Substrate free radicals are intermediates in ligninase catalysis

(lignin biodegradation/Phanerochaete chrysosporium/radical coupling/spin-trapping/hydroperoxides)

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ABSTRACT The H2O2-requiring ligninase of the basidiomycete Phanerochaete chrysosporium oxidatively cleaves both lignin and lignin model compounds between Cα and Cβ (C-1 and C-2) of their aliphatic side chains. Previous work has demonstrated a reaction mechanism by which ligninase oxidizes aromatic substrates to their cation radicals, which then undergo side chain cleavage to yield carbon-centered free radicals. These carbon-centered radicals add O2 to give substrate peroxy radicals that react further to yield the hydroxylated and carboxylated end products usually seen in experiments with ligninase. To investigate this radical mechanism, we have now designed three dimeric lignin models: 1-(3,4-dimethoxyphenyl)-2-phenylethanol (I), 1-(3,4-dimethoxyphenyl)-2-phenylpropanol (II), and 1-(3,4-dimethoxyphenyl)-2-methyl-2-phenylpropanol (III). The following results were obtained when these models were oxidized by ligninase: (i) methyl groups at Cα of the substrate favored Cα-Cβ cleavage versus Cα oxidation to the ketone. (ii) GC/MS and HPLC analysis showed that II gave a radical coupling dimer, 2,3-diphenylbutane, as a major (26% yield) reaction product under anaerobic conditions. The anaerobic oxidation of III yielded 2,3-dimethyl-2,3-diphenylbutane. (iii) Spin-trapping experiments with nitrobenzene showed that model II oxidation produced α-methylbenzyl radicals, whereas model III oxidation gave α,α-dimethylbenzyl radicals. (iv) TLC and iodometric determinations showed that III gave cumene hydroperoxide as a major (21% yield) reaction product in air. These findings demonstrate that carbon-centered and peroxy radicals at Cα are major products of Cα-Cβ cleavage by ligninase.

Lignin, a complex polymer of phenylpropanoid subunits, resists biodegradation by most organisms. Basidiomycetes that cause white-rot of wood, however, are efficient lignin-degraders, and one of these fungi, Phanerochaete chrysosporium, secretes a ferric hemoprotein ligninase that oxidatively cleaves both natural lignin and synthetic lignin model compounds between Cα and Cβ (C1 and C2) of their aliphatic side chains (1-5). This cleavage reaction is a major one in fungal lignin depolymerization (6-9). Recent work has demonstrated several properties of ligninase relevant to the mechanism of Cα-Cβ cleavage: (i) H2O2 is a cosubstrate, and the ligninase heme undergoes spectral transients during catalysis that resemble those of peroxidase compounds I and II (10). (ii) Certain methoxybenzenes that resemble the aromatic substructures of lignin are oxidized to their cation radicals by ligninase (11). (iii) Substrate free radicals are observable by spin-trapping techniques during the Cα-Cβ cleavage of a lignin model dimer, and the trapping of these radicals suppresses end product formation (12). These results demonstrate a mechanism of enzymatic Cα-Cβ cleavage similar to that published for the one-electron oxidation of phenylalkanols by Fenton’s reagent or peroxydisulfate (13).

That is, compound I or II of ligninase is proposed to oxidize an alkoxylated ring of the substrate by one electron, yielding a cation radical that then fragments between Cα and Cβ to give a benzaldehyde and a carbon-centered free radical. Addition of O2 to the carbon-centered radical then yields a peroxyl radical, the precursor of various oxygenated end products (12, 14).

Our purpose was to assess directly the importance of this free radical mechanism in Cα-Cβ cleavage by ligninase. We therefore designed lignin models to answer the following questions: (i) Is Cα-Cβ cleavage more prevalent in models with electron-donating substituents at Cβ than in models without them? Such substituents will stabilize a radical at Cβ (13). (ii) Do appropriately designed lignin models yield products, not seen in experiments with the customary β-1- and β-0-4-linked models, that are characteristic of free-radical reactions? Such products would include dimers from coupling of carbon-centered radicals or peroxides from the addition of O2 to these radicals. If the free-radical pathway is a significant one in substrate oxidation, they should occur at appreciable levels in the overall stoichiometry. (iii) Do ESR experiments show that the enzymatically produced radicals have structures consistent with those predicted from the structures of the model substrates? Our results show that the reaction mechanism previously proposed (12, 14) is substantially correct: carbon-centered and peroxy radicals are major products of Cα-Cβ cleavage by ligninase.

