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Mineralization of ^{14}C-Ring-Labeled Synthetic Lignin Correlates with the Production of Lignin Peroxidase, not of Manganese Peroxidase or Laccase

JUANA PEREZ† AND THOMAS W. JEFFRIES*

Institute for Microbial and Biochemical Technology, Forest Products Laboratory, Forest Service, U.S. Department of Agriculture, One Gifford Pinchot Drive, Madison, Wisconsin 53705-2398

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Recently, Mn(II) has been shown to induce manganese peroxidases (MnPs) and repress lignin peroxidases (LiPs) in defined liquid cultures of several white rot organisms. The present work shows that laccase is also regulated by Mn(II). We therefore used Mn(II) to regulate production of LiP, MnP, and laccase activities while determining the effects of Mn(II) on mineralization of ring-labeled synthetic lignin. At a low Mn(II) level, *Phanerochaete chrysosporium* and *Phlebia brevispora* produced relatively high titers of LiPs but only low titers of MnPs. At a high Mn(II) level, MnP titers increased 12- to 20-fold, but LiPs were not detected in crude broths. *P. brevispora* formed much less LiP than *P. chrysosporium*, but it also produced laccase activity that increased more than sevenfold at the high Mn(II) level. The rates of synthetic lignin mineralization by these organisms were similar and were almost seven times higher at low than at high Mn(II). Increased synthetic lignin mineralization therefore correlated with increased LiP, not with increased MnP or laccase activities.

Lignin peroxidases (LiPs), manganese peroxidases (MnPs), and laccases are three families of enzymes that have been implicated in the biological degradation of lignin. LiP production was first shown with *Phanerochaete chrysosporium* (11, 38) and has been demonstrated with *Phlebia radiata* (2, 20, 33), *Trametes versicolor* (*Coriolus versicolor*) (3, 18, 30, 40), and at least five other white rot fungi (4, 9, 32, 40). MnP was likewise first shown with *P. chrysosporium* (10, 15, 35, 36), and it is also produced by *P. radiata*, *Phlebia tremellosa* (3), and *T. versicolor*, among other white rot organisms (17). Laccase is not produced by *P. chrysosporium* but is readily demonstrated with various white rot fungi, including *Phlebia* and *Trametes* spp. (27, 29).

LiP and MnP are glycosylated, extracellular heme proteins. Several different LiP isoenzymes are found in the extracellular culture fluids of *P. chrysosporium*. They abstract single electrons from phenolic and nonphenolic aromatic rings, thereby forming cation radicals from the latter (23). Subsequent cleavage reactions (13) partially depolymerize methylated lignins (38). LiP oxidizes phenolic substrates to phenoxy radicals (37). *P. chrysosporium* also forms several isoenzymes of MnP. These generate Mn(III) from Mn(II) and H_2O_2. Mn(III), in turn, can oxidize phenols to phenoxy radicals (12, 36). Laccases are extracellular, nonheme, copper-containing proteins that catalyze the one-electron oxidation of phenols to phenoxy radicals. Phenoxy radicals, however formed, can give rise to degradative reactions in certain phenolic model compounds (21, 27, 29).

It is difficult to determine the roles of each of these enzymes in lignin biodegradation because complete catalytic depolymerization of lignin has not yet been demonstrated in vitro with a cell-free enzyme system. However, lignin mineralization can be assayed in vivo by using ^{14}C-labeled lignins (5, 28), so it is possible to correlate $^{14}CO_2$ production with the presence or absence of individual enzymes, provided that the enzymatic activities can be regulated differently. Bonnarme and Jeffries (3) recently showed that Mn(II) induces MnP while repressing LiP production by *P. chrysosporium* and other white rot fungi. Mn(II) is therefore a differential effector of these two enzyme families.

