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Roles of Manganese and Organic Acid Chelators in Regulating Lignin Degradation and Biosynthesis of Peroxidases by *Phanerochaete chrysosporium*

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We studied the effect of manganese and various organic chelators on the distribution, depolymerization, and mineralization of synthetic ^{14}C-labeled lignins (DHP) in cultures of *Phanerochaete chrysosporium*. In the presence of high levels of manganese [Mn(II) or Mn(III)], along with a suitable chelator, lignin peroxidase (LiP) production was repressed and manganese peroxidase (MnP) production was stimulated. Even though partial lignin depolymerization was observed under these conditions, further depolymerization of the polymer to smaller compounds was more efficient when low levels of manganese were present. LiPs were prevalent under these latter conditions, but MnPs were also present. Mineralization was more efficient with low manganese. These studies indicate that MnP performs the initial steps of DHP depolymerization but that LiP is necessary for further degradation of the polymer to lower-molecular-weight products and mineralization. We also conclude that a soluble Mn(II)-Mn(III) organic acid complex is necessary to repress LiP.

Manganese is present in virtually all woody tissues. It is most abundant in barks, where its concentration ranges between 46 and 1,305 ppm, but it is also prevalent in heartwood at concentrations between 5 and 221 ppm (32). Mn is interesting in that it possesses seven positive oxidation states, some of which are potentially strong oxidants (27). The chemistry of Mn has been reviewed elsewhere (24).

Manganese appears to play an important role in biological lignin degradation. It is essential for the activity of manganese peroxidase (MnP), an enzyme implicated in the depolymerization of lignin (31), and it regulates the production of MnP (3, 6), laccase, and lignin peroxidase (LiP) in *Phanerochaete chrysosporium* and in other white rot fungi (22). MnP oxidizes Mn(II) to Mn(III); the latter species is chelated by organic acids, and the resulting Mn(III) complex diffuses away from the enzyme active site to act as a diffusible oxidant (13, 28). Certain organic acids are important in MnP-catalyzed reactions. α-Hydroxy acids stabilize Mn(III) and also appear to facilitate the dissociation of Mn(III) from the MnP active site, thus stimulating the enzyme activity (29). Mn(II) is also an essential component of the MnP catalytic cycle: although other donors can reduce compound I of LiP to compound II, only Mn(II) is capable of reducing MnP compound II to its original state, thereby allowing the enzyme to initiate new substrate oxidations (28, 29).

Manganese is also involved in several reactions catalyzed by LiP. Popp et al. (23) reported the oxidation of Mn(II) to Mn(III) by LiP in the presence of veratryl alcohol and oxalic acid, both of which are natural metabolites of *P. chrysosporium*. The oxidation of veratryl alcohol by LiP is enhanced by Mn(II), but only in the presence of an certain organic acid, which is a potential chelator of Mn(III) (5). At pH 3, with lactate buffer, 100 μM Mn(II) enhances the oxidation of veratryl alcohol. The formation of Mn(III)-lactate complex is detected at the end of this reaction. Manganese does not have such an effect in glycine-HCl or sodium acetate buffer. Bono et al. (5) hypothesized that these results imply complexation of Mn(II) by lactate. In contrast to these findings, 100 μM of Mn(II) did not influence the oxidation of veratrylglycerol β-guaiacyl ether by LiP. Schmidt et al. (25) observed a pronounced decrease in the formation of ring cleavage compounds in the oxidation of 3,4-dimethoxybenzyl methyl ether in the presence of 0.2 and 1 mM of Mn(II). Such effects were more pronounced as the pH was increased from 3 to 5.

Biomimetic studies have been done to clarify the role that manganese plays in lignin biodegradation. Forrester et al. (11) reported that Mn(III) is a ligninolytic agent but that it requires a suitable chelator. In addition, colloidal Mn(III) acetate is able to oxidize a nonphenolic diarylpropane model and veratryl alcohol, leading to a reduction of Mn(III) to Mn(II) (15). Chemically generated Mn(III) complexes such as malonate or Mn(III) lactate have been shown to oxidize phenolic MnP substrates (12, 30). Furthermore, electrochemical studies have demonstrated that several organic acid complexes of Mn(III) are able to oxidize lignin model compounds (8). These investigators suggest that the stability of the manganese complexes would be an important key in their role as effective mediators.

Two recent reports have demonstrated that either MnP or LiP can depolymerize lignin in vitro (14, 31), but the roles that individual enzymes play in in vivo degradation have not yet been clarified.

We previously reported that manganese has an important role in mineralizing synthetic lignins (16). The highest levels of mineralization appear in the absence of Mn(II). We observed that with *P. chrysosporium* and other white rot fungi, the addition of high levels of Mn(II) reduces the LiP titer and increases MnP. In addition, mineralization rates decrease when Mn and MnP levels increase (3). All our previous studies were performed with tartrate buffers. In those studies, small but significant changes in medium pH occurred after the addition of Mn(II). This observation prompted us to investigate further the role of manganese ion

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and organic acids (potential chelators) in enzyme production, lignin depolymerization, and mineralization.

