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REACTIVITY OF DIFFERENT OXIDASES WITH LIGNINS AND LIGNIN MODEL COMPOUNDS

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Key Word Index—Lignins; lignin model compounds: oxidases; peroxidase; laccase; pH activity differences; syringaldazine.

Abstract—Oxidase activities toward lignins and lignin model compounds failed to produce low molecular weight products. Peroxidase and laccase react similarly, and can be distinguished when both enzymes are present on the basis of pH activity differences with syringaldazine as substrate.

The three oxidases, laccase (EC 1.10.3.2), peroxidase (EC 1.11.1.7), and tyrosinase (EC 1.10.3.1) have been implicated in the degradation of lignins [1–5], despite little direct evidence in support of this function [6–8]. Young and Steelink [4] proposed a two-step oxidation catalysed by peroxidase which resulted in side chain cleavage of lignin monomer α-methylsyringyl alcohol. In this reaction sequence, α-methylsyringyl alcohol is converted to acetosyringone and then to a quinone. Hartenstein *et al.* [8] found evidence for this reaction sequence with α-methylguaiacyl alcohol and peroxidase. Both studies indicated a similar reaction sequence catalysed by peroxidase with industrial lignins, but Hartenstein *et al.* [8] found no evidence for depolymerization. It is important to determine whether laccase and tyrosinase catalyse both reaction sequences and depolymerize lignin molecules.

Lignin model dimers guaiacylglycerol-β-guaiacyl ether and veratrylglycerol-β-guaiacyl ether represent the predominant linkage connecting phenylpropyl units and dehydrodiapocynol represents one of the biphenyl linkages comprising softwood and hardwood lignins [9, 10]. Activities of oxidases toward lignin dimers provides insight into the biochemical mechanisms of lignin degradation.

Much of the work with oxidase production by fungi has been done *in vivo*, using spot tests or color changes of growth media. Neither of these approaches distinguishes the three oxidases from each other [11–15]. A sensitive assay for distinguishing peroxidase from laccase in the presence of the latter is needed if the separate roles of these enzymes are to be understood.

Evidence is presented here for similar reactivities of laccase and peroxidase toward lignins and lignin model compounds, but none was obtained for the production of stable low MW compounds. Tyrosinase was different with respect to its reactivity. Distinction can be made between laccase and peroxidase using pH activity differences.

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RESULTS AND DISCUSSION

The conversion by laccase of α-methylguaiacyl alcohol to acetoguaiacone and then to quinone occurred optimally at pH 4.0 and 5.2, respectively, while oxidation of α-methylsyringyl alcohol and acetosyringone occurred optimally at pH 4.6 and 4.0, respectively (Fig. 1). These

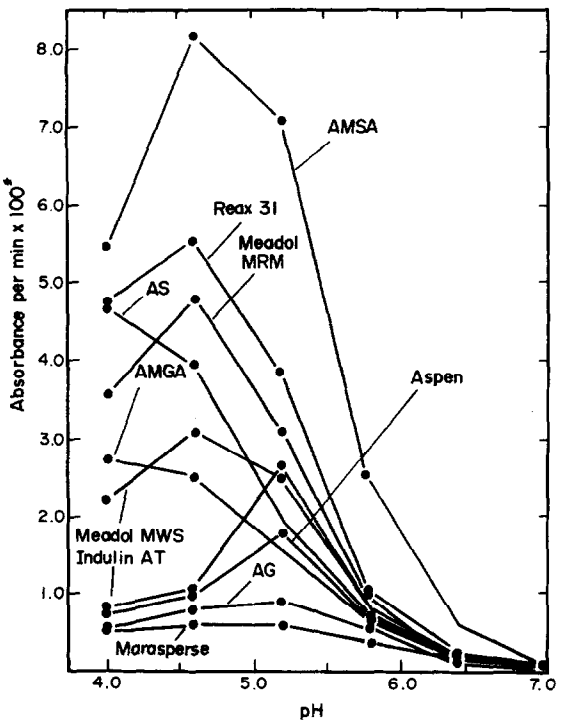

Fig. 1. Laccase activity at various pHs with 20 mM acetoguaiacone (AG), acetosyringone (AS), α-methylguaiacyl alcohol (AMGA), α-methylsyringyl alcohol (AMSA), or 20 μl 0.5% lignin in dimethylsulfoxide in a final volume of 2.0 ml 0.1 M sodium acetate (4.0–5.8) or potassium phosphate (5.8–7.0). * The changes in absorbancy for acetoguaiacone were 10 times less and for α-methylsyringyl alcohol 10-fold more than indicated by the scale.

observations suggest that side chain cleavage from lignin might be catalysed by laccase under acidic conditions.

Relatively strong spectrophotometric signals at 300 and 380 nm, indicating a reaction between laccase and lignin, were obtained up to pH 7.0 for reax 31, meadol MRM, meadol MWS and indulin AT, and weak signals were obtained for aspen cellulase and marasperse, while no signals were obtained with sitka spruce and DHPs (Fig. 1). These findings are similar to those obtained for horseradish peroxidase [8] though the reactions proceeded optimally at higher pHs.

