ORIGINAL ARTICLE

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Origin of free radicals produced from the syringyl end groups in lignins

Abstract Enzyme lignin was prepared from reed; it was acid-hydrolyzed and reduced with $NaBH_4$. Spin concentrations of radicals produced by oxidation of the lignins with $K_3[Fe(CN)_6]$ or H_2O_2 were determined from electron spin resonance spectra. The radicals were observed by the oxidation of sinapyl alcohol. It was found that the two radicals observed by the oxidation of dioxane lignins were deprotonated 2,6-dimethoxy-p-benzosemiquinone and 6-hydroxy-2-methoxy-p-benzosemiquinone, and that they were produced from syringyl end groups. The production rate of radicals from syringyl end groups possessing an α-carbonyl group was greater than that possessing an α-hydroxyl group. It was suggested that stilbene-type syringyl end groups were produced from phenylcoumaran moieties in lignins by acidolysis. The radicals produced from guaiacyl end groups were not observed by the oxidation process.

Key words ESR · Lignin · Acidolysis · Reduction · Syringyl end group

Introduction

It has been found that two kinds of electron spin resonance (ESR) signals were observed by the oxidation of lignins in alkaline solutions: One was the signal S due to 2,6-dimethoxy-p-benzosemiquinone radical (radical S) and the other was assigned to be the signal G due to 5-hydroxy-2-methoxy-p-benzosemiquinone radical (radical G).[1,2] The results suggested that the radicals were produced by the oxidative cleavage of syringyl or guaiacyl end groups from lignin molecules. Because the linkage types of the end groups in lignins are complicated, elucidation of the production mechanisms of the radicals is difficult. Several mechanisms were proposed in previous papers.[1,2] It was suggested

that the chemical surroundings around $C\alpha$ and $C\beta$ carbons of a syringyl or a guaiacyl end group in lignins were important for the radical production.[2]

In this study the effects of acidolysis and reduction of enzyme lignin from reed on the production of the radicals were examined using an ESR measurement. From the results, it was found that the radical G was a deprotonated 6-hydroxy-2-methoxy-p-benzosemiquinone and was produced from syringyl end groups in lignins.

Materials and methods

Reagents and lignin preparation

Coniferyl alcohol, sinapyl alcohol, syringaldehyde, and methoxyhydroquinone were obtained from the Aldrich Chemical Company, and sinapyl alcohol was used after purification by thin-layer chromatography on cellulose plates (Merck) with water saturated with benzene as the mobile phase.[3] 2,6-Dimethoxy-p-hydroquinone was prepared by the method of Bailey and Dence.[4] 2,6-Dimethoxy-p-benzoquinone and methoxybenzoquinone were prepared by oxidizing 2,6-dimethoxy-p-hydroquinone and methoxyhydroquinone, respectively, with silver oxide. Enzyme lignin (LE) were obtained from the stem of a reed (*Phragmites communis* Trinius) as reported by Sorvari et al.[5]

Acidolysis of LE

Enzyme lignin (2g) was dissolved in $500\,cm^3$ dioxane-2mol/dm^3 HCl (9:1) and refluxed for 3h. After hydrolysis, large amounts of ether petronium ether (1:1) were added to the solution to extract dioxane; large amounts of water (pH 2) were then added to the aqueous residue, and the solution was filtered. The resultant precipitate was dissolved in a small amount of 90% aqueous acetonitrile, and the filtrate was poured into water (pH 2). The solution was filtered with a membrane filter (0.2 µm), and the precipitate (LAc) was dried in vacuo over P_2O_5 for 72h at room temperature.

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Reduction of LE and LAc

Enzyme lignin or acid-hydrolyzed LE (LAc) (300 mg) was dissolved in ethanol water (1:1; 200 cm^3), and the solution was made alkaline with NaOH (pH 11) in argon. NaBH$_4$ (150 mg) was added to the solution, which was left for 24 h. Excess borohydride was then destroyed with dilute HCl, and the solution was filtered. The resultant precipitate was dissolved in a small amount of 90% aqueous acetonitrile, and the filtrate was poured into water (pH 2). The solution was filtered with a membrane filter and the precipitate [NaBH$_4$-reduced LE (LER) or NaBH$_4$-reduced LAc (LAcR)] was dried in vacuo over P$_2$O$_5$ for 72 h at room temperature.

