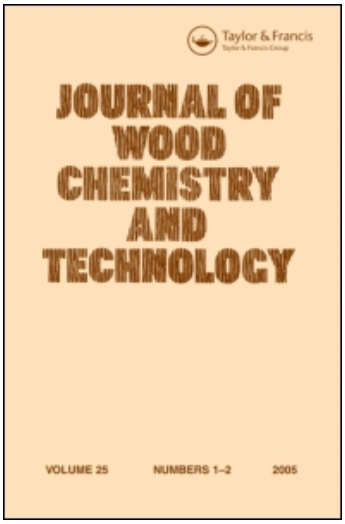

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STUDIES ON THE NATURE OF LIGNIN - CARBOHYDRATE BONDING

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ABSTRACT

A lignin - carbohydrate complex (LCC) fraction which was initially extracted with dimethyl sulfoxide from birch residual wood meal after stepwise MWL extraction (total yield 28%), contained about 7% galacturonic acid together with about 4% glucuronic acid. Although galacturonic acid residue is well known to be a component of hardwood xylan, it also suggests the possibility of the existence of pectic substance in this LCC fraction.

The nature of galacturonic acid in this LCC fraction was characterized by means of two purified pectinases, endo-polygalacturonase and endo-pectin lyase. It was concluded that most of the carbohydrate in this LCC fraction, especially xylan, is combined with lignin through pectic substance.

INTRODUCTION

The lignin - carbohydrate complex has long been one of the most attractive but tough targets for wood chemists. Based on information from gel filtration chromatography, electrophoresis, sugar analysis and so on, the existence of co-valent bonds between lignin and carbo-

hydrate became widely accepted, and many types of bonding structures have been proposed. However, none of them have been clearly established so far.

In this connection, lignin - carbohydrate complexes (LCC) treated with hemicellulases or a mixture of hemicellulases and cellulases seem to be suitable materials to be investigated. This is because lignin - carbohydrate bonds, in other words, carbohydrate residues directly bound to lignin, should be highly concentrated in those materials. Eriksson et al.[1] have examined the nature of carbohydrate residues in thus treated spruce LCC and concluded that at least arabinose and galactose residues must be contributing to lignin - carbohydrate bonding. This was because some arabinose and galactose residues were reduced to alditols by $NaBH_4$ but remained in the lignin fraction, even though those residues were glycosidically linked to the hemicellulose frame work. Recently, they further reported that all types of neutral sugar residues in hemicellulose are, to some extent, bound to lignin probably by benzyl ether bonds[2], and that 4-O-methylglucuronic acid seems to link to lignin by benzyl ester bonds.[2] Lundquist et al.[3] found that the major part of carbohydrate in milled wood lignin (MWL) was readily removed by a mild alkaline treatment, but was considerably stable to a mild acidic treatment. Benzyl ester bonding between uronic carboxyl groups and lignin was thought to be in accord with these observations.

The authors have studied the characterization of MWL and LCC sequentially extracted from birch wood meal and found that a considerable amount of galacturonic acid (7.6% on LCC) is contained in LCC fraction. Although galacturonic acid residue has been reported to be a component of hardwood xylan,[4,5] it also indicates the possible presence of pectin, polygalacturonic acid, in LCC. In this paper, the nature of galacturonic acid residue in

LCC was examined by purified pectinase treatments and discussed in relation to the lignin - carbohydrate bonding structure.

EXPERIMENTAL

Preparation of Lignin - Carbohydrate Complex (LCC)

LCC was extracted from the residual wood meal of MWL preparation (yield:28.06% based on Klason lignin content of original wood meal. LCC-I) by DMSO extraction for 21 days with occasional shaking (Fig. 1). Then, LCC-II was extracted by another 188 days extraction. Yields of these two LCC fractions were 11.7% and 4.2%, respectively, based on the residual wood meal after MWL preparation.

Pectinase Treatment

Table 1 shows the list of pectinases used in this study. The first two commercial enzymes, such as Pectinase (Tokyo kasei) and Pectolyase (Kikkoman) were mainly used in the preliminary experiment. Endo-polygalacturonase (endo-PG) and endo-pectin lyase (endo-PL) were purified pectinases isolated from culture extract of Aspergillus japonicus by Ishii.[6,7] The natures of these two enzymes will be discussed later. Application conditions of pectinases were also summarized in Table 1.

Gel Filtration

Pectinase treated samples were concentrated under reduced pressure and lyophilized to dryness, and then dissolved again in methylcellosolve - water (1 : 1, by volume) mixture. 1 ml of the solution was subjected to gel chromatography on a Sepharose CL-6B column (2.5cm x

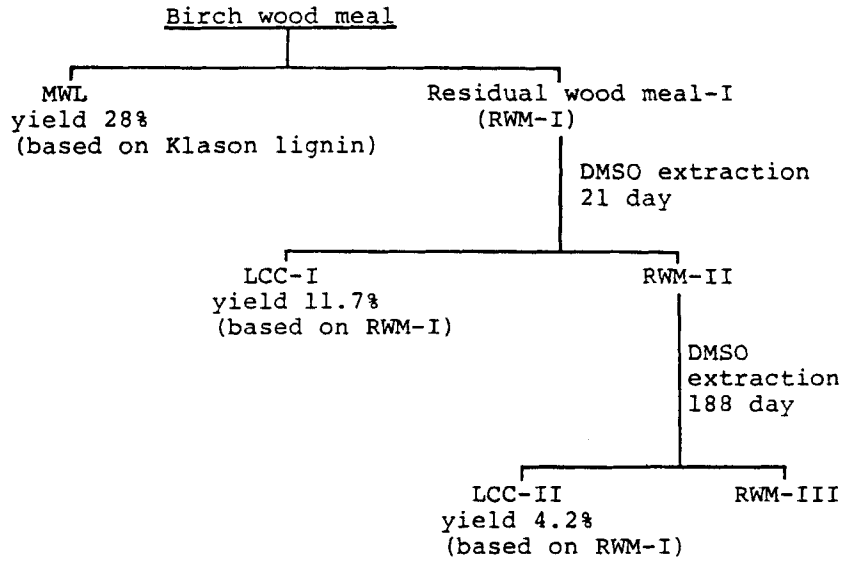

FIGURE 1 Scheme for preparation of Lignin-Carbohydrate Complex

TABLE 1

List of Pectinases

Pectinase	Treatment Condition
Commercial	
Pectinase(Tokyo Kasei)	pH4.0(acetate buffer), 20 °C, 1-10day
Pectolyase(Kikkoman)	pH4.5, 5.0(acetate buffer), 40 °C, 6hr - 10day
Purified	
Endo-polygalacturonase (endo-PG)	pH4.5, 5.0(acetate buffer), 40 °C, 40hr 300unit*1/50mg LCC-I
Endo-pectin lyase (endo-PL)	pH4.5, 5.0(acetate buffer), 40 °C, 40hr 30unit*2/50mg LCC-II

*1 1 unit corresponds to the amount of endo-PG which liberates 1μmole of aldehyde groups per minute.

*2 1 unit corresponds to the amount of endo-PL which increases the absorbance of reaction mixture at 235nm by 1.0 per minute.

50cm). The flow rate was kept at 1.5ml/10min. The distribution curves of carbohydrate were determined by the phenol - sulfuric acid method (480nm) and that of lignin by UV absorption at 280nm. Based on the distribution curves of carbohydrate and lignin, pectinase treated LCC-Is were divided into three fractions. Namely, fraction-I : elution volume 55ml(void volume) - 90ml, fraction-II : 90ml - 145ml and fraction-III : 165ml - 205ml.

Analysis of Carbohydrate Components

Original LCC-I and each fraction of pectinase treated LCC were hydrolyzed by 4% sulfuric acid at 120 °C for 1 hr, and then subjected to neutral sugar analysis by the alditol acetate method. G.l.c. conditions for alditol acetate were as follows. Column: stationary phase; Gaschrom Q, liquid phase; EGA 0.2% + EGS 0.2% + silicone GE XF-1150 0.4%, 0.3cm x 2m. Column temp.: 180 °C. Carrier gas(N_2): 1.4kg/cm^2. H_2: 7.5kg/cm^2. Detector: FID. Acid sugar components, such as galacturonic acid and glucuronic acid were analyzed by the dithioacetal-TMS method proposed by Honda.[8] G.l.c. conditions were as follows. Column: glass capillary OV-I, 0.28mm x 50m. Column temp.: 225 °C.

RESULTS AND DISCUSSION

Characteristics of LCC-I

As discussed by Sarkanen[9] in "Lignins", all of the protolignin in the cell wall is present in some sense as lignin - carbohydrate complex. Both of milled wood lignin (MWL) and lignin - carbohydrate complex (LCC) by Björkman's method are parts of lignin fragments formed by vibratory ball milling, and a principal difference of

these two lignin preparations is the carbohydrate content. The latter amounts to less than 10% for MWL and more than 80% for LCC.

The authors concluded in the previous paper[10] that MWLs sequentially extracted up to 28.06% (total yield, based on Klason lignin) were originating mainly from the compound middle lamella region of hardwood cell walls, although it is not consistent with the Goring's recent findings.[11] This might be due to a very high lignin concentration in that region. In other words, fracturing of lignin by vibratory ball milling seems to be enough to be extracted as MWL. However, in the case of lignin in secondary wall region, it might not be the case. Not only lignin, but also carbohydrate chains must be fractured enough before aqueous dioxane extraction. In any case, the selective extraction of compound middle lamella lignin as MWL seems to suggest the possibility of separate extraction of LCC from different cell wall regions.

LCC-I and -II were extracted sequentially from the residual wood meal of MWL preparation, and the further extraction of LCC is now going on (Fig. 1). Yield of LCC-I initially extracted by 21 days extraction was 11.7% on residual wood meal, but the amount of net lignin in LCC-I accounted for only about 1.3% of original wood meal or 5% of its Klason lignin content. By the prolonged extraction up to 188 days, LCC-II was obtained at only 4.2% yield on residual wood meal or about 1.4% on original Klason lignin content. Because of these very low yields, LCC-I and -II seem to be attributable to a rather minor fraction of cell wall lignin. If this is true, secondary wall lignin is relatively resistant to MWL and LCC preparations.

As part of continuing research on inhomogeneity of the chemical structure of lignin in different regions of the cell wall, the authors, in this study, focused on the

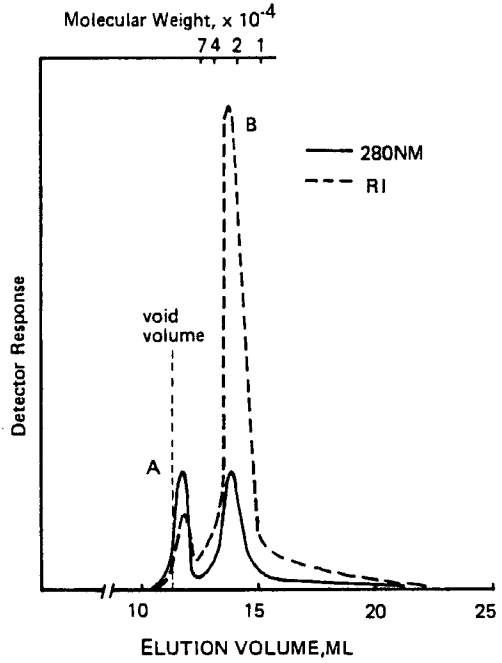

FIGURE 2 GPC Curves of LCC-I by Toyosoda G2000SW Column
column: 7.5x60cm, eluent: water

characterization of LCC-I, especially on the nature of lignin - carbohydrate bonding. Fig. 2 shows the gel permeation chromatograms of LCC-I. Besides the main peak appearing at an elution volume of 13.8ml, a small peak was found at 11.8ml. Here, curves for refractive index (RI) and UV absorption at 280nm correspond to the distribution of total substance and lignin, respectively. The comparison of these two distribution curves suggest quite different characteristics of the fractions. That is, in spite of the similar intensities of UV absorption at 280 nm, RI intensity for the peak of lower molecular weight region (peak B) was much greater than that for the higher molecular weight peak (peak A). Carbohydrate should be predominant in peak B, but a comparatively higher lignin

content could be anticipated for peak A. The potential contribution of enzyme protein to UV absorption at 280 nm was assumed to be negligible because of the very low enzyme dosage applied.

Although LCC-I is not homogeneous as shown here, it appears that the fraction of peak B is most representative of the nature of LCC-I. Molecular weight ($\bar{M}w$) of peak B was determined to be 25,000 in water and 16,500 in acetate buffer by means of the calibration curve of FITC dextrans. The higher molecular weight ought to reflect the expansion of LCC molecules in water. The contributions of lignin and carbohydrate fractions to the molecular weight of LCC-I in acetate buffer might be estimated depending on the relative composition of each fraction in LCC-I (Table 2) to be about 3,000 and 13,000, respectively. It is interesting that the molecular weight of the lignin fraction is only about one fourth of the typical MWL. Chemical compositions of LCC-I were as follows: Lignin 19.7%, neutral sugars 68.9%, uronic acids 11.4% (galacturonic acid 7.6% and glucuronic acid 3.8%).

Pectinase Treatment of LCC-I

In the preliminary experiment, the authors found that carbohydrate content in LCC-I was remarkably reduced by treatment with commercial pectinases. Although commercial pectinases are not pure enough to conclude anything about the presence or the nature of pectin in LCC-I, the results were suggestive of the important role of pectin for lignin - carbohydrate bonding.

Then, two kinds of purified pectinases, endo-polygalacturonase (endo-PG) and endo-pectin lyase (endo-PL), were applied to this study. Homogeneities of these two enzymes were confirmed by gel chromatography, ultra-

TABLE 2

Sugar Composition of LCC-I Before and After Pectin Lyase Treatment

Sample	Lignin	Neutral sugar	Acidic sugar	Relative composition of neutral sugars					
				Rha.	Ara.	Xyl.	Man.	Gal.	Glu.
LCC-I	19.7%	68.9%	11.4%	0.8%	2.3%	69.2%	12.9%	3.1%	11.7%
PL- *1 treated LCC-I	71.0*2	29.0*2	-	6.8 (0.7)	15.3 (1.5)	4.2 (0.4)	12.5 (1.2)	47.7 (4.8)	13.5 (1.3)

*1: Fraction-I (lignin fraction) obtained by Sepharose CL-6B gel filtration chromatography
*2: Determined from Sepharose CL-6B gel filtration curves
( ) : based on total sugar content in LCC-I

centrifugal analysis, disc electrophoresis and so on by Ishii.[6,7] These enzymes were found to be specifically active to pectin, that is polygalacturonate. In other words, no cellulase or hemicellulase activities were observed for these two pectinases.[12] Reconfirmation of the specific activities of these two enzymes was achieved by showing that no monomeric neutral sugars could be detected in the reaction mixtures of pectinase treatments of LCC-I. Confirmation of the specific activity of the enzyme is very important to correctly interpret the results of enzyme treatment.

Other features of these two enzymes were also examined carefully by Ishii.[6,7] Endo-PG preferably hydrolyzed pectic acid or low methoxy pectin and released reducing groups in a random manner, yielding a mixture of mono-, di- and tri-galacturonic acids as the end products. Optimum pH for this enzyme was pH 4.5, but sufficiently high activity was observed between pH 4.0 and 5.6.[6] Endo-PL, on the other hand, was most active to high methoxy pectin and was almost inactive to low methoxy (below 45%) pectin. By endo-PL treatment, a new structure with a characteristic absorption at 235nm is formed in the reaction mixture from galacturonic acid units in pectin. This corresponds to the changes shown in Fig. 3. Optimum pH for this enzyme was pH 5.[7]

Compared with the fractionation curves of original LCC-I (Fig. 4), endo-PL treated sample showed surprising changes in the carbohydrate distribution (Fig. 5). About 90% of carbohydrate which originally appeared at the void volume fraction together with lignin disappeared, and at the same time a new peak appeared at the lower molecular weight region ($\bar{M}w$ ca. 2,000). Some amount of carbohydrate was also recovered as a water insoluble precipitate. It proved to be almost pure xylan. However, with respect to lignin, it was difficult to see

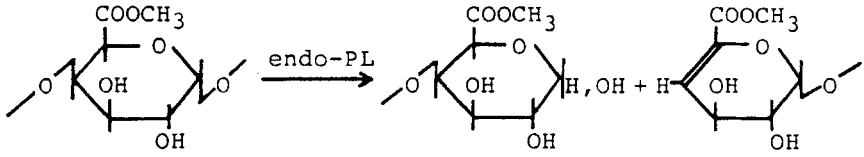

FIGURE 3 Depolymerization Mechanism of Pectin by Endo-PL Treatment

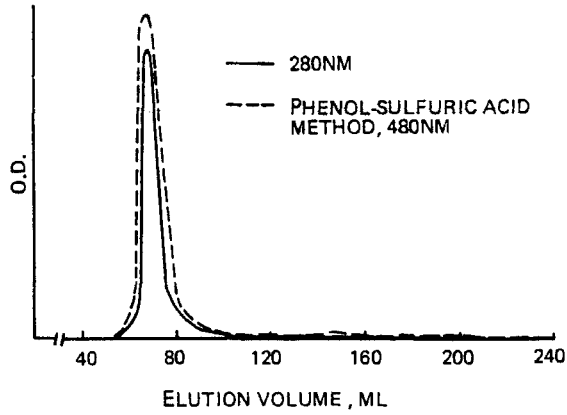

FIGURE 4 Gel Filtration Curves of LCC-I

column: Sepharose CL-6B, 2.5cm X 50cm
eluent: methylcellosolve/water = 1/1

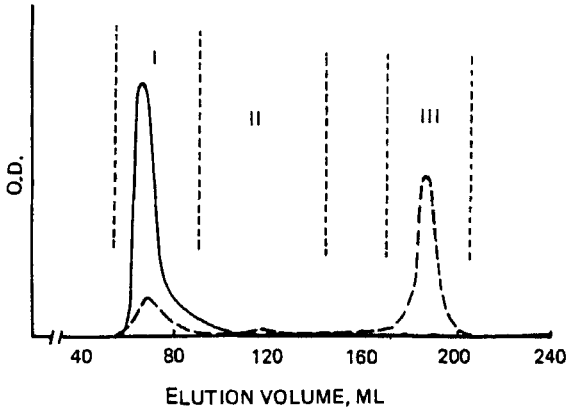

FIGURE 5 Gel Filtration Curves of LCC-I After Endo-PL Treatment

column: Sepharose CL-6B, 2.5cm X 50cm
eluent: methylcellosolve/water = 1/1

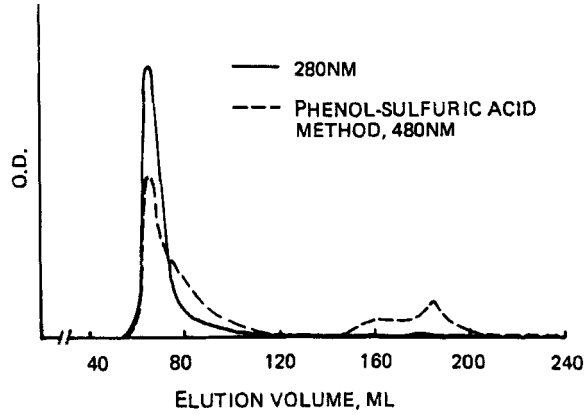

FIGURE 6 Gel Filtration Curves of LCC-I After Endo-PG Treatment

column: Sepharose CL-6B, 2.5cm X 50cm
eluent: methylcellosolve/water = 1/1

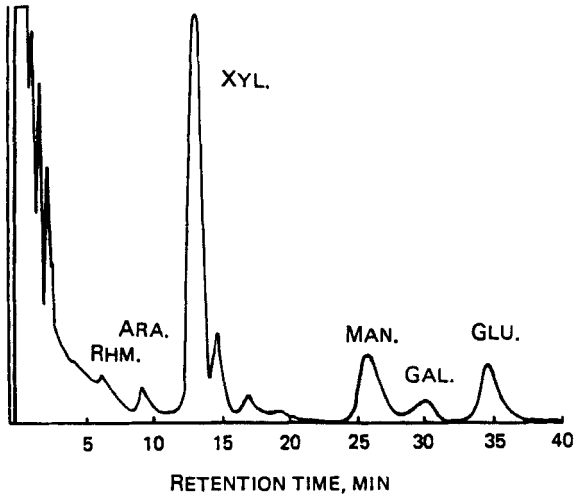

FIGURE 7 Gas Chromatogram of Hydrolysate of LCC-I as Alditol Acetates

column: stationary phase;Gaschrom Q
liquid phase;EGA 0.2%+EGS 0.2%
+Silicone GE XF-1150 0.4%
0.3cm x 2m
column temp.: 180 ℃, carrier gas(N_2):
1.4kg/cm^2, H_2: 7.5kg/cm^2

any apparent change in its distribution curve. These facts mean that there must be some pectin in LCC-I, and it plays a very important role to bind lignin and carbohydrate. A small quantity of galacturonic acid residue has also been observed in xylan, and as a partial structure of xylan, O-β-D-xylopyranosyl-(1-3)-O-α-L-rhamnopyranosyl-(1-2)-O-(α-D-galactopyranosyluronic acid)-(1-4)-D-xylose was reported by Shimizu et al.[4] Since endo-PL is specifically active to α-(1-4) linkage of galacturonic acid residues, it is not conceivable that galacturonic acid residue in xylan is also involved in the reaction at this enzyme treatment.

Furthermore, the lignin fraction of endo-PL treated LCC-I still appeared at the void volume fraction, although the molecular weight must be greatly reduced by the loss of carbohydrate during enzyme treatment. In this case, it is likely that LCC-I appearing at the void volume does not behave as a single molecule, but as an associated molecule. In other words, fractionation curves shown in Fig. 5 do not exhibit the true molecular weight distribution of endo-PL treated LCC-I. However, the degree of carbohydrate loss from lignin in LCC-I by endo-PL treatment is apparent, because the liberated carbohydrate appeared at a different elution volume in this gel chromatogram.

Endo-PG also shows similar but less effect on the fractionation curves (Fig. 6). About 55% of carbohydrate disappeared from the void volume fraction by this enzyme treatment. Even with a higher dosage of endo-PG, the removal of carbohydrates did not increase markedly. A higher effect of endo-PL compared with endo-PG suggests that pectin in LCC-I is highly esterified, so-called high methoxy pectin. Since pectin is distributed exclusively in primary cell wall, it seems reasonable to conclude that LCC-I must originate in the compound middle lamella.

