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Fungal degradation of recalcitrant nonphenolic lignin structures without lignin peroxidase

EWALD SREBOTNIK*, KENNETH A. JENSEN, JR., AND KENNETH E. HAMMEL†

Institute for Microbial and Biochemical Technology, Forest Products Laboratory, U.S. Department of Agriculture, Madison, WI 53705

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ABSTRACT **Lignin peroxidases (LiPs) are likely catalysts of ligninolysis in many white-rot fungi, because they have the unusual ability to depolymerize the major, recalcitrant, nonphenolic structures of lignin. Some white-rot fungi have been reported to lack LiP when grown on defined medium, but it is not clear whether they exhibit full ligninolytic competence under these conditions. To address this problem, we compared the abilities of a known LiP producer, *Phanerochaete chrysosporium*, with those of a reported nonproducer, *Ceriporiopsis subvermispora*, to degrade a synthetic lignin with normal phenolic content, a lignin with all phenolic units blocked, and a dimer, 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol, that represents the major nonphenolic structure in lignin. *P. chrysosporium* mineralized all three models rapidly in defined medium, but *C. subvermispora* showed appreciable activity only toward the more labile phenolic compound under these conditions. However, in wood, its natural environment, *C. subvermispora* mineralized all of the models as rapidly as *P. chrysosporium* did. Defined media therefore fail to elicit a key component of the ligninolytic system in *C. subvermispora*. A double-labeling experiment with the dimeric model showed that a LiP-dependent pathway was responsible for at least half of dimer mineralization in wood by *P. chrysosporium* but was responsible for no more than 6–7% of mineralization by *C. subvermispora* in wood. Therefore, *C. subvermispora* has mechanisms for degradation of nonphenolic lignin that are as efficient as those in *P. chrysosporium* but that do not depend on LiP.**

White-rot fungi are the only organisms known that have evolved the specialized extracellular mechanisms needed to degrade lignin, a nonhydrolyzable plant cell wall polymer of phenylpropane-based subunits. In intact woody tissues, lignin functions as a barrier to microbial attack on the cellulose and hemicelluloses that account for most terrestrial fixed carbon. Fungal ligninolysis, which releases wood polysaccharides and lignin fragments into the biosphere for further metabolism to CO_2, is therefore an essential link in the carbon cycle (1, 2).

Lignin peroxidases (LiPs) are generally considered the primary catalysts of fungal ligninolysis. These extensively characterized, unusually oxidizing peroxidases (3–6) employ free radical chemistry to cleave the propyl side chain of lignin substructures (7–10) and have been shown to depolymerize lignin *in vitro* (11). LiPs have the unusual ability to cleave the recalcitrant nonphenolic units that comprise ≈90% of lignin (12, 13) and appear tailor-made for the biochemically formidable task of ligninolysis.

However, it is not clear that LiP is needed for ligninolysis in all white-rot fungi. Some of these basidiomycetes have been reported to degrade pure synthetic lignins in liquid culture without expressing detectable LiP activity (14–16), and some apparent nonproducers—e.g., *Dichomitus squalens* and *Ceriporiopsis subvermispora*—are aggressive decayers that selectively remove lignin from wood (17, 26).

One possibility is that the cleavage of nonphenolic structures is not important for fungal ligninolysis in wood. Some white-rot fungi might avoid the need for LiP by using weaker enzymatic oxidants that they are known to produce, such as manganese peroxidases or laccases, to cleave the labile phenolic structures that are minor constituents of lignin (14–16). These enzymes are unable to oxidize nonphenolic lignin but can cleave phenolic lignin model compounds (18, 19). Manganese peroxidases depolymerize phenolic lignin to a limited extent *in vitro* (11, 20).

