Manganese-Dependent Cleavage of Nonphenolic Lignin Structures by Ceriporiopsis subvermispora in the Absence of Lignin Peroxidase

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Many liginolytic fungi appear to lack lignin peroxidase (LiP), the enzyme generally thought to cleave the major, recalcitrant, nonphenolic structures in lignin. At least one such fungus, Ceriporiopsis subvermispora, is nevertheless able to degrade these nonphenolic structures. Experiments showed that wood block cultures and defined liquid medium cultures of C. subvermispora rapidly depolymerized and mineralized a "C-labeled, polyethylene glycol-linked, high-molecular-weight β-O-4 lignin model compound (model I) that represents the major nonphenolic structure of lignin. The fungus cleaved model I beteen Cα, and Cβ, to release benzyl fragments, which were shown in isoie trapping experiments to be major products of model I metabolism. The Cα-Cβ cleavage of β-O-4 lignin structures to release benzyl fragments is characteristic of LiP catalysis, but assays of C. subvermispora liquid cultures that were metabolizing model I confirmed that the fungus produced no detectable LiP activity. Three results pointed, instead, to the participation of a different enzyme, manganese peroxidase (MnP), in the degradation of nonphenolic lignin structures by C. subvermispora. (i) The degradation of model I and of exhaustively methylated (nonphenolic), "C-labeled, synthetic lignin by the fungus in liquid cultures was almost completely inhibited when the Mn concentration of the medium was decreased from 35 µM to approximately 5 µM. (ii) The fungus degraded model I and methylated lignin significantly faster in the presence of Tween 80, a source of unsaturated fatty acids, than it did in the presence of Tween 20, which contains only saturated fatty acids. Previous work has shown that nonphenolic lignin structures are degraded during the MnP-mediated peroxidation of unsaturated lipids. (iii) In experiments with MnP, Mn(II), and unsaturated lipid in vitro, this system mimicked intact C. subvermispora cultures in that it cleaved nonphenolic β-O-4 lignin model compounds between Cα, and Cβ, to release a benzylic fragment.

The white-rot fungi principally responsible for lignin degra-duction produce a variety of enzymes that are thought to attack this recalcitrant polymer. Lignin peroxidases (LiPs) are able to oxidize the most resistant nonphenolic structures that make up about 90% of the lignin in wood (1). The immediate products of these reactions are lignin cation radical intermediates that undergo a variety of spontaneous degradative reactions, of which the most important is Cβ-Cα cleavage to release benzaldehyde products (6, 9, 15, 19, 20, 35). Two other enzymes, manganese peroxidases (MnP) and laccases, are also thought to function in liginolysis, but by themselves they oxidize only the more labile phenolic structures that constitute about 10% of the lignin (5, 14, 28, 40).

Given that nonphenolic lignin structures are so preponderant in lignin, the ability to degrade them is probably an important component of fungal liginolysis. It is therefore significant that many white-rot fungi produce no detectable LiP when they grow on defined laboratory media (26) yet, in some cases, retain liginolysis activity under these conditions (30, 34). Research has shown that one of these fungi, Ceriporiopsis subvermispora, is a rapid and selective delignifier (27, 37) that can degrade nonphenolic lignin structures when it grows in wood specimens (36). Although it has recently been shown that C. subvermispora possesses lip-like genes, it remains to be determined whether these genes are ever expressed or whether the proteins they encode actually have LiP activity (31). The apparent lack of LiP activity in C. subvermispora suggests that this fungus uses other mechanism to degrade nonphenolic lignin structures.

To address this question, we have found conditions under which C. subvermispora degrades high-molecular-weight (MW) nonphenolic lignin structures efficiently in a defined liquid culture medium that can be assayed easily for LiP activity. By using this culture system, we have identified some of the major cleavage metabolites that C. subvermispora generates when it degrades a high-MW polyethylene glycol (PEG) -linked lignin model compound that represents the major nonphenolic β-O-4 structure of lignin (13). The results show that C. subvermispora cultures which express no detectable LiP activity are nevertheless able to cleave benzylic fragments from macromolecular B-O-4 lignin structures as LiP-producing fungi do. This cleavage reaction requires Mn and may be a consequence of MnP-mediated lipid peroxidation.

MATERIALS AND METHODS

Organism, chemicals, and enzymes. C. subvermispora FP-90031 was obtained from the Center for Forest Mycology, USDA Forest Products Laboratory, and was maintained on, yeast extract-malt extract-peptone-glucose agar slants.

