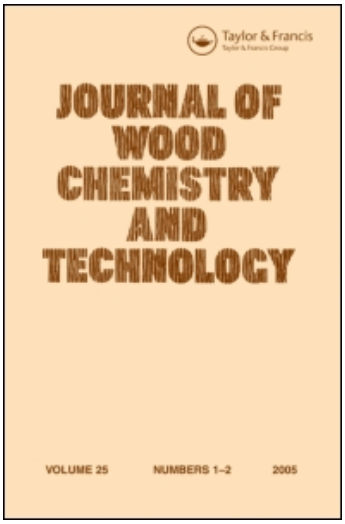

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LOCATION OF LIGNIN-BONDED PECTIC POLYSACCHARIDES

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ABSTRACT

The hypothesis that galactose and arabinose pectic substances are distributed with lignin throughout wood cell walls was tested by analyzing a high-yield loblolly pine kraft pulp that had been separated into middle-lamella-rich and secondary-wall-rich fractions. The fractions were first treated with a polysaccharidase enzyme mixture to obtain "enzyme lignins." The structures of the galactose and arabinose oligomers remaining attached to the lignin were determined by methylation analysis. The 1→4 linked galactose structure, characteristic of pectic materials, was present in significant amounts in the middle-lamella-rich fraction, but to only a very small extent in the secondary-wall-rich fraction. In contrast, the pectic arabinan 1→5 linked structure was a significant proportion of the arabinose from both fractions. The 1→5 linked arabinose structure can arise from a linkage of arabinoxylan to lignin through the 5 position of the arabinose substituent as well as from pectic structures. After partial acid hydrolysis, approximately half the remaining arabinose in the secondary-wall-rich fraction was attached as monomeric units to lignin through the 5 position.

INTRODUCTION

Methylation analyses of carbohydrates bonded to lignin in pine normal wood have indicated that the well-established cell wall polysaccharide structures are generally involved and that the primary hydroxyl group of the sugar monomers is preferred as the position of linkage to lignin.[1] A notable exception to the usual polysaccharide structure is the abundance of galactose and arabinose units with β-D-(1→4) and α-L-(1→5) glycosidic links, respectively. These structural patterns are characteristic of the pectic group substances (Fig. 1).

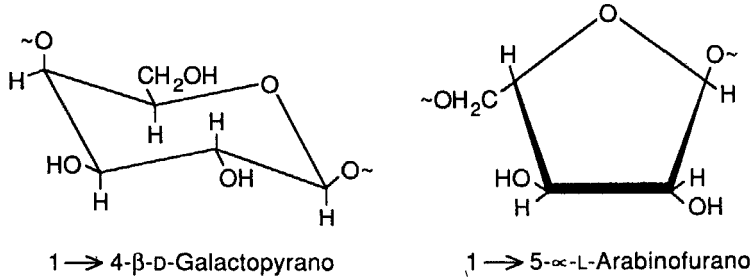

Figure 1. Pectic structures of galactose and arabinose.

The middle lamella region of differentiating cells is known to be rich in pectic substances.[2,3] However, the composition of different regions of lignified cells has been studied only recently. In a study of lignified spruce cell walls,[4] an increase in arabinose and galactose content in the compound middle lamella was observed and was attributed by the authors to the presence of "pectins."

Considerable evidence indicates that the pectic structures of arabinose and galactose are intimately associated with lignin. They have been detected with softwood lignins,[1,5] with residual lignin in kraft pulps,[6,7] and with kraft lignins from pulping liquors.[8,9] They survive kraft pulping but dissolve if the associated lignin is oxidized with chlorite.[9] The 1→4 linked galactan structure is present in a high proportion in compression wood, which is also rich in lignin. A strong correlation has been noted between galactose, arabinose, and xylose content and the lignin content of various tissue fractions separated by density.[10]

It has been suggested that calcium in the cambial tissue is important for the lignification of the cell wall[11] and that pectic acid serves a regulatory role with the concentration of calcium ion. It was subsequently shown[12] that the pectic components, galacturonic acid and rhamnose, are mostly degraded or removed during maturation of spruce cell walls. It was suggested that this degradation is related to liberation of calcium ions during lignification. The presence or absence of the unique "pectic" galactan and arabinan structures is also related to the question of how lignin is biosynthesized. These structural linkages are contained within complex cell wall (pectic) carbohydrates that also contain galacturonic acid and rhamnose [12] and references therein). The galactose and arabinose structures remain after lignification and appear to be involved in lignin-carbohydrate bonding. If these structures

have a role in lignin biosynthesis, they should be found with lignin in the secondary wall and in the compound middle lamella.