An alternative hypothesis is that white-rot fungi previously thought to lack LiP may in fact produce the enzyme but only on wood, their natural substrate. Polymerase chain reaction analysis with degenerate primers has demonstrated the presence of LiP-like genes in both *C. subvermispora* and *D. squalens* (D. Cullen, personal communication) as has Southern blot hybridization with a LiP gene from *Phanerochaete chrysosporium* in the case of *C. subvermispora* (21). However, it remains unclear whether these LiP-like genes are ever expressed or actually code for LiP activity.

We have designed experiments to distinguish between these hypotheses and report that neither of them explains ligninolysis by *C. subvermispora* when it grows on wood. This fungus attacks recalcitrant nonphenolic lignin structures as rapidly and as extensively as the known LiP producer *P. chrysosporium* does but without significant participation by LiP.

MATERIALS AND METHODS

Organisms and Reagents. *P. chrysosporium* (ATCC 24725) and *C. subvermispora* (FP-90031) were obtained from the Center for Forest Mycology, Forest Products Laboratory. 1-(4-Ethoxy-3-methoxy[*ring*-^{14}C]phenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (**I**) was prepared by ethylating 3-hydroxy-1-(4-hydroxy-3-methoxy[*ring*-^{14}C]phenyl)-2-(2-methoxyphenoxy)propan-1-one with CH_3CH_2I/K_2CO_3 in *N,N*-dimethylformamide and then reducing the product with $NaBH_4$. 4-Ethoxy-3-methoxy[*ring*-^{14}C]benzaldehyde (**II**) was prepared by ethylating labeled vanillin with CH_3CH_2I. Unlabeled **I** and **II** were prepared by the same procedures from unlabeled precursors. Labeled and unlabeled 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propan-1-one (22), [*ring*-^{14}C]vanillin (23), and synthetic [*ring*-^{14}C]guaiacyl lignin (23) were obtained from T. K. Kirk (Forest Products Laboratory). An exhaustively methylated [*ring*-^{14}C]guaiacyl lignin was prepared by treating the unmodified polymer with diazomethane as reported (11).

Mineralization Experiments. *Liquid cultures. P. chrysosporium* was grown from conidiospore inocula as described

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Abbreviation: LiP, lignin peroxidase.

*Permanent address: Abteilung für Mykologie, Institute für Biochemische Technologie und Mikrobiologie, Technische Universität Wien, A-1060 Vienna, Austria.
†To whom reprint requests should be addressed.

at 39°C and under O_2 in stationary cultures that contained 10 ml of minimal low trace element medium with ammonium tartrate (1.1 mM) as the nitrogen source (24). *C. subvermispora* was grown from blended mycelial inocula (15) in the same medium but with the following changes: The pH of the medium was 5.0 rather than 4.5, the cultures were grown under air rather than O_2 for the first 7 days, and the temperature was 29°C. ^{14}C-labeled lignins (53 μg; 792 Bq) or **I** (95 μg; 792 Bq) were added to the cultures in 0.75 ml of sterile H_2O after 6 days of growth for *P. chrysosporium* and after 7 days for *C. subvermispora*. The cultures were flushed with O_2 at intervals thereafter and vented $^{14}CO_2$ was trapped in ethanolamine for quantitation by scintillation counting (23).

Solid wood cultures. Solutions containing 53 μg of [^{14}C]lignin (792 Bq, in *N,N*-dimethylformamide) or 95 μg of [^{14}C]**I** (792 Bq, in methanol) were pipetted onto the end grain of autoclaved, vacuum-dried birch (*Betula papyrifera*) sapwood blocks (approximately 15 × 15 × 15 mm), and the solvents were evaporated under vacuum. The wood blocks were then infiltrated with a sterile 0.36% (wt/vol) solution of potato dextrose broth (*C. subvermispora*) or with minimal medium (*P. chrysosporium*) (24) for 1–2 min using a vacuum aspirator pump. Under these conditions, a final water content between 75% and 85% of the wood dry weight was achieved, and the loss of radiolabeled substrate was <5%. The wood blocks were placed on Teflon spacers over pregrown 7-day cultures of the desired fungus on potato dextrose agar (*C. subvermispora*) or vermiculite soaked with minimal medium (*P. chrysosporium*) in 125-ml flasks. The wood blocks were then inoculated with agar plugs from plate cultures of the fungi and incubated at 29°C (*C. subvermispora*) or 39°C (*P. chrysosporium*). Colonization of the wood blocks was apparent by eye within 48 h of inoculation. The flask headspaces were flushed periodically with air (*C. subvermispora*) or O_2 (*P. chrysosporium*) and vented $^{14}CO_2$ was trapped for quantitation (23). In some experiments, *P. chrysosporium* was grown as *C. subvermispora* was, in wood blocks over potato dextrose agar and under air at 29°C. These cultures gave mineralization rates similar to those obtained with the procedure described above.