ESR measurements

Lignin (20 mg) was dissolved in 2 cm^3 of a buffer solution (pH 12.5 or 13.0, KCl-NaOH) or in 2 cm^3 of a NaOH solution 0.5 mol/dm^3 in air in the presence or absence of oxidants; the solution was then introduced into a quartz flat cell. The conditions of the ESR measurements were the same as those reported previously.[2] ESR spectra were obtained every 5 min up to 120 min.

Results and discussion

Four lignin preparations were compared: enzyme lignin (LE); acid-hydrolyzed LE (LAc); NaBH$_4$-reduced LE (LER); and NaBH$_4$-reduced LAc (LAcR).

Oxidation with K$_3$[Fe(CN)$_6$]

It has been shown that two ESR signals (signals S and G) are observed by the oxidation of lignins with K$_3$[Fe(CN)$_6$].[1,2] Figure 1 shows the changes with reaction time of the spin concentrations of the radicals S and G obtained by the oxidation of LE and LER with K$_3$[Fe(CN)$_6$]. The signal S was observed first and was replaced by the signal G over time. It was found that the rate of radical production increased with increased pH. The rate of radical production in LER was smaller than that in LE. Therefore, the spin concentrations in LER were shown also in the reaction in NaOH solution (0.5 mol/dm^3). The production and destruction of the radical S were fast in the reaction in NaOH solution (0.5 mol/dm^3); therefore, the change of the radical S in LER was not shown. It has been shown that NaBH$_4$ reduces the α-carbonyl group to an α-alcohol group.[6,7] The results suggest that the rates of radical production from end groups containing α-alcohol group in lignins are smaller than the rates of production from those containing α-carbonyl group by oxidation with K$_3$[Fe(CN)$_6$]. The spin concentration of the radicals was not influenced much by the reduction.

Figure 2 shows the change with reaction time of the spin concentrations of the radicals S and G produced by the
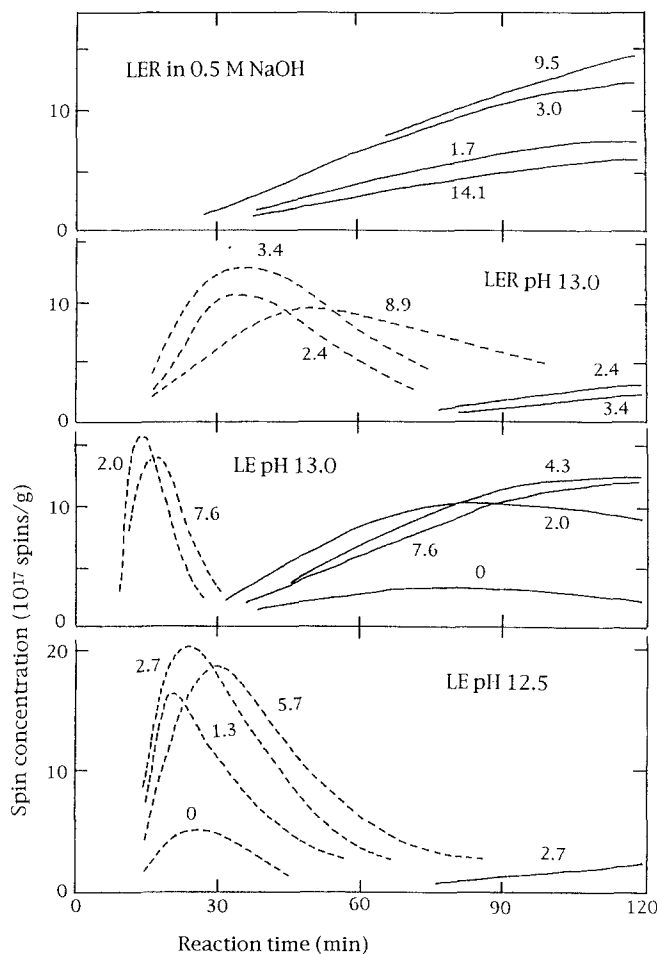

