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Role of Organic Acid Chelators in Manganese Regulation of Lignin Degradation by *Phanerochaete chrysosporium*

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ABSTRACT

Nitrogen, carbon, and manganese are potent regulators of lignin degradation, but although nitrogen and carbon elicit a generalizated response when cells are starved, manganese is a relatively specific regulator of lignin and manganese peroxidase (LiP and MnP, respectively). At high manganese levels, MnP is induced, and LiP is repressed. At low Mn levels, MnP is repressed, and LiP is induced. Organic acid chelators are very important in attaining LiP repression with high Mn. Both mineralization and lignin depolymerization are regulated by manganese in the presence of organic acid chelators. As long as the chelators keep Mn(II) and Mn(III) in solution, repression is observed, but eventually, dismutation reactions cause the formation and precipitation of Mn (IV) as MnO_2. Repression is immediately relieved, and depolymerization and mineralization proceed at a high rate.

Index Entries: *Phanerochaete chrysosporium*; organic acid; chelator; manganese; lignin peroxidase; manganese peroxidase; lignin degradation.

INTRODUCTION

Lignin in its various forms is the second most abundant biopolymer in nature. Lignin biodegration is slow, and biological and environmental factors are critical in determining its turnover in the earth's carbon cycle.

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Lignin not only imparts rigidity to woody tissues, but because it is recalcitrant, also protects the cellulose and hemicelluloses from degradative enzymes. The heterogeneous and complex nature of lignin polymer makes it resistant to enzymatic attack. In nature, lignin is degraded mainly by basideomycetous fungi. The commonly occurring white-rot fungus *Phanerochaete chrysosporium* is the principal organism employed for studies of lignin biodegradation. The extracellular lignin-degrading system of this microorganism is expressed in response to growth limitation, and nitrogen, carbon, or sulfur starvation can be used to trigger induction (*1,2*). The two primary components of this system are lignin peroxidase (LiP) (*3*) and manganese peroxidase (MnP)(*4*).

LiP is a 41,000–42,000 mol-wt glycoprotein that contains about 15% carbohydrate and a heme prosthetic group. It catalyzes the oxidation of aromatic rings to cation radicals (*5*). The most important reactions that follow this first step are the cleavage of $C\alpha$—$C\beta$ bonds, β—O—4 bonds, and aromatic rings. Also, in the absence of oxygen, LiP can bring about radical coupling of lignin-related compounds (*6*).

MnP is a high spin, ferric, pentacoordinate hemeprotein of molecular mass 46,000. It oxidizes Mn(II) to Mn (III), the latter species is chelated by organic acids, and the Mn(III) complex diffuses away from the enzyme active site to act as an oxidant. This enzyme is responsible for the oxidation of a variety of lignin substructures and simple phenols (*7–10*). Two recent papers have demonstrated that either LiP or MnP can depolymerize lignin in vitro (*11,12*), but the roles that individual enzymes play in vivo have not yet been clarified.

Manganese—present in virtually all woody tissues—seems to play an important role in the lignin degrading system and in lignin biodegradation. It has previously been reported that the highest levels of mineralization of synthetic lignins (DHP) appear in the absence of Mn(II). The addition of high levels of Mn(II) reduces the LiP titers and increases MnP (*13,14*). It has recently been reported that organic acid chelators play an important role in determining the effects of Mn(II) and Mn(III) on the regulation of MnP and LiP (*15*). Organic acids are necessary to stabilize MnP-generated Mn(III) in order to attain regulation (*16*). The present article describes the roles of such organic acids in maintaining (or inhibiting) MnP and LiP activities. Lignin depolymerization, mineralization, and distribution of the degradation products in the culture were also followed as a function of manganese concentration and enzymatic activity.

MATERIAL AND METHODS

Organism and Culture Conditions

P. chrysosporium BKM-F 1767 (ATCC 24725) was employed for all studies. For mineralization and regulatory studies, the fungus was

cultivated according to Bonnarme and Jeffries (*13*), but 20 m*M* polyacrylic acid (PAA) was used as buffer (pH 4.5). Mn(II) was added as $MnSO_4$ after 48 h of growth. The levels of Mn(II) present in the media after addition were as follows: low Mn(II) 6 μM (0.3 ppm), and high Mn(II) 720 μM (40 ppm). Mn(II) was oxidized to Mn(III) (*7*), which subsequently disproportionated to Mn(II) and Mn(IV) (*15*). For in vitro assays, Mn(II) was added as $MnSO_4$ and Mn(III) as Mn(III) acetate. Unless assayed and specifically reported, the molar activities of these species are not known. Organic acids were added to the media as sodium salt (pH 4.5) to a final concentration of 5 m*M* after 48 h of growth.