Given the facts that the galactose and arabinose polymer structures have not been conclusively determined for different lignified cell wall regions and that the 1→4 and 1→5 links, respectively, are present in lignified wood, a reasonable hypothesis to test is whether the pectic polysaccharide structures are intimately associated with lignin wherever it has been formed. An attempt has been made[13] to locate pectic substances in the sapwood cell walls of beech and spruce using staining techniques and electron microscopy. "In some cases" the authors obtained "a reaction over the whole secondary wall with good distributions in the middle lamella."[13] Earlier electron microscopic work[14] on onion roots using a stain considered specific for pectin indicated that although the pectin was concentrated in the middle lamella and near the primary wall surface, it was also observed throughout the cell wall.

In the present work, a very high-yield loblolly pine (*Pinus taeda*) kraft pulp was separated into middle-lamella-rich and secondary-wall-rich fractions. The separate fractions were treated with polysaccharidases and then analyzed for the carbohydrate structures retained with the lignin residue.

RESULTS AND DISCUSSION

A middle-lamella-rich fraction, termed crill, was isolated from a high-yield loblolly pine kraft pulp.[15] This lignin-enriched material was then treated with a commercially available mixture of cellulases containing some hemicellulases to produce a crill enzyme lignin that contained fragments of polysaccharides chemically bonded to lignin. The sugar analyses of the crill before and after enzyme treatment are given in Table 1. The content of galactose, arabinose, and xylose in the crill before enzyme treatment was somewhat enriched compared to that in the unfractionated pulp. After enzyme treatment, the content of galactose and arabinose was exceptionally high compared with that of other similarly prepared enzyme lignins from wood[1] or bleachable-grade kraft pulp.[6] Enrichment in galactose after enzyme treatment is partially caused by a lack of suitable hydrolyzing enzymes for these structures in the cellulase-hemicellulase mixture produced by *Trichoderma reesei*.[16] This observation was confirmed with the enzyme preparation used in the present investigation. The enrichment is an advantage in this particular study because the interest centers on the pectic-type galactan structure.

TABLE 1
Sugar Analysis of Loblolly Pine Lignin Samples

Pulp Fraction	Klason Lignin (%)	Total Sugar Content (%)	Percentage of Total Sugar				
			Galactose	Arabinose	Xylose	Mannose	Glucose
Crill	42.1	55.9	4.2	3.4	11.8	6.3	74.3
Crill Enzyme Lignin	80.9	5.2	32.8	20.6	8.8	11.0	26.7
Secondary Wall	–	80.3	2.0	1.0	10.0	6.2	80.9
Secondary Wall Enzyme Lignin	–	8.6	9.9	2.6	9.2	16.4	62.4
Partially Hydrolyzed Enzyme Lignin	–	7.3	5.9	1.1	5.7	14.9	72.5

A pulp with the middle lamella and primary wall portions largely removed was prepared by isolating the long-fiber fraction from the same pulp used for the crill preparation. The pulp was treated with oxygen in dilute sodium hydroxide solution. Treatment with oxygen gas has been shown to topochemically remove lignin from the outside of fiberized pulp tracheids.[17] Removal of lignin was monitored by ultraviolet microscopy. Two treatments were necessary to remove a majority of middle lamella and cell corner lignin. After the second treatment, the primary wall was removed by extensive beating in a PFI mill.[18] The remaining long-fiber fraction was then treated with polysaccharidases to give the "secondary wall enzyme lignin," the chemical composition of which is given in Table 1. Compared to the crill enzyme lignin, the carbohydrate portion of the secondary wall enzyme lignin is much richer in glucose and mannose and poorer in galactose and arabinose.

The two enzyme lignin samples were then submitted to methylation analysis; the results are shown in Table 2. A majority of the galactose from the crill enzyme lignin gave the 2,3,6-tri-o-methyl derivative that arises from the 1→4 linked unbranched units, whereas the majority of galactose from the seconday wall enzyme

TABLE 2
Methylation Analysis of Loblolly Pine Lignin Samples

Alditol and Position of Methylation	Area (%) of All Chromatographed Products[a]		
	Crill Enzyme Lignin	Secondary Wall Enzyme Lignin	Partially Hydrolyzed Secondary Wall Enzyme Lignin
Galactitol			
2,3,4,6	<1%	1.4	1.4
2,4,6	3.0	3.9	2.5
2,3,6	22.3	0.8	0.8
2,3	3.8	0.2	0.2
2,4	1.4	1.7	1.2
Arabinitol			
1,2,3,4	–	– (0.1)	0.3 (0.6)
2,3,5	1.8	0.6	0.2
2,3	13.2	1.1	0.4
Xylitol			
1,2,3,5	–	0.6	0.4
2,3,4	<1%	1.4	1.1
2,3	4.6	4.2	3.8
3	0.8	1.0	0.8
2	0.8	2.0	0.6
Mannitol			
1,2,3,5,6	–	0.4	0.9
2,3,4,6	–	2.0	1.9
2,3,6	6.6	5.7	6.2
2,3	1.8	1.9	2.0
Glucitol			
1,2,3,5,6	–	0.3	1.1
2,3,4,6	–	1.9	2.0
2,3,6	17.1	60.0	60.6
2,3	2.5	2.1	1.9
hexaOAc	–	0.7	0.2
Totals	81.7	93.9	90.5