Reactions of laccase with reax 31 and meadol MRM lignins in dialysis sacs failed to yield measurable low MW products. Despite the production of brown products within the sacs, no UV absorbing material passed outside of them. These observations suggest that depolymerization did not proceed to the point of yielding monomeric or low polymeric products having a MW less than *ca* 500 (benzoylated sacs) or 15000 (ordinary sacs). Possibly low MW products were formed but were quickly repolymerized. Hartenstein *et al.* [8] obtained similar results with horseradish peroxidase. Ishihara and Miyazaki [16] observed simultaneous polymerization and depolymerization reactions being catalysed by laccase, though polymerization predominated.

Both laccase and peroxidase reacted with the lignin model dimer, guaiacylglycerol-β-guaiacyl ether and showed strong increases in UV absorbance at 255 and 300 nm (Table 1). No evidence for production of guaiacol from lignin model dimers was found through analysis of reaction products by GLC. Gierer and Opara [7] concluded that laccase and peroxidase function mainly in oxidative coupling reactions. They obtained no evidence for cleavage of ether bonds in lignin model dimers, including guaiacylglycerol-β-guaiacyl ether.

The polyphenoloxidase tyrosinase reacted with the guaiacyl and syringyl alcohols though it reacted only slightly with acetosyringone and not at all with acetoguaiacone (Table 1). These findings suggest that tyrosinase does not react like laccase or peroxidase in the two step side-chain cleavage reaction postulated by Young and Steelink [4] to be important in lignin depolymerization. Tyrosinase is unreactive toward the lignin dimers and lignins (Table 1).

Table 1. Reactions of lignins and lignin model compounds with oxidases (pH range; pH optimum)*

Compound	Laccase	Tyrosinase	Peroxidase + MeOOH
Monomers			
α-Methylguaiacyl alcohol	+ (4.0–7.0; 4.0)	+ (5.8–8.8; 7.6)	+ (4.0–8.8; 7.6)
Acetoguaiacone	+ (4.0–6.4; 5.2)	0	+ (4.0–7.0; 5.2)
α-Methylsyringyl alcohol	+ (4.0–7.0; 4.6)	+ (4.0–8.8; 7.0)	+ (4.0–8.8; 8.8)
Acetosyringone	+ (4.0–7.0; 4.0)	+ (5.8–8.8; 8.2)	+ (4.0–7.0; 4.6)
Dimers			
Guaiacylglycerol-β-guaiacyl ether	+ (4.0–7.0; 4.6)	0	+ (4.0–8.8; 7.0)
Veratrylglycerol-β-guaiacyl ether	0	0	0
Dehydrodiapocynol	+ (4.0–7.0; 4.6)	0	+ (4.0–8.8; 7.0)
Lignins			
Reax 31	+ (4.0–6.4; 4.6)	0	+ (4.0–8.8; 7.0)
Meadol MWS	+ (4.0–7.0; 4.6)	0	+ (4.0–8.8; 7.6)
Indulin AT	+ (4.0–7.0; 5.2)	0	+ (4.0–8.8; 6.4)
Marasperse	+ (4.0–7.0; 4.6)	0	+ (4.0–8.8; 5.8)
DHP	0	0	0
Sitka spruce	0	0	0
Aspen cellulase	+ (4.0–6.4; 5.2)	0	+ (4.0–8.8; 7.0)
Meadol MRM	+ (4.0–7.0; 4.6)	0	+ (4.0–8.8; 7.0)

* + indicates compound reacted; 0 indicates no reaction.

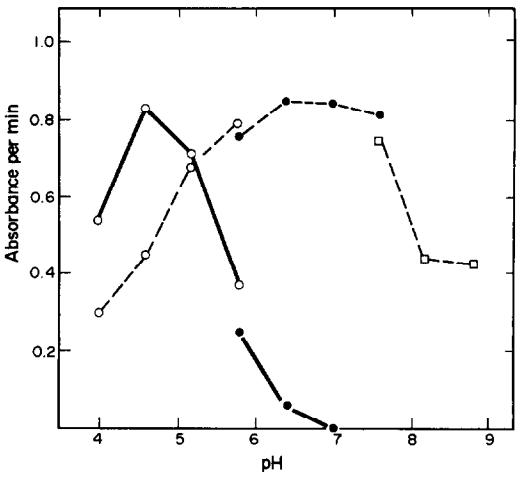

Fig. 2. Activity of laccase (solid line) and peroxidase (broken line) with 3.3×10^{-4} M syringaldazine in 30% ethanol and 3.8 mM methyl hydrogen peroxide 0.1 M Na acetate (hollow circles), potassium phosphate (solid circles), or Tris(hydroxymethyl)amino methane-HCl (squares).

The substrate specificity of laccase, peroxidase and tyrosinase have been investigated previously [17–20]. The distinction between peroxidase and laccase was not elucidated when both enzymes were present, though tyrosinase was distinguishable from peroxidase and laccase with tyrosine as substrate.