For enzyme production, cells were grown with PAA for 48 h; 5 m*M* of malonate were then added with low or high levels of Mn(II). Control cultures were grown with low and high Mn(II) in absence of malonate. Complete sets (four cultures) were harvested on day 7 and day 8. This timing of harvesting coincided with the transition from MnP-generated Mn(III) to MnO_2 precipitated in the pellets.

Enzyme Purification and Isoelectric Focusing

Enzyme purification was performed as described elsewhere (*17*). Analytical isoelectric focusing was performed with a pH gradient of 3–6 by using a thin-layer polyacrylamide gel (Servatil Precotes 3-6; Serva Fine Biochemicals, Inc., Westbury, NY). Before the samples were loaded, the gel was first focused for 5 min at 4 kV and for 5 min at 8 kV. Gels were stained with Coomasie blue. Isoenzymes were designated according to the most common nomenclature (*17*).

Enzyme Assays

LiP and MnP activities were assayed in controls to which unlabeled DHP (55 μg/flask) was added to the cultures. LiP was measured as described by Tien and Kirk (*3*), and MnP according to Paszczynski et al. (*4*). In order to determine the effects of Mn(II), Mn(III), and chelators on veratryl alcohol oxidation, assays were performed at pH 3 in 25 m*M* sodium 2,2-dimethylsuccinate (DMS). Final concentrations were: 5 m*M* of chelators and 40 ppm of Mn(II) or Mn(III). In order to determine the stability of LiP, isoenzyme H1 (p*I* 4.7) at 39°C in polyacrylic acid (PAA), pH 4.5 or 5.5 in presence of malonate (5 m*M*) plus Mn(II) or Mn(III) (40 ppm) were used. Sodium 2,2-dimethylsuccinate (DMS) was used as control. Assays were performed in 25 m*M* DMS, pH 3, at 25°C.

Degradation Studies

[^{14}C] DHP Mineralization: Synthetic lignin produced from ^{14}C-ring-labeled-coniferyl alcohol [DHP] (*18*) was added to each flask (50,000 dpm; 55 μg/flask) after 48 h of growth. Production of CO_2 was followed as described in Keyser et al. (*2*). Control samples were obtained by adding DHP

Table 1
Effects of Mn(II) and Mn(III) on the Relative Rates of Veratryl Alcohol Oxidation in the Presence of Different Chelators

		Control[a]	
		no Mn + Mn(II)	+ Mn(III)
Malonate	115	100	84
Oxalate	8	29	0
Tartrate	118	119	104
Pyrophosphate	132	138	115

[a]Enzymatic activity in control assays without chelator was 544 nmol/min·mL (= 100%).

to cultures that had been killed by steam sterilization. All incubations and extractions were carried out in the same manner as with the live cultures.

DHP depolymerization: Mycelial-bound, dioxane water solubles and insolubles were followed according to the methods of Chua et al. (*19*) and Faix et al. (*20*). Molecular size distribution of the DMF solubles was determined by fractionating the residual lignin on a Sephadex LH-60: LH-20 (1:1) column. DMF-LiCl was used to elute the samples according to the method of Hammel and Moen (*11*).

Identification of Mn(IV)

The presence of Mn(IV) (MnO_2) was determined by three tests:

1. Production of bubbles in the presence of H_2O_2;
2. Formation of a blue color in the presence of benzidine; and
3. Disappearance of color in the presence of dithionite (*7,15,20*).

RESULTS

Influence of Mn(II), Mn(III), and Organic Acids in Veratryl Alcohol Oxidation and LiP Stability

LiP activity decreased in cultures when 40 ppm of Mn(II) and organic acids were added to the media. In order to know if this decrease was owing to an inhibition of LiP activity by Mn(II) or Mn(III), the veratryl alcohol oxidation titers were followed. Table 1 shows that neither the organic acids (malonate or tartrate) nor pyrophosphate (an effective Mn[II] chelator) affect veratryl alcohol oxidation in vitro by purified LiP. Oxalate inhibits this activity. Neither Mn(II) nor Mn(III) seems to affect significantly the rate of veratryl alcohol oxidation in the presence of Mn(III) chelators.