[a] –, not identified; values in parentheses are from end group analysis.

lignin gave the 2,4- and 2,4,6-tri-O-methyl derivatives that arise from 1→3, 1→6 branched arabinogalactan. On this basis, it is apparent that the 1→4 linked galactan structure in loblolly pine is associated to a large extent with the compound middle lamella lignin and to only a very small extent with secondary wall lignin. The fractionation procedure is not perfect, and it is possible that no 1→4 linked galactan is associated with the lignin of the secondary wall of normal *P. taeda*.

The methylation results for arabinose are less clearly interpreted. The major arabinose methylated product from the crill and secondary wall enzyme lignins was the 2,3-di-O-methyl derivative. This is surprising because arabinose in arabinogalactans and in arabinoxylans should be present mostly as terminal units. In addition, reports of methylation analyses on the arabinogalactans from pines[19] indicated that the 2,5-di-O-methyl-L-arabinitol derivative might have been expected in significant amounts from the secondary wall enzyme lignin. The major difference between the methylation results of the two enzyme lignins was in the total quantity of arabinose, which was present to a greater extent in the crill. The ratio of 2,3-di-O-methyl derivative to the terminal 2,3,5-tri-O-methyl derivative indicates that proportionately more of the 1→5 linked structure was present in the crill.

The major methylated products from glucose, mannose, and xylose reflected the proportions present in the starting materials. No major structural differences existed between the products from the secondary wall and those from the compound middle lamella.

In addition to their participation in pectic polysaccharides, arabinose and galactose have been implicated as possible "cross-links" between softwood lignin and cell wall polysaccharides through their positions as monomeric substituents in 4-O-methyl glucuronoarabinoxylans and galactoglucomannans, respectively. The furanosidic linkage of the arabinose substituent makes it exceptionally labile toward acid hydrolysis. A selective dissolution of arabinose and xylose from a spruce enzyme lignin preparation upon mild acid hydrolysis has been demonstrated.[20] Reducing arabinose end units were also formed in the solid during the partial hydrolysis. These observations were explained by postulating a glycosidic linkage to a xylan backbone and a nonglycosidic linkage to lignin. Methylation analysis has shown that galactose bonded 1→6 and arabinose bonded 1→5 are present in spruce[5,21] and pine[1,8] normal wood. One explanation for these structures is that they are present as cross-links from polysaccharides to lignin.

The unexpected finding of major proportions of 2,3-di-o-methyl arabinose from methylation analysis of the secondary wall material in this study prompted further investigation. If the 2,3-di-o substitutents arise from 1→5 linked arabinans, they should be susceptible to mild acid hydrolysis. If they are a cross–link with lignin, they should be retained as a new reducing end after a partial hydrolysis. The nonreducing terminal units should be hydrolyzed readily.

The secondary wall enzyme lignin preparation was submitted to acid hydrolysis under conditions selective for hydrolysis of the arabinofuranoside units.[20] Sugar, end group, and methylation analyses were performed on the reduced, partially hydrolyzed secondary wall enzyme lignin. The sugar analyses are shown in Table 1. The major losses upon partial acid hydrolysis were in quantities of arabinose, xylose, and galactose. An analysis of the partial hydrolyzate showed that the major monomeric sugar loss was arabinose. Xylose and galactose were lost primarily as oligomers that may or may not have been still attached to solubilized lignin fragments. The total yield loss during the partial acid hydrolysis was 15% to 20%.

End group analysis was performed by deuterium labeling of the reducing end units.[22] The results are shown in Table 3. The largest change in apparent chain length was in arabinan. After partial acid hydrolysis, about every other arabinose unit was a reducing end unit. These end units must be linked to some component of the solid by a nonglycofuranosidic linkage (presumably an ether link). The gas chromatogram from a methylation analysis of the partially hydrolyzed enzyme lignin showed only one new product when compared with the chromatogram of the unhydrolyzed lignin methylation products. That product was identified as 5-o-acetyl-1,2,3,4-tetra-o-methyl arabinitol, which comes from the reducing end unit. As observed previously,[1,6] the amount of the methylated end unit observed by gas chromatography was less than that predicted by end group analysis. The chromatographic yield was presumably low because of the volatility of this highly methylated product with consequent losses during sample preparation. The concentration of arabinitol end units as determined by deuterium analysis is given in parentheses in Table 2.