Fig. 1. Change, with time, of the spin concentrations of the radical S (*dotted line*) and the radical G (*solid line*) produced by the oxidation of enzyme lignin (LE) or an NaBH$_4$-reduced LE (LER) with K$_3$[Fe(CN)$_6$] at 20°C. Numbers indicate the concentration (mmol/dm^3) of K$_3$[Fe(CN)$_6$]. Value of 10^{17} spins corresponds to 28 µg radical S and 25 µg radical G, respectively

oxidation of LAc and LAcR with K$_3$[Fe(CN)$_6$]. The rate of radical production in LAcR also was smaller than that in LAc. It is known that acidolysis cleaves the α-ether and β-ether linkages and ester bondings.[8] Therefore, it is expected that the molecular weight of residual lignins after acidolysis decreases and the number of the end groups per unit weight of the lignins increases, resulting in increased radical formation. However, the spin concentration in LAc did not increase compared with that of LE, but the rate of radical formation in LAc increased. The rate of radical S production in LAcR at pH 13.0 with K$_3$[Fe(CN)$_6$] (13.1 mmol/dm^3) was not monotonous, suggesting that in LAcR there are two or more types of syringyl end groups that produce the radical S. The production and destruction of the radical S in LAc at pH 13.0 or in LAcR in NaOH solution (0.5 mol/dm^3) also were fast; therefore, its change is not shown in Fig. 2.

Oxidation with H$_2$O$_2$

Figure 3 shows the change, with reaction time, of the spin concentration of the radical S produced by oxidation of the
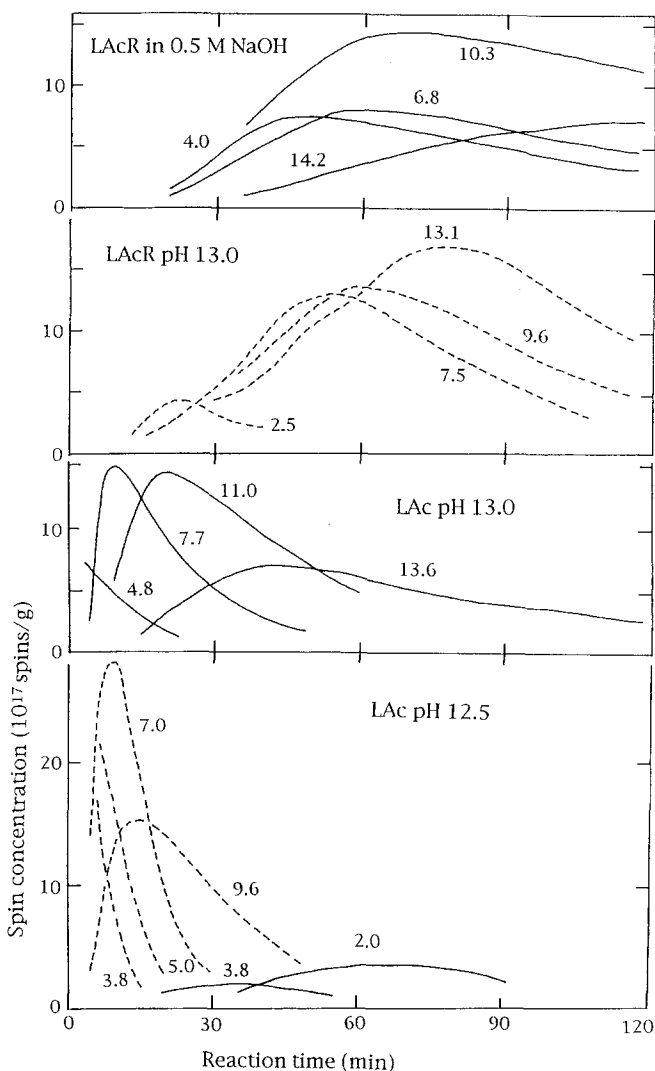
Fig. 2. Change, with time, of the spin concentrations of the radical G (*solid line*) and the radical S (*dotted line*) produced by the oxidation of acid-hydrolyzed LE (LAc) or an $NaBH_4$-reduced LAc (LAcR) with $K_3[Fe(CN)_6]$ at 20°C. Numbers indicate the concentration (mmol/dm³) of $K_3[Fe(CN)_6]$