The present research examined the relationship between the mineralization of a ^{14}C-ring-labeled dehydrogenative polymerisate of coniferyl alcohol ([^{14}C]DHP lignin) and extracellular LiP, MnP, and laccase activities. We examined two organisms known to be effective lignin degraders in wood, *P. chrysosporium* and *Phlebia brevispora* (1, 34, 40). We obtained widely different titers of LiP, MnP, and laccase by using various Mn(II) concentrations in defined liquid media. DHP mineralization was highest at low Mn(II) levels and correlated with increasing LiP activity rather than with increasing MnP or laccase.

MATERIALS AND METHODS

Fungi. *P. chrysosporium* BKM-F-1767 (ATCC 24725) and *P. brevispora* HHB-7030-Sp. were obtained from the culture collection in the Center for Forest Mycology Research at the Forest Products Laboratory, U.S. Department of Agriculture, Forest Service, Madison, Wis.

Culture conditions. Fungi were maintained at 4°C on yeast malt peptone glucose agar slants grown at 30°C. Agitated cultures were grown in 2-liter Erlenmeyer flasks containing 750 ml of defined liquid basal medium or in 125-ml flasks with 40 ml of medium. $MnSO_4$ was added after 48 h of growth. The levels of Mn(II) in parts per million (ppm [μg/liter]) were the following: low Mn(II), 0.35 ppm; high Mn(II), 39.8 ppm. The details of these methods have been previously described (3).

Inocula were grown in Roux flasks containing 75 ml of basal medium without Tween 20 (Sigma Chemical Co., St. Louis, Mo.) or veratryl alcohol. These cultures were not agitated and were grown at 30°C for 10 days. Unwashed mycelial mats were broken by two 15-s pulses in a Waring blender. Nine milliliters of each of these homogenates was used as an inoculum for the 2-liter flask cultures, and 0.5 ml

* Corresponding author.
† Present address: Departamento de Microbiologia, Facultad de Farmacia, Universidad de Granada, Granada, Spain.

was used for the 125-ml flask cultures. *P. chrysosporium* cultures were incubated at 30 or 39°C; *P. brevispora* cultures were incubated at 30°C. Cultures were shaken at 120 rpm for 2-liter flasks and at 180 rpm for 125-ml flasks. After 48 h of incubation, cultures were flushed daily with oxygen for 1 min. In experiments with 2-liter flasks, the gas flow rate was 10.5 liters/min (760 mm Hg [~101 kPa], 21°C); for 125-ml flasks, the gas flow rate was 6.5 liters/min. Mycelial pellets were formed in all cultures.

Protein assays. Protein concentrations were determined by the colloidal gold method as described in the supplier's guidelines (Diversified Biotech, Newton Center, Mass.).

Enzyme assays. LiP and MnP activities were measured by the methods of Tien and Kirk (37) and Paszczynski et al. (35), respectively, as previously described (3). The substrates were veratryl alcohol (for LiP) and vanillylacetone (for MnP), with H_2O_2 for each. Laccase activity was measured by monitoring the oxidation of 2,2-azinodi-3-ethylbenzothiazoline-6-sulfuric acid (ABTS) in the absence of H_2O_2 by the method of Niku-Paavola et al. (33). Calculation of enzymatic activity used an extinction coefficient of 29,300 M^{-1} cm^{-1} for oxidized ABTS.

Laccase activity did not interfere with MnP assays, as determined by controls using vanillylacetone without H_2O_2, and MnP did not interfere with the laccase assay using ABTS. This latter control consisted of ABTS plus Mn(III). As expected, Mn(III) oxidized ABTS in a stoichiometric manner; hence, ABTS could be oxidized by MnP and Mn(II) when H_2O_2 was provided (12). However, in the absence of exogenous H_2O_2, there was no additional Mn(II)-dependent oxidation of ABTS. All enzymatic activities were expressed as nanomoles per minute per milliliter.

Enzyme assays were performed by using supernatant solutions from cultures that were simultaneously being assayed for DHP mineralization. Separate control cultures with or without unlabeled DHP (0.001 mg/ml) were also assayed.