Carbohydrate compositions of fraction I and III(see Figures) from endo-PL treated LCC-I were analyzed by means of the alditol acetate method and compared to that of original LCC-I. Fig. 7 shows a gas chromatogram of hydrolyzate of original LCC-I. It is quite obvious that xylose unit is the predominant sugar component of LCC-I, and that mannose, glucose, galactose, arabinose and rhamnose are also contained in relatively small amounts. Fraction I from LCC-I thoroughly treated with endo-PL showed, on the other hand, a completely different gas chromatogram (Fig. 8). Xylose was no longer a main component. Galactose and arabinose units were also present in considerable quantities. Fraction III which was liberated from lignin by endo-PL treatment, was mainly composed of xylose units (Fig. 9). As shown in Table 2, contents of rhamnose, arabinose and galactose units did not change appreciably during endo-PL treatment, if experimental error for endo-PL treated LCC-I was taken into account. Mannose and glucose contents decreased to one tenth of their original contents. The most striking change is in the xylose content. Indeed, 69.2% in original LCC-I decreased to only 0.4% after endo-PL treatment. Here, it is suggested that most of the xylan and main part of the glucomannan in LCC-I should be connected to lignin by means of pectin. Of considerable interest is to know whether pectin binds to lignin directly or through other hemicellulose. Based on removal of the major part of xylan by a weak alkaline treatment, alkaline labile benzyl ester bonds between lignin and glucuronic acid residue have been proposed for the lignin - carbohydrate bonding.[2] However, if this is so, the almost total removal of xylan by pectinase treatment can not be explained. In other words, a part of pectin may be bound to lignin directly by an alkaline labile, probably benzyl ester bond.

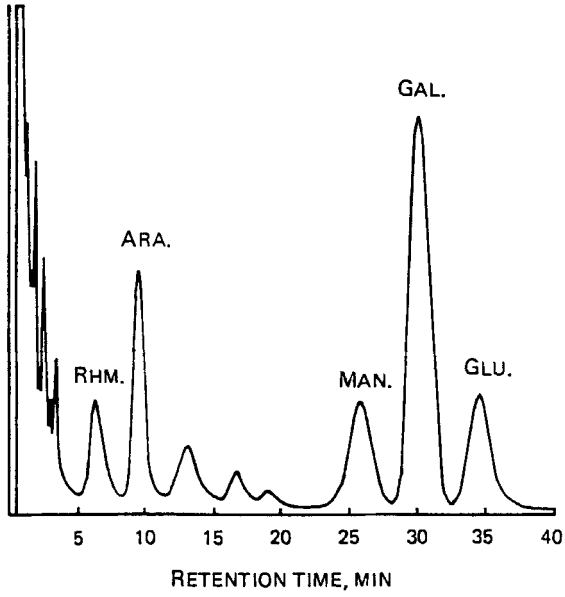

FIGURE 8 Gas Chromatogram of Hydrolysate of Fraction-I from LCC-I After Endo-PL Treatment

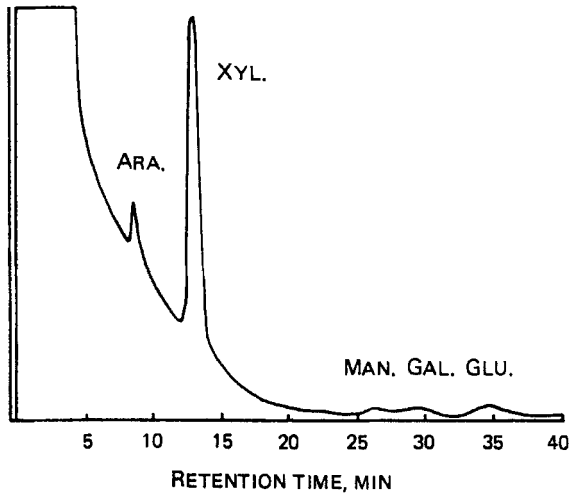

FIGURE 9 Gas Chromatogram of Hydrolysate of Fraction-III from LCC-I After Endo-PL Treatment

Results obtained so far are not applicable to the total lignin, but to a portion of lignin originating in the compound middle lamella. However, further isolation and characterization of LCC fractions will afford valuable information to evaluate the homogeneity of lignin.

CONCLUSIONS

1. A small quantity of pectin is present in the initially extracted lignin - carbohydrate complex fraction from birch wood meal. Therefore, it should have originated in the compound middle lamella region.
2. Pectin in that particular LCC is so-called high methoxy pectin in which the carboxyl group of galacturonic acid residue is highly methylesterified.
3. Most of the xylan in this LCC binds to lignin through pectin, and a benzyl ester bond between lignin and the carboxyl group of galacturonic acid residue is probable for the lignin - pectin bonding structure.

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