Fig. 3. Change, with time, of the spin concentrations of the radical S produced by oxidation of the lignins with H_2O_2 at pH 13.0 and 20°C. Numbers represent the concentration (mmol/dm³) of H_2O_2

lignins with H_2O_2 at pH 13.0. The radical G was not observed after the oxidation with H_2O_2. Production of the radical S in LER was low and slow compared with that of LE. It has been suggested[1,2] that the radical S was produced from 2,6-dimethoxy-p-hydroquinone formed by the Dakin reaction.[4,9] The data support the fact that the rate of the Dakin reaction of α-hydroxyl groups is lower than that of α-carbonyl groups.[10]

A large amount of radical S was produced at the beginning of LAc oxidation. The spin concentration decreased once and then increased again. The data suggest that there are two or more mechanisms for radical S production by the oxidation of LAc. Hence a different type of syringyl end group, which does not present in LE, may be produced by the acidolysis. Although analyses of low-molecular-weight compounds obtained by acidolysis were studied,[8] there was little chemical specification about the end groups in residual
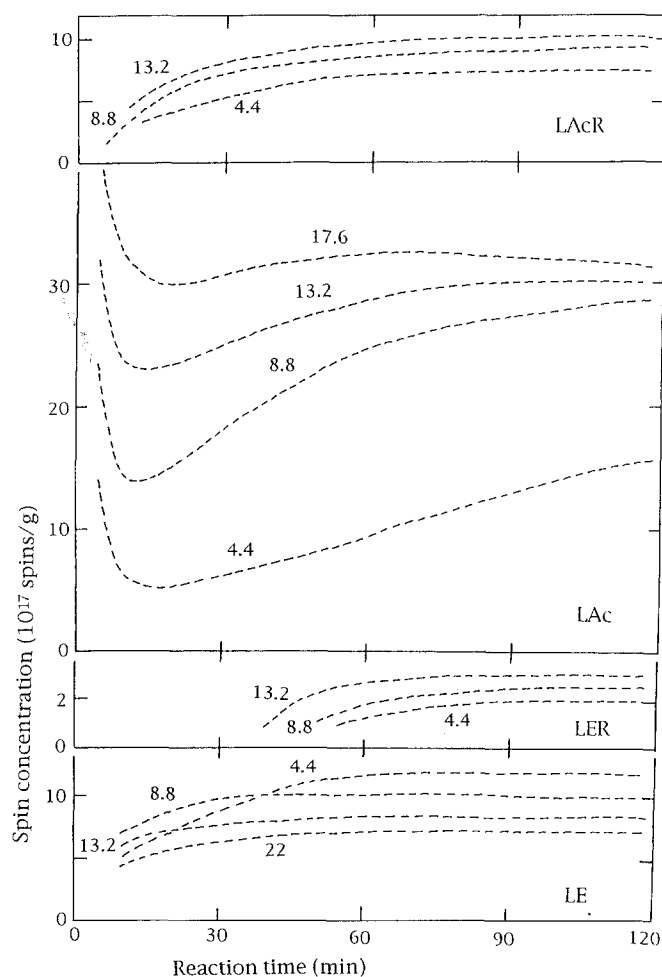
lignins obtained by acidolysis.[11] However, from the dimeric acidolysis products, the syringyl end groups producing the radical S that are formed by the acidolysis can be assumed. Among the acidolysis products, stilbene-type syringyl end groups originated from phenylcoumaran type moieties[8] are the most probable end groups to produce the radical S. The amount of spin concentration of the radical S produced in LAcR was larger than that in LER, which suggests that a different type of syringyl end group, not reduced by $NaBH_4$, presents in LAc.