Enzyme purification and fast protein liquid chromatography (FPLC) profiles. Cultures (2 liters) were grown with low and high levels of Mn(II). Extracellular culture fluids were concentrated by ultrafiltration with a membrane that retains proteins having an apparent molecular weight greater than 10,000 (YM 10; Amicon Corp., Lexington, Mass.). The extracellular polysaccharides were precipitated by adding one-third volume of ice-cold acetone and removed by glass wool filtration. The supernatant solutions were dialyzed against 10 mM sodium acetate, pH 6.0, and then concentrated to 4.0 ml by ultrafiltration (Amicon PM 10). Isoenzymes in the crude extracts were separated by FPLC (6).

Isoelectric focusing. Isoenzymes were separated by analytical isoelectric focusing. FPLC fractions were concentrated (Centricon-10 microconcentrators, 10,000 molecular weight cutoff; W. R. Grace & Co., Danvers, Mass.). Analytical isoelectric focusing was performed with a pH gradient of 3 to 6 by using a thin-layer polyacrylamide gel (Servalyt Precotes 3-6; Serva Fine Biochemicals, Inc., Westbury, N.Y.). Before the samples were loaded, the gel was first focused for 5 min at 4 kV and for 5 min at 8 kV. The pIs were determined by focusing for 5 min at 4 kV and for 5 min at 8 kV. Analytical pI standards were run with each sample. Amyloglucosidase from *Aspergillus oryzae* (pI, 3.55), trypsin inhibitor from soybean (pI, 4.55), β-lactoglobulin A from bovine milk (pI, 5.13), and carbonic anhydrase B from bovine erythrocytes (pI, 5.85) were used as standards. Gels were stained with Coomassie Blue (PhastGel Blue; Pharmacia, Inc., Piscataway, N.J.). From their relative FPLC retention times and isoelectric points, the LiP isoenzymes were designated according to the nomenclature of Farrell et al. (6).

Molecular weight determination. Culture supernatant solutions were denatured by mixing them 1:1 with denaturing buffer (20 mM Tris hydrochloride, pH 8, 2 mM EDTA, 5% sodium dodecyl sulfate, 10% β-mercaptoethanol) and heating the mixtures at 99°C for 5 min. Proteins were separated on a continuous polyacrylamide gradient gel from 10 to 15% (PhastGel; Pharmacia). Coomassie blue was used to stain the proteins.

[^{14}C]DHP mineralization. Assays of lignin-oxidizing activity were conducted in triplicate with 125-ml flask cultures. Synthetic, ring-labeled [^{14}C]DHP (9.2×10^5 dpm/mg) was prepared from ^{14}C-ring-labeled coniferyl alcohol (24, 25); 45,000 dpm was added to each flask as an aqueous suspension after 48 h of cultivation. Production of $^{14}CO_2$ was measured as previously described (24, 25, 28).

Statistical procedures. Enzymatic activities and rates of DHP mineralization were determined in triplicate or quadruplicate parallel cultures. Complete experiments were duplicated for *P. chrysosporium* and *P. brevispora*. Values reported are averages from replicate flasks. Standard deviations were less than 10% of the averages.

RESULTS

Correlation of DHP mineralization with enzyme activities in *P. chrysosporium*. The onset of and increase in LiP activity correlated with the production of $^{14}CO_2$, and the presence or absence of MnP seemed to have little relationship to DHP mineralization. When grown at either 30 or 39°C, *P. chrysosporium* oxidized [^{14}C]DHP to $^{14}CO_2$ about six times faster with low Mn(II) than with high Mn(II) (Fig. 1a). With low Mn(II), the onset of DHP mineralization occurred on day 5 or 6, and DHP mineralization continued at a high rate until day 9 or 10. With low Mn(II), LiP activity was readily detected in supernatant solutions after 4 days of cultivation (Fig. 1b). LiP activity accumulated until day 9 and then fell. No MnP activity was observed in low-Mn(II) cultures until day 6 or 7 (Fig. 1c). At high Mn(II), DHP mineralization began at approximately day 4 or 5 but proceeded much more slowly. No LiP activity was observed in culture supernatant solutions at high Mn(II). At high Mn(II), high levels of MnP were observed after day 4 or 5, and titers remained high until at least day 12. The maximum MnP titer of *P. chrysosporium* increased almost 20-fold at high Mn(II) compared with at low Mn(II). Even so, the rate of DHP mineralization decreased dramatically.