Table 2
Stability of Purified LiP at pH 4.5 and 5.5
and in the Presence and Absence of Malonate, Mn(II), and Mn(III)

Condition	Time, h					
	0	1	2	3	5	18
DMS pH 4.5	100[a]	104	115	120	100	102
PAA pH 4.5	156	166	173	173	170	157
PAA pH 5.5	139	164	164	166	152	148
PAA pH 4.5+Mal	150	126	157	152	137	145
PAA pH 4.5+Mal+Mn(II)	148	153	162	165	149	0
PAA pH 4.5+Mal+Mn(III)	123	39	9	0	0	0
PAA pH 5.5+Mal	131	144	160	160	138	95
PAA pH 5.5+Mal+Mn(II)	118	138	150	154	92	0
PAA pH 5.5+Mal+Mn(III)	105	66	46	33	0	0

[a]One hundred percent of activity was 372 nmol/min·mL.

The stability of LiP (Table 2) to pH 4.5 and to 5.5 was also studied. The latter is reached when high levels of Mn(II) along with a chelator are added to the media. The enzyme was stable in sodium 2,2-dimethylsuccinate (DMS) at pH 4.5 and PAA at pH 4.5 and 5.5 for 18 h. PAA was used as buffer, because that acid had been chosen for the in vivo studies. PAA *per se* seems to stimulate veratryl alcohol oxidation. pH did not show drastic effect on this enzymatic activity. In the presence of Mn(II), the enzyme was stable during the first 5 h of incubation, but a lack of activity was detected after 18 h at any pH. Mn(III) strongly destabilized LiP after 1 h of incubation.

Effect of Malonate and Manganese on Stability of LiP and MnP Isoenzymes

Complete sets of cultures grown with low Mn(II) and high Mn(II) in the absence or presence of malonate were harvested on days 7 and 8. This timing of harvesting coincided with the exhaustion of Mn(III) and precipitation of Mn(IV) in the pellets (*15*). The isoenzyme profile obtained from low-Mn malonate-free solutions on days 7 and 8 show that LiP H_8(p*I* 3.5) was predominant, but H_6 (p*I* 3.7) and H_2 (p*I* 4.4) were also evident (PL7 and PL8 in Fig. 1). The addition of 5 m*M* of malonate to low-Mn cultures had little effect: H_8, H_6, and H_2 could also be detected. In these cultures, a weak band corresponding to MnP (p*I* 5.1) was observed (ML7 and ML8 in Fig. 1).

On day 7, the Mn(II)/Mn(III) complex was stable in both sets of cultures (high-Mn/malonate-containing and high-Mn/malonate-free) as could be observed by the presence of red supernatant solutions and white pellets. LiP activities and LiP isoenzymes were still negligible (PH7 and

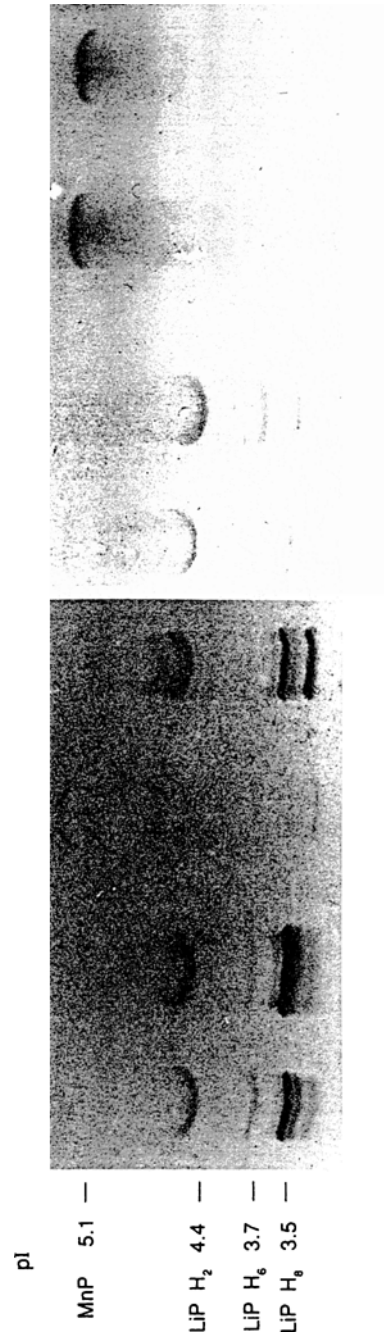

Fig. 1. Isoelectrofocusing of concentrated extracellular fluid from *P. chrysosporium* cultures grown in PAA (P) buffer and PAA plus malonate (M): with low Mn (L) or high Mn(H). Cultures were harvested on day 7 (7) or day 8 (8). For example, PL7 = 1PAA, low Mn, day 7.