Oxidation of sinapyl alcohol

For confirming the asssumption mentioned above, coniferyl alcohol and sinapyl alcohol were oxidized with $K_3[Fe(CN)_6]$ or H_2O_2. No ESR signal was observed when coniferyl alcohol was oxidized, whereas the signal S was observed after oxidation of sinapyl alcohol with H_2O_2. When sinapyl alcohol was oxidized with $K_3[Fe(CN)_6]$, three ESR signals were observed (Fig. 4). The signal observed at an early stage was due to the demethoxylated radical, which was the
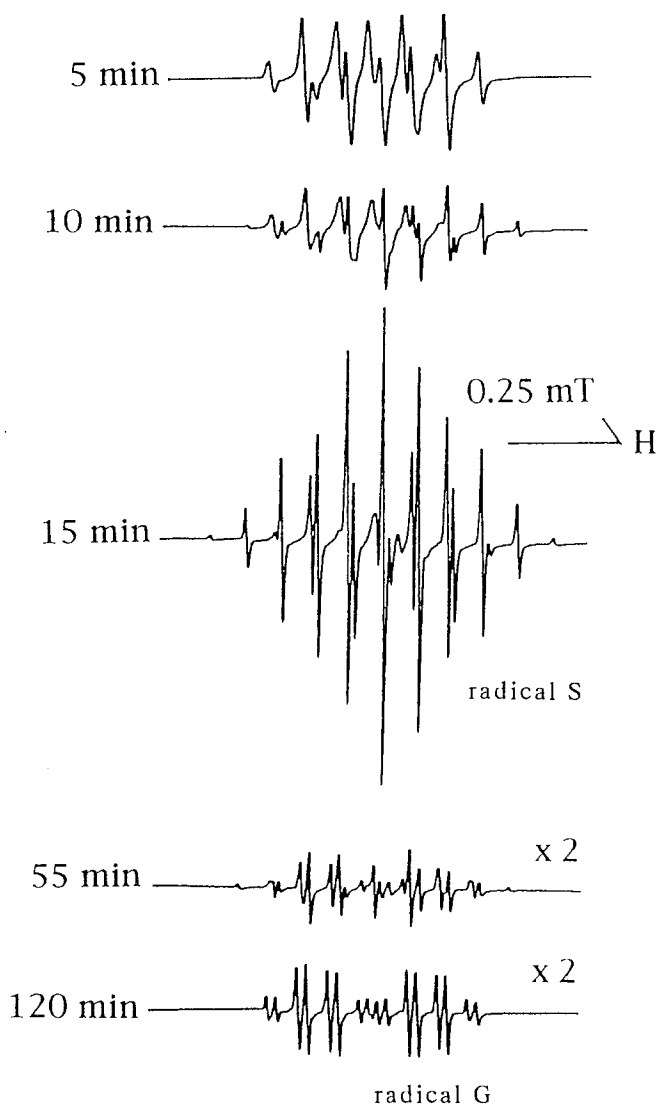
Fig. 4. Electron spin resonance (ESR) spectra observed by the oxidation of sinapyl alcohol (1 mg) with $K_3[Fe(CN)_6]$ (5.7 mmol/dm³) in 1 cm³ solution at pH 12.5 and 20°C

Fig. 5. Change, with time, of the spin concentration of radical S, produced by the oxidation of sinapyl alcohol with H_2O_2 at pH 13.0 (bottom), and radical G, produced by the oxidation of sinapyl alcohol with $K_3[Fe(CN)_6]$ at pH 12.5 (top) and 20°C. Numbers represent the concentration (mmol/dm³) of H_2O_2 (bottom) or $K_3[Fe(CN)_6]$ (top)

same produced by the oxidation of syringaldehyde or syringic acid with $K_3[Fe(CN)_6]$.[1] Signal G was observed after oxidation of sinapyl alcohol with $K_3[Fe(CN)_6]$ (Fig. 4); it was also observed when methoxy-p-hydroquinone was oxidized with O_2 or $K_3[Fe(CN)_6]$.[1] The data suggest that radical G is not a deprotonated 5-hydroxy-2-methoxy-p-benzosemiquinone[1] but a deprotonated 6-hydroxy-2-methoxy-p-benzosemiquinone radical anion, and that radical G is produced from syringyl end groups in lignins.

Figure 5 shows the change in spin concentration of radicals S and G. The change in spin concentration of radical S was not shown during oxidation with $K_3[Fe(CN)_6]$ because radical S destruction was fast. The change is not simple and is similar to those of lignin oxidation. The results reflect a complicated mechanism for the radical production.