P. chrysosporium produced more LiP and more MnP at 30 than at 39°C. With low Mn(II), the rate of DHP mineralization was greater at 30 than at 39°C. At high Mn(II), there was no increase in DHP mineralization at the lower temperature.

The presence of DHP had no influence on enzymatic activities (data not shown).

***P. chrysosporium* isoenzyme profiles.** LiP was not observed in crude supernatant solutions of high-Mn(II)-grown cells, but its presence could be demonstrated in larger-scale (2-liter) 6-day-old cultures after concentration and fractionation. At low Mn(II), the dominant enzymes were LiP1, LiP2, and LiP6, with lesser amounts of LiP3 and LiP5 and a trace of LiP4. MnP1 and MnP2 were produced in small amounts (Fig. 2a). At high Mn(II), MnP1 and MnP2 were the dominant isoenzymes. MnP3 was also present, while LiP2 was the major LiP isoenzyme. Traces of LiP3 and LiP1 were also observed (Fig. 2b). The pIs in samples from low Mn(II) levels were 3.5, 3.7, 4.4, and 4.7, which corresponded to

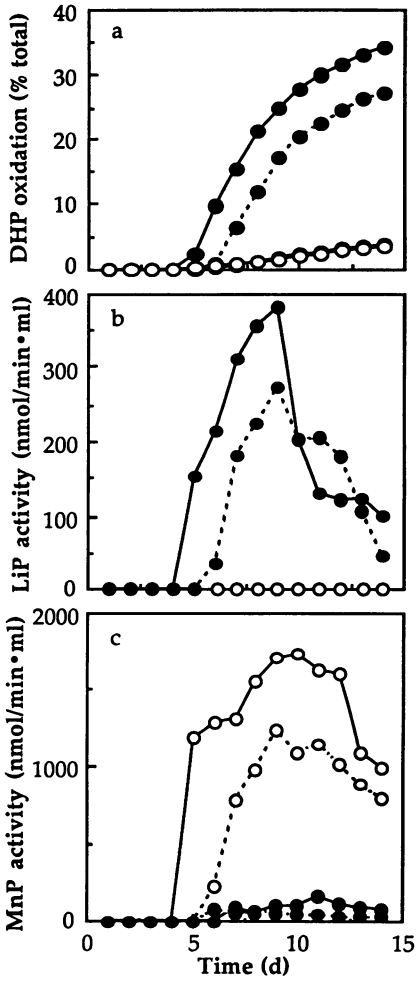

FIG. 1. Comparison of the rates of DHP mineralization (a) and LiP (b) and MnP production (c) by *P. chrysosporium* grown at low Mn(II) (●) or high Mn(II) (○) levels at 30 (——) and 39°C (– – –).