MATERIALS AND METHODS

Reagents and Organic Syntheses. 1-(3,4-Dimethoxyphenyl)-2-phenylethanone was made by acylating 1,2-dimethoxybenzene with phenylacetic acid in polyphosphoric acid at 70°C (15). 1-(3,4-Dimethoxyphenyl)-2-phenylethanol (model I) was prepared by reducing this ketone with NaBH4 in CH3OH at room temperature and purified by preparative TLC on silica gel (12). 1H NMR (CDCl3) δ (ppm), 3.00 (2H, d, β-CH2), 3.83, 3.86 (6H, two singlets, =OCH2 × 2), 4.83 (1H, α-CH), 6.79-6.87 (3H, m, aromatic), 7.14-7.32 (5H, m, aromatic). Mass spectrum, m/z (relative intensity): 258 (M⁺), 168 (8), 167 (100), 139 (33), 124 (7), 108 (5), 91 (9), 77 (3). 1-(3,4-Dimethoxyphenyl)-2-phenylpropanol (model II) was prepared from 1-(3,4-dimethoxyphenyl)-2-phenylethanone in three steps: (i) CH3I/KH/N,N-dimethylformamide at room temperature, (ii) NaBH4/CH3OH at room temperature, and (iii) preparative TLC (12). 1C5 Model II (1.13 mCi/mmol−1, 1 Ci = 37 GBq) was made by including 14CH3I (ICN) in the methylation reaction. 1H NMR (CDCl3) δ (ppm), 1.09 (3H, d, γ-CH3), 2.96-3.03 (1H, m, β-CH); 3.86, 3.89 (6H, two singlets, =OCH2 × 2), 4.63 (1H, d, α-CH), 6.82-6.93 (3H, m, aromatic), 7.21-7.43 (5H, m, aromatic). Mass spectrum, m/z (relative intensity) 272 (M⁺, 1), 168 (5), 167 (100), 139 (22), 124 (8), 108 (3), 105 (4), 77 (5). 1-(3,4-Dimethoxyphenyl)-2-methyl-2-phenylpropanol (model III) was made by (i) acylation of 1,2-dimethoxybenzene with α,α-dimethylphenylacetic acid (K & K) in

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polyphosphoric acid at 70°C (15), (ii) purification of the resulting ketone by preparative TLC (12), (iii) reduction with NaBH4 in CH3OH at room temperature, and (iv) purification again by preparative TLC. 1H NMR (CDCl3) δ (ppm): 1.32, 1.33 (6H, two singlets, γ-CH3), 3.65, 3.85 (6H, two singlets, -OC6H5 × 2), 4.69 (1H, s, α-CH), 6.40 (1H, m, aromatic), 7.22–7.39 (5H, m, aromatic). Mass spectrum, m/z (relative intensity) 286 (M+2, 2), 168 (10), 167 (100), 139 (26), 124 (6), 119 (4), 108 (4), 105 (3), 91 (8), 77 (4).

meso- and dl-2,3-diphenylbutane were prepared by Kolbe electrolysis of 2-phenylpropionic acid in N,N-dimethylformamide (16). 2,3-Dimethyl-2,3-diphenylbutane was obtained from Pfaltz & Bauer (Waterbury, CT) and cumene hydroperoxide was from Matheson. Other reagents were bought from Aldrich.

Enzyme Production and Use. P. chrysosporium Burds. (ATCC 24725) was grown in stationary culture (9), and its ligninase H8, the predominant ligninase species under these growth conditions, was purified by ion-exchange HPLC on Pharmacia MonoQ (17). Aerobic and argon-purged dimer cleavage reactions were set up and monitored spectrophotometrically at 308 nm as described (12). Typical reaction mixtures included for TLC, GC/MS, or HPLC analysis (1.0 or 3.0 ml, room temperature) contained 40 mN Na tartrate (pH 3.0), 70–100 μM lignin model, 200 μM H2O2, and 0.5–1.0 μM ligninase.