Double-Labeling Experiments. Birch blocks (8 or 10 replicates) were infiltrated with a mixture of **I** (0.29 μmol per block) and **II** (8.33 μmol per block), with **I** radiolabeled in half the set and **II** labeled in the other half, such that the total ^{14}C per block was set at a known value of $>10^3$ Bq. The blocks were then inoculated by vacuum infiltration with conidiospores (*P. chrysosporium*) or blended mycelium (*C. subvermispora*) in potato dextrose broth and were incubated over pregrown cultures on potato dextrose agar at 29°C under air as described above for the mineralization studies. This procedure gave a more rapid and synchronous onset of $^{14}CO_2$ evolution than did the inoculation method used for the mineralization experiments, with the result that rates were linear by days 6–7. Mineralization was determined over a 24-h period preceding day 7 or 8, after which the blocks in each category were pooled, pulverized in an electric coffee mill, and extracted with methanol in a Soxhlet apparatus for 3 h. Polymeric material was removed from the extracts by gel filtration on Sephadex LH20 in *N,N*-dimethylformamide (11), and the low molecular weight products were analyzed by reversed-phase HPLC and scintillation counting (25).

RESULTS AND DISCUSSION

P. chrysosporium and *C. subvermispora* showed distinctly different patterns of lignin mineralization when grown in liquid culture. *P. chrysosporium* degraded unmodified (phenolic) and exhaustively methylated (nonphenolic) lignins rapidly and to similar extents (Fig. 1), which is consistent with observations that this fungus secretes LiP in liquid culture (12, 13, 24) and that LiP depolymerizes exhaustively methylated lignin (11). *P. chrysosporium* also exhibited high degradative activity (>30% mineralized in 2 days) toward the lignin model dimer **I**, which represents the major nonphenolic arylglycerol-β-aryl ether subunit of the polymer (Fig. 2). The high degradative rates obtained with this basidiomycete reflect its thermotolerant nature and the fact that the culture conditions used were developed for it specifically (24).

C. subvermispora, although it was a slower degrader, mineralized the unmodified lignin nearly as extensively as *P. chrysosporium* did. However, unlike *P. chrysosporium*, *C. subvermispora* mineralized the nonphenolic lignin far less than the phenolic polymer (Fig. 1) and also showed low biodegradative activity toward model dimer **I** (<10% mineralized in 20 days). The same results were obtained with *C. subvermispora* under a variety of culture conditions—e.g., in high-nitrogen potato dextrose broth medium rather than minimal medium, on agar rather than liquid medium, or under air rather than O_2 (data not shown). These results, which are consistent with the reported lack of LiP in *C. subvermispora* (15), could be taken to indicate that the primary mechanism for ligninolysis in this fungus involves attack on phenolic units of the polymer.

However, further work showed that cultures grown under the usual laboratory conditions are unreliable predictors of ligninolytic competence. In wood, *C. subvermispora* mineralized unmodified lignin, nonphenolic lignin, and lignin model dimer **I** as rapidly as *P. chrysosporium* did (Fig. 3). The ability of *C. subvermispora* to degrade nonphenolic lignin was significantly increased under these conditions, whereas *P. chrysosporium* mineralized both lignins more slowly than it did in defined medium (Fig. 1). These results, unlike those obtained in liquid cultures, agree with previous ultrastructural observations and chemical analyses, which show that *C. subvermispora* and *P. chrysosporium* delignify wood at similar rates (17, 26). Most important, the data show that growth of *C. subvermispora* on its natural substrate enhances the expression of a previously undetected degradative mechanism that can attack recalcitrant nonphenolic lignin structures.