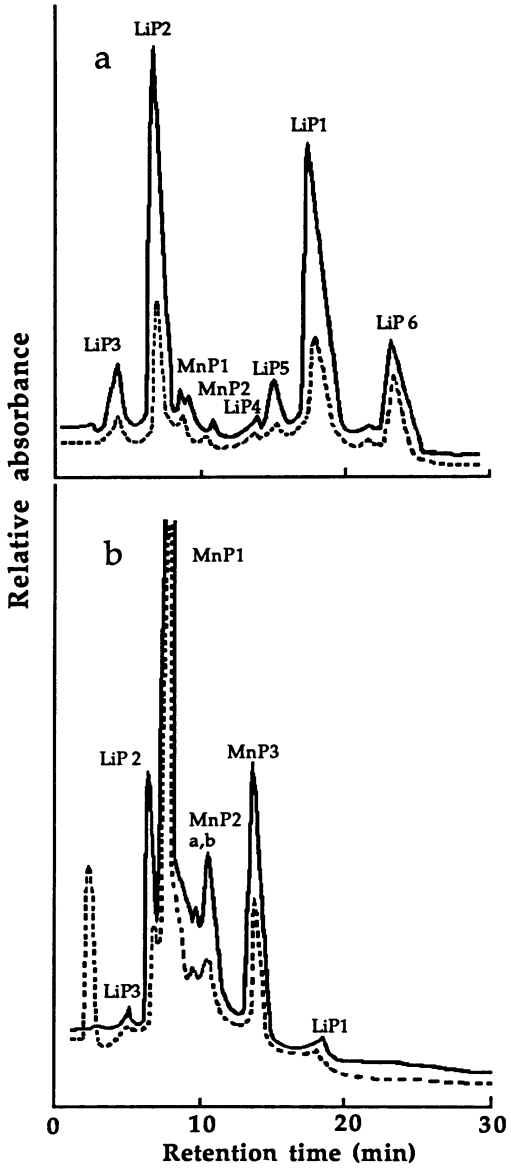

FIG. 2. Isoenzymes from extracellular culture solutions of *P. chrysosporium* grown with low (a) or high (b) Mn(II). ——, A_{405}; – – –, A_{280}.

LiP1, LiP4, LiP2, and LiP3. In cultures with high Mn(II) levels, six different bands were detected, with the following pIs: 4.9, 4.6, 4.4, 4.2, 3.7, and 3.5. The main one was pI 4.9, which corresponded to MnP1. *P. chrysosporium* did not produce detectable levels of laccase.

Correlation of DHP mineralization with enzyme activities of *P. brevispora*. Significant DHP mineralization was observed in low-Mn(II) cultures of *P. brevispora* beginning on about day 5 (Fig. 3a). This continued at a relatively constant rate until at least day 20. By comparison, the rate of DHP mineralization was only about one-seventh as rapid with high Mn(II). *P. brevispora* produced LiP at low Mn(II) along with low levels of MnP and laccase (Fig. 3b), but it formed much higher levels of extracellular MnP and laccase at high Mn(II) (Fig. 4a and b). *P. brevispora* oxidized DHP at a significant rate before the appearance of LiP in the supernatant solution. Even so, as in the case of *P. chrysosporium*, DHP mineralization correlated with LiP activity rather than MnP or laccase activities. *P. brevispora* reached a total cumulative $^{14}CO_2$ release of about 23%.

Production of laccase was regulated by Mn(II) in parallel with that of MnP (Fig. 4). MnP and laccase activities increased 12- and 7-fold, respectively, with cultivation at high Mn(II), while the DHP mineralization rate was less than one-sixth of that observed at low Mn(II).

***P. brevispora* isoenzymes.** Six different proteins were detected by isoelectric focusing with the following pIs: 3.3, 3.5, 3.7, 4.0, and 5.2. By sodium dodecyl sulfate gel electrophoresis, four major bands were visualized with molecular weights of 22,000, 44,000, 50,000, and 66,000.

Laccase activity was associated with two proteins separated by FPLC. One of these (Lac1) did not have an associated heme absorption; it had a pI of 5.2. The other (Lac2) showed a heme absorption and was also associated with MnP activity. Only one protein with LiP activity was detected. This had an associated heme absorption. It eluted at the same position of the salt gradient as LiP1 of *P. chrysosporium*. We designated it PBLiP1 (Fig. 5). Two minor heme proteins (HP1 and HP2) were observed along with two other nonheme proteins (P1 and P2).

