolyzable. From these and the previous results, it is concluded that 70% to as many as 90% of the L-C bonds in MWEL are resistant to cleavage under soda pulping conditions.

Summary

Milled-wood enzyme lignin, the unfractionated lignin residue obtained by polysaccharidase digestion of vibratory ball-milled wood, contains all of the L-C bonds in wood with the possible exception of glycosides. Mild alkaline treatment, 0.1N sodium hydroxide at 20°C for 90 min, removed 10 to 20% of the residual carbohydrates in loblolly pine, Sitka spruce, red pine, and aspen MWEL's. These labile carbohydrates were rich in xylose and the predominant bond type was proposed to be xylan-uronic acid ester linkages to lignin. The alkali-labile bonds could be effectively cleaved in wood by pretreatment with caustic before MWEL prep-

aration. The average number of sugar units comprising the polysaccharide fragments attached to MWEL was determined by borohydride reduction of end groups. Assuming one L-C bond per saccharide fragment, an average L-C bond frequency was calculated to be one for every 36 phenyl propane units. Mild-alkali labile L-C bonds were calculated to have a frequency of about one for every 200 phenyl propane units. Hardwood and softwood MWEL's had similar L-C bond frequencies, but compression wood MWEL contained more L-C bonds than normal wood and most of these were to galactose. Loblolly pine MWEL, borohydride-reduced to inhibit alkaline degradation of the residual carbohydrates, was treated under soda pulping conditions. Most of the L-C bonds, from 70% to as many as 90%, were found to be resistant to alkaline cleavage. The stability of these bonds is significant in the selective removal of lignin or stabilization of hemicellulose in high-yield pulping and in the removal of residual lignin in bleaching and is the subject of further research at this laboratory.

Experimental

Wiley-milled wood was acetone/water (9/1) and ethanol/toluene (1/2) extracted, dried, and milled in a vibratory ball mill. The milled wood was digested twice with Onazuka SS enzyme mixture (Yakult Biochemicals Company Ltd.), filtered through a 400-mesh screen, and digested a final time at pH 4.6 and 48°C. Microscopic examination of the MWEL's revealed the absence of identifiable wood cells and fragments.

Typical mild alkaline treatment of 1.0g MWEL was at 20°C in 100 ml 0.1N sodium hydroxide with stirring for 90 min under nitrogen. The mixture was neutralized with acetic acid to pH 6.5 and centrifuged at 25,000 G. The pellet was washed twice with water and then freeze-dried. The washings and liquor were combined and the volume reduced by vacuum evaporation. Soda cooking was with 2.0 g of reduced MWEL in 40 ml of 0.5N sodium hydroxide in a stainless steel bomb tumbled in an oil bath at 160°C for 3 hr. Workshop was as above.

Borohydride reduction of the MWEL

was carried out with 400 mg MWEL, 100 mg of sodium borohydride, and 100 mg of sodium hydroxide in 50 ml of water at 20°C for 2 hr with stirring. The mixture was neutralized with acetic acid and freeze-dried. The soluble fraction obtained after mild alkaline treatment of MWEL was borohydride-reduced with excess borohydride at pH 12 for 1 hr.

Total sugars were measured after sulfuric-acid-catalyzed hydrolysis by a photometric method (17). Sugar ratios were determined by paper chromatography.

Infrared spectra were run on a Beckman IR-12 as KBr disks.

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