To examine the hypothesis that *C. subvermispora* produces LiP in wood, we infiltrated compound **I** into wood blocks, colonized the blocks with fungi, and then analyzed the resulting metabolites. The rationale for this experiment is that the biochemically unusual C_α–C_β fission reaction accomplished by LiP is unlikely to be duplicated by other mechanisms. Given **I** as the substrate, LiP yields **II** as the C_α–C_β cleavage product (2). **II** is rapidly reduced to 4-ethoxy-3-methoxybenzyl alcohol (**III**) by *P. chrysosporium* (27), and in the work reported here we found this also to be the case

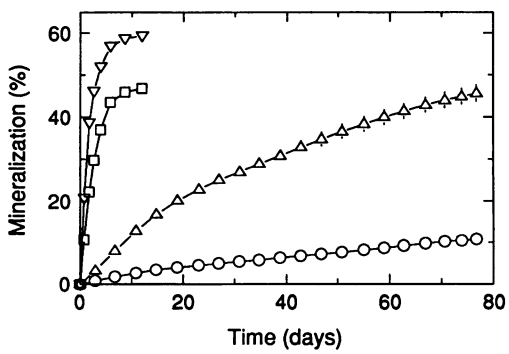

FIG. 1. Mineralization of synthetic [*ring*-^{14}C]guaiacyl lignin by *P. chrysosporium* (▽) and *C. subvermispora* (○) and of an exhaustively methylated preparation of the same lignin by *P. chrysosporium* (□) and *C. subvermispora* (△) in liquid culture. Error bars show 1 SD of the sample for eight replicate cultures and, where not shown, are smaller than the size of the data points.

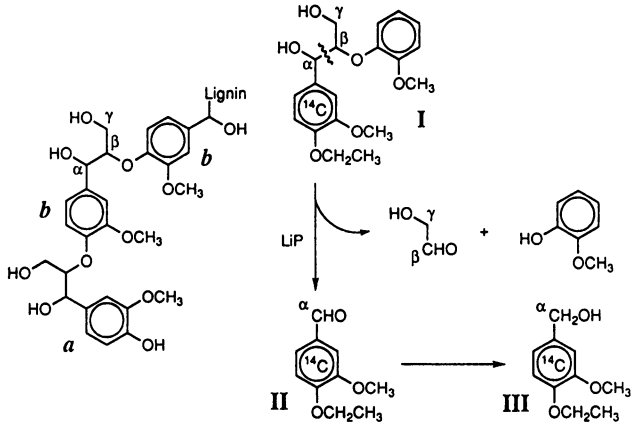

FIG. 2. (*Left*) Principal intermonomer linkages of lignin showing phenolic (*a*) and nonphenolic (*b*) units. (*Right*) Structure of nonphenolic lignin model I, showing radiolabel location, site of cleavage by LiP to give II, and fungal reduction of II to give III.

for *C. subvermispora* (data not shown). Therefore, the formation of III from I in fungal cultures would demonstrate the presence of active LiP (Fig. 2). This approach showed clearly that *P. chrysosporium* metabolized I to III in wood, in agreement with previous experiments done in defined medium (27, 28). However, no C_α–C_β fission products were detectable in the *C. subvermispora* cultures.

The possibility remained that C_α–C_β fission products were in fact formed by *C. subvermispora* in wood but were degraded too rapidly to permit detection. To address this problem, we performed double-labeling experiments in which wood block cultures of the fungi were given a mixture of I and II to mineralize. All of the cultures received identical mixtures of the two compounds, but I was ^{14}C-labeled in half the set and II was labeled in the other half, so that the fates of I and II in the added mixture could be monitored independently. The blocks were then colonized with *P. chrysosporium* or *C. subvermispora* and, once linear rates of mineralization for I and II had been established, the cultures were harvested and extracted for metabolite analysis.

The contribution made at harvest time toward the mineralization of I by a LiP-dependent pathway (I → II → III → CO_2) was then assessed for each fungus as follows. The fraction of CO_2 derived solely via C_α–C_β cleavage of I is a quantity, x, that can be derived from four experimental measurements: a, the rate of CO_2 evolution from II immediately before harvest; b, the amount of extractable III derived from II; c, the rate of CO_2 evolution from I immediately before harvest; and d, the amount of extractable III derived from I. The relationship between the amount of I-derived III in the wood block (d) and the rate of I-derived CO_2 evolution that is produced via II (xc) must be the same as the relationship between the amount of II-derived III in the block (b) and the rate of II-derived CO_2 evolution (a). This is so because any III that is produced from I via C_α–C_β cleavage and reduction must mix with the III that is derived from the reduction of exogenous II. That is, $xc/d = a/b$, and $x = ad/bc$.

An important feature of this experimental design is that it requires only reproducible, as opposed to quantitative, extraction of III from the cultures. HPLC analyses showed that, for both fungi, the total amount of III extractable from the ^{14}C[I] cultures agreed with the total amount extractable from the ^{14}C[II] cultures within an error of ±20%. A limitation of this approach should also be noted: we must assume that mineralization of I by the two fungi is a consequence of initial attack by extracellular ligninolytic mechanisms and not of intracellular oxidation. For *P. chrysosporium*, previous work

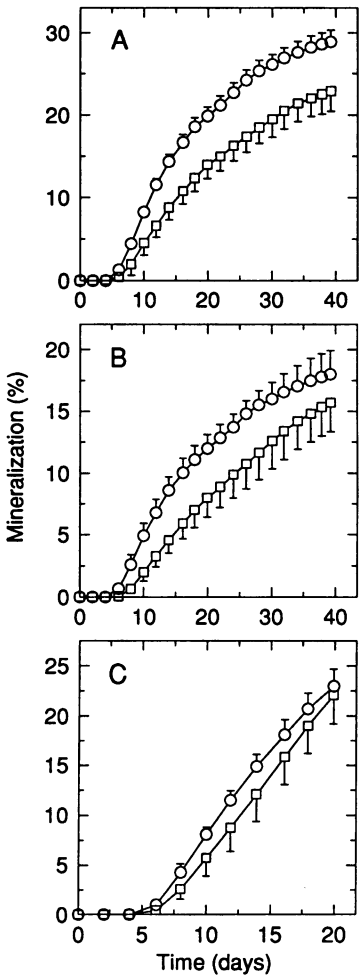

FIG. 3. Mineralization of *ring*-^{14}C-labeled synthetic guaiacyl lignin (*A*), exhaustively methylated lignin (*B*), and lignin model I (*C*), by *C. subvermispora* (five replicates; ○) and *P. chrysosporium* (six replicates; □) in wood. Error bars show 1 SD of the sample.

shows this assumption to be reasonable (2, 27). For *C. subvermispora*, the assumption is supported by our mineralization data obtained with I and with exhaustively methylated polymeric lignin. Since both fungi mineralized the nonphenolic high molecular weight substrate at the same rate (Fig. 3*B*), the presence of an additional intracellular mechanism in *C. subvermispora* specifically for the mineralization of low molecular weight lignin models should have caused this fungus to mineralize I more rapidly than *P. chrysosporium* did. However, the two fungi also mineralized I at the same rate (Fig. 3*C*).