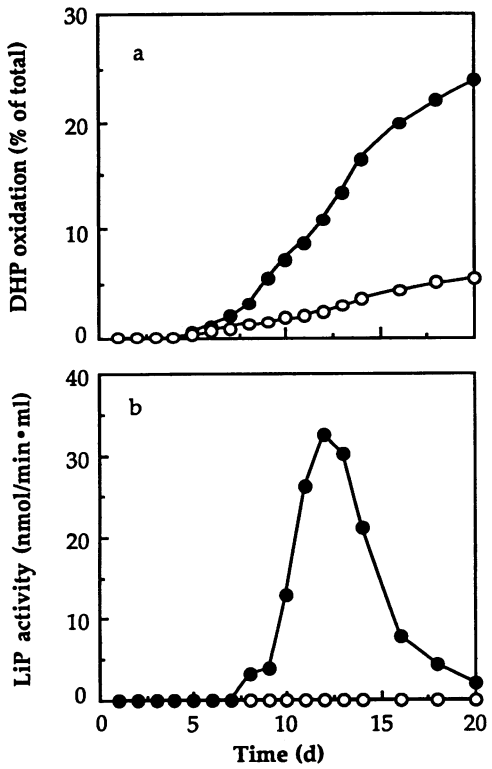

FIG. 3. Comparison of the rates of DHP mineralization (a) and LiP production (b) by *P. brevispora* grown at 30°C with low (●) or high (○) Mn(II).

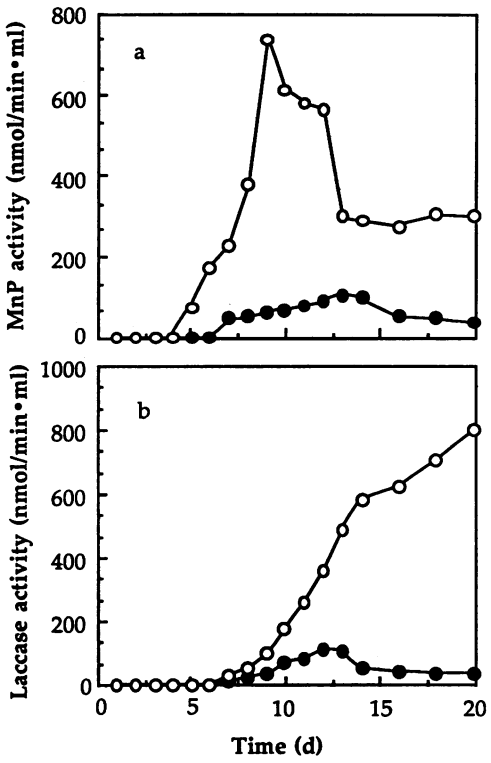

FIG. 4. Comparison of MnP (a) and laccase production (b) by *P. brevispora* grown at 30°C with low (●) or high (○) Mn(II).

Comparison between *P. chrysosporium* and *P. brevispora*. The overall rate of DHP mineralization by *P. brevispora* was 72% of that observed with *P. chrysosporium*, even though *P. chrysosporium* produced about 12 times as much extracellular LiP activity as *P. brevispora* at low Mn(II). *P. chrysosporium* produced more than twice as much MnP as *P. brevispora* at high Mn(II) (Table 1).

DISCUSSION

These results confirm the observations of Bonnarme and Jeffries (3) with respect to the Mn(II) regulation of MnP and LiP in *P. chrysosporium* and extend them to include Mn(II) regulation of laccase activity and DHP mineralization by *P. brevispora*. Our results suggest that LiP rather than MnP or laccase is associated with the mineralization of ^{14}C-ring-labeled DHP.

The effects of Mn(II) on DHP mineralization by *P. chrysosporium* have been studied previously. Jeffries et al. (16) showed that the rate of DHP mineralization decreased with increasing Mn(II). At 38 ppm of Mn(II), the rate of $^{14}CO_2$ production by *P. chrysosporium* was only about one-sixth the rate observed when Mn(II) was left out of the medium altogether.