Enzyme lignin was prepared similarly from deodar (*Cedrus deodara* Loud.), acid hydrolyzed, and reduced.
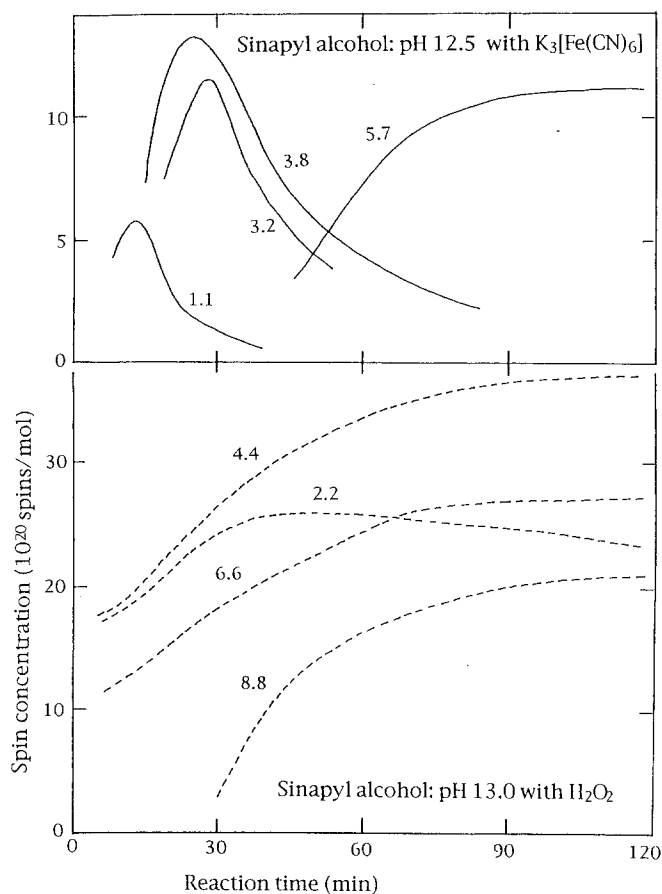
Almost none of the lignins from deodar showed ESR signals (not shown), which suggests that radical G is not produced from guaiacyl end groups in lignins.
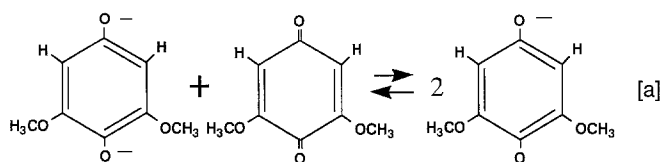
When 2,6-dimethoxy-p-hydroquinone and 2,6-dimethoxy-p-benzoquinone were dissolved in a buffer solution in argon in the dark, an ESR signal due to radical S was observed. This suggests that 2,6-dimethoxy-p-hydroquinone and 2,6-dimethoxy-p-benzoquinone form a charge transfer complex (quinhydrone-type complex) resulting in radical production.[12] Table 1 shows the concentration dependence of the radical at equilibrium on the initial concentrations of hydroquinone and benzoquinone. From the data it was found that equilibrium constants of the following reaction were 0.0053 and 0.0147 at pH 13.0 and 12.5, respectively.

The mixture of methoxy-p-hydroquinone and methoxy-p-benzoquinone in argon did not show an ESR signal of methoxy-p-benzosemiquinone radical[1] but did show a broad (0.04 mT) and weak signal due to a charge transfer complex. This fact also is why radical formation from guaiacyl end groups cannot be observed with oxidation.

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Table 1. Concentration of 2,6-dimethoxy-p-benzosemiquinone and equilibrium constant in Eq. 1 at 25°C

Measurement	Concentration for five assays				
	1	2	3	4	5
pH 13.0					
H (mmol/dm^3)	0.41	0.18	0.16	0.34	0.17
B (mmol/dm^3)	2.44	1.35	2.44	0.22	0.34
S (mmol/dm^3)	0.056	0.040	0.035	0.023	0.015
K (10^{-3})	3.4	7.5	3.5	7.7	4.2
pH 12.5					
H (mmol/dm^3)	0.40	0.32	0.22	0.26	0.24
B (mmol/dm^3)	0.92	0.77	0.81	0.18	0.12
S (mmol/dm^3)	0.068	0.052	0.050	0.025	0.020
K (10^{-3})	14.2	12.4	16.3	15.1	15.8