PEG-linked lignin model compound I (Fig. 1A) was synthesized with a "C license, at Cα as described previously (13). The model contained approximately 0.9 β-O-4 dimer per 8,000-average-MW PEG chain. Its specific activity was 1.0 × 10⁶ mCi mg of total polymer⁻¹ and 0.1 mCi mmol of attached β-O-4 dimer⁻¹. α-L-(["Cl]l-[(4-ethoxy-3-methoxyphenyl)-2-[(4-ethoxyphenoxy)propane]-1,3-diol (model V, 0.1 mCi mmol⁻¹, [Fig. 1B]) (23, 36). α-[("Cl]-4-ethoxy-3-methoxybenzaldehyde (compound IV, 5.0 × 10⁵ mCi mmol⁻¹, [Fig. 1A]) (25, 36), and

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Excavating methylated, 8-\(^{14}\)C-labeled, synthetic guaiacl lignin (0.01 mCi mmol of phenylpropane substructures) (8, 16) were prepared by minor modifications of the methods described in the references cited.

TWEEN 20 and Tween 80, from Pierce Chemical Co. (Rockford, Ill.), were surfactant-Amps grade. All of the other chemicals used were reagent grade.

Recombinant \textit{Phanerochaete chrysosporium} MnP (isozyme H4) was expressed in cultures of \textit{Apergillus oryzae} and partially purified by anion-exchange chromatography on DEAE-BioGel A (Bio-Rad, Hercules, Calif.) as previously described (38). The preparation had a specific activity of 2 × 10\(^{4}\) U mmol of hemoprotein in the 2,6-dimethoxyphenol oxidation assay (see below). Crude \textit{C. subvermispora} MnP consisted of concentrated, dialyzed extracellular medium from static liquid cultures of the fungus that were grown with Tween 80 under the conditions described below for biodegradation experiments.

**Ligninolytic activity of \textit{C. subvermispora} in wood block cultures.** Lignin blocks (approximately 3 cm\(^{3}\), six replicates per experimental condition) were infused with 8-\(^{14}\)C-labeled model I (1.0 × 10\(^{10}\) to 1.1 × 10\(^{10}\) dpm per block) as described previously (13). When isotope trapping experiments were done, unlabeled compound IV (5 mg) was infused into each block with the labeled model I. The blocks were then inoculated and adjusted to 85% water content by infusing them with a blended suspension of \textit{C. subvermispora} mycelium in 0.36% (w/v) potato dextrose broth. The inoculated blocks were placed on Teflon spacers over pregrown \textit{C. subvermispora} cultures on potato dextrose agar in 125-ml Erlenmeyer flasks (13). The flasks were fitted with gassing manifolds, incubated at 30°C, and flushed daily with sterile, moist air to vent evolved CO\(_2\), which was trapped in an ethanolicamine-containing cocktail for quantitation by scintillation counting.

To obtain the 8-\(^{14}\)C-labeled metabolites produced from model I, wood block cultures were harvested after 6 to 8 days of incubation, pulverized in an electric coffee mill, and extracted with methanol in a Soxhlet apparatus for 3 h. N,N-Dimethylformamide (10 ml) was added to each methanol extract, the solutions were concentrated to approximately 5 ml in a rotary vacuum evaporator, and the samples were then centrifuged to remove insoluble material. At this stage, the samples contained 65 to 75% of the 8-\(^{14}\)C initially added to the cultures; the remainder consisted of evolved 8-\(^{14}\)CO\(_2\) and insolubles. Each sample was subjected to gel permeation chromatography (GPC) on a column (33.5 by 1.9 cm) of Sephadex LH20 in N,N-dimethylformamide. Fractions (1.5 ml) were collected, and a 100-µl portion of each sample was assayed for 8-\(^{14}\)C by scintillation counting.

To identify labeled metabolites, the GPC fractions that eluted from the column at the positions of lignin monomers and dimers (between 55 and 85 ml) were pooled, evaporated to dryness, and redissolved in 2.0 ml of acetonitrile-water-H\(_2\)PO\(_4\) (150:850:1). The samples were filtered, and a 0.6-ml portion of each was subjected to reversed-phase high-performance liquid chromatography (HPLC) on a Hamilton PRP-1 column (150 by 4.1 mm; 5-µm particle size) at ambient temperature and a flow rate of 1.0 ml min\(^{-1}\). Metabolites were eluted from the column with acetonitrile-water-H\(_2\)PO\(_4\) (150:850:1) for 15 min, followed by a linear gradient to acetonitrile-water-H\(_2\)PO\(_4\) (650:350:1) between 15 and 52 min. The eluate was monitored spectrophotometrically at 280 nm, and fractions (1.0 ml) were collected for quantitation of 8-\(^{14}\)C by scintillation counting.

**Ligninolytic activity of \textit{C. subvermispora} in liquid medium.** Static liquid cultures of \textit{C. subvermispora} were grown in an N-limited medium described previously (18), except that the pH was 5.8 rather than 4.5. Precultures of the fungus were prepared by removing the mycelium from an agar slant, homogenizing it in approximately 25 ml of sterile water in a Waring blender, and inoculating the resulting suspension at a rate of 5% (vol/vol) into 50 ml of medium in a 2,800-ml Fernbach flask. The precultures were grown under air at 30°C for 8 to 10 days, after which they were homogenized and inoculated into fresh medium at a rate of 5% (vol/vol). When low-Mn cultures were to be grown, MnSO\(_4\) was omitted from the medium at this stage. Medium prepared by this procedure contains approximately 5 µM Mn because of trace levels of the metal in the other medium constituents (3).

The newly inoculated medium was dispensed in 10-ml portions into 125-ml Erlenmeyer flasks (four to six replicates per experimental condition), which were incubated under air at 30°C for 8 days, at which time degradation experiments were commenced by adding 8-\(^{14}\)C-labeled model I (4.7 × 10\(^{4}\) to 5.3 × 10\(^{5}\) dpm) in 1 ml of sterile water to the surface of each mycelial mat. When experiments to assess the effect of Tween surfactants were done, this 1-ml addition also contained the surfactant at a concentration of 1%, thus giving a final Tween concentration of 0.09% in the cultures. When isotope trapping experiments were done, 3 ml of unlabeled compound IV was also included in each water addition. Experiments on the degradation of methylated, 8-\(^{14}\)C-labeled synthetic lignin (C. subvermispora) (2.3 × 10\(^{10}\) to 2.4 × 10\(^{10}\) dpm per culture) were conducted in the same way as experiments with model I, except that the lignin was added to the cultures as a soluble suspension in sterile water or in Tween solution. Incubation of all cultures was continued at 30°C, and the 8-\(^{14}\)CO\(_2\) evolved from the labeled substrates was vented, trapped, and quantitated as described above for the wood block cultures.

To obtain the 8-\(^{14}\)C-labeled metabolites produced from model I, liquid cultures including the mycelium were harvested after 6 to 8 days of incubation, pooled, combined with 2 volumes of methanol, and shaken overnight at ambient temperature. The methanol-water fraction was collected by filtration through glass wool, and 10 ml of N,N-dimethylformamide was added. The sample was then concentrated to approximately a 5-ml volume by rotary vacuum evaporation at 30°C and centrifuged to remove insoluble material. At this stage, the samples contained about 65% of the 8-\(^{14}\)C initially added to the cultures; the remainder consisted of evolved 8-\(^{14}\)CO\(_2\) and insolubles. GPC and HPLC analyses were conducted as described above for the wood block experiments.

**Confirmation of product identifications.** HPLC fractions that contained metabolite II, III, or IV (Fig. 1A) were collected and pooled. Each sample from a culture without an isotope trap was then spiked with 10 ng of the appropriate unlabeled standard. Samples from cultures with a trap did not receive these additions because they already contained enough of compound II, III, or IV for detection by spectrophotometric detection during HPLC. The samples were then extracted into dichloromethane, evaporated to dryness, and modified chemically as follows.

Samples corresponding to metabolite II were reduced with disoburyl ammonium hydride (1 ml of a 1.5 M solution in toluene) at 0°C for 1 h. Excess reductant was then decomposed by adding 1.0 M HCl dropwise until H\(_2\) evolution ceased, after which the reduced samples were resuspended in 15 ml of water and extracted four times with 15 ml of dichloromethane. The pooled organic extracts were pooled, concentrated, and resuspended in 2.0 ml of acetonitrile-water-H\(_2\)PO\(_4\) (150:850:1). The samples were filtered, and a 0.6-ml portion of each was subjected to reversed-phase high-performance liquid chromatography on a Hamilton PRP-1 column (150 by 4.1 mm; 5-µm particle size) at ambient temperature and a flow rate of 1.0 ml min\(^{-1}\). Metabolites were eluted from the column with acetonitrile-water-H\(_2\)PO\(_4\) (150:850:1) for 15 min, followed by a linear gradient to acetonitrile-water-H\(_2\)PO\(_4\) (650:350:1) between 15 and 52 min. The eluate was monitored spectrophotometrically at 280 nm, and fractions (1.0 ml) were collected for quantitation of 8-\(^{14}\)C by scintillation counting.

**FIG. 1.** Products obtained when 8-\(^{14}\)O-4-linked lignin structures were oxidized by \textit{C. subvermispora} cultures (A) and via MnP-mediated lipid peroxidation in vitro (B). Dotted arrows indicate chemical oxidoreductions that were performed to confirm product identifications. DiBal-H, dibutylborohydridimide; DDO, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.
were dried over Na2SO4 and concentrated to dryness by rotary vacuum evaporation.

Samples corresponding to metabolite III were redissolved in 2.0 ml of dichloromethane, and 25 mg of 2,3-dichloro-5,6-dicyanobenzoquinone was added. The mixtures were stirred at ambient temperature for 4 h, and the dichloromethane was then evaporated from the oxidized samples under a stream of argon.

Samples corresponding to metabolite IV were redissolved in 1.0 ml of 95% ethanol and placed in an ice bath. NaBH4 (20 mg) was added slowly with stirring, and the samples were then removed from the ice bath for an additional 4 h of stirring at ambient temperature. Excess reductant was decomposed with HCl, and the samples were worked up as described above for the reduction of metabolite II.

The oxidized and reduced samples derived from metabolites II to IV were redissolved in 0.6 ml of acetonitrile-water-HPO (15:85:0.1), filtered, and subjected to reverse-phase HPLC analysis on a Hamilton PRP-1 column as described above. For all three metabolites, the radiochromatogram HPLC peak (resulting from model I cleavage) and the UV absorbance HPLC peak (due to the unlabeled standard) behaved identically after chemical treatment.

**Enzyme assays.** LiP activity in *C. subvermispora* culture medium or dialyzed culture medium (concentrate was assayed spectrophotometrically at 308 nm by monitoring the oxidation of veratraldehyde to veratraldehyde in the presence of H2O2 at ambient temperature (39). MnP activity in *C. subvermispora* culture medium was assayed spectrophotometrically at 469 nm by monitoring the H2O2 and Mn(II)-dependent oxidation of 2,6-dimethoxyphenol to 2,2',6,6'-tetramethoxydiphenyloquinone in sodium tartrate buffer at pH 4.5 and ambient temperature (41). An extinction coefficient of 49.6 M−1 cm−1 was used to quantitate diphenyloquinone formation, and 1 U of MnP activity was defined as the amount of enzyme that catalyzed the oxidation of 1 µmol of 2,6-dimethoxyphenol per min.

**Lignin model cleavage by the MnP-lipid peroxidation system.** The cleavage of B- or 4-linked lignin structures during MnP-mediated lipid peroxidation was assayed under aerobic conditions by a modification of a procedure described previously (42). Complete reaction mixtures (2.0 ml, ambient temperature) contained α- 14C-labeled model V (1.4 × 104 dpm), Tween 80 or Tween 20 (1.0%), MnSO4 (0.4 mM), and sodium tartrate (10 mM, pH 4.5). Reactions were initiated with 0.3 U of recombinant *P. chrysosporium* MnP or crude *C. subvermispora* MnP, and an additional 0.3 U of enzyme was added at 24-h intervals for a total reaction time of 120 h.

A portion (0.5 ml) of each reaction mixture was then filtered and subjected to reversed-phase HPLC on a C4 column (250 by 4.6 mm, 10-µm particle size; Vydac 218TP). The column was eluted at 1.0 ml min−1 and ambient temperature with methanol-water-HPO (100:900:1) for 5 min. followed by a 45-min linear gradient to methanol-water-HPO (700:300:1). Fractions (0.5 ml) were collected and analyzed for 14C by scintillation counting. Product identifications were obtained by gas chromatography-electron impact mass spectrometry of pooled and extracted HPLC fractions (24).

**RESULTS**

Degradation of PEG-linked model I by *C. subvermispora* in wood blocks. 14C-labeled lignin model I was degraded rapidly by *C. subvermispora* in wood block cultures. With 23% of the compound mineralized in 6 days (Fig. 2). GPC analysis of the remaining soluble 14C-labeled material in the cultures showed that the model was partially depolymerized to fragments that corresponded in size to lignin dimers and monomers (Fig. 3). After 6 days, these degradation products accounted for approximately 15% of the model I initially supplied. Products smaller than model I but larger than its attached β-O-4 moiety were also evident in the GPC analysis, which could indicate that *C. subvermispora* in wood specimens is able to cleave the PEG polymer backbone, but it is also possible that the result simply reflects repolymerization of 14C-labeled degradative metabolizes with extractives in the wood blocks.

HPLC analysis of the low-MW products from the GPC analysis showed that they consisted of a complex mixture. Most of the components were not identified, but one of them coeluted with a standard of 4-ethoxy-3-methoxybenzoic acid methyl ester (compound II in Fig. 1A) (Table 1). The structure of metabolite II was confirmed by reducing it with diisobutylaluminum hydride and showing that the resulting product coeluted with a standard of 4-ethoxy-3-methoxybenzyl alcohol (compound III in Fig. 1A) when it was reanalyzed by HPLC. These results indicated that *C. subvermispora* can oxidize non-phenolic β-O-4 linked lignin structures to give benzylic cleavage products.

An isotope trapping experiment was then conducted to determine whether more 14C labeling of benzylic metabolizes could be obtained in *C. subvermispora* wood block cultures. The cultures were supplied with 14C-labeled model I and with a large excess of unlabeled 4-ethoxy-3-methoxyberraldyde (compound IV), the initial product expected if model I were to be cleaved between Cα and Cβ by a cation radical mechanism. Under these conditions, the fungus mineralized model I at a much lower rate than it did in the absence of compound IV, giving 7.1% in 8 days (Fig. 2). GPC analysis of the remaining soluble labeled metabolizes gave a result similar to that obtained in cultures with no isotope trap, except that more monomeric products and fewer dimeric ones accumulated in cultures that included compound IV (Fig. 3). The mineralization and GPC results are both consistent with an isotope trapping effect. The isotope trap was also mineralized in these experiments: when it rather than model I was supplied with an α-14C label, 20 to 30% was evolved as 14CO2 in 7 days (data not shown).

HPLC analysis of the low-MW products from GPC showed that methyl ester II, alcohol III, and aldehyde IV were major products of model I degradation by *C. subvermispora* (Fig. 1A and Table 1). The reason that 14C accumulated in these three products rather than in aldehyde IV alone was that the fungus converted much of unlabeled isotope trap IV to alcohol III and to a smaller quantity of methyl ester II, as shown by spectrophotometric monitoring of the HPLC eluate (data not shown). All of the product identifications were confirmed by converting the metabolizes chemically to other products and then showing that the derivatives coeluted with the expected standards by HPLC. Metabolize II was reduced to alcohol III with diisobutylaluminum hydride, metabolite III was oxidized to aldehyde IV with 2,3-dichloro-5,6-dicyanobenzoquinone, and metabolite IV was reduced to alcohol III with NaBH4 (Fig. 1A).
Selection of conditions for liquid cultures. One explanation for the ability of *C. subvermispora* to cleave benzylic fragments from model I was that the fungus might produce LiP in wood block cultures. Since there are no reliable methods to assay LiP in wood specimens, it was necessary to find a defined liquid culture medium in which the degradation of model I could be replicated and then to use conventional assays to determine which enzymes were produced by those cultures.

We observed that *C. subvermispora* mineralized model I more rapidly in N-limited liquid medium than in medium with nonlimiting N (data not shown). This result agrees with an earlier observation that high N levels inhibit the mineralization of synthetic lignin by *C. subvermispora* (34). In N-limited medium, the mineralization rate was significantly enhanced by the addition of Tween 80, a polyoxyethylene surfactant that contains unsaturated fatty acids. No stimulation was obtained with Tween 20, which contains only saturated fatty acids (Fig. 4A). We also found that the fungus was virtually unable to mineralize model I when the Mn concentration in the medium was decreased from 35 µM (the basal level) to approximately 5 µM. This result could not be attributed to growth inhibition under low-Mn conditions, because the average dry weight of the mycelium in a basal-Mn culture with Tween 80 (54 mg) was not greatly different from that of a low-Mn culture with Tween 80 (45 mg). In the presence of Tween 80, basal Mn, and limiting N, the liquid medium cultures mineralized model I at rates that were variable from experiment to experiment but similar to those observed in wood block cultures (Fig. 2, 4A, and 5).

Tween 80 addition also enhanced the ability of *C. subvermispora* cultures to mineralize exhaustively methylated synthetic lignin, which contains only nonphenolic structures (Fig. 4B). In this case, Tween 20 also stimulated the rate, but only about half as well as Tween 80. In the absence of Tween, the cultures degraded nonphenolic lignin very slowly, as reported previously (36). The mineralization of nonphenolic lignin by cultures that contained Tween 80 was almost completely inhibited when the Mn concentration of the medium was decreased from 35 µM to approximately 5 µM.

**Degradation of PEG-linked model I by *C. subvermispora** in liquid medium. Since *C. subvermispora* liquid cultures that contained limiting N, Tween 80, and basal Mn exhibited relatively high degradative activity towards model I, we investigated them to determine whether they would cleave model I to give benzylic metabolites as the wood block cultures did. In this experiment, the cultures mineralized 15% of the model I in 8 days (Fig. 5), at which point GPC analysis showed that they had cleaved it to give dimeric and smaller fragments with a yield of about 10% (Fig. 6). There was little production of metabolites intermediate in size between model I and its attached β-O-4-linked moiety, which suggests that *C. subvermispora* in liquid culture attacks the aromatic portion of the model without cleaving the polyoxyethylene linkages of its PEG portion.

When the low-MW material was analyzed by HPLC, the results showed a complex mixture dominated by polar products that were not retained on the HPLC column. However, a peak corresponding to methyl ester II was also observed in the chromatogram (Fig. 1A and Table 1). To confirm the identification, the radiolabeled peak was collected and reduced with diisobutyl aluminum hydride, after which it chromatographed identically to an alcohol III standard (Fig. 1A).

We then conducted an isotope trapping experiment to determine whether *C. subvermispora* liquid cultures could produce a significant yield of benzylic cleavage metabolites from 14C-labeled model I. When unlabeled aldehyde IV was included in the cultures as an isotope trap, the mineralization of model I was initially slower than it was in the absence of the trap. However, after 4 days of incubation, the cultures with the isotope trap mineralized compound I more rapidly than the cultures without the trap did, giving a total of 21% in 8 days (Fig. 5). The isotope trap was also mineralized in these experiments: when it rather than model I was supplied with an α-14C

![Image](image-url)
label, nearly 50% was evolved as $^{14}$CO$_2$ in 7 days (data not shown).

GPC analysis of the fungal metabolites generated from model I in the presence of an isotope trap demonstrated that approximately 10% of the model was cleaved to dimeric and monomeric fragments, with the monomers more prevalent than they were in the experiment without the trap. The production of low-MW metabolites was inhibited approximately threefold when Tween 80 was omitted from the cultures (data not shown) and was inhibited almost completely in low-Mn cultures (Fig. 6).

HPLC analysis of the low-MW products obtained in cultures with Tween 80, basal Mn, and isotope trap IV showed that ester II was a major metabolite and that a smaller quantity of alcohol III also accumulated (Fig. 1A and Table 1). Aldehyde IV was not detected, but this finding was not surprising because the fungus converted virtually all of unlabeled aldehyde trap IV to ester II and alcohol III, with the ester predominating (data not shown). The identities of metabolites II and III were confirmed by chemical oxidoreductions as outlined in Fig. 1A.

Enzyme assays. No LiP activity was detected by the conventional spectrophotometric assay (39) in direct samples from C. subvermispora liquid cultures that were metabolizing model I. The same result was obtained when dialyzed, 100-fold-concentrated extracellular fluid from cultures with Tween 80 and basal Mn was assayed by this procedure. The sensitivity of the spectrophotometric assay was sufficient to conclude that soluble LiP activity in the C. subvermispora cultures was less than 0.01 U liter$^{-1}$, compared with a typical level of 20 U liter$^{-1}$ in static ligninolytic P. chrysosporium cultures grown in similar medium (18).

MnP activity was present in all C. subvermispora liquid cultures that were metabolizing model I (Table 2). We did not make an exhaustive survey of MnP levels under the various culture conditions employed, but assays of pooled samples from replicate cultures indicated that Mn(II) and Tween stimulated MnP activity as reported earlier (32-34). For reasons that remain unclear, isotope trap IV also enhanced MnP activity.

Oxidation of a nonphenolic lignin model during MnP-mediated lipid peroxidation. In previous work, we showed that P. chrysosporium MnP can degrade nonphenolic lignin structures in vitro when it peroxidizes unsaturated fatty acids (2), but we did not look for or observe $\alpha$-$\beta$ cleavage in those experiments. Therefore, we repeated the in vitro study with crude
and recombinant MnPs and used model V as the substrate (Fig. 1B). The products were analyzed by an HPLC method that was optimized to detect benzylic cleavage products. Model I was also oxidized successfully by the system in vitro, but for reasons yet to be determined, the oxidation rates were only 20 to 30% of those found with model V (data not shown).

The results showed that crude C. subvermispora MnP and recombinant P. chrysosporium MnP oxidized dimer V to give the same product distribution when the reactions were done in the presence of Mn(II) and Tween 80 (Fig. 7). No oxidation of the lignin model occurred when MnP or Mn(II) was omitted or when unsaturated lipid (Tween 80) was replaced by saturated lipid (Tween 20) (data not shown). These results agree with our earlier ones, which also showed that the reaction requires O2 and is inhibited by the free-radical scavenger butylated hydroxytoluene (2).

Gas chromatography-electron impact mass spectrometry analysis of the major HPLC peaks (Fig. 7) showed that they consisted of uncleaved ketone VI and three cleavage products (Fig. 1B): 1-(4-ethoxy-3-methoxyphenyl)propane-1,2,3-triol (compound VII), 1-(4-ethoxy-3-methoxyphenyl)-1-oxo-propane-2,3-diol (compound VIII), and 4-ethoxy-3-methoxybenzoic acid (compound IX). The mass spectra of the cleavage products were as follows. Tri(methylsilyl) ether of compound VII: m/z (relative intensity) 458 (M+1, 1), 253 (100). Di(methylsilyl) ether of compound VIII: m/z (relative intensity) 384 (M+1, 10), 268 (88), 253 (10), 205 (38), 204 (11), 179 (100), 151 (17). Tri(methylsilyl) ester of compound IX: m/z (relative intensity) 268 (M+1, 100), 253 (79), 225 (43), 209 (31), 181 (9), 179 (10).

Aldehyde IV was not found as a cleavage product but cannot be ruled out as an intermediate because experiments with it as a substrate in the MnP-lipid peroxidation system showed that it was oxidized to acid IX (data not shown). Compounds VII to IX accounted for approximately 25% of the products formed from model V.

**TABLE 2. MnP activities in C. subvermispora liquid cultures**

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>MnP activity (U liter⁻¹)</th>
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<tr>
<td></td>
<td>Minus isotope trap IV</td>
</tr>
<tr>
<td>35 μM Mn minus Tween 80</td>
<td>72⁺</td>
</tr>
<tr>
<td>5 μM Mn plus Tween 80</td>
<td>20⁺</td>
</tr>
<tr>
<td>35 μM Mn plus Tween 80</td>
<td>140⁺, 194⁺</td>
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* Experiment 1.
⁺ Experiment 2.
DISCUSSION

It is well established that LiP-producing fungi such as *P. chrysosporium* and *Trametes versicolor* cleave nonphenolic \( \beta-O-4 \)-linked structures between C_{7} and C_{8} to give benzylic products (6, 10, 11, 19), but little is known about the reactions that LiP-negative fungi use to degrade these structures. Our initial approach to this problem was to characterize the cleavage–metabolizes that *C. subvermispora* forms when it degrades a low-MW, \( \beta-O-4 \)-linked lignin dimer, because models of this type are easy to synthesize and have provided much valuable information about ligninolytic mechanisms in LiP-producing fungi. However, in our first study, we found that wood block cultures of *C. subvermispora* degrade low-MW lignin models rapidly without accumulating diagnostic cleavage metabolites. These negative results led us to conclude, incorrectly as it turns out, that *C. subvermispora* produces insignificant levels of benzylic fragments when it degrades nonphenolic lignin structures (36).

In a second attempt to solve the problem, we developed a new, high-MW, \( \beta-O-4 \)-linked lignin model compound (model I) (13) and analyzed the products of its degradation by *C. subvermispora*. The rationale for changing to a macromolecular model was that the low MW of lignin model dimers makes them susceptible to intracellular uptake and metabolism by pathways that are unrelated to ligninolysis. Intracellular metabolism, if it accounts for a major portion of total degradation, could mask the production of extracellular metabolizes that arise via ligninolytic mechanisms. The results presented here show that the new approach was productive: *C. subvermispora* produced detectable levels of a benzylic cleavage metabolite, methyl ester II, when it degraded model I in wood blocks or in liquid culture (Fig. 1A and Table 1).

To determine whether *C. subvermispora* could produce a significant yield of benzylic cleavage products, we performed iso*tope trapping experiments which showed that \(^{14}C\)-labeled benzylic metabolizes II to IV were produced at high levels when \(^{14}C\)-labeled model I was supplied to the cultures in the presence of excess, unlabeled aldehyde IV (Fig. 1A and Table 1). The data also indicate that the benzylic metabolizes were intermediates in the mineralization of PEG-linked model I, first because experiments with \(^{14}C\)-labeled compound IV showed that *C. subvermispora* mineralized it rapidly (data not shown) and second because the addition of excess, unlabeled compound IV inhibited the production of \(^{14}C\)CO_{2} from \(^{14}C\)-labeled model I during the initial stage of degradation (Fig. 2 and 5).

Although the results are straightforward, we must note a limitation of isotope trapping experiments: since the trap must be added to cultures at a high concentration (1.5 mM in our liquid cultures), it is conceivable that it acts as an unpredictable modulator of fungal metabolism and not just as a passive sink for \(^{14}C\). Indeed, it is evident that compound IV acted as more than a simple isotope trap in *C. subvermispora* liquid cultures because it stimulated the mineralization of model I after a brief lag (Fig. 5) and also enhanced MnP levels in the cultures (Table 2). Nonetheless, the trapping results establish for the first time that a ligninolytic pathway which yields benzylic cleavage products exists and can be elicited at high levels in *C. subvermispora*.

The presence of this pathway was unexpected because the biological cleavage of nonphenolic lignin structures to give benzylic products is generally considered a unique property of LiP, which was not detectable in these experiments. Unless *C. subvermispora* produces a mycelium-bound LiP that remains undetected because it is not released into the surrounding medium, we can conclude from our results that LiP was not involved in model I degradation by this fungus.

Instead, the data presented a role for MnP. The cleavage of model I, the mineralization of model I, and the mineralization of methylated synthetic lignin were all strongly inhibited in low-Mn liquid cultures (Fig. 4 and 6). Typically, low Mn levels have the opposite effect in LiP-producing fungi: they enhance both ligninolytic activity and LiP levels (3, 29). Mn(II) is an obligatory cosubstrate for MnP (5, 22, 41) and also stimulates the production of this enzyme in *C. subvermispora* and other white-rot fungi (3, 4, 33, 34). Previous work has shown that Mn(II) stimulates lignin degradation in another LiP-negative white-rot organism that produces MnP, *Dichomitus squalens* (30).

Although MnP does not oxidize nonphenolic lignin structures such as model I during normal turnover with H_{2}O and Mn(II), these structures are slowly cooxidized when MnP peroxidizes unsaturated fatty acids. These findings have led us to the hypothesis, also proposed by others (12), that lipid peroxidation plays a role in fungal ligninolysis. Our results obtained with *C. subvermispora* support this possibility because model I degradation by the fungus was stimulated by Tween 80 (Fig. 4A), which contains esterified, unsaturated fatty acids. By contrast, Tween 20, which contains only saturated fatty acids, did not enhance the mineralization of model I in vivo. For this reason, we do not think the stimulatory effect of Tween 80 on model I mineralization was due merely to its surfactant properties, which it shares with Tween 20.

The results we obtained with methylated synthetic lignin in *C. subvermispora* cultures were less clear-cut in that both Tween 20 and Tween 80 stimulated mineralization, but this result is not surprising. Methylated lignin, unlike model I, is insoluble in water, and it is likely in this case that the Tween surfactants increased the bioavailability of the lignin. However, even here Tween 80 had a significantly greater stimulatory effect than Tween 20 (Fig. 4B).

Evidence for the participation of unsaturated lipid peroxidation in ligninolysis was also obtained in cell-free reactions. A system consisting of MnP, Mn(II), and Tween 80 cleaved models I and V to give a benzylic product, acid IX, which is closely related to in vivo metabolizes II to IV (Fig. 1). The in vitro reaction also cleaved the models \( \beta-O-4 \)-aryl linkage to give two products that we did not attempt to trap in fungal cultures, phenylglycerol VII and ketol VIII.

The occurrence of these products suggests either that the MnP-lipid system operates by oxidizing nonphenolic lignin structures to aryl cation radical intermediates (20) or that it generates an oxy radical that adds to an aromatic ring of the lignin structure (21). Lipid peroxy and alkyl radicals, which are generated during lipid peroxidation (7), appear to be the most likely oxidants of lignin in the MnP-lipid system. However, our data do not rule out the possibility that a nonlipid species, perhaps the MnP heme or \( \cdot \) OH, acts as the proximal oxidant of lignin in the system. Moreover, work is needed to characterize the MnP-lipid system and to determine whether it actually plays a role in fungal ligninolysis, but presently it provides the simplest explanation for the ability of *C. subvermispora* to cleave nonphenolic lignin structures.

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