The effects of Mn(II) on LiP have also been studied. In addition to the recent report of Bonnarme and Jeffries (3), Kirk et al. (26) examined the effects of trace elements on LiP production by *P. chrysosporium* and found that increasing Mn(II) from 1.6 to 11.2 ppm increased crude LiP activity by 1.8-fold. This is not consistent with the findings of Bonnarme and Jeffries, who reported that increasing Mn(II) from 1.6 to 8 ppm decreased LiP production by about threefold. The difference is possibly attributable to aspects of cultivation and assay. Kirk et al. used 10 mM 2,2-dimethylsuccinate (pH 4.5), whereas Bonnarme and Jeffries (and the present study) used 20 mM sodium tartrate. The buffer and pH used can affect ligninolytic activity by this organism (7). The buffer could also affect Mn(II) availability.

Kern (22) has recently reported that the addition of 1.0 g of solid MnO_2 (11.5 mM) to cultures of *P. chrysosporium* at the

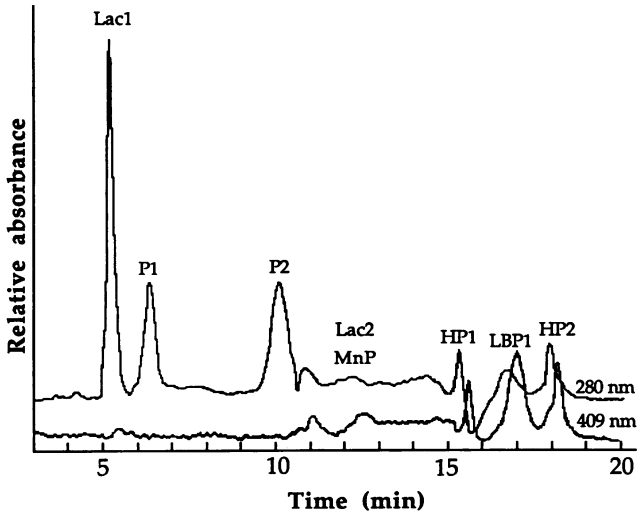

FIG. 5. Isoenzymes from extracellular culture solution of *P. brevispora* grown with low Mn(II). The tracing for A_{409} trails that for A_{280} by approximately 20 s.

TABLE 1. Effect of Mn(II) on maximum enzymatic activities and DHP mineralization rates of white rot fungi

Organism	Enzyme activity (nmol/min per ml) of[a]:						DHP mineralization[a,b]	
	LiP		MnP		Laccase			
	Low Mn(II)	High Mn(II)	Low Mn(II)	High Mn(II)	Low Mn(II)	High Mn(II)	Low Mn(II)	High Mn(II)
P. chrysosporium	380 (9)	ND[c]	88 (6)	1,725 (10)	ND	ND	2.9 (7–13)	0.43 (7–13)
P. brevispora	32 (12)	ND	59 (13)	734 (9)	109 (12)	801 (20)	2.1 (8–14)	0.32 (8–14)

[a] Number(s) in parentheses is the day (or range of days) on which maximum activity was observed.
[b] Activity given in percentage of total $^{14}CO_2$ released per day.
[c] ND, Activity not detected in crude broths.

onset of ligninolytic activity improves production and stability of the enzymes. Control cultures contained 0.2 mM (11 ppm) Mn(II), as was found in our medium. The addition of MnO_2 led to a qualitatively different isoenzyme profile. LiP and MnP were detected in both control and Mn(IV)-supplemented cultures. No significant difference was found in the mineralization of [^{14}C]DHP under these two conditions. Because of the insolubility of MnO_2 in water and because the net effect of metabolic activity is to oxidize Mn(II) to Mn(IV), the effect of the addition of MnO_2 on the concentration of Mn(II) in solution was probably negligible. Kern did not examine the cultivation of *P. chrysosporium* at very low Mn(II) levels (less than 11 ppm or 0.2 mM), so the regulatory effect of Mn(II) was not shown.