Analytical Methods. TLC, ESR spectrometry, and NMR spectrometry were done as reported (12). GC/MS was performed on ether extracts of reaction mixtures, also as in ref. 12, except that a Supelco (Bellefonte, PA) SP2340 (polar silica) capillary column was used for GC. The mass spectra of ligninase reaction products were identified by comparison with the spectra of authentic standards and by a computer search (INCOS system; Finnigan, San Jose, CA) of the Environmental Protection Agency/National Institutes of Health Mass Spectral Data Base.

Reversed-phase HPLC was done on a Gilson HPLC system, using a 0.46 × 25 cm Vydac (Hesperia, CA) 201TP54 (C18) column. Various CH3OH/H2O mixtures were used as eluants at a flow rate of 1.0 ml/min−1. Elution was monitored at 255 nm and recorded on a Hewlett–Packard 3380 integrator. When veratraldehyde and 1-[3,4-dimethoxyphenyl]-2-phenylethanone resulting from model I oxidation were to be determined, samples of reaction mixture (100 μl) were eluted stepwise with CH3OH/H2O (35:65), 15 min, followed by CH3OH/H2O (50:50). Veratraldehyde eluted at 8.0 min, and the ketone eluted at 19.4 min. Authentic standards were used for external calibration, and the two compounds were quantitated from their peak areas.

When mixtures of veratraldehyde, 1-14C]phenylethanol, and 1-14C]acetophenone resulting from 14C-labeled model II cleavage were to be analyzed, samples of reaction mixture (100 μl) that contained unlabeled 1-phenylethanol and acetophenone to mark the peak positions were eluted isocratically with CH3OH/H2O (35:65). Fractions were collected every 20 sec, upon which 1-phenylethanol and acetophenone were quantitated by scintillation counting in a modified Bray’s cocktail (18) using a Packard Tri-Carb 4530 scintillation counter. Veratraldehyde was determined from its peak area as described above.

When the meso- and dl-2,3-14C]diphenylbutanes resulting from 14C-labeled model II cleavage were to be determined, samples of reaction mixture were diluted with an equal volume of CH3OH, and 100 μl of this mixture was chromatographed in CH3OH/H2O (77.5:22.5). Authentic nonradioactive standards of the diphenylbutanes were included to identify the radioactive peaks. Fractions were collected and their 14C contents were determined as described above.

When the yields of the different 14C-containing cleavage products were to be determined, the total amount of veratraldehyde produced in each reaction was used in calculations as a measure of the quantity of 14C-labeled model II cleaved.

Cumene hydroperoxide in CH3Cl2 extracts of model III reactions was determined iodometrically according to ref. 19. It was visualized as a purple spot on TLC plates by spraying with 0.8% KI in 80% acetic acid, followed by 1% starch solution (20).

RESULTS AND DISCUSSION

The lignin model dimers used in this investigation (Fig. 1) represent the β-1 linkage of lignin. Model II in particular closely resembles naturally occurring β-1-linked substructures (1). The models were also constructed to facilitate product analysis, in that no alkoxyl groups were included on their Cβ-linked aromatic rings. This tactic prevents a second attack by ligninase on reaction products that have become hydroxylated at Cβ—i.e., benzyl alcohol and α-alkylbenzyl alcohols are not substrates for ligninase, whereas analogous alcohols that have ring methoxyls are substrates (12). Finally, dimers I, II, and III were synthesized with 0, 1, and 2 methyl groups, respectively, at Cβ, to determine whether Cα-Cβ cleavage is favored by the presence of electron-donating substituents at Cα.