MH7 in Fig. 1). MnP isoenzymes could be detected in both sets of cultures, even though activities were stronger in malonate-containing cultures.

On day 8, in high-Mn/malonate-free cultures, the pellets turned dark, and the supernatant solutions became clear. In these solutions, LiP activity and LiP isoenzymes appeared strongly for the first time (PH 8 in Fig. 1). In contrast, in those cultures with high Mn(II) plus malonate, the pellets remained white and the supernatant solutions red (because of the stabilization of Mn[III] by malonate). LiP activity and LiP isoenzymes remained repressed, whereas MnP activity and MnP isoenzymes were elevated (MH8 in Fig. 1).

Effect of Malonate and Manganese on Lignin Biodegradation

In order to determine whether the dramatic changes in protein correlated with differences in mineralization, distribution, and depolymerization of DHPs, the ^{14}C was recovered as:

1. CO_2 (mineralization);
2. DMF solubles;
3. DMF-insoluble fraction; and
4. The mycelial bound fraction.

The distribution of radioactivity among these four fractions as well as the molecular size distribution of the DMF-soluble fraction were then determined (Figs. 2 and 3).

When low levels of Mn were added to the cultures, good mineralization rates were attained in the presence or absence of malonate. In malonate-free cultures with high Mn, only a 5% of mineralization was reached on day 7. However, the mineralization increased drastically to 18% on day 8 (when Mn[III] had precipitated as MnO_2). In cultures containing high Mn and malonate, the mineralization did not increase from day 7 to day 8. In these same cultures, Mn(III) stayed in solution, and LiP was not induced (*see* Fig. 1). The biggest difference among these four conditions was that in cultures receiving only low Mn, the largest fraction was mineralized, whereas in cultures receiving high Mn, the largest recovered fraction was bound to the mycelia.

Differences were also observed in molecular size distribution of the DMF-soluble fraction. When the DHP samples from cultures with high Mn, but no malonate were sized, a progressive decrease in the molecular size distribution pattern was observed with samples incubated for 7 and 8 d (Fig. 2). Conversely, when malonate chelated the high levels of MnP-generated Mn(III), depolymerization remained the same on days 7 and 8 (Fig. 3).

The DMF-soluble fractions of DHP incubated with cultures containing malonate showed an initial shift from the highest-molecular-weight