We now report a regulatory effect of soluble Mn ions on LiP production. MnP-generated Mn(III) can be stabilized by using organic acids as chelators. In cultures without any chelator, LiP is repressed only until manganese precipitates as MnO_2. In contrast, when a chelator is added, LiP is repressed as long as the Mn(II)-Mn(III) complex is in solution. Conversely, MnP is stimulated by chelators in the presence of high levels of Mn(II).

MATERIALS AND METHODS

Organism and culture conditions. *P. chrysosporium* BKM-F 1767 (ATCC 24725) was used for all studies. Cultures were maintained on yeast extract-malt extract-peptone glucose slants at 39°C (3).

For mineralization and regulatory studies, *P. chrysosporium* was cultivated in basal medium containing the following per liter of medium: 10.0 g of glucose, 2.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $CaCl_2$, 1 mg of thiamine, 0.5 g of Tween 20, 2.5 mM veratryl alcohol, 20 mM polyacrylic acid (PAA) (2,000 molecular weight; Aldrich) adjusted to pH 4.5 with KOH, and 70 ml of trace elements (3). Filter-sterilized medium (40 ml) was dispensed into sterile rubber-stoppered 125-ml flasks and inoculated with 5.5×10^4 conidiospores per ml. Cultures were incubated at 39°C with shaking at 180 rpm (2.5-cm-diameter stroke). After 48 h of growth, cultures were flushed daily for 1 min with pure oxygen (gas flow rate, 6.5 liters/min). Mn(II) was added as $MnSO_4$ after 48 h of growth. The levels of Mn(II) present in the medium after addition were as follows: low Mn(II), 6 μM (0.3 ppm); and high Mn(II), 720 μM (40 ppm).

The indicated concentrations of organic acids were added to the described medium after 48 h of growth. In every case, organic acids were added as the sodium salt adjusted to pH 4.5.

All buffers were added as their sodium salts at pH 4.5 before inoculation, except for PAA, which was added as the potassium salt.

Enzyme purification and assays. To determine enzyme profiles, we grew 750-ml cultures in 2-liter flasks at 39°C and 120 rpm. Cultures were harvested on days 7 and 8. Mycelial pellets were filtered and extracellular fluid was concentrated by ultrafiltration (Amicon YM 10; 10,000-M_r cutoff). Samples were partially purified in a QMA anion-exchange column (Waters) equilibrated with 10 mM sodium acetate (pH 6). Proteins were eluted with a gradient of 0.6 M sodium acetate (pH 6). Collected fractions were dialyzed against 10 mM sodium acetate (pH 6) and concentrated with an ultrafiltration membrane (Centricon 10; 10,000-M_r cutoff). Final volumes were 1 ml in samples collected on day 7 and 2 ml in samples collected on day 8.

Isoenzymes were analyzed by fast protein liquid chromatography (FPLC) (10). Because more protein was formed in the presence of malonate, sensitivity of the detector was lowered to one-half of the level used on cultures grown without malonate. A_{280} (for protein) and A_{409} (for heme group) were monitored, but only A_{280} profiles have been illustrated to make the results easier to interpret.

LiP and MnP activities were assayed in control cultures identical to experimental cultures. To avoid any problems with gratuitous induction, cold dehydrogenative polymerizate (DHP) (0.055 mg per flask) was added to cultures not receiving labeled DHP.

LiP was measured as described by Tien and Kirk (26) and MnP as described by Paszczynski et al. (21). From day 4 or 5 up to day 8 or 9, an initial decrease at 310 nm was monitored for at least 2 min in supernatant solutions having high Mn(II) levels.

Protein assays. Proteins were measured by Bio-Rad protein assays.

Lignin degradation studies. [^{14}C]DHP mineralization. Assays of lignin-oxidizing activity were conducted in triplicate under the conditions specified above. Synthetic lignin produced from ^{14}C-ring-labeled coniferyl alcohol (19) was added to each flask on day 2 as an aqueous suspension (~50,000 dpm; 55 μg per flask). Production of CO_2 was monitored as described by Keyser et al. (18).

DHP depolymerization. On the harvesting day, and after $^{14}CO_2$ had been trapped, the pHs of the cultures were adjusted to 2.0 to 2.5 with 2 N HCl. Dioxane (1:1 [vol/vol]) was added, and the cultures were agitated at 25°C overnight. Mycelial pellets were separated by centrifugation, and 1 ml of each supernatant solution was used to measure the dioxane-water DHP solubles. Mycelia were solubilized in 5 ml of TS-1 tissue solubilizer (RPI Corp., Elk Grove, Ill.) and incubated at 50°C overnight. Radioactivity in pellets was determined by scintillation counting after adding 5 ml of toluene solution (7, 9). The efficiencies of the solutions were corrected individually by adding internal standards.

Dioxane-water solutions were adjusted to between pH 6.5 and 7 and vacuum evaporated at 35°C. *N,N*-Dimethylformamide (DMF) (2 ml) was then added to the flask, which was shaken to remove the DMF solubles. Flasks were then rinsed with water-HCl (2.5 ml) to remove the insoluble materials, and an aliquot was taken to measure the radioactivity.

Molecular size distribution of the DMF solubles was determined by fractionating the residual lignin on a column (28 by 2.5 cm) of Sephadex LH-60 plus LH-20 (mixed 1:1) (20). DMF-LiCl (0.1 N) was used to elute the samples (14). Fractions (2 ml) were collected, and radioactivity was determined in each fraction by using Poly-Fluor (Packard) as the scintillation cocktail. Polystyrenes were used for calibrating the column, except that veratraldehyde was used as the 166-molecular-weight marker.

RESULTS

Enzymatic studies. The effects of buffers, pH, and organic acids on the enzyme activities and the expression of the isoenzymes were studied.

(i) Effect of buffers and pHs on enzyme expression. In repeating experiments previously reported (3, 22), we observed pH changes of 0.5 to 1.0 unit in tartrate-buffered medium after the addition of high levels of Mn(II) to the cultures. The observed shift in pH from 4.5 to pH 5.2 and 5.5 did not affect cell growth or MnP activity, but in those cultures, LiP activity was not detected.

We decided to assess the most common buffers used for the cultivation of *P. chrysosporium*. These included the following: 2,2-dimethylsuccinic acid (DMS) (20 mM), trans-aconitic acid (TAA) (10 mM), sodium acetate (20 mM), and PAA (20 mM).

With DMS and TAA, pHs were well maintained. High Mn(II) levels delayed the appearance of LiP activity by 1 day compared with low levels of Mn(II). MnP titers were elevated in the presence of either of the buffers when high Mn(II) was used, but the enzyme reached a peak on day 5 and then decreased drastically (Table 1). With Na acetate buffer, the pH changed in the presence of low and high

TABLE 1. Production of LiP and MnP in different buffers

Buffer (mM)	Mn(II)	nmol/min/ml on day:					
		5		6		7	
		LiP	MnP	LiP	MnP	LiP	MnP
TAA (10)	Low	253	280	394	460	386	222
	High	0	1,211	238	802	350	222
DMS (20)	Low	193	112	232	169	309	170
	High	184	260	347	80	356	73
Na acetate (20)	Low	39	44	92	20	150	20
	High	0	552	0	810	0	608

Mn(II), probably because of the metabolization of acetate by the fungus, but LiP remained repressed and MnP remained induced at high Mn levels (Table 1).

We chose 20 mM PAA for further experiments because the pH was well maintained in this buffer and the enzymes were more stable. In PAA medium with high Mn(II), the pH decreased to between 4.1 and 4.2; with low Mn(II), the pH ranged between 4.3 and 4.5. Results from enzyme activities in PAA buffer are detailed in subsequent figures.

We observed that when 40 ppm Mn(II) was added to the media containing TAA, DMS, or PAA buffer, the initial appearance of LiP activity occurred immediately after a darkening of the mycelial pellets. This darkening was attributed to precipitation of MnO_2. Such precipitation was observed only in the pellets and never in the extracellular medium. The diagnostic tests used to identify the brown color as MnO_2 were (i) production of bubbles in the presence of H_2O_2, (ii) formation of blue color in the presence of benzidine, and (iii) disappearance of the color in the presence of hydroquinone (12). In cultures containing organic acids along with the buffer, the supernatant solutions with high Mn were orange-brown and the pellets were white. Red to red-brown is associated with Mn(III) in solution (24). The disappearance of such color from the supernatant solution and the sharp decrease in the A_{266} (data not shown) after the addition of dithionite (23) led us to conclude that the color observed in the supernatant solutions was due to Mn(III). Thus, as long as Mn(II)-Mn(III) complex remained in solution, LiP was not observed in the extracellular broth. Once Mn(III) had precipitated on the pellets as MnO_2, LiP was secreted.

When 5 or 10 mM of organic acid was added to PAA-buffered medium along with high Mn levels, the pH increased to between pH 5.2 and 5.3, and repression of LiP was observed. Since no such shift in pH was observed upon the addition of organic acid to low-Mn(II) cultures, we concluded that the shift was attributable primarily to the oxidation of organic acids by Mn(III).

To clarify whether the pH shift observed was responsible for LiP repression, we assayed supernatant solutions from cells cultivated at different pHs (Fig. 1a). LiP activity was not repressed by elevated pH in the presence of either low or high Mn alone (i.e., without buffer). At an initial pH of 5.0, the pH of the cultures rose from 4.9 to 5.1 with low or high Mn. At an initial pH of 4.5, the pH ranged from 4.1 to 4.4 with low Mn and from 4.0 to 4.4 with high Mn. With high levels of Mn, LiP production was delayed while the Mn(II)-Mn(III) complex was in solution (Fig. 1a), but precipitation of MnO_2 occurred rapidly, and LiP was formed soon thereafter. Three-times-higher levels of MnP were detected at pH 5 than at pH 4.5 (Fig. 1b). Thus, we observed that the pH alone did not repress LiP and that high levels of Mn(II) alone did not cause dramatic changes in pH.

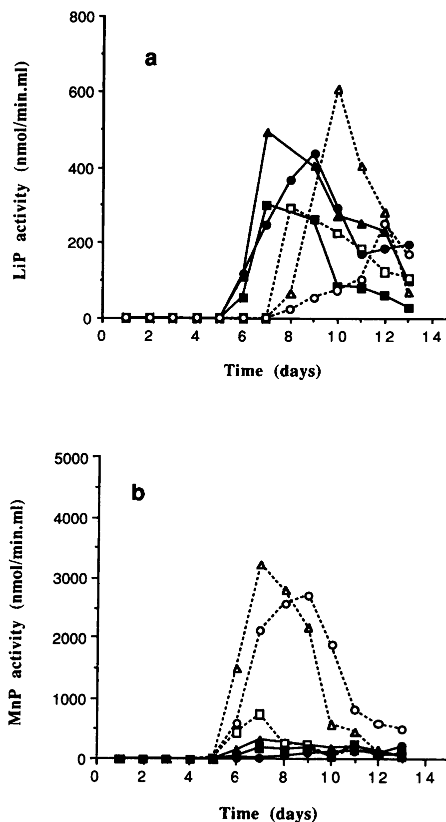


FIG. 1. Production of LiP (a) and MnP (b) by *P. chrysosporium* grown in PAA buffer (20 mM) at pH 4.5 with low Mn (——■——) or high Mn (- - -□- - -); in PAA at pH 5.0 with low Mn (——▲——) or high Mn (- - -△- - -); or in PAA plus malonate (10 mM) and low Mn (——●——) or high Mn (- - -○- - -).

(ii) Effect of malonate on enzyme expression. We studied the influence of a recognized Mn(III) chelator, malonate, on manganese solubility and enzyme activity. When 10 mM Na malonate was added to medium, MnP activity increased and LiP activity decreased. In a control without malonate but with high Mn, the appearance of LiP was delayed for 2 days while Mn(III) remained in solution. When malonate was added, the delay was longer. After this delay, LiP activity increased by the end of the experiment (12 days). At the same time, the fungal pellets turned brown because of the eventual consumption of malonate by Mn(III) oxidation and the disproportionation of Mn(III) to Mn(IV) oxide (discussed below) (Fig. 1a).

(iii) Effect of malonate and manganese on isoenzyme stability. To stabilize the Mn(III) generated by MnP, we grew cells with PAA (20 mM) for 48 h; 5 mM of malonate was then added along with low and high levels of Mn(II). Control cultures were grown with high and low levels of Mn in the absence of malonate. A complete set of four cultures each was harvested on days 7 and 8. This timing of the harvesting coincided with the transition from Mn(III) in solution to MnO_2 precipitated on the pellets.

The FPLC isoenzyme profiles obtained from low-Mn, malonate-free supernatant solutions on days 7 and 8 are shown in Fig. 2a and b. The addition of 5 mM malonate to

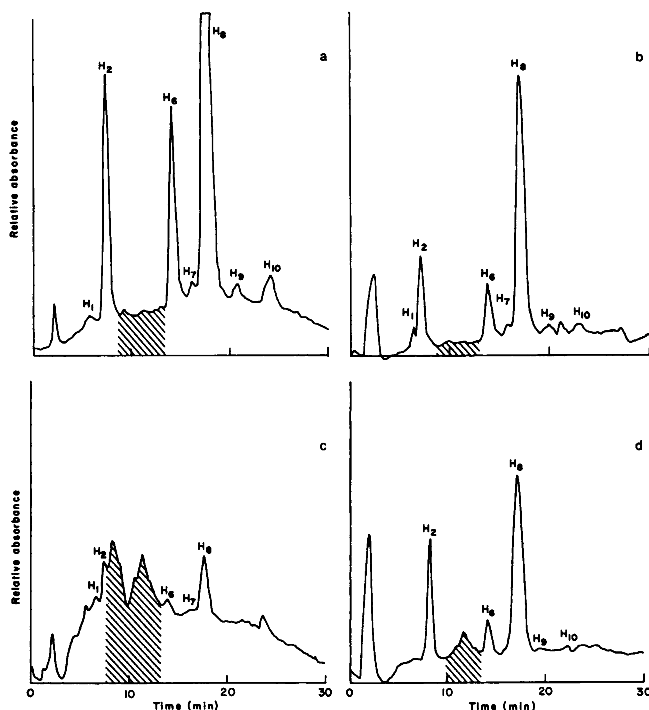


FIG. 2. Isoenzymes from extracellular cultures of *P. chrysosporium* grown with low Mn (a and b) or high Mn (c and d) in PAA buffer. Cultures were harvested on day 7 (a and c) or day 8 (b and d). Shaded areas indicate MnP isoenzymes. The pH of cultures was 4.2 on the day of harvest. Protein levels injected to the column were 255 μg (a), 88 μg (b), 80 μg (c), and 70 μg (d).

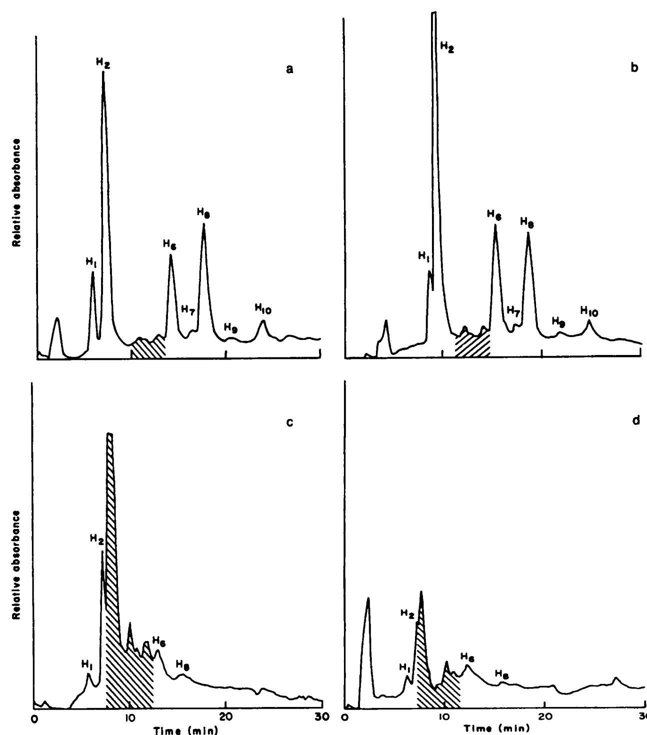


FIG. 3. Isoenzymes from extracellular cultures of *P. chrysosporium* grown with low Mn (a and b) or high Mn (c and d) in PAA buffer plus malonate (5 mM). Cultures were harvested on day 7 (a and c) or day 8 (b and d). Shaded areas indicate MnP isoenzymes. On the day of harvest, cultures with low Mn had pH 4.7 and cultures with high Mn had pH 4.9. Protein levels injected were 363 μg (a), 180 μg (b), 343 μg (c), and 330 μg (d). Because more protein was injected, the absorbance sensitivity was lowered to one-half of that shown in Fig. 2.

cultures receiving only low levels of Mn(II) had little effect on the isoenzyme profiles (Fig. 2a and b and 3a and b). However, some differences were observed: isoenzyme H_2 predominated in cultures receiving malonate, whereas H_8 was prevalent in control cultures. Moreover, malonate stimulated production of all LiP and MnP isoenzymes.

When a high level of Mn(II) was added to the medium without malonate, the FPLC profiles changed drastically in cultures harvested on days 7 and 8. On day 7 (Fig. 2c), isoenzymes H_2, H_6, H_7, H_8, and H_{10} were repressed in high-Mn, malonate-free medium, while MnP isoenzymes were elevated. On day 8 (Fig. 2d), after the Mn(III) had precipitated in the pellets, the profiles from supernatant solutions of high-Mn cultures greatly resembled the profiles of supernatant solutions from low-Mn, malonate-free cultures (Figs. 2b and d). Specifically, the LiP isoenzymes H_2, H_6, and H_8 increased quickly and the MnP isoenzymes decreased. These changes in isoenzyme profiles observed before and after precipitation of MnO_2 were not attributable to changes in pH because the pH remained stable throughout. The increase in LiP isoenzyme from day 7 to day 8 was not observed in those cultures in which Mn(III) was stabilized by the addition of malonate (Fig. 3c and d). These dramatic changes in isoenzyme profiles corresponded with the precipitation of Mn as MnO_2, further implicating the pivotal role of this event.

(iv) Effect of other chelators on enzyme production. Malonate was not unique in its ability to stabilize Mn(III), repress LiP, and increase MnP production. A number of other carboxylic acids had similar effects. When lactate, malate, tartrate, citrate, or oxalate (5 mM each) was added on day 2 to the medium containing 20 mM PAA, LiP was repressed and MnP was induced, as can be observed in Fig. 4a and b. Each mono-, di-, or tricarboxylic acid was able to stabilize the Mn(III) and consequently affect production of the peroxidases.

Degradation studies. (i) Effect of pH, manganese level, and malonate on DHP mineralization. When low levels of Mn were added to the cultures, good mineralization rates were attained in all cases. In controls with pH 5.0, delays in MnO_2 precipitation and in DHP mineralization were observed. This delay was observed also in LiP production (Fig. 1a and 5). In controls with high Mn and pH 4.5, a delay of 2 days was observed, but after that time, the same levels of mineralization were reached. With malonate and high Mn, only 17% mineralization was reached on day 12. In contrast, 40% mineralization was reached with malonate and low Mn.

(ii) Effect of different chelators on DHP mineralization. In the presence of three different organic chelators (5 mM) plus PAA (20 mM), the mineralization rate decreased when high Mn was present. The organic acids assayed were oxalate, tartrate, and citrate. In each case, a decrease in $^{14}CO_2$ release correlated with a decrease in LiP activity and an increase in MnP activity. The mineralization rates after 12 days oscillated between 45 and 40% when low Mn plus organic acid was added and between 8 and 11% with high Mn and organic acid (data not shown).

(iii) Molecular weight distribution of degraded DHP. To

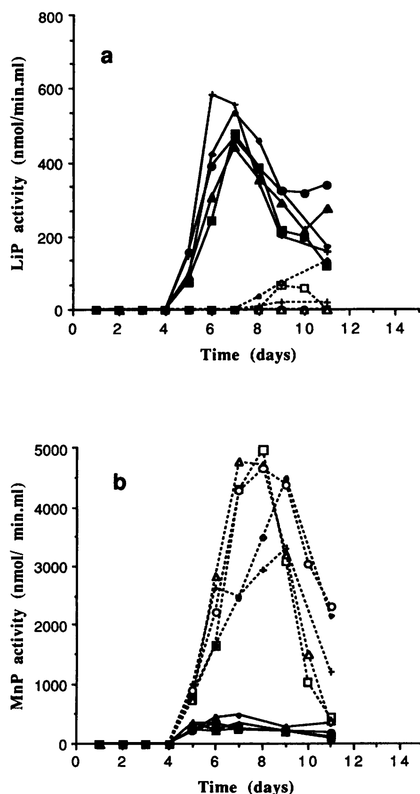


FIG. 4. Production of LiP (a) and MnP (b) by *P. chrysosporium* in PAA buffer (20 mM) plus 5 mM different organic acids: oxalate, low Mn (——■——) and high Mn (---□---); citrate, low Mn (——▲——) and high Mn (---△---); tartrate, low Mn (——●——) and high Mn (---○---); lactate, low Mn (——◆——) and high Mn (---◇---); malate, low Mn (——+——) and high Mn (---+---).

determine whether the dramatic changes in protein profiles correlated with differences in the depolymerization patterns observed with DHP, cultures receiving high or low levels of Mn, with or without 5 mM malonate, were incubated with ^{14}C-labeled DHP and harvested on days 7 and 8. The ^{14}C was recovered as $^{14}CO_2$ (mineralization) and, in DMF-soluble and DMF-insoluble fractions, along with the mycelia (Table

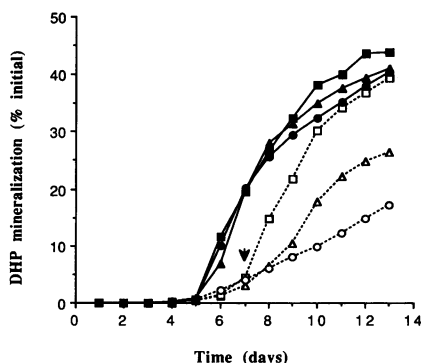


FIG. 5. DHP mineralization by *P. chrysosporium* grown in PAA buffer to two different pHs (4.5 and 5.0) and PAA (pH 4.5) plus malonate (10 mM) and low or high Mn levels. Symbols are the same as those used in Fig. 1.

TABLE 2. Distribution of radioactive DHP in cultures

Culture	Distribution (% initial)			
	Mineralization	Mycelia	DMF insolubles	DMF solubles
PAA, low Mn, day 7	33.5	3.7	9.9	23.2
PAA, high Mn, day 7	5.1	51.4	6.0	18.4
PAA, high Mn, day 8	16.3	31.8	5.2	22.3
PAA, high Mn, day 12	39.2	5.0	10.9	17.3
Malonate, low Mn, day 7	25.4	6.2	13.6	16.4
Malonate, high Mn, day 7	6.3	50.0	9.4	15.1
Malonate, high Mn, day 8	5.6	51.1	10.2	13.3
Malonate, high Mn, day 12	17.2	26.1	8.6	20.8
Control[a]	0	8.7	22.2	34.9

[a] DHP was added on day 6 after cells were killed by autoclaving.

2). The data from days 7 and 8 in low-Mn(II) cultures were similar, and for the sake of simplicity, only day 7 is shown in Fig. 6. The biggest difference among these four conditions was that in cultures receiving only low Mn, the largest fraction of the recovered radioactivity was mineralized, whereas in cultures receiving high Mn, the largest recovered fraction was bound to the mycelium.

There were also dramatic differences in the molecular size

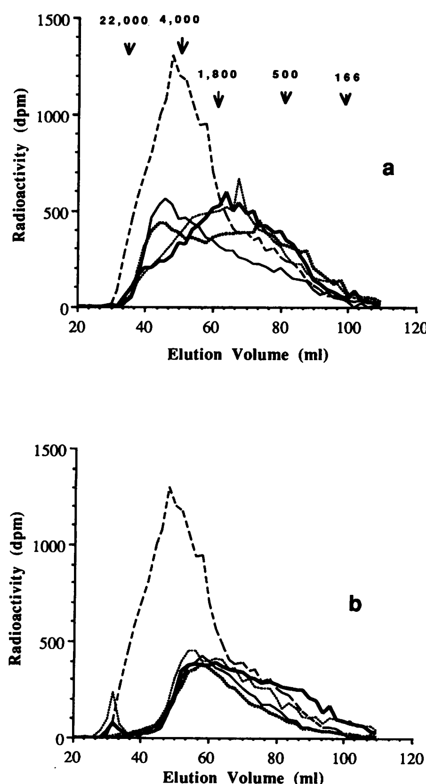


FIG. 6. Depolymerization of DHP in cultures of *P. chrysosporium* grown in PAA buffer (20 mM) (a) and PAA buffer plus malonate (b). Cultures with high Mn were harvested on day 7 (——), day 8 (········), and day 12 (·········). Cultures with low Mn were harvested the same days. Because the depolymerization profiles were very similar, for the sake of simplicity, only day 7 is shown (——). The DHP was added to the control culture on day 6 after the cells were killed by autoclaving. Control cultures were harvested on day 8 (------).

distributions of the DMF-soluble fractions. These were apparent not only between the DHP samples incubated with cultures receiving high and low Mn, but also between cultures having only 20 mM PAA present as the buffer and those receiving PAA plus 5 mM malonate.

When DHP samples were sized from cultures with high Mn but no malonate, a progressive decrease in the molecular size distribution pattern was observed with samples incubated for 7, 8, and 12 days, i.e., from just before MnO_2 precipitation until several days later. The final sample showed similarities to the sample taken from the corresponding low-Mn culture at day 7 (cf. Fig. 6a and b).

The molecular size distribution of DMF solubles in cultures containing malonate, regardless of whether low or high Mn was employed, showed a shift from highest-molecular-weight to lower-molecular-weight compounds, compared with that of samples without malonate. However, in cultures without malonate, the largest amount of low-molecular-weight products was also detected in low-Mn(II) cultures. In contrast to cultures without chelator and high Mn, when malonate chelated the high levels of MnP-generated Mn(III), depolymerization remained the same on days 7 and 8. Depolymerization began on day 12, but did not reach that obtained with low Mn(II) levels (Fig. 6b).

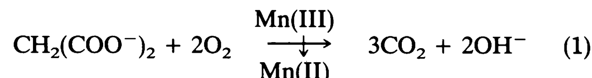
DISCUSSION

The original objective of the present experiments was to use Mn as a regulator of LiP and MnP expression in vivo and to observe the effects of these cultivation conditions on the size distribution of residual DHP. While repeating our earlier experiments (3, 4), we observed significant changes in the pHs of the various cultures. We therefore decided to use other buffers in which the pH was well maintained. Surprisingly, we noticed that with some of these other buffers, Mn(II) only delayed the appearance of LiP until MnO_2 had precipitated in the pellets. After that time, LiP activity was detected at even higher levels. This onset of LiP activity paralleled by mycelial darkening was previously studied by Kern (17). He observed that when dark mycelia were resuspended in fresh medium containing ammonium tartrate, the pellets turned white and the secretion of the enzymes ceased until the mycelia became dark again. Using two different analytical methods, Kern (17) detected high concentrations of Mn together with low amounts of iron and cobalt at the periphery of the pellets. No accumulation of Mn in cells inside the pellets was detected. He suggested (17) that the addition of MnO_2 mimics the natural accumulation of MnO_2 observed in areas of lignin degradation by white rot fungi (2). Kern (17) also suggested that MnO_2 plays an important role in lignin biodegradation by shielding LiP from damage by H_2O_2 in the vicinity of protein secretion.

Our findings support Kern's suggestion (17) that the MnO_2 produced in cultures results from disproportionation of Mn(III) formed upon enzymatic oxidation of Mn(II) by MnP. Our findings are consistent with the hypothesis that the further disproportionation of Mn(III) to Mn(IV) plus Mn(II) occurs spontaneously whenever a suitable chelator is not present to stabilize Mn(III). We observe here that it is the disappearance (or absence) of soluble Mn(II) or Mn(III) complexes from the culture that enables the derepression of LiP. We further hypothesize that whereas Mn(II) is the apparent inducer of MnP, simultaneous repression of LiP is due to the presence of soluble Mn(II) or Mn(III) complexes of simple organic acids.

Because one of the objectives of the present research was to investigate the regulatory effect of Mn on LiP production, we decided to avoid the disproportionation of Mn(III) to MnO_2 by adding organic acids to the medium. Organic acids stabilize Mn(III) by forming various ill-defined complexes (8, 24). We chose malonic acid in our studies because the malonate-Mn(III) complex is able to oxidize free phenolic lignin substructures (30). Malonic acid was also described as the most efficient ligand from among the α-hydroxy acids assayed (8).

When the chelators were added, the red color of the supernatant solution was stable for a longer time and the darkening of the pellets was delayed. This was an obvious consequence of Mn(III) stabilization. However, the organic acids may themselves be oxidized by the Mn(III) ions which they coordinate. The pH of the cultures increased to between 5.3 and 5.4 when organic acids were added. Conversely, in the plain buffer, in the presence of Mn, the pH decreased to between 4.2 and 4.3. The explanations for these changes differ. Empirical experiments with trivalent manganese have shown that the metal is able to oxidize organic acids such as malonic acid in the presence of oxygen according to the following equation:



$$CH_2(COO^-)_2 + 2O_2 \xrightarrow[\text{Mn(II)}]{\text{Mn(III)}} 3CO_2 + 2OH^- \quad (1)$$

In addition, Mn(III) can be regenerated by MnP according to:

$$2H^+ + 2Mn(II) + H_2O_2 \xrightarrow{\text{MnP}} 2Mn(III) + 2H_2O_2 \quad (2)$$

Reaction 2 is catalytic; therefore, the organic acid oxidation leads to a continuous increase in the pH, even in the presence of buffer. Consequently, the Mn(III)-organic acid complex is gradually decomposed because of the oxidation of the ligand (24, 27). Probably the decomposition of the complex is due in part also to its oxidation by H_2O_2 (1). In our experiment, this slow decomposition led to a slow increase of LiP activity (Fig. 1a) and an apparent mineralization of the DHP (Fig. 5). Conversely, in the absence of a suitable ligand, the manganic ion formed as consequence of MnP activity undergoes a fast disproportionation according to the following reaction:

$$2Mn(III) + 2H_2O \leftrightarrow Mn(II) + MnO_2 + 4H^+ \quad (3)$$

This reaction leads to an insignificant decrease in the pH (to 4.2 to 4.3) and to a drastic onset of LiP production and mineralization.

FPLC profiles of low-Mn cultures with and without malonate were very similar, and all the isoenzymes described by other investigators (10) are present. H_8 is the predominant isoenzyme in cultures without malonic acid, as it is in cultures with PAA as the buffer (10). However, H_2 is the major isoenzyme in cultures containing malonate as it is in cultures containing tartrate (22).

FPLC profiles of high-Mn cultures harvested on day 7 showed an increase in MnP isoenzymes and repression of LiP. When malonate was used as the Mn(III) chelator, the profiles resembled those reported by other investigators (3, 22).

The necessity of a suitable chelator to stabilize the enzyme-generated Mn(III) and to facilitate the diffusion of Mn(III) away from the active site (29) is supported by these experiments because the depolymerization presumably resulting from the MnP was observed only in cultures containing malonate.

Regarding the size distribution of DHP, in cultures in which only about 5% mineralization was attained, about 50% of the DHP was bound to the mycelia (not extractable with dioxane-water). Chua et al. (7) have pointed out that the characteristics of this bound material are not really known and that this fraction could contain the highest-molecular-weight lignin. This possibility seems to be excluded in our experiments because when MnO_2 precipitated, the mycelium-bound lignin disappeared at the same time that mineralization occurred. Our results therefore support previous suggestions (7) that binding precedes degradation and that the bulk of the bound DHP consists of partially degraded reactive material. It is possible that uptake of the lignin subproducts occurs only when Mn is present at very low levels. The fact that more low-molecular-weight compounds are detected at low Mn levels supports this hypothesis. It is also possible that MnP modifies DHP by creating a group(s) that is able to react strongly with the extracellular glucan. This matrix has been reported to consist of a supporting structure for the retention of extracellular enzymes. This regulatory effect of Mn on LiP, MnP, and mineralization can be extended to other organic acids that are able to chelate Mn(III), as could be appreciated when other ligands were added. Our results are therefore consistent with a role for MnP performing initial depolymerization of lignin mediated by a Mn(II)-Mn(III)-organic acid complex, and then more extensive degradation of the lignin oligomers by LiP.

In summary, the present results show that as long as Mn is in solution [as a chelator-stabilized equilibrium mixture of Mn(II)-Mn(III)], repression of LiP isoenzymes and stimulation of MnP occurs. MnP isoenzymes are able to depolymerize DHP only when organic acids and manganese are present, and similar depolymerization has been observed at both low and high MnP levels. We speculate that MnP performs the depolymerization of the highest-molecular-weight products. The MnP decomposition products are not taken up into the cells and remain bound to the mycelia. The chelator is gradually oxidized by Mn(III). Subsequent reoxidation by MnP to Mn(III) results eventually in disproportionation to MnO_2. The LiP isoenzymes appear rapidly once the Mn(II)-Mn(III) has precipitated as MnO_2. This observation correlates with the onset in depolymerization to lower-molecular-weight product and subsequent mineralization of DHP by the fungus. We conclude that probably the MnO_2 deposits (2) naturally produced by MnP help to precipitate Mn(II) and that this removal of Mn facilitates more efficient degradation of lignin by LiP.

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