Our observations are that LiP activity correlates with DHP mineralization in both *P. chrysosporium* and *P. brevispora*. Both $^{14}CO_2$ release and LiP levels increase at low Mn(II), and both activities fall while MnP and laccase rise at high Mn(II). However, correlative evidence such as this cannot provide proof that LiP is required for lignin degradation or that MnP or laccase are not. Lignin biodegradation is a multistep process ([^{14}C]DHP $\rightarrow$ $^{14}CO_2$) involving an unknown number of extracellular and intracellular enzymes. Our data suggest that LiP is required and that it could be rate limiting at high Mn(II). At the same time, our data suggest that MnP and laccase are not rate limiting for these organisms under any of the conditions examined.

It is possible, however, that Mn(II) affects intracellular respiratory activity or other enzymes not assayed here. The time of onset of [^{14}C]DHP mineralization preceded the detection of all three enzymes. Moreover, the rate of $^{14}CO_2$ release was not proportional to LiP production by the two different organisms. The level of extracellular LiP detected with *P. brevispora* was only 1/12 that of *P. chrysosporium*, while the rate of [^{14}C]DHP mineralization by *P. brevispora* was about 72% of that observed with *P. chrysosporium*. Hatakka et al. (14) obtained DHP mineralization rates with *P. radiata* similar to our results with *P. brevispora*, and their extracellular LiP titers were in the same range as ours (10 to 30 nmol/min per ml). So, the discrepancy we observed between extracellular LiP activities produced by *P. chrysosporium* and *P. brevispora* is probably not anomalous. It is apparent, then, that some factor other than LiP determines the rate of lignin mineralization in different systems.

There are several possible reasons for the difference in total extracellular LiP activity observed between *P. chrysosporium* and *P. brevispora*. First, the enzymes produced by *P. brevispora* might have been cell bound. In *P. radiata*, MnP and laccase activities are extracellular, but LiP is associated with the periplasmic fraction (19). By analogy, it is possible that *P. brevispora* oxidized DHP via cell-bound LiP. If so, the titers would have appeared to be lower than they actually were. Second, we used veratryl alcohol oxidation and vanillylacetone oxidation assays developed for *P. chrysosporium* to determine LiP and MnP in *P. brevispora*. It is possible that the *P. brevispora* LiP has a lower affinity, a different pH optimum, or different activity against veratryl alcohol than does the LiP of *P. chrysosporium*, again making the titers appear lower. Third, one or more of the other proteins produced by *P. brevispora* might have been responsible for DHP mineralization, or they might have enhanced the activity of the PBLiP1.

Arguments have been made for MnP as the enzyme responsible for primary lignin depolymerization. The active species that oxidizes phenolic moieties is Mn(III), not MnP (41), and diffusion of this small, probably chelated (8) molecule into the wood would occur much more readily than diffusion of MnP. The ability of laccase to depolymerize certain phenolic lignin substructure model compounds has also been cited as evidence for its role in lignin degradation (21). In contrast to MnP and laccase, which apparently can attack and depolymerize only the phenolic portions of lignin, LiP appears to be capable of oxidizing nonphenolic lignin substructures (31) and substituting nonphenolic aromatic rings (39). We examined only the production of $^{14}CO_2$ from DHP. We did not analyze for DHP depolymerization, so it is possible that even though we did not observe high rates of $^{14}CO_2$ production when cells were grown on high Mn(II), DHP degradation or depolymerization might have occurred without ring oxidation.

It is possible that maximal DHP mineralization rates are achieved at intermediate Mn(II) levels at which both LiP and MnP (or laccase) are present in abundance. Indeed, previous studies using such intermediate levels have obtained DHP mineralization better than that reported here. Our objective was to examine the roles of these enzyme families, so we used Mn(II) concentrations that would form one or the other predominantly. Further clarification of the roles of these three enzymes in lignin biodegradation could be achieved by the use of specific inhibitors, by specific gene inactivation, or through the use of lignin model compounds in cell-free enzyme systems.

In conclusion, we have shown that Mn(II) regulates mineralization of ring-labeled DHP along with the production of LiP, MnP, and laccase activities by *P. chrysosporium* and *P. brevispora* and that the mineralization of ring-labeled DHP correlates with the appearance of LiP and not of MnP or laccase.

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