For *P. chrysosporium*, the fraction of I mineralization attributable to C_α–C_β cleavage was determined on day 8 in two independent experiments, giving values of 0.56 (Fig. 4 *A* and *B*) and 0.51 (data not shown). That is, at the time of workup, at least half of the compound I being mineralized was routed through a pathway that depends on the principal cleavage reaction catalyzed by LiP. This finding alone is significant because, although LiP has been detected immunocytologically in fungus-colonized wood (30–32), it has not previously been shown to degrade lignin-related structures in this environment. Actually, the total contribution made by LiP toward the degradation of I was probably somewhat greater than our numbers indicate, because this enzyme catalyzes several minor fragmentation reactions in arylglycerol-β-aryl ether structures besides C_α–C_β cleavage (2, 27).

For *C. subvermispora*, the fraction of I mineralization attributable to C_α–C_β cleavage on day 7 was far lower, giving

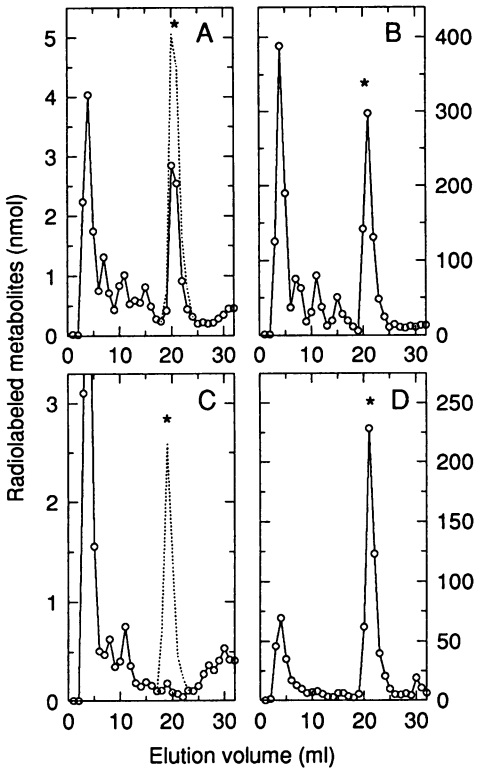

FIG. 4. HPLC analysis of metabolite III (*) formed by *P. chrysosporium* from precursors I (*A*) and II (*B*) and by *C. subvermispora* from I (*C*) and II (*D*) in double-labeling experiments. Amount of III expected if I were degraded exclusively via C_α–C_β cleavage is indicated by dotted curves in chromatograms *A* and *C*. In chromatograms *A* and *C*, most of the ^{14}C consisted of unmetabolized I, which eluted at 36 min and is not shown. Identity and quantity of [^{14}C]III in all experiments were confirmed by oxidizing the collected HPLC peak with 2,3-dichloro-5,6-benzoquinone (29) and subjecting it again to HPLC: both the labeled and unlabeled product then eluted together at 38 min, the elution time for authentic II. Variables *a–d* (see *Results and Discussion*) were calculated on a per culture basis and had the following values: *P. chrysosporium*, a = 500 nmol/day, b = 643 nmol, c = 9.9 nmol/day, d = 7.1 nmol; *C. subvermispora*, a = 725 nmol/day, b = 484 nmol, c = 7.9 nmol/day, d = 0.3 nmol.

values of 0.06 (Fig. 4 *C* and *D*) and 0.07 (data not shown) in two independent experiments. The contribution of LiP toward the mineralization of I was therefore ≈8 times greater in *P. chrysosporium* than it was in *C. subvermispora*. Since the absolute rates of I mineralization were essentially the same for the two fungi (Fig. 3), we conclude that *C. subvermispora* has a LiP-independent mechanism for the degradation of nonphenolic lignin structures and that this mechanism is as efficient as the LiP-dependent process in *P. chrysosporium*. This does not mean that LiP is never expressed by *C. subvermispora*, but it is evident that additional mechanisms of attack, perhaps involving benzylic hydrogen abstraction from C_α of the lignin propyl side chain (33) or direct attack by an oxyradical on the aromatic ring, must be invoked to explain the degradation of lignin by this basidiomycete.

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