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Secretion of Ligninolytic Enzymes and Mineralization of ^{14}C-Ring-Labelled Synthetic Lignin by Three *Phlebia tremellosa* Strains

TAMARA VARES, OUTI NIEMENMAA, AND ANNELE HATAKKA*

Division of Microbiology, Department of Applied Chemistry and Microbiology, FIN-00014 University of Helsinki, Finland

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Production of ligninolytic enzymes by three strains of the white rot fungus *Phlebia tremellosa* (syn. *Merulius tremellosus*) was studied in bioreactor cultivation under nitrogen-limiting conditions. The Mn(II) concentration of the growth medium strongly affected the secretion patterns of lignin peroxidase and laccase. Two major lignin peroxidase isoenzymes were expressed in all strains. In addition, laccase and glyoxal oxidase were purified and characterized in one strain of *P. tremellosa*. In contrast, manganese peroxidase was not found in fast protein liquid chromatography profiles of extracellular proteins under either low (2.4 μM) or elevated (24 and 120 μM) Mn(II) concentrations. However, H_2O_2- and Mn-dependent phenol red-oxidizing activity was detected in cultures supplemented with higher Mn(II) levels. Mineralization rates of ^{14}C-ring-labelled synthetic lignin (i.e., dehydrogenation polymerizate) by all strains under a low basal Mn(II) level were similar to those obtained for *Phanerochaete chrysosporium* and *Phlebia radiata*. A high manganese concentration repressed the evolution of $^{14}CO_2$ even when a chelating agent, sodium malonate, was included in the medium.

Since the discovery of lignin peroxidase (LiP) in the white rot fungus *Phanerochaete chrysosporium* (44), the production of this enzyme or the corresponding activity has been reported in a number of other white rot fungi (5, 7, 11, 17, 18, 29, 31, 46–48). LiP is believed to be one of the key enzymes in lignin biodegradation by white rot fungi (22). Active delignifiers in species of the genus *Phlebia*, such as *Phlebia radiata* 79 (30) and *Phlebia brevispora* (34), secrete one to three LiP isoenzymes, one to two manganese peroxidase (MnP) isoenzymes, and, in addition, a laccase of the phenoloxidase type (EC 1.10.3.2). H_2O_2-producing activity has also been detected in *P. brevispora* (43). Since the *Phlebia* species were reported to be among the most promising fungi for biopulping (1, 4, 9, 32, 40, 41) and feed pretreatment (41), ligninolytic enzyme systems of these fungi are of special interest with respect to possible biotechnological applications. However, one species, *Phlebia tremellosa* 2845 (ATCC 48745), appeared to produce either very low levels of LiP (3) or no LiP at all (3, 5, 42), even though it is a selective lignin degrader and has been studied extensively, e.g., for improving the in vitro digestibility of aspen wood (4, 40, 41). Recently, however, production of LiP isoenzymes by *P. tremellosa* 2845 was detected in our laboratory (17). The purpose of the work presented here was to characterize in more detail ligninolytic enzymes from three other *P. tremellosa* strains since the earlier results were so controversial. In addition, since more evidence for the regulative role of Mn(II) in ligninolytic systems of white rot fungi has been obtained recently (5, 6, 34–37) and since different fungi show dissimilar responses to elevated Mn(II) concentrations (16, 37), the influence of Mn(II) on secretion patterns of extracellular lignin-modifying enzymes in *P. tremellosa* and on the ability of this fungus to degrade ^{14}C-ring-labelled synthetic lignin (dehydrogenation polymerizate [DHP]) was also examined.

* Corresponding author. Mailing address: Division of Microbiology, Department of Applied Chemistry and Microbiology, P.O. Box 27, FIN-00014 University of Helsinki, Finland. Phone: 358-0-7085279. Fax: 358-0-7085212. Electronic mail address (Internet): AHATAKKA @VIIKKI.HELSINKI.FI.

MATERIALS AND METHODS

Fungi. *P. tremellosa* (Schrad.: Fr.) Nakas. *et* Burds. (28) strains 79-16, 77-51, and 76-24 were obtained from E. Parmasto (Institute of Zoology and Botany, Estonian Academy of Sciences, Tartu). *P. radiata* 79 (ATCC 64658) and *P. chrysosporium* BKM 1767 (ATCC 24725) were cultivated as reference organisms in studies on synthetic lignin mineralization. Fungi were maintained on malt agar (2%, wt/vol) slants. Cultures were inoculated with a mycelial suspension as described previously (47).

