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New Preparations of Lignin Polymer Models under Conditions that Approximate Cell Wall Lignification

I. Synthesis of Novel Lignin Polymer Models and their Structural Characterization by ^{13}C NMR

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Summary

Guaiacyl-type lignin polymer models were prepared from coniferin by the action of ß-glucosidase and peroxidase, with hydrogen peroxide generated *in situ* through the action of oxygen and glucose oxidase on the glucose liberated from the coniferin, Polylignols were also prepared from coniferyl alcohol using procedures modified to more closely correspond to conditions prevailing in the cell wall environment. The structure of these novel polylignols approximated that of native lignin more closely than did the structure of polylignols prepared by the conventional method from coniferyl alcohol.

Introduction

Dehydrogenation polymers of monolignols (DHPs) are widely used as models of lignin in the cell wall. Most DHPs are prepared by polymerizing monolignols in homogeneous reaction media using either hydrogen peroxide and peroxidase or laccase and oxygen to initiate the polymerization (Freudenberg 1968). These DHPs, however, have structures that differ from the structures of milled wood lignins (MWLs), no doubt, because the conditions of polymerization differ dramatically from those in the cell wall. The cell wall environment, wherein native lignins are assembled, is heterogeneous at the nanoscale level. This significant difference suggests explorations of the changes that occur in DHP structure when the synthesis environment is modified to more closely approximate that of native lignins.

Freudenberg and coworkers suggested that monolignol glucosides function as intermediates in the biosynthesis of lignin in plant cell walls (Freudenberg *et al.* 1955). The difficulty of detecting glucosides and ß-glucosidase in certain hardwood species, however, led Sarkanen (1971) to suggest that the role of the glucosides is to act as a reservoir to augment the precursor supply in the Dignifying cells, and that free monolignols alone or derivatives other than the glucosides act as Signification intermediates in specific cases. In more recent studies (Terashima *et al.* 1993), it was shown that when different kinds of radio-label led monolignols glucosides were administered to dignifying tissue of various plants, the monolignol components were incorporated into the lignin in specific morphological regions of the cell wall. Microautoradiography indicated incorporation in a biologically regulated manner depending on the kind and age of the cells (Terashima *et al.* 1993). When monolignols glucosides enriched with carbon 13 at specific side chain carbons were administered to growing stems of various trees, the newly formed lignins were shown to be carbon 13 enriched at the side chain carbons corresponding to those of the glucosides (Terashima *et al.* 1991; Xie and Terashima 1991). The effective and biologically-regulated incorporation of monolignols as their glucosides has been observed in every plant examined including monocotyledons h-em which the isolation of glucosides has not been reported (Xie and Terashima 1993). If monolignols are generated from their glucosides by ß-glucosidase immediately prior to and at the site of polymerization in the cells in which they are synthesized, it may be quite difficult in some cases to isolate the glucosides to demonstrate their participation as signification intermediates. Based on the various studies, a proposal concerning the mech-

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anism of biogenesis of macromolecular lignin in the plant cell wall has been developed (Terashima 1990; Terashima *et al.* 1993). In the proposed mechanism, it is postulated that the monolignols are synthesized in a Golgi apparatus (Fukuda and Komamine 1985) and supplied to the Dignifying cell wall as their stable and water-soluble derivatives, the glucosides. It is suggested that the glucosides are hydrolyzed by ß-glucosidase to monolignols and glucose, and the latter is oxidized by glucose oxidase and oxygen to give hydrogen peroxide and gluconolactone, which, in turn, is subsequently hydrolyzed to gluconic acid. Monolignols polymerization is then brought about by the action of peroxidase and the hydrogen peroxide generated *in situ.*

The presence of ß-glucosidase in Dignifying wood tissue has been observed (Freudenberg *et al. 1952,* 1954, 1955). Though the presence of glucose oxidase has not been reported in wood cell walls, it is not unreasonable to postulate its occurrence. Glucose oxidase occurs in various fungi and plays an important role in the biodegradation of lignin (Kelley and Reddy 1988). Furthermore, the combination of glucose oxidase and glucose as a generating system for hydrogen peroxide is effective in the preparation of DHP from coniferyl alcohol (Tollier *et al.* 1991). Gross *et al.* (1977) proposed a hydrogen peroxide generation mechanism in horseradish involving malate dehydrogenase, malate, NADH, monophenol, Mn^{++}, and peroxidase, and showed that coniferyl alcohol was polymerized to give a lignin-like DHP. The characterization of the DHP produced was rather limited, however. Another possible mechanism for generation of hydrogen peroxide from oxygen by amine oxidase has been suggested (Federico and Angelini 1986), though its participation in lignification has not been demonstrated.