The detection by Harkin *et al.* [21] and Harkin and Obst [22] of laccase *in vivo* using syringaldazine is limited in use by a number of factors. Peroxidase activity can only be detected in the absence of laccase, it is often difficult to detect color changes against a darkened plate or pigmented fungus, results are purely qualitative, and production of extracellular hydrogen peroxide by the fungi [23] may prevent distinction between laccase and peroxidase, despite failure of the authors to detect its production. For studies on wood decay basidiomycetes *in vitro*, where pH adjustments can be made, syringaldazine can be used to detect peroxidase in the presence of laccase.

Fig. 2 illustrates the pH-activity relationship for laccase from *Coriolus versicolor* and horseradish peroxidase, a suitable electron acceptor, and syringaldazine. No laccase activity occurred above pH 7.0, while at pH 4.6 with no added electron acceptor, only laccase reacted. No activity occurred with tyrosinase at any pH [22]. This proved to be the case with laccase enzymes isolated from *Fomes annosus* (Fr.) Cke., *C. versicolor* (L. ex Fr.) Quel, *Phellinus pini* (Thore ex Fr.) A. Ames, *F. everhartii* (Ell. and Gell.) v. Schr. and Spauld and *F. connatus* (Weinm.) ex Fr. Gil. Laccase from *C. hirsutus* (Wolf. ex Fr.) Quel exhibited slight activity at pH 7.6 but not above. Fahraeus and Rheinhammar [24] also found laccase from *C. versicolor* to be inactive above pH 7.0. These observations on pH are interesting in that laccases isolated from

soil ascomycetes show a pH range from 3 to 9 and optima from pH 3 to 8, depending upon substrate [25].

Tyrosinase exhibited distinct reactivities from laccase and peroxidase. Despite identical reactivities of laccase and peroxidase toward lignins and lignin model compounds, no spectrophotometric evidence for depolymerization was found. Oxidase activities toward these molecules in cell-free systems seem insufficient for lignin catabolism and other enzymes may be required for depolymerization. In related studies, no strong correlations were found between the presence of the oxidases in microorganisms and their ability to degrade lignins [26].

EXPERIMENTAL

Tyrosinase (mushroom) and horseradish peroxidase (type VI) were purchased from Sigma, and acetoguaiacone and acetosyringone from Aldrich. α-Methylguaiacyl alcohol, α-methylsyringyl alcohol and dehydrodiapocynol were gifts from Dr. Carlton Dence, meadol MRM and MWS hardwood soda lignins from Dr. Conrad Schuerch (both of this College), sitka spruce cellulase and aspen cellulase lignins from Dr. John R. Obst (Forest Products Lab., Madison, WI, U.S.A.), marasperse hardwood lignosulfonate lignin from American Can Co., reax 31 and indulin AT Kraft pine lignins from Westvaco Co., and dehydrogenated polymerizates (DHPs) guaiacylglycerol-β-guaiacyl ether and veratrylglycerol-β-guaiacyl ether from Dr. William J. Connors (Forest Products Lab). The benzoylated dialysis membranes were prepared according to ref. [27] and excluded passage of molecules of MW greater than *ca* 500 as determined with dyes.

Laccase was isolated from *C. versicolor* according to ref. [24] with the following changes. *p*-Toluidine was used as the inducer [28] and after the initial ammonium sulfate precipitation the dialysed soln was lyophilized and stored at −15°. Upon resuspension, the solution was dialysed for 2 days against 4 changes each of 4 l. distilled water.

Lignin model compounds were reacted with peroxidase and 3.8 mM methyl hydrogen peroxide, laccase, or tyrosinase in a total vol. of 2.0 ml at 25° in a Gilford spectrophotometer and in a Perkin–Elmer scanning spectrophotometer. Reactions with α-methylguaiacyl alcohol, α-methylsyringyl alcohol and dimers were followed at 300 nm, while reactions with acetoguaiacone, acetosyringone and lignin were monitored at 380 nm [4].

The production of low MW products was investigated with 0.05% reax 31 and meadol MRM lignins in 0.05 M K-Pi with benzoylated and ordinary dialysis sacs for 4 hr at pH 4.6. The sacs containing 10 ml reaction medium were dialysed during the reaction period against 50 ml 0.05 M K-Pi dialysing soln. Both the reaction mixture and external dialysis soln were monitored for increase in absorbance and spectral shifts.

Lignins and lignin dimers in MeOH were reacted with the oxidases in the scanning spectrophotometer. Reactions with 0.05% lignins in DMSO were followed at 300 and 380 nm in both spectrophotometers.

The production of guaiacol from the lignin dimers, guaiacylglycerol-β-guaiacyl ether and veratrylglycerol-β-guaiacyl ether by oxidases was monitored with a Varian Series 2100 gas chromatograph, employing a column of SAE 30 chromasorb G. Reactions were run for 2 hr or overnight, H_2O was evapd at 45°, 0.2 ml EtOH was added, and 0.005 ml was injected. The gas chromatograph was programmed from 80° to 230° at 10° min with detector and injector ports at 275°.

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