Cultivation conditions. *P. tremellosa* strains were grown immobilized on polypropene carriers in bioreactors (working volume, 2 liters) (19) with low-nitrogen asparagine ammonium nitrate dimethylsuccinate medium (pH 4.5) (15) supplemented with 56 mM glucose, 1.0 mM veratryl alcohol, and 0.05% (wt/vol) Tween 80 at 28°C as described earlier (19). The Mn(II) levels used were as follows: (i) basal, 2.4 μM (0.12 ppm); (ii) 10-fold (or 10×), 24 μM (1.2 ppm); and (iii) 50-fold (or 50×), 120 μM (6 ppm) with 10 mM sodium malonate added (for cultivations with DHP, see below). *P. tremellosa* 76-24 was grown in 100-ml conical flasks on a rotary shaker at 180 rpm to obtain extracellular culture liquid for protein purification. The culture liquids for protein purification from strains 79-16 and 77-51 were produced in bioreactors.

Enzyme assays. Activities of lignin-modifying enzymes were determined with a Shimadzu 160A spectrophotometer as described previously (26). LiP was assayed by monitoring the oxidation of veratryl alcohol (Aldrich; vacuum distilled prior to use) in the presence of H_2O_2 at pH 3.0 (45). Laccase activity was measured with syringaldazine (EGA-Chemie) at pH 5.0, and MnP activity was monitored at 520 nm in the presence of H_2O_2 by using phenol red (Merck) at pH 4.5 (26). Control assays without Mn(II) in the reaction mixture were also carried out to verify the Mn-dependent (i.e., MnP) activity. H_2O_2-

producing glyoxal oxidase (GLOX) activity was measured in a reaction coupled with horseradish peroxidase (type II; Sigma) and expressed as nanomoles of H_2O_2 per minute per milliliter. The substrates used were methylglyoxal (Sigma) for GLOX and phenol red for horseradish peroxidase (21). LiP and laccase activities were expressed in nanokatals, and MnP activity was expressed as the increase in A_{520} (ΔA_{520}) per minute per milliliter.

Enzyme purification. Extracellular culture liquids of strains 79-16 and 77-51 were collected from 13-day bioreactor cultures supplemented with the basal Mn(II) concentration (LiP activities, 0.9 and 1.2 nkat ml^{-1}, respectively). Culture liquid of strain 76-24 was obtained from selected 14-day shake flask cultures with large mycelial pellets (17), also grown under the basal Mn(II) level (LiP activity, 1.0 nkat ml^{-1}). Extracellular proteins of *P. tremellosa* 77-51, cultivated with 50× Mn(II) and 10 mM sodium malonate, were purified from 9-day bioreactor cultures (LiP activity undetectable). Culture liquids were kept at −20°C prior to enzyme purification. Purification procedures using anion-exchange chromatography (Sepharose-Q Fast-Flow) and a fast protein liquid chromatography apparatus (Pharmacia) have been described in detail previously (46, 47). Proteins were eluted with an NaCl gradient in 25 mM sodium acetate buffer (pH 5.5).

Gel electrophoresis and IEF. Purified ligninolytic enzymes were analyzed by discontinuous polyacrylamide gel electrophoresis (PAGE; sodium dodecyl sulfate [SDS]-PAGE and native PAGE) on 16 by 20 cm 10% gels (23). Isoelectric focusing (IEF) was carried out on 0.5-mm polyacrylamide gels over a pH range of 2.8 to 5.5 obtained by using the following mixture of carrier ampholytes: 20% ampholytes with a pH range 3.5 to 10.0 and 80% ampholytes with a pH range 2.5 to 5.0 (Pharmacia LKB Biotechnology). A Pharmacia low-pI calibration kit was used as a reference protein mixture. The pH gradient was determined more precisely with a Multiphor surface electrode (Pharmacia LKB). Laccase activity in gels was visualized by staining with 0.1 M guaiacol in 0.1 M citrate-phosphate buffer (pH 5.0). LiP isoenzymes were stained with phenol red by the method adopted for MnP activity (33), except that gelatin and $MnSO_4$ were omitted from the reaction mixture. H_2O_2-producing GLOX activity was demonstrated by staining the gel with 86.2 mM sodium 2,2-dimethylsuccinate (pH 6.0) containing 10 mM methylglyoxal (Sigma), 0.031 mM *o*-dianisidine (Fluka), and 17 μg of horseradish peroxidase ml^{-1}.