H, initial concentration of 2,6-dimethoxyhydroquinone; B, initial concentration of 2,6-dimethoxybenzoquinone; S, concentration of 2,6-dimethoxybenzosemiquinone at equilibrium; K, equilibrium constant.
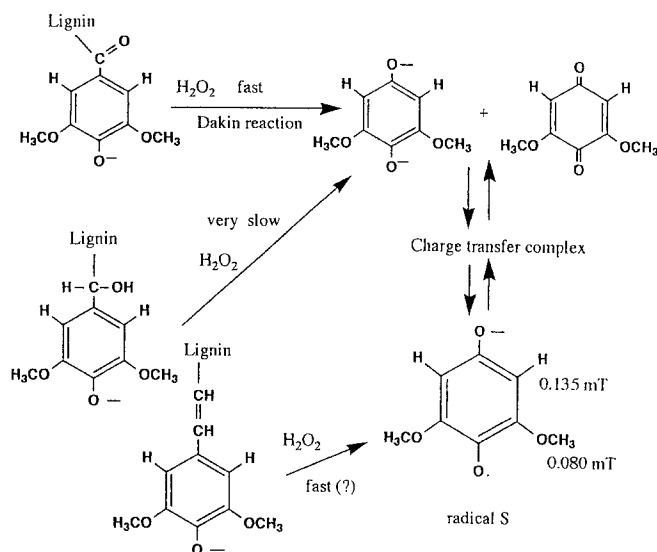

Schema [1]

Schema [a]

Schema [2]

Conclusions

The results of our studies led to the following conclusions about radical formation.

Oxidation with H_2O_2: Deprotonated 2,6-dimethoxy-p-hydroquinone and 2,6-dimethoxy-p-benzoquinone are produced by the Dakin reaction from syringyl end groups that contain α-carbonyl and α-hydroxyl groups and form a charge transfer complex. Deprotonated 2,6-dimethoxy-p-benzosemiquinone radical anion is produced from this complex. Because the Dakin reaction of the α-carbonyl group is faster than that of the α-hydroxyl group, the rate of radical production from the α-carbonyl group is greater than that from the α-hydroxyl group. The reaction rate of the syringyl end group, which contains stilbene type moieties, is fast; but the reaction mechanism is unknown.

Oxidation with $K_3[Fe(CN)_6]$: Phenoxyl radicals are produced at the syringyl end groups in lignins and are cleaved from lignins to produce the radicals S and G. The mechanism of this radical formation is unknown. The rates of radical production in three types of linkage are similar to those of the reaction with H_2O_2.

Guaiacyl end groups in lignins do not produce observable amounts of radical(s) by oxidation with H_2O_2, $K_3[Fe(CN)_6]$, O_2, or KO_2.
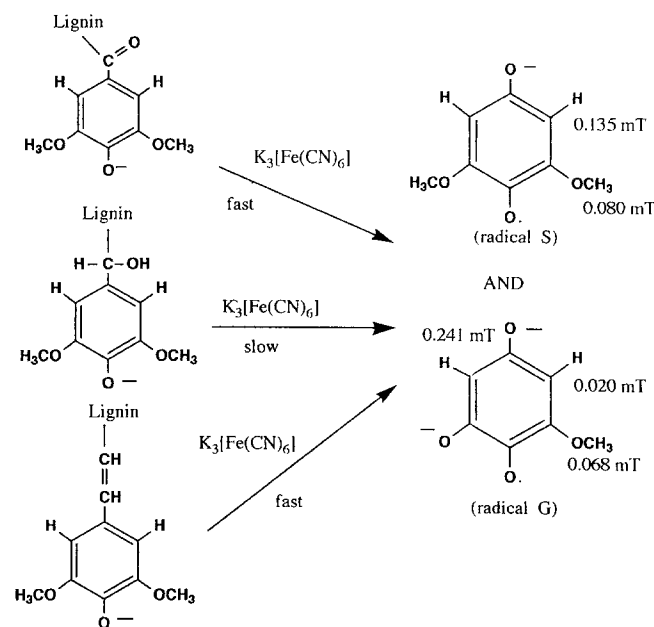
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