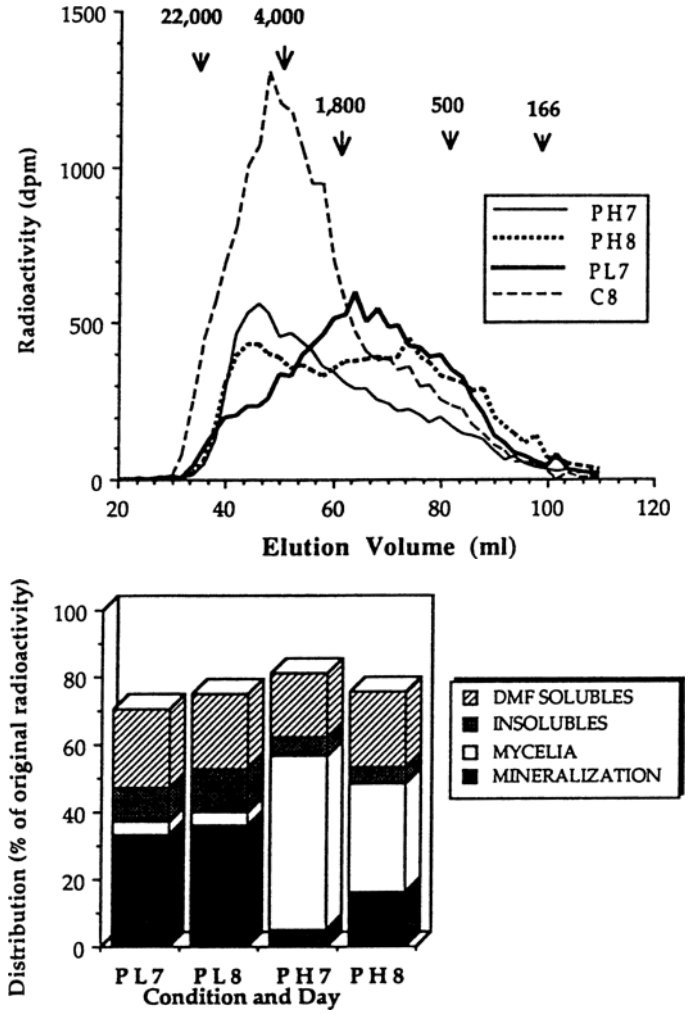

Fig. 2. Molecular-weight distribution of DMF-soluble DHP in cultures of *P. chrysosporium* grown in PAA buffer (P) and low Mn(L) or high Mn (H), and harvested on day 7 (7) or day 8 (8). In the control (C8), DHP was added on day 6 after killing the cells by autoclaving. Bar graphs represent the distribution of DHP in those supernatant solutions.

toward lower-molecular-weight compounds (Fig. 3) compared to that of samples without malonate. However, the largest amount of low-molecular-weight compounds was detected in cultures containing low and no malonate (Fig. 2).

In the control cultures, < 10% of the initial radioactivity was asso-associated with the mycelia, approx 20% was insoluble, and the balance was soluble in DMF. No mineralization occurred in these cultures (data not shown).

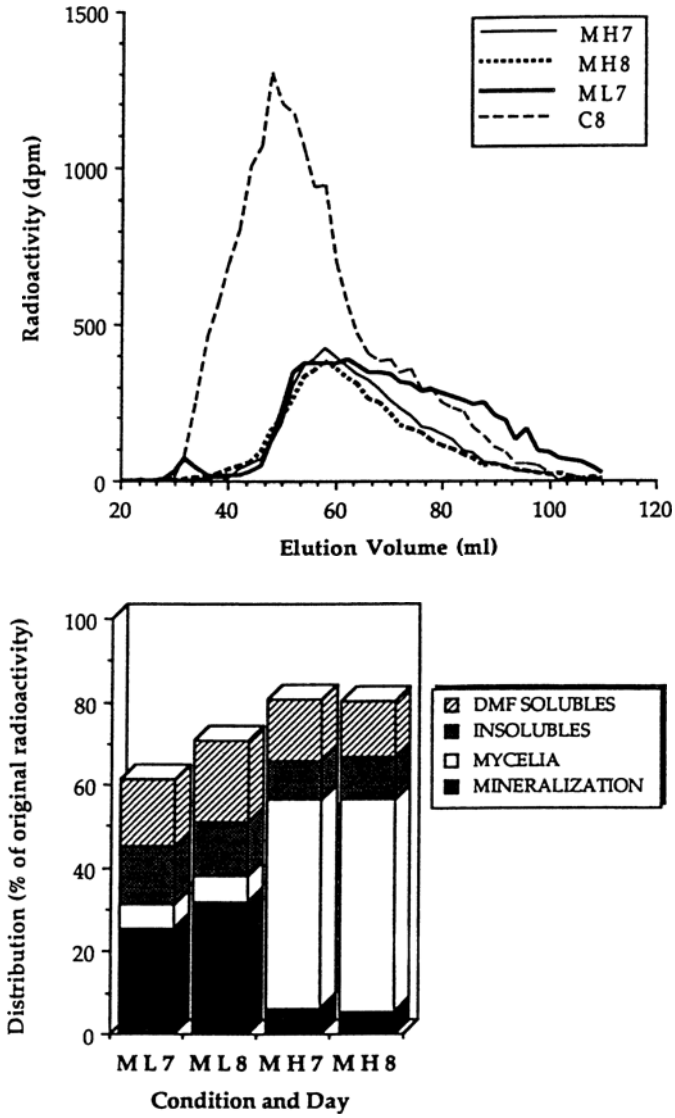

Fig. 3. Depolymerization of DMF-soluble DHP in cultures of *P. chrysosporium* grown in PAA buffer plus malonate (M). Legends are the same as those shown in Fig. 2.

DISCUSSION

One of the objectives of the present research was to investigate the previously reported (*13,14*) negative regulatory effect of Mn on LiP production. When high Mn(II) levels were added to the media, LiP was repressed as long as Mn was in solution. However, as pellets in the

cultures darkened owing to the disproportionation of Mn(III) to MnO_2, LiP was produced. That disproportionation of Mn(III) was avoided by adding organic acids to the cultures. Organic acids stabilize Mn(III) by forming various ill-defined complexes (*16,21*). The influence of several organic acids and pyrophosphate, and presence of high levels of Mn(II) or Mn(III) on the veratryl alcohol oxidation were assayed (the most common assay for LiP activity). As previously reported (*22,23*), oxalic acid inhibits veratryl alcohol oxidation. Malonate was chosen because it does not have any effect on this activity and because the malonate–Mn(III) complex is able to oxidize free phenolic lignin structures (*10*). Malonic acid was also described as the ligand most able to form Mn(II)/Mn(III) complexes from among the α-hydroxy acids assayed (*21*). However, it was observed that LiP is not stable in the presence of Mn(II) and, more significantly, in the presence of Mn(III). Therefore, it was not clear that the absence of LiP was attributable to repression. In order to determine whether the absence of LiP activity was the result of the destabilization of the enzyme by manganese or the repression of protein formation, the proteins were studied in supernatant solutions.

Isoenzyme profiles of low-Mn cultures with and without malonate were very similar to the isoenzymes described by other authors (*13,17*). In low-Mn, malonate-free cultures, good levels of mineralization were observed, and the molecular size distribution of DMF solubles showed a high rate of depolymerization on day 7. A similar profile was obtained on day 8. In low-Mn cultures with malonate, neither an increase in LiP nor mineralization of DHP was observed as compared to low-Mn, malonate-free cultures. The molecular size distribution of DMF solubles showed a shift from the highest-molecular-weight compounds toward low-molecular-weight compounds. This shift was presumably owing to the MnP activity, because a similar result was obtained in cultures with high Mn wherein no LiP was detected. This initial shift in size distribution is only attained in the presence of organic acid. These results are consistent with the stabilization of Mn(III) by the organic acid, so that the complex can serve as a diffusable oxidant (*24*). The accumulation of mycelial-bound DHP degradation products in the presence of high Mn(III) and the rapid conversion of these mycelial-bound products to CO_2 following disproportionation of Mn(III) suggests that MnP-generated Mn(III) properly chelated with an organic acid carries out the first depolymerization of the highest-molecular-weight compounds. Because the disappearance of complexed, soluble Mn(II)/Mn(III) corresponds with the appearance of LiP, these results are consistent with this complex acting as the effective repressor of LiP.

When high levels of Mn(II) were added to malonate-free cultures, a repression of all LiP isoenzymes and a significant increase of MnP were observed on day 7. At this point, the Mn(II)/Mn(III) complex is still in solution. Only 5% of mineralization had been attained, and no depolymerization was observed. About 50% of the ^{14}C was bound to the mycelia.

On day 8, when Mn(III) had precipitated as MnO_2, LiP isoenzymes appeared rapidly, and the isoenzyme profile resembled those obtained with low Mn. This onset of LiP activity paralleled by mycelial darkening was previously studied by Kern (*25*). He suggested that the addition of MnO_2 mimics the natural accumulation of this compound in areas of lignin biodegradation (*26*). The findings presented here support Kern's suggestion that MnO_2 produced in cultures results from disproportionation of Mn(III) formed on enzymatic oxidation of Mn(II) by MnP, but do not support his conclusion that MnO_2 stabilizes LiP in cultures. It is proposed that the onset of LiP is mainly owing not to the presence of MnO_2, but to the disappearance (or absence) of soluble Mn(II) or Mn(III) complexes. The onset of LiP, on day 8, directly correlated with an increase in mineralization and depolymerization, and decrease in binding to mycelia compounds. These results support previous suggestions that binding precedes degradation (*19*) and that the bulk of the bound DHP consists of partially degraded reactive materials. It may be possible that the uptake of the lignin subproducts occurs when Mn is present at very low levels. This hypothesis is supported by the fact that low-molecular-weight compounds are always detected at low-Mn levels. In cultures containing high Mn and malonate, neither this derepression of LiP nor increase of mineralization or depolymerization was observed.

The present results are therefore consistent with MnP being formed in the presence of Mn(II) and chelated Mn(III) carrying out initial depolymerization of the highest-molecular-weight lignin compounds. Those products are not taken up into the cells. Rather, they remain bound to the mycelia. The chelator is gradually oxidized by Mn(III), and Mn(III) undergoes disproportionation to MnO_2 (*27*). LiP isoenzymes are derepressed rapidly once the Mn(II)/Mn(III) complex has disappeared. This onset correlates with the onset in depolymerization to the lowest molecular products, the release of $^{14}CO_2$ owing to mineralization, and to the observed decrease in bound mycelia compounds.

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