GC/MS Product Identifications. All three model compounds were oxidized by ligninase in the presence of H2O2.
either in air or under argon (Fig. 1). GC/MS analysis of both aerobic and anaerobic reactions showed that II and III gave veratraldehyde as the only methoxylated product [mass spectrum, m/z (relative intensity): 256 (M+, 5), 166 (7), 165 (100), 151 (9), 95 (18), 79 (10), 77 (15), 51 (13)]. Spectrophotometric measurements at 308 nm (12) showed that II and III were quantitatively cleaved between C₆ and C₈. Model I, by contrast, gave not only veratraldehyde but also the ketone, 1-(3,4)-dimethoxyphenyl-2-phenylethanone [mass spectrum, m/z (relative intensity): 256 (M+, 5), 166 (7), 165 (100), 151 (9), 95 (18), 79 (10), 77 (15), 51 (13)]. HPLC analysis showed that the molar ratio of aldehyde to ketone resulting from cleavage of I, both in air and in argon-purged reactions, was ≈1:1. To summarize, methylation of the substrate at C₆ favors cleavage versus C₈ oxidation to the ketone.

GC/MS analysis of aerobic and anaerobic reactions showed that the benzylc (nonmethoxylated) cleavage products from dimers I–III included those hydroxylated or carbonylated at the carbon derived from C₉ of the original dimer (Fig. 1). Model I gave benzyl alcohol [mass spectrum, m/z (relative intensity): 108 (M+, 78), 107 (49), 91 (70), 77 (100), 77 (49), 51 (15)] and 2-phenylethanol [mass spectrum, m/z (relative intensity): 106 (M+, 100), 105 (88), 78 (77), 77 (93), 51 (46), 50 (23)]. Dimer II yielded 1-phenylethanol [mass spectrum, m/z (relative intensity): 122 (M+, 46) 107 (100), 105 (7) 79 (93), 78 (20), 77 (52), 52 (9), 52 (6), 51 (28), 50 (10)] and acetophenone [mass spectrum, m/z (relative intensity): 120 (M+, 35), 106 (7), 105 (98), 78 (9), 104 (13), 77 (100), 74 (8), 52 (6), 51 (55), 50 (26)]. Model III gave 2-phenyl-2-propanol [mass spectrum, m/z (relative intensity): 136 (M+, 15), 122 (7), 121 (100), 105 (6), 91 (7), 78 (11), 77 (19), 60 (6), 51 (15)].

The oxygenated products were expected in view of previous work with β-1-linked lignin models, but they were not the only ones that we observed here: GC/MS analysis showed that symmetrical diphenylalkanenes also resulted from C₉-C₈ cleavage of II and III (Fig. 1). Under argon, but not in air, II yielded meso- and dl-2,3-diphenylbutane, with the same total ion count detected in the mass spectrum of each isomer [mass spectrum, m/z (relative intensity): 210 (M+, 2), 106 (6), 105 (100), 104 (23), 103 (13), 79 (17), 78 (7), 77 (26), 51 (9)]. Model III, under the same conditions, gave 2,3-dimethyl-2,3-diphenylbutane [mass spectrum, m/z (relative intensity): 238 (M+, 0.1), 120 (9), 119 (100), 118 (10), 103 (7), 91 (39), 79 (5), 78 (5), 77 (9)]. We conclude from these results that C₉-C₈ cleavage of II yields α,α-methylbenzyl radicals, whereas cleavage of III yields α,α-dimethylbenzyl (cumyl) radicals. Under anaerobic conditions, these carbon-centered radicals couple to give the observed diphenylalkanenes. We found no 1,2-diphenylethane in reactions with model I, a result consistent with the relative instability of the primary (benzyl) radical proposed to result from C₉-C₈ cleavage of this dimer. It is noteworthy that another enzyme, soybean lipoxygenase, catalyzes an anaerobic dimerization of linoleic acid, which was interpreted to show the involvement of carbon-centered substrate radicals (21). Although soybean lipoxygenase (22) and liginase (3, 5) both contain iron, only liginase has been reported to contain heme.

Spin-Trapping of Carbon-Centered Substrate Radicals. In an attempt to trap the carbon-centered radicals that result from C₉-C₈ cleavage, we performed experiments with models I–III, H₂O₂, and ligninase in the presence of the spin-trap nitrosobenzene. The ESR spectrum obtained from nitrogen-purged model II reactions with this spin trap was shown by computer simulation to be that of a nitrosobenzene spin adduct with a single β-hydrogen (aH = 14.0 G, aCH = 5.0 G, aCH₂ = 3.4 G, aCH₃ = 1.1 G) (Fig. 2a). Anaerobic model III reactions, by contrast, gave a spin adduct with no β-hydrogens (aH = 12.6 G, aCH = 2.5 G, aCH₂ = 1.0 G) (Fig. 2b). We attribute these spectra to α-methylbenzyl phenyl nitrooxide and α,α-dimethylbenzyl phenyl nitrooxide, respec-

![Fig. 2.](https://example.com/fig2.png)
concentrations of radioactive aerobic reaction mixtures. Concentrations in profile anaerobic reaction contained of products and matography mCi monitored 14C enzymatic reaction, 1.5I of Reversed-phase CL of 246 HPLC of CH3OH.

The results demonstrate that carbonylation to give acetophenone is O2 dependent, which is consistent with a peroxy radical mechanism. The small amount of acetophenone formed during argon-purged reactions may have been due to residual O2 in the reaction mixture. Hydroxylation to give 1-phenylethanol is less affected by the availability of O2, but the alcohol might result either from self-reactions of α-methylbenzyperoxy radicals (aerobic mechanism) or from oxidation of α-methylbenzyl radicals to carboxim ions, followed by addition of H2O (anaerobic mechanism).

The yield of the radical coupling dimers, meso- and dl-2,3-diphenylbutane, was revealed by HPLC in 77.5% CH3OH. These compounds accounted for 26% of all liberated phenethyl moieties under anaerobic conditions. The two isomers occurred at equal concentrations, as expected for products of a nonstereoselective radical coupling reaction (Fig. 3, Lower). These results show that carbon-centered substrate radicals are major products of Cα-Cβ cleavage by Phanerochaete ligninase. The recovery of radiolabel from the HPLC column in this experiment was 78%—i.e., some unidentified reaction products failed to elute.

**Cumene Hydroperoxide from Cleavage of Model III.** TLC analysis showed that aerobic ligninase reactions containing Model III yielded veratraldehyde, 2-phenyl-2-propanol, and cumene hydroperoxide as products (Fig. 1). Spraying of the TLC plates with KI and starch solutions confirmed the identification of cumene hydroperoxide (Fig. 4). The hydroperoxide was not detected by GC/MS, because it decomposed in the ionization chamber to give acetophenone and 2-phenyl-2-propanol. Since cumene hydroperoxide was the only product of model III oxidation that gave a positive reaction with KI, iodometric determinations done on whole CH2Cl2 extracts of the enzyme reactions provided an estimate of how much cumene hydroperoxide was produced during Cα-Cβ cleavage. In the aerobic reaction, 15 µM reaction were as follows: D, 6 µM dl-2,3-diphenylbutane; E, 6 µM meso-2,3-diphenylbutane. The 2,3-diphenylbutane standard used to mark the elution positions also contained an impurity, 1,2-diphenylpropane, which is apparent at ~9.5 min.

**Fig. 3.** Reversed-phase HPLC of the products from cleavage of 14C, model II by P. chrysosporium ligninase. —, elution profiles monitored at 255 nm. ---, radioactivity of the collected fractions. The initial concentration of 14C, model II (specific activity, 1.13 mC/mmmol) present in these reactions was 95 µM. (Upper) Chromatography in 35% CH3OH of an aerobic reaction mixture. The products and their concentrations in the assay mixture at completion of the enzymatic reaction were as follows: A, 79 µM veratraldehyde; B, 28 µM 1-phenylethanol; C, 49 µM acetophenone. This reaction mixture contained some unreacted II, which did not elute from the column in 35% CH3OH. Similar results were obtained when anaerobic reaction mixtures were chromatographed in 35% CH3OH (column profile not shown), except that the products and their concentrations in the assay mixture at completion of the enzymatic reaction were as follows: A, 95 µM veratraldehyde; B, 22 µM 1-phenylethanol; C, 9 µM acetophenone. The unidentified minor radioactive peaks apparent at 8.0 min and 9.7 min appeared only in aerobic reaction mixtures. (Lower) Chromatography in 77.5% CH3OH of an anaerobic reaction mixture. The products and their concentrations in the assay mixture at completion of the enzymatic reaction were as follows: D, 6 µM dl-2,3-diphenylbutane; E, 6 µM meso-2,3-diphenylbutane. The 2,3-diphenylbutane standard used to mark the elution positions also contained an impurity, 1,2-diphenylpropane, which is apparent at ~9.5 min.

**Fig. 4.** TLC of cumene hydroperoxide from model III oxidation by P. chrysosporium ligninase in air. Model III (70 µM) was oxidized at room temperature in a 3.0-ml reaction mixture that contained 40 mM Na tartrate (pH 3.0), 170 µM H2O2, and 0.5 µM ligninase. The mixture was then extracted (four times) with 1 vol of CH2Cl2. The combined extracts were dried over Na2SO4, concentrated by rotary evaporation, and subjected to TLC on silica gel in Skellysolve B (hexanes)/ethyl acetate (4:1). The TLC plate was then sprayed with KI and starch solutions. Left lane, cumene hydroperoxide standard; center lane, cumene hydroperoxide standard and CH2Cl2 extract of model III oxidation; right lane, CH2Cl2 extract of model III oxidation.
cumene hydroperoxide was detected per 70 μM model III cleaved. The argon-purged reaction gave 4 μM per 70 μM. That is, a significant number of the α,α-dimethylbenzyl moieties released by cleavage of III acquired hydroperoxyl groups in an O₂-dependent reaction. The presence of the hydroperoxide in reactions done under argon may have been due to residual O₂ in the cuvette. Exogenous cumene hydroperoxide would not replace H₂O₂ as an oxidant for ligninase in our experiments.

The occurrence of cumene hydroperoxide as a major cleavage product from III indicates that α,α-dimethylbenzylperoxyl radicals are intermediates. Singlet oxygen can oxidize some compounds to hydroperoxides (26), but there is no evidence that ligninase can produce singlet oxygen, and our observation of carbon-centered substrate radicals in ligninase reactions favors the involvement of peroxyl radicals. α,α-Dimethylbenzylperoxyl radicals are expected products from the addition of O₂ to the α,α-dimethylbenzyl radicals that result from C₃-C₆ cleavage (25). A radical mechanism is also supported by our finding that 2,3-dimethyl-2,3-diphenylbutane, a product of anaerobic radical coupling reactions, occurred in argon-purged experiments but was absent in aerobic ones. Conversely, cumene hydroperoxide was present at higher levels in aerobic experiments than in anaerobic ones. In ESR experiments with III, ligninase, and the spin-trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), we obtained a nine-line spectrum (a = 7.1 G, a_H (2) = 4.2 G; data not shown) indicative of 5,5-dimethylpyrroolidone(2)-oxy(1), which has been observed as a decomposition product of DMPO-peroxyl radical adducts (27, 28). This result also is most consistent with the formation of substrate peroxyl radicals, which were presumably trapped by DMPO prior to formation of the decomposition product.

The reductant that generates cumene hydroperoxide from the α,α-dimethylbenzylperoxyl radical is not known, but it could be the resting state or compound II of ligninase. Alternatively, the peroxyl radical might abstract a hydrogen atom from a second molecule of the substrate, model III, but we have not obtained evidence for such a reaction. Abstraction of the benzylic hydrogen at C₃, which is expected to be the most weakly bound hydrogen (29), would probably result in the formation of 1-(3,4-dimethoxyphenyl)-2-methyl-2-phenylpropanone, which was not detected as a product.

According to our results, the β-1-linked substructures of natural lignin will yield secondary peroxyl radicals when cleaved by ligninase in air. Secondary hydroperoxides that might result from these radicals evidently do not occur as stable products; alcohols and ketones are obtained instead from the initial attack on the β-1 linkage. Indeed, our HPLC results with II in air show that nearly all (97%) of its C₆-linked cleavage products can be accounted for in 1-phenylethanol and acetophenone. It is only with a model that gives tertiary carbon-centered radicals—i.e., model III, that we observe the relatively stable tertiary hydroperoxide, which we interpret as a product of the peroxyl radical.

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