Incorporation of Peroxidase into Synthetic Lignin

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A synthetic lignin was produced by using coniferyl alcohol, horseradish peroxidase, and H_2O_2. The lignin contained 9.4% protein which was contributed by the peroxidase, probably by cross-linking of the tyrosine residues from the protein to the monomeric or polymeric intermediates during the reaction. Solid-state NMR results indicated that the peroxidase had been incorporated into the lignin and that it was an integral part of the whole polymer and not merely a contaminant.

INTRODUCTION

The formation of lignin in plant tissues allows for structural integrity, transportation by vessel elements of water and nutrients, and transpiration in the intact plant. Lignin is commonly associated with and/or incorporated into the hemicellulose of the cell wall matrix of selected tissues and organs of the plant. Past (Freudenberg et al., 1963; Gagnaire and Robert, 1977; Tanahashi and Higuchi, 1981) and recent (Fry, 1986) research have shown that peroxidase is involved in the polymerization of lignin from basic monomeric phenylpropane units. Coniferyl alcohol is thought to be one of the common precursors used for the synthesis of lignin in vivo. This alcohol has frequently been used to synthesize lignin in vitro, by use of commercial peroxidase preparations, and the methods used have recently been summarized (Wood and Kellogg, 1988).

Although the methods vary, the polymer produced is usually verified by NMR. Little mention is made of the protein content in these synthetic polymers or in the naturally occurring lignins, and when mentioned, the protein is usually considered a contaminant. The present research describes the synthesis of a lignin polymer using coniferyl alcohol as the hydrogen donor, commercial horseradish peroxidase, and H_2O_2. It further shows that the peroxidase is incorporated into the lignin polymer and is an integral part of the polymer.

MATERIALS AND METHODS

Reagents. Coniferyl alcohol was obtained from Aldrich Chemical Co. (Milwaukee, WI), horseradish peroxidase from Calbiochem-Behring Corp. (La Jolla, CA), and H_2O_2 from Fisher Scientific (Springfield, NJ). The peroxidase contained 65.6 purpurogallin units/mg. Dialysis tubing ($^5/_8$ in.) was obtained from A. H. Thomas Co. (Philadelphia, PA) and had a molecular weight exclusion of 20 000.

Lignin Synthesis. Twenty milliliters of a 2 mg/mL solution of peroxidase was prepared in 0.05 M phosphate buffer (pH 6.7), placed in the water-washed dialysis tubing, and tied off. Coniferyl alcohol (200 mg) was dissolved in 200 mL of the above buffer and the dialysis bag allowed to float in this solution in a 250-mL Erlenmeyer flask. Ten micromoles of H_2O_2 was added to the flask, and the contents were mechanically stirred continuously during the entire reaction. Additional 10-μmol aliquots of H_2O_2 were added to the flask every 12 h, and the reaction was allowed to proceed for 48 h. During this period, as the polymer began to form and precipitate, the dialysis bag was rotated to mix the contents of the bag.

After 48 h, the contents in the dialysis bag were transferred to a centrifuge tube and centrifuged at 37000g for 20 min. The polymer was washed with H_2O and centrifuged twice, as above, to remove the residual peroxidase, buffer, and H_2O_2. The precipitate was resuspended in H_2O and freeze-dried until used.

Analytical Procedures. Nitrogen analyses were determined by the micro-Kjeldahl procedure (McKenzie and Wallace, 1954), and amino acid analyses (Wilkinson et al., 1968) were performed on a Beckman Model 121 amino acid analyzer.

Solid-state NMR spectra were obtained on coniferyl alcohol, horseradish peroxidase, and the synthesized lignin by using a Bruker MSL 300 NMR spectrometer operating at 75.47 MHz for carbon-13. The spectra were obtained by using the CP/MAS (Schaefer and Stejskal, 1979) technique. Approximately 50 mg of each material was spun in a 4 mm diameter zirconium oxide rotor at 6 kHz utilizing a 2.6-μs 90° pulse and a 1.5-ms contact time. Data were accumulated for 4K, 8K, and 20K scans, respectively. Spectra were referenced externally to hexamethylbenzene at 132.3 ppm relative to TMS.

Table I. Amino Acid (AA) Profiles of Peroxidase and Synthetic Lignin

amino acid	peroxidase native[a]	peroxidase adjusted[b]	synthetic lignin[c]	AA in lignin/AA in peroxidase, %
Lys	27.7 ± 1.9	56.8	4.8 ± 0.3	8.5
His	12.4 ± 0.8	25.4	1.5 ± 0.1	5.9
Arg	36.8 ± 2.1	75.5	7.0 ± 0.4	9.3
Asp	63.7 ± 5.4	130.6	14.0 ± 1.1	10.7
Thr	36.9 ± 3.1	75.7	7.4 ± 0.5	9.8
Ser	32.4 ± 1.8	66.4	7.0 ± 0.6	10.5
Glu	38.3 ± 2.4	78.5	8.4 ± 0.6	10.7
Pro	24.3 ± 1.9	49.8	5.1 ± 0.4	10.2
Gly	21.0 ± 1.8	43.1	4.8 ± 0.4	11.1
Ala	25.1 ± 2.3	51.5	5.3 ± 0.4	10.3
Cys	8.4 ± 0.5	17.2	1.5 ± 0.1	8.7
Val	32.9 ± 1.9	67.5	6.6 ± 0.6	9.8
Met	7.3 ± 0.4	15.0	1.6 ± 0.1	10.7
Ile	21.2 ± 1.2	43.5	4.5 ± 0.3	10.3
Leu	43.0 ± 3.0	88.2	9.3 ± 0.6	10.5
Tyr	24.8 ± 1.4	50.9	1.1 ± 0.1	2.2
Phe	31.4 ± 1.8	64.4	6.6 ± 0.6	10.2
totals	487.6 ± 33.7	1000.0	96.5 ± 6.8	$\bar{x}$ = 9.4

[a] Milligrams of amino acid per gram of dry matter of commercial horseradish peroxidase ± SD from two determinations. [b] Milligrams of amino acids adjusted to 1 g of protein. [c] Milligrams of amino acids per gram of dry matter of synthetic lignin ± SD from two determinations.

RESULTS AND DISCUSSION

During the course of the 48-h reaction, the white polymer precipitated along the sides of the dialysis tubing, hence the need to occasionally rotate the bag. The total yield of the polymer was 95 mg. This method of lignin synthesis was chosen since it produced the highest molecular weight polymer compared to other methods (Tanahashi and Higuchi, 1981), although total yield of the polymer was sacrificed (Kirk and Brunow, 1988). Any soluble oligomers below

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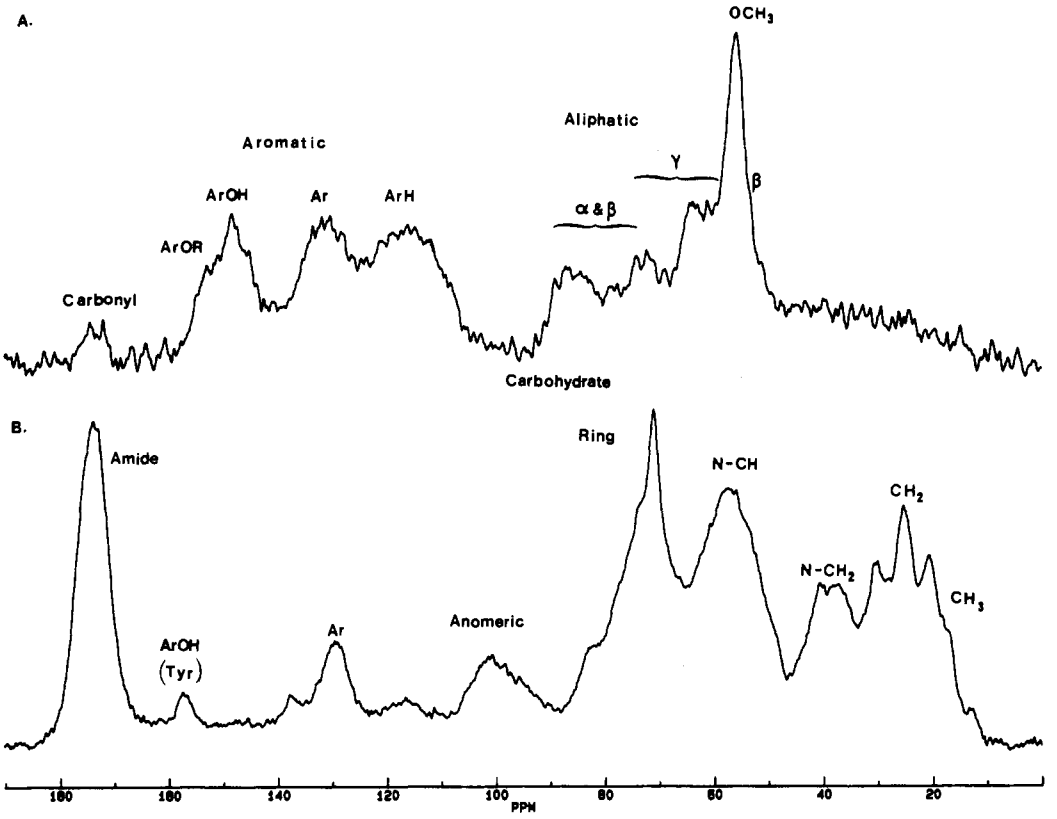

Figure 1. ^{13}C CP/MAS spectra of (A) synthetic lignin from coniferyl alcohol, showing aromatic and aliphatic regions (Ar, aromatic; α, β, γ, aliphatic side chain carbon in increasing distance from the aromatic ring), and (B) peroxidase, showing amino acid and carbohydrate signals of the glycoprotein.

20 000 molecular weight passed through the dialysis tubing, leaving the insoluble peroxidase–oligolignols within the dialysis tubing.

The results of the amino acid analyses are in Table I. The native or commercial peroxidase contained 48.8% amino acids, presumably peroxidase protein, on a dry matter (DM) basis. The N content of the commercial preparation was 9.2%, and the N recovered as amino acids was 6.7%, indicating that 27.2% of the N was nonprotein N. The synthetic lignin contained 1.3% N and 9.7% amino acids on a DM basis. To validly compare the individual amino acids in the peroxidase with those in the synthetic lignin, it was necessary to adjust the native peroxidase amino acids to 100% or 1000 mg of amino acids/g of protein or DM (Table I). The ratio of the amino acids in the lignin to the adjusted peroxidase value for a given amino acid gave a correct value for each amino acid incorporated into the lignin as part of the peroxidase molecule. The peroxidase protein in the lignin has about the same ratio of amino acids as the native peroxidase with a few notable exceptions (Table I). The tyrosine ratio is greatly reduced, which indicated that the cross-linking between the protein and the lignin occurred through the oxidative coupling of the protein tyrosine residues during lignin formation. This oxidative coupling of tyrosine residues by peroxidase to other proteins has been shown in other plant systems (Fry, 1986) to form dityrosine or isodityrosine bridges. It is highly probable that the free radicals produced by peroxidase during this reaction would also cause these tyrosine residues to react or cross-link to the coniferyl moieties of the lignin polymer and indeed cause the actual polymerization of the alcohol.

The solid-state NMR spectra of the synthetic lignin (Figure 1A) gave clear indications that it was a polymeric lignin. In the aliphatic region of the spectrum there were carbon signals for γCH_2s in the 60–67 ppm range, αCHs at 76, 81, and 86–88 ppm, and β on the shoulder of the aromatic methoxyl ($ArOCH_3$) at 55 and also 84 ppm. These aliphatic signals plus the broad envelopes of signals for protonated aromatics, nonprotonated nonoxygenated aromatics, and oxygenated aromatics centered at 116, 130, and 148 ppm, respectively, satisfy all the requirements for the compound to be classified as polymeric lignin by NMR (Nimz, 1978). In addition to these signals for lignin, there were also signals around 172 ppm. These signals would seem to be indicative of the presence of residual protein as the peroxidase (Figure 1B) also contained signals at the same position. These signals have been previously alluded to (Lüdemann and Nimz, 1973) as being due to aliphatic ester carbonyl carbons of lignin or due to polyuronic ester groups. Since the precursor alcohol was not oxidized to this extent and the system lacks carbohydrate, neither of these explanations would be valid in this case. A more likely explanation is that the peroxidase has been incorporated into the lignin and that it is an integral part of the whole polymer. The peroxidase incorporated into the lignin polymer was still enzymatically very active; however, due to the insoluble nature of the polymer, exact activity and kinetic studies have not yet been reported and are currently being pursued.

This incorporation of peroxidase into the lignin polymer, which is normally deposited within the hemicellulose of the cell wall matrix, might explain the presence of the residual cell wall peroxidase so often found in plants (Evans, 1990).

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Received for review September 6, 1990. Accepted December 18, 1990.

Registry No. Lignin, 9005-53-2; peroxidase, 9003-99-0; coniferyl alcohol 458-35-5.