Two types of deuterium labeling experiments were performed for the end group analysis of the partially hydrolyzed secondary wall enzyme lignin samples. The recommended method[22] for maximum accuracy is reduction with sodium borohydride, complete acid hydrolysis, and reduction of the monomers with sodium

TABLE 3
Apparent Chain Length of Secondary Wall Oligomers Remaining With Lignin

Oligomer	Before Partial Hydrolysis	After Partial Hydrolysis	
Arabinan	16	2.0	1.8[a]
Xylan	8	9.5	
Mannan	12	9.4	
Galactan	(100)[b]	(100)	
Glucan	24	20	

[a]Apparent chain length based on new reducing end units generated by partial acid hydrolysis.
[b]Maximum reliable chain length determination is 20, reference.[22] Chain lengths are "apparent;" large numbers reflect a very small amount of free-reducing-end units.

borodeuteride. However, if the initial reduction of the enzyme lignin is performed with sodium borohydride, a reduction after the partial hydrolysis with sodium borodeuteride followed by complete acid hydrolysis and subsequent reduction with sodium borohydride will indicate how many new reducing end units were formed in the partial hydrolysis step. Analysis by the recommended procedure (borohydride first, borodeuteride second) gives the total of new and old end units in the partially hydrolyzed lignin. The results, included in Table 3, indicated that some monomeric free-reducing-end units were present in the secondary wall enzyme lignin. The monomeric units attached to lignin would be expected to survive the mild acid hydrolysis. Other free-reducing-end units that would be dissolved in the mild acid hydrolysis would be those that were a part of an arabinan or sequence of arabinose units most likely linked 1→5. A reliable, quantitative determination of the amount of monomeric reducing end arabinose present in the lignins was not possible considering the small quantity present and the magnitude of potential errors. However, the data suggest that approximately a third of the arabinose reducing end units survived the partial acid hydrolysis.

Interestingly, both the 2,3 and 2,3,5 methyl substituted arabinitol units were present after mild acid hydrolysis, although in reduced quantity. The 2,3-di-o-methyl derivative could come from a carbohydrate-to-lignin arabinose cross–link

as well as from 1→5 linked arabinan oligomers. The 2,3,5-tri-o-methyl derivative that comes from the nonreducing terminal arabinose structure should have been eliminated during the partial acid hydrolysis. Either the partial hydrolysis was not quite complete because of inhibited accessibility or perhaps some structural factor in the lignin-carbohydrate macromolecule increased the acid resistance of some furanosidic bonds.

CONCLUSIONS

The unique (1→4) linkage of galactose units is characteristic of complex carbohydrates associated with pectin rather than that of softwood cell wall arabinogalactans or galactoglucomannans. This linkage predominates in the galacto–oligomers remaining with the lignin in a compound middle-lamella-enriched fraction of loblolly pine. In contrast, much less galactose is associated with secondary wall lignin, and the (1→4) linkage is a minor proportion of that amount. Thus, although various interpretations are possible for the observation of some (1→4) linked galactose in the secondary wall, it appears that this linkage is not uniquely associated with lignin and probably has no role in lignin deposition.

Arabinose, which is also a pectic polysaccharide component, is also present in much larger quantities in the compound middle-lamella-enriched pulp fraction. However, in contrast with galactose, the unique "pectic" structure (1→5 linkage) is predominant among the arabinose structures of the secondary wall enzyme lignin. After a mild acid hydrolysis to hydrolyze the glycofuranosidic linkages, half the remaining arabinose was present as a monomeric unit attached at the 5 position. Although the attachment could be to another sugar, the present evidence and our knowledge of cell wall polysaccharide structures favor attachment to lignin. The results are consistent with previous suggestions that arabinose functions as a cross–link between lignin and wood hemicelluloses.

EXPERIMENTAL

A high-yield kraft pulp, kappa number 142, was prepared from loblolly pine chips using 14% active alkali, 25% sulfidity, 5:1 L/W, 90 min to and 10 min at 160°C. The pulp was refined in a 12-in. (30.48 cm) refiner, beaten in a PFI mill for 5,000 revolutions, and size screened in a Bauer–McNett classifier. Material passing through 200 mesh was collected as crill. The long-fiber fraction was further treated as follows to obtain "secondary wall" material.

The long-fiber fraction was treated with oxygen for 30 min at 120°C, under 180 lb/in^2 (1,240 kPa) total pressure, with 10% NaOH on pulp at 3% consistency. This treatment gave a 116 kappa number pulp that still contained lignin on the fiber surfaces and cell corners. The oxygen treatment was repeated using the conditions of the first treatment. The resultant pulp had a kappa number of 92. The pulp was then beaten in the PFI mill for 30,000 revolutions, dispersed in a blender at 1% consistency, and reclassified. The longest fiber fraction was saved for enzymolysis.

Enzyme treatments, end group analyses, and methylation analyses were performed as previously reported.[1,6,22] The partial acid hydrolysis was performed using the conditions described in reference.[20]

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