The possible participation of monolignols glucosides as an intermediate suggested an *in vitro* method for preparation of DHPs from glucosides, which could well represent a closer approximation of the natural process for the formation of macromolecular lignin in the cell wall. This is the first of the two avenues pursued in the work described in this report. The intent was to confirm that a DHP can be formed from monolignols glucosides in the presence of ß-glucosidase, peroxidase, glucose oxidase, and oxygen, and to compare the structure of this DHP with that of a DHP prepared from a free monolignols by the conventional method, as well as with the structure of a MWL. The study was then extended to include inquiry concerning the degree to which the presence of a soluble polysaccharide would modify the results. All products of the polymerization reactions were acetylated before ^{13}C NMR analysis. Nimz and Lüdemann (1976) have shown that the use of acetates provides better resolution for characterization of lignin and DHP by ^{13}C NMR.

Materials and Methods

Coniferin and coniferyl alcohol

Coniferin was synthesized according to the procedure for preparation of radio-labelled precursors for lignin biosynthesis (Terashima *et al.* 1986). Coniferyl alcohol was prepared by the method of Quideau and Ralph (1992).

Milled wood lignin

The MWL was prepared from loblolly pine wood meal according to the procedure described by Björkman (1956).

Preparation of DHP from coniferin

Method A (DHP-CFN1)

Coniferin (1500 mg). ß-glucosidase (50 mg, 5.5 u/mg from almond, Sigma Chemical Co., St. Louis, MO. U.S.A.), glucose-oxidase (6 mg, 150u/mg frum *Aspergillus niger,* Sigma Chemical Co., Type 11), and peroxidase (6 mg. 96u/mg from horseradish, Sigma Chemical Co., Type 11) were dissolved in water (150ml). Oxygen was bubbled into the solution with stirring at 35°C. After a few hours, the initial pH of about 5-5.3 had declined to about 3.1-3.5. After 9h, the pH was adjusted to 5.0 with dilute sodium hydroxide, and amounts of ß-glucosidase, glucose oxidase, and peroxidase identical to those used at the beginning of the reaction were added. After 94 h, the precipitate was collected by filtration to yield 553 mg of crude DHP, representing 67.5% recovery of the coniferyl alcohol moiety. A mixture of dichloroethane and ethanol (2 : 1, v/v, 5ml) was added to the dried, crude DHP, and the soluble fraction was added drop by drop to dry ether (100ml) with stirring to precipitate the majority of the DHP (430 mg, 54.5% of the coniferyl alcohol moiety in the glucoside).

Method B (DHP-CFN2)

Coniferin (500mg) was dissolved in acetic acid-sodium acetate buffer (0.2 M solution. pH 5.0, 50ml). Konyak mannan (500mg, obtained in Nagoya as a powder from a manufacturer of konyak, a food made from powdered tubers of konyak plants, *Amorphophallus konjac* C. Koch) was dissolved in hot water (50ml) to give a viscous gel. After cooling, ß-glucosidase (50mg), glucose oxidase (5mg), and peroxidase (1mg) dissolved in water (3ml) were added to the gel and mixed thoroughly. The solution of coniferin was added slowly over a period of 24h to the mannan gel containing the mixture of enzymes at 30°C. After remaining at room temperature for 10 days, most of the water in the reaction mixture was removed by blowing a stream of air onto it. The residual mass was extracted twice with dioxane-water (96:4, v/v, 100ml), The dioxane solution was concentrated under reduced pressure to dryness. The residue was dissolved in dichloroethane-ethanol (2:1, v/v, 5ml). and poured into dry ether (100ml) with stirring to precipitate DHP (188mg, 71.4% based on coniferyl alcohol moiety).

Preparation of DHP from coniferyl alcohol

Method C (conventional preparative method, DHP-CAL0)

Coniferyl alcohol (200mg) was dissolved in acetone (1ml), to which was added quickly warm phosphate buffer (pH 6.5, 200ml). After cooling, peroxidase (Sigma Co. type II, 4mg) dissolved in phosphate buffer was added to the solution of coniferyl alcohol. Using a micro pump, the coniferyl alcohol solution in buffer and dilute hydrogen peroxide (200 ml, 0.025%) were added slowly (about 3 ml/h) and simultaneously to a flask containing a solution of peroxidase (1mg in 2ml of the same buffer) with stirring. The mixture was stirred for 24 h after completion of the addition. The precipitate was collected by centrifugation and washed with water.

Purification by the same procedure as in method A gave 116mg (58%) of cream-colored DHP.

Method D (DHP-CAL1)

Coniferyl alcohol (900 mg) was dissolved in 2ml of acetone and a warm-water solution of pectin (citrus fruits, Sigma Chemical Co., 200mg in 450ml) was added. After cooling, the pH was adjusted to 5.0 by addition of sodium bicarbonate. Into a l000ml round bottomed flask mounted to a rotary evaporator rotating at 20rpm under reduced pressure at 35°C, a solution of coniferyl alcohol containing pectin, is dilute aqueous solution of hydrogen peroxide (0.0076%, 450ml), and an aqueous solution of peroxidase (2mg in 450ml) were added slowly using a micro pump. The process was continued for 12 h, removing water continuously from the flask to keep the reaction mixture in a slurry state. The thick slurry formed was evaporated to dryness and extracted with dioxane-water (96:4, v/v). Purification by the procedure used in method A gave 650mg (72%) of cream-colored DHP.

Method E (DHP-CAL2)

Except for the use of a larger amount of pectin (2000mg) and lower pH (3.5), a procedure almost identical to method D was used. The yield of purified DHP (235mg, 26%) was low. The major part of the polymer was insoluble in dioxane-water. This fraction was thought to be a DHP-pectin complex.

Acetylation of DHPs

DHP (100mg) was dissolved in dry pyridine (1ml), and acetic anhydride (0.75ml) was added. The mixture was kept at room temperature for 1 day. It was then poured into water (30ml) and the precipitate was collected by filtration, washed with water, and dried under vacuum,

^{13}C NMR

Samples (about 50-150mg) were dissolved in acetone-d_6 (0.3ml in 5mm tube), and ^{13}C NMR spectra were obtained with a Bruker WM-250 spectrometer (Bruker Instruments, Inc., Billerica, MA, U. S. A.) equipped with a 5 mm broad-band probe tuned to 63 MHz. Generally, overnight acquisitions of about 40,000 transients (16K data pts) were needed for acceptable signal/noise ratios in the spectra. A pulse width of 60° and a relaxation delay of 1s were used. An exponential multiplication of 4Hz was applied prior to Fourier transformation. For purposes of comparison, the spectra were normalized on the basis of the resonance at 112 ppm, which is assigned to the aromatic carbon at position 2 of the guaiacyl ring; almost no bonding occurs at this position during the formation of guaiacyl lignin (Tomimura and Terashima 1979),

Results and Discussion

The major dimeric substructures in lignin are shown in Figure 1 as their acetates. Differences in the content of these substructures are considered to be useful indicators of structural differences between DHP and lignin. Though the ^{13}C NMR spectra were not recorded under quantitative analytical conditions, they were determined under the same analytical condi-

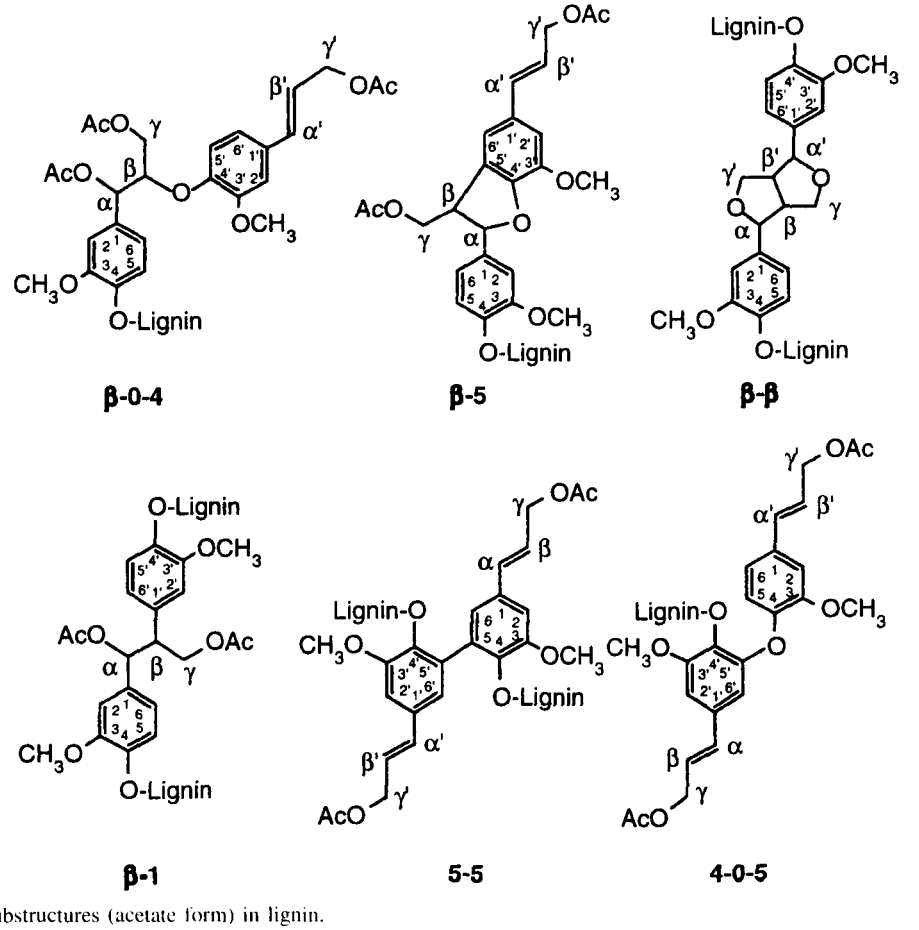

Fig. 1. Dimeric substructures (acetate form) in lignin.

Table 1. Relative intensities of signals of side chain carbons and acetyl carbonyls based on the intensity of guaiacyl ring C-2 at 112ppm

Signal assignment	Signal No. (ppm)	CFN1	CFN2	CAL0	CAL1	CAL2	MWL
Guaiacyl ring C2	6 (112)	100	100	100	100	100	100
Cα (β-O-4, threo)	11 (75.4)	34	34	20	50	56	60
Cα (β-O-4, erythro)	12 (74.6)	50	62	27	70	51	62
Cα (α-O-4 · β-O-4)	9 (80.6)	45	43	34	60	73	69
Cβ (β-O-4)	10 (80.2)	43	48	25	70	56	69
Cγ (β-O-4, α-O-4, β-5)	15 (65.5)	211	202	232	220	123	100
Cγ (β-O-4)	18 (63.1)	64	67	36	65	49	91
Cγ (α-O-4 · β-O-4)	16 (64.2)	29	38	27	45	32	83
Cγ (α-O-4 · β-O-4)	17 (63.6)	61	64	50	93	90	110
Cα (β-5)	7 (88.5)	50	44	48	35	56	27
Cβ (β-5)	20 (51.1)	66	62	61	45	73	43
Cγ (β-5)	14 (65.9)	88	90	95	68	98	50
Cα (β-β)	8 (86.1)	50	84	52	95	76	19
Cβ (β-β)	19 (55.3)	77	115	73	119	93	29
Cγ (β-β)	13 (72.4)	64	100	48	98	83	25
Acetyl CO (primary)	3 (170.9)	146	121	121	145	93	203
Acetyl CO (secondary)	4 (170.0)	88	77	39	65	49	118
Acetyl CO (phenolic)	5 (168.9)	46	41	38	90	63	97

tions. Therefore, it is possible to compare the relative content of specific structural moieties from the signal intensities in the spectra normalized on the basis of the intensity of an aromatic ring signal.

^{13}C *NMR Spectra of DHPs and lignin*

The spectra of the acetate of DHPs and MWL are shown in Figure 2. Assignments of the predominant signals were based on the chemical shift data of model compounds and DHPs (Nimz and Lüdemann 1976; Landucci and Ralph 1993). In Table 1, intensities of the major signals of side chain carbons and acetate carbonyl carbons are listed for making comparison easy, though they are not quantitative values. The intensity of the signals assigned to the C_α carbon of a ß-O-4 structure (75.4 and 74.6ppm), the C_α of an et-O-4·ß-O-4 structure (80.6 ppm), the C_β of a ß-O-4 structure (80.2 ppm), the Cγ of a ß-O-4 structure (63.1 ppm) and the Cγ of an α-O-4 structure (64.2 and 63.6ppm) are higher in the spectra of CFN1 and CFN2 (Fig. 2A and 2B), which are prepared from coniferin, than the intensities of the corresponding signals in the reference spectrum of CAL0 (Fig. 2C) which was prepared from coniferyl alcohol by the conventional method. This suggests a higher level of ß-O-4 in the novel polylignols relative to the reference material. The relative contents of primary, secondary, and phenolic hydroxyl groups also reflect the relative abundance of ß-O-4 linkages. These can be estimated by comparing the signal intensities of their acetate carbonyls at 170.9, 170.0, and 168.9 ppm respectively (Signal No. 3, 4 and 5 in Fig. 2 and Table 1). The higher intensity of the signals at 170.0ppm in the spectra of the acetates of CFN1 and CFN2 (Fig. 2A and 2B) in comparison to the spectrum of CAL0 (Fig. 2C) indicates that the former contain larger amounts of secondary hydroxyl groups than the latter. Because the ß-ß and ß-5 structures do not contain secondary hydroxyl groups, a higher content of secondary hydroxyl groups can indicate a greater abundance of ß-O-4 structure relative to the contents of ß-5 and ß-ß structures in CFN1 and CFN2. The high ß-O-4 and low ß-5 and ß-ß content in MWL is reflected well in the relative signal intensities (Fig. 2F and Table 1). Clearly the structures of the novel DHPs prepared from coniferin more closely approximate native lignin than does the structure of the conventional DHP. These findings are consistent with the results of linkage-type analysis by thioacidolysis, which will be reported in part 2 of this series of papers (Terashima *et al.* 1996).

The spectrum of the acetate of CAL0 (Fig. 2C) is compared with the spectra of DHPs prepared from coniferyl alcohol in the presence of pectin under dehydrating conditions (spectrum D, CAL1 and spectrum E, CAL2) and the spectrum of a MWL (Fig. 2F). The intensities of the signals assigned to Cα, Cß and Cγ of the ß-O-4 structure (75.4, 74.6, 80.2, 63.1ppm) and the CZ-O-4·ß-O-4 structure (80.6, 64.2 and 63.6ppm) were higher in spectra D (CAL1) and E (CAL2) than those in spectrum C (CAL0). But the intensities of signal No. 4 (secondary acetyl carbonyls) were low in all DHPs prepared from coniferyl alcohol. The intensities of signals assigned to the ß-5 structure in CAL1 is lower than that in CAL0, though the intensities of the signals assigned to the ß-ß structure seem to be high in CAL1 and CAL2. The structures of these new DHPs resemble that of MWL more than the structure of conventional DHP does. However, in most of the

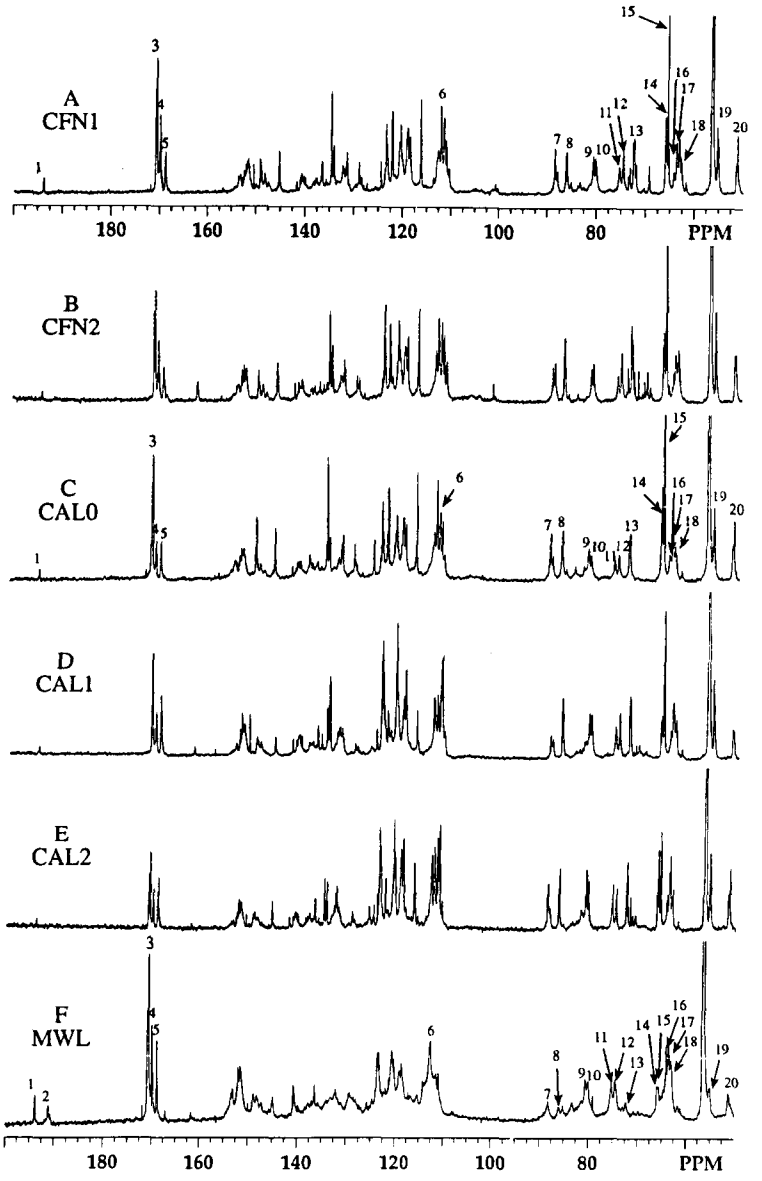

Fig. 2. ^{13}C-NMR spectra of acetates of DHPs and MWL. A: DHP-CFN1 prepared from coniferin by method A. B: DHP-CFN2 prepared from coniferin by method B. C: DHP-CAL0 prepared from coniferyl alcohol by conventional method C. D: DHP-CAL1 prepared from coniferyl alcohol by method D. E: DHP-CAL2 prepared from coniferyl alcohol by method E. F: MWL of loblolly pine.

respects noted above, the CFN1 represented closer approximations to the MWL spectra than did the spectra of CAL0, CAL1 and CAL2.

Differences in structure between DHPs and MWL

The structures of the novel DHPs prepared in this work by alternative methods more closely approximate the structures of MWL than does the structure of DHP prepared from coniferyl alcohol using the conventional method. Considerable differences remain, however, between the structures of DHPs and MWL as seen in the NMR spectra. In addition to the difference in linkage types between the monolignols, it is likely that differences exist between the natural processes and the *in vitro* processes with respect to modification of the side chain to give α- and γ-carbonyl groups as revealed in Figure 2. The coniferaldehyde carbonyl at 193.8ppm (signal No.1) is more pronounced in the MWL than in any of the polylignols. The α-carbonyl at 191.2ppm (signal No. 2) is found only in the spectra of MWL (Fig. 2F). Oxidation of the side chain during isolation of MWL (Pew 1957; Chang *et al.* 1975) may contribute to the observed difference. However, the solid state NMR spectra of wood tissue from ginkgo trees, in which cell wall lignin was specifically enriched with carbon

13 at the α-position of the side chain, showed an enhanced α-carbonyl resonance (Xie and Terashima 1991). This indicates that an oxidation mechanism other than the hydrogen peroxide-peroxidase system may participate in formation of protolignin in the cell walls. It is not clear whether other differences in the spectra, such as the resonances at about 85.7 ppm and 114.7 ppm, which occur only in the spectrum of MWL (Fig. 2F), are the result of differences in linkage types, in monomer composition (for example the presence of p-hydroxy phenylpropane or syringylpropane units in MWL), or in side-chain structures.

In the current series of experiments, the issue of spatial organization was not addressed. However, some differences in NMR spectra were observed between CFN1 and CFN2 prepared by method A and B, which differ in that procedure B included konyak mannan. Furthermore, the major part of the polylignol prepared in the presence of a large amount of pectin was complexed to pectin, and it was found that the fraction containing pectin had an NMR spectrum showing closer approximation to MWL than that of the dioxane soluble fraction (Terashima *et al.*, unpublished results). These results indicate that even for *in vitro* preparations of DHPs, the effect of the carbohydrate matrix must be considered. This problem will be dealt with in a future publication.

Conclusion

1. A guaiacyl type lignin polymer model can be prepared from coniferin by the action of ß-glucosidase and peroxidase, with hydrogen peroxide generated *in situ* through the action of oxygen and glucose oxidase on the glucose liberated from the coniferin.
2. Structural characterization by ^{13}C NMR indicates that the structure of this DHP represents closer approximations to the structure of MWL than does the structure of DHP prepared by the conventional method.
3. DHPs prepared from coniferyl alcohol in the presence of pectin under dehydrating conditions result in polylignols, the structure of which also approximated that of MWL more closely than did the structure of conventional DHP. However, the DHP prepared from coniferin provided a closer approximation than the DHP from coniferyl alcohol.

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