Mineralization of ^{14}C-ring-labelled DHP. Synthetic lignin (DHP) with a molecular mass of 4,000 to 10,000 was polymerized from ^{14}C-ring-labelled coniferyl alcohol (8) and added as an *N,N*-dimethylformamide–aqueous suspension (1:20) to give approximately 50,000 dpm in a total volume of 10 ml per 100-ml flask. Cultures were also supplemented with 3.3 mg of nonlabelled guaiacyl-type DHP obtained from G. Brunow (Department of Chemistry, University of Helsinki, Finland) to enhance the production of ligninolytic enzymes. Stationary cultivations were carried out in quadruplicate under a 100% oxygen atmosphere at 28°C with low-nitrogen asparagine ammonium nitrate dimethylsuccinate medium supplemented with the basal Mn(II) level. The influence of Mn(II) both alone and in the presence of a chelator on DHP mineralization was studied in *P. tremellosa* 77-51 by using (i) a 50-fold concentration of Mn(II) and (ii) a 50-fold Mn(II) concentration with 5 mM sodium malonate. The evolution of $^{14}CO_2$ was monitored as described previously (15).

RESULTS

Secretion of ligninolytic enzymes. Activities of extracellular ligninolytic enzymes were monitored until days 35 to 45 of growth in cultivation with both basal Mn(II) medium and medium with a 10-fold Mn(II) level and until days 9 to 12 in bioreactors supplemented with 50× Mn(II) and 10 mM chelator (Fig. 1). It should be noted that the dynamics of enzyme activities in the fungi studied varied greatly between the strains and from batch to batch when the same strain was used. However, some general features were noted, i.e., laccase activity always appeared first and reached maximal values earlier than LiP activity, clearly decreasing with increasing LiP activity and vice versa. Correspondingly, with strong repression of LiP activity by increasing (10- and 50-fold) Mn(II) concentrations, the titers of laccase activity achieved maximal values (Fig. 1g to i).

Mn-dependent peroxidase was assayed by oxidizing phenol red in the presence of Mn(II) and H_2O_2 (Fig. 1d to f). Parallel control measurements in the absence of Mn(II) were also carried out. However, this method is not very specific for detecting MnP in crude culture liquid since other peroxidases, including LiP, and laccase (2, 39) may interfere in the assay. When *P. tremellosa* strains were cultivated under the basal Mn(II) content, phenol red-oxidizing activity (Fig. 1d to f) coincided very closely with LiP activity (Fig. 1a to c) and was not dependent on the presence of Mn(II) in the assay. In comparison with the basal Mn(II) level, the 10-fold Mn(II) content did not notably increase the phenol red-oxidizing activity in cultures, although the activity observed was mostly Mn dependent. In bioreactors with the addition of 50× Mn(II) and malonate, phenol red-oxidizing activity obviously corresponded to the laccase activity with respect to both time of expression and total titers. Taking into account these observations, we could not directly attribute to MnP the phenol red-oxidizing activity monitored in our cultivations (Fig. 1d to f). To verify the production of MnP by the fungi, purification of extracellular proteins from the 9-day culture supplemented with 50× Mn(II) and 10 mM malonate was performed as shown below.

Separation and characterization of ligninolytic enzymes. Profiles of extracellular proteins from the three *P. tremellosa* strains grown under the basal Mn(II) level were similar (Fig. 2), except that two hemeproteins containing LiP activity (H1 and H2) were detected in strains 79-16 and 76-24 (Fig. 2a and c) and only one LiP was found in strain 77-51 (Fig. 2b, H1). Lower titers of LiP activity were obtained in strain 79-16 than in strains 77-51 and 76-24 (Fig. 2). This was probably caused by an unexpected loss of activity from culture fluid in strain 79-16 after thawing. Low levels of laccase activity were detected at the elution volumes of 260 to 300 ml and 250 to 350 ml in strains 79-16 and 77-51, respectively (data not shown). No MnP activity was observed in any elution profiles. GLOX activity was not assayed in these separations.

GLOX, LiP, and laccase activities were separated from the extracellular liquid of the 9-day *P. tremellosa* 77-51 culture grown under the 50-fold Mn(II) concentration with the addition of 10 mM sodium malonate (Fig. 3). On the day of harvest, we did not observe darkening of the mycelia caused by the precipitation of MnO_2 as the result of Mn(III) disproportionation, which indicated that chelated manganese complexes were present in the solution (35, 36). Although under these conditions LiP activity was hardly detectable in the crude culture broth (Fig. 1a to c), it was detected in concentrated and dialyzed samples and eluted as hemeprotein H1 (Fig. 3a) corresponding to the H1 isoenzyme purified previously from

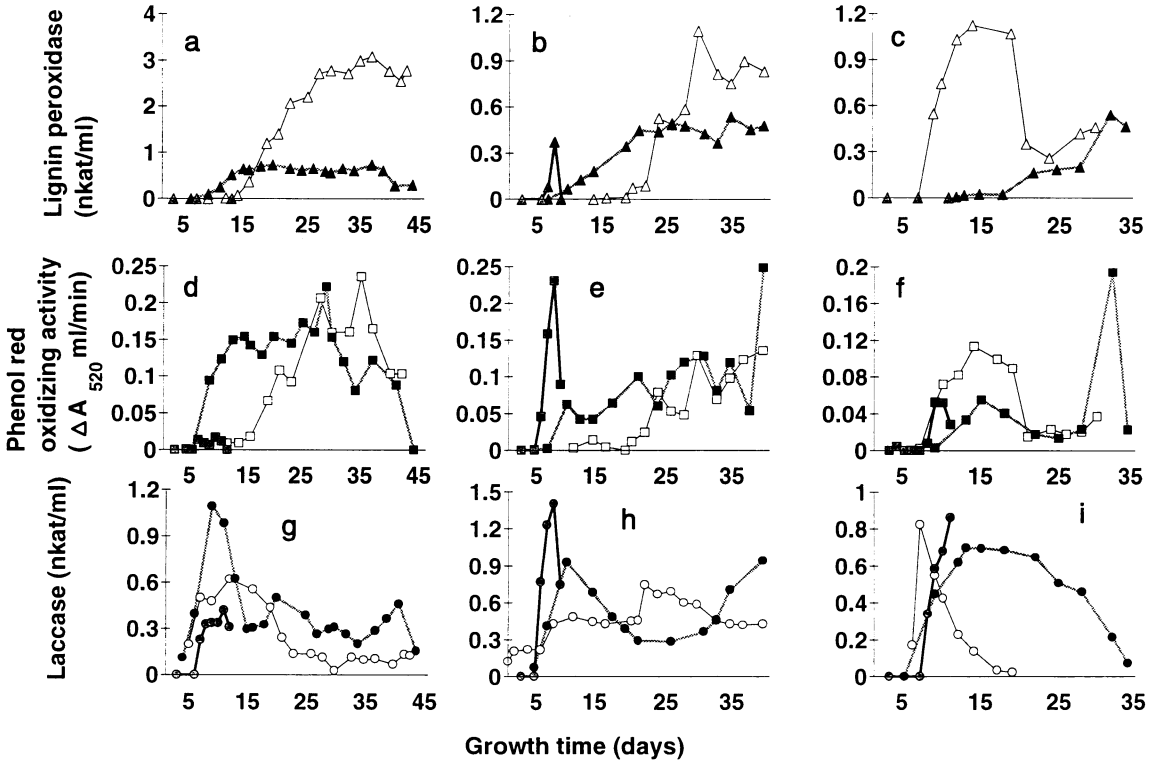

FIG. 1. Enzyme activities in cultures of *P. tremellosa* 79-16 (a, d, and g), 77-51 (b, e, and h), and 76-24 (c, f, and i) supplemented with Mn(II) at various levels. (a to c) LiP activity at basal (△), 10× (▲), and 50× (◬) Mn(II) levels; (d to f) phenol red-oxidizing activity in the presence of Mn(II) and H_2O_2 at basal (□), 10× (■), and 50× (▨) Mn(II) levels; (g to i) laccase activity at basal (○), 10× (●), and 50× (◍) Mn(II) levels.

the low-Mn(II) culture of the same strain (Fig. 2b). Evidence for MnP activity was not confirmed in this separation, despite the Mn(II)- and H_2O_2-dependent oxidation of phenol red found in crude, concentrated, and dialyzed culture liquid. Fractions with GLOX activity had relatively low protein contents and were eluted before a sharp protein peak designated P1 in Fig. 3a. Comparing profiles of extracellular proteins obtained from *P. tremellosa* 77-51 grown under low and high Mn(II) levels (Fig. 2b and 3a, respectively) revealed the presence of a new protein peak (P3) after LiP protein peak P2 (Fig. 3a), which apparently was not associated with A_{405}. Laccase activity coincided with the protein peak designated P4 in Fig. 3a.

SDS-PAGE and IEF analyses of the purified enzymes produced by the various *P. tremellosa* strains are shown in Fig. 4 and 5, and the results are summarized in Table 1. Although only one hemeprotein (H1) with LiP activity was observed in elution profiles of strain 77-51, two major protein bands with pI values of 3.75 and 3.25 (Fig. 5A, lane 4) showing H_2O_2-dependent oxidation of phenol red in the absence of Mn(II) (results not shown) were found when IEF was used. Also, in some SDS-polyacrylamide gels, a weak band with a molecular mass of 41 kDa was detected. Thus, we conclude that all strains produced at least two major LiP isoenzymes, designated H1 and H2, with molecular masses of 45 and 41 kDa and isoelectric points of 3.75 and 3.25, respectively. The character of some minor protein bands with molecular masses of 38 and 39 kDa obtained by SDS-PAGE (Fig. 4B, lanes 1 and 2, lower protein bands) is not clear. Heterogeneity of LiP H2 (pI 3.25), which resolved into two close bands, can also be observed by IEF (Fig. 5A, lane 5). By SDS-PAGE, GLOX was found to be a

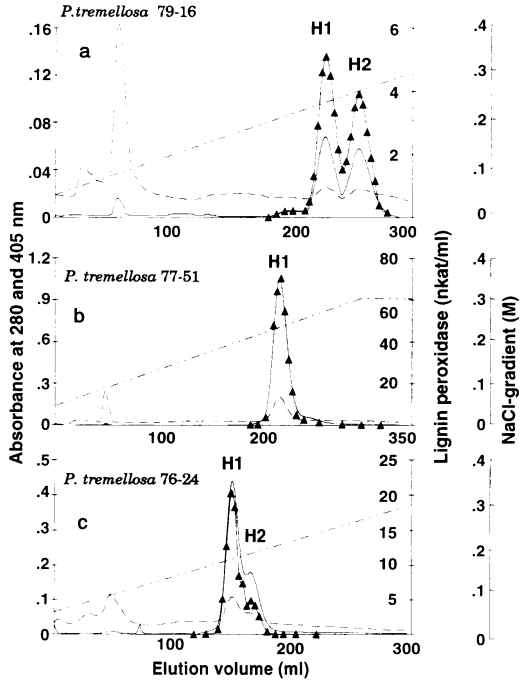

FIG. 2. Profiles of extracellular proteins separated from 13- to 14-day cultures of three (a to c) *P. tremellosa* strains supplemented with the basal concentration of Mn(II). Symbols: – – – –, A_{280}; ——, A_{405}; — · · —, NaCl gradient; ▲, LiP activity.

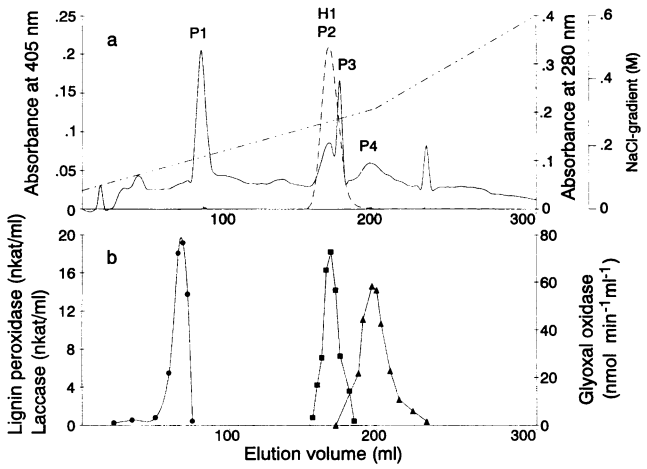

FIG. 3. Profiles of extracellular proteins separated from a 9-day culture of *P. tremellosa* 77-51 supplemented with the 50-fold Mn(II) level and 10 mM malonate. (a) Symbols: ——, A_{280}; – – –, A_{405}; — · · —, NaCl gradient; (b) symbols: ■, LiP activity; ▲, laccase activity; and ●, GLOX activity.

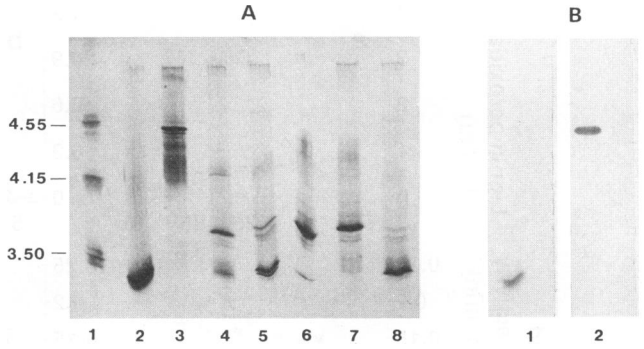

FIG. 5. IEF of ligninolytic enzymes separated from *P. tremellosa* strains. (A) Proteins stained by silver staining. Lanes: 2 to 4, strain 77-51 laccase, GLOX, and LiP H1 respectively; 5 and 6, strain 76-24 LiP H2 and LiP H1, respectively; 7 and 8, strain 79-16 LiP H1 and LiP H2, respectively. Isoelectric points of standard proteins (lane 1) are indicated on the left. (B) Lanes: 1 and 2, activity staining of laccase and GLOX, respectively, from strain 77-51. For details, see Materials and Methods.

nearly homogeneous protein with a molecular mass 67 kDa (Fig. 4A, lane 3), but IEF analysis revealed a minor protein band with a pI value slightly lower than 4.5 measured for the major protein (Fig. 5A, lane 3; Fig. 5B, lane 2). Laccase had a molecular mass of 67 kDa and an isoelectric point of 3.25 (Fig. 5A, lane 2; Fig. 5B, lane 1). All characterized proteins were shown to be monomers by native PAGE. Major proteins of the protein peaks P1 and P3 (Fig. 3a) had molecular masses of 26 and 97 kDa, respectively. Protein P3 neither oxidized phenol red in the presence or absence of Mn(II) and H_2O_2 nor showed H_2O_2-producing activity with methylglyoxal, glucose, or cellobiose as substrates in coupled reactions with horseradish peroxidase.

Laccase purified from *P. tremellosa* 77-51 had a pH optimum of 3.9 to 4.7 for syringaldazine, unexpectedly showing in this range two similar peaks at around 4.0 and 4.5 (data not shown). It is difficult to interpret this result since laccase was found to be a homogeneous monomer when analyzed by gel electrophoresis but appeared as a double band when analyzed by IEF, which is not seen in Fig. 5A, line 2, because of overloading of this particular sample. GLOX had a pH optimum 5.5 to 6.5 for methylglyoxal, with maximal activity near pH 6.0.

Mineralization of ^{14}C-ring-labelled DHP. When cultivated under the basal Mn(II) level, all three *P. tremellosa* strains degraded synthetic lignin (i.e., DHP) to $^{14}CO_2$ with efficiency equal to that of *P. chrysosporium* BKM 1767 or *P. radiata* 79 (Fig. 6a). *P. tremellosa* strains mineralized more than 40% of radiolabelled DHP to $^{14}CO_2$ after 33 to 35 days of growth in cultures supplemented with a low (2.4 μM) Mn(II) content. The patterns of DHP mineralization were similar in all fungi cultivated under the basal Mn(II) level, with the most intensive evolution of $^{14}CO_2$ occurring during days 8 to 16 (Fig. 6a). In contrast, cultures of *P. tremellosa* 77-51 grown with 50× Mn(II) or 50× Mn(II) plus chelator showed a drastically different pattern of lignin mineralization (Fig. 6b). Production of $^{14}CO_2$ was very low until ca. day 16, but then lignin

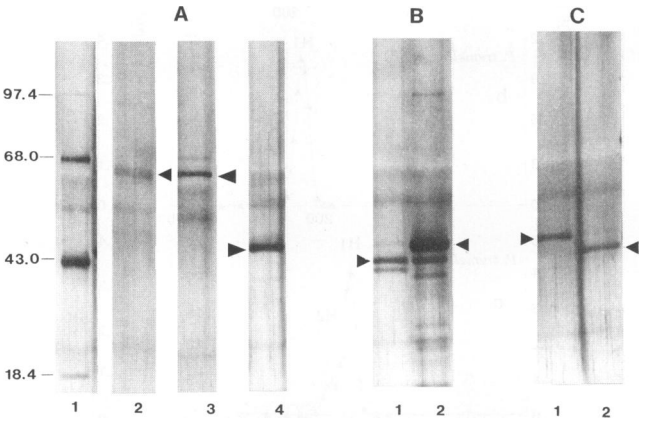

FIG. 4. SDS-PAGE of ligninolytic enzymes separated from *P. tremellosa* strains (protein bands are indicated by arrows). (A) strain 77-51. Lanes: 2, laccase; 3, GLOX; 4, LiP H1. (B) Strain 76-24. Lanes: 1, LiP H2; 2, LiP H1. (C) Strain 79-16. Lanes: 1, LiP H1; 2, LiP H2. Molecular mass standards (lane A1) are indicated on the left in kilodaltons.

TABLE 1. Ligninolytic enzymes of *P. tremellosa*

P. tremellosa strain	Enzyme	Molecular mass (kDa)	pI
79-16	LiP H1	45	3.75
	LiP H2	41	3.25
	Laccase	ND[a]	ND
77-51	LiP H1	45	3.75
	LiP	41	3.25
	Laccase	67	3.25
	GLOX	67	4.5
76-24	LiP H1	45	3.75
	LiP H2	41	3.25
	Laccase	ND	ND
2845[b]	Mt-L1[c]	35–36	3.1
	Mt-L2[c]	38–39	3.5
	Mt-L3[c]	40	4.0
	MnP	ND	ND
	Laccase	ND	ND

[a] ND, not determined.
[b] From reference 17.
[c] Heme proteins showing LiP activity.

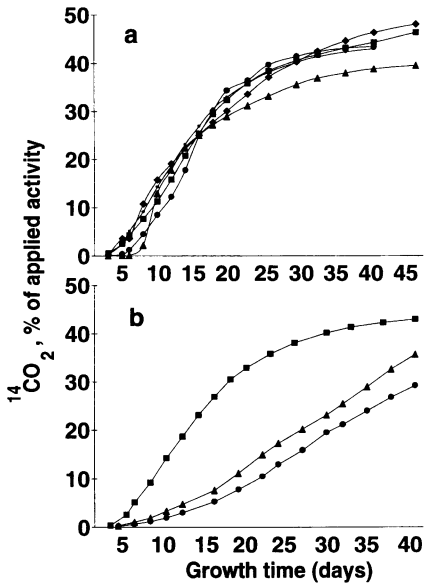

FIG. 6. Mineralization of ^{14}C-ring-labelled DHP by fungi. (a) evolution of $^{14}CO_2$ under the low basal Mn(II) level by *P. radiata* 79 (■), *P. chrysosporium* BKM 1767 (▲), *P. tremellosa* 79-16 (●), *P. tremellosa* 77-51 (◆), and *P. tremellosa* 76-24 (·); (b) evolution of $^{14}CO_2$ by the cultures of *P. tremellosa* 77-51 supplemented with 50-fold Mn(II) (●) and 50-fold Mn(II) plus malonate (▲) in comparison with culture grown under basal Mn(II) (■).

mineralization became more intensive up to the end of cultivation. When the medium was supplemented with 50× Mn(II) or 50× Mn(II) plus chelator, 29% ± 2% and 36% ± 5%, respectively, of applied activity was evolved as $^{14}CO_2$ by day 41. Also, maximal rates of lignin mineralization under these conditions were lower than those in cultures with the basal Mn(II) content (Table 2).

DISCUSSION

The production patterns of LiP and laccase in three *P. tremellosa* strains resembled those which have been reported for *P. radiata* 79 (19), *P. radiata* L 12-41, and *P. tremellosa* 2845 (17) under similar cultivation conditions. In all of the fungi mentioned above, laccase activity appeared early (days 1 to 5) and usually reached its maximum level rapidly before decreasing simultaneously with increasing LiP activity. The same order of appearance of the enzymes has been detected in *P. radiata* under various cultivation conditions, such as in stationary flasks with different amounts of veratric acid (17, 24, 26) or veratryl alcohol (30) and in bioreactor cultivations supplemented with different concentrations of veratryl alcohol (19). These results further show that under growth conditions which enhance LiP activity, e.g., by the addition of aromatic compounds or flushing with oxygen, the level of laccase activity decreases. In our experiments with different concentrations of Mn(II) in the medium, a close relationship in the expression of laccase and LiP was also observed. Strong repression of LiP activity by the higher Mn(II) levels caused, in turn, an increase in laccase activity in all strains. Although stimulation of laccase production by Mn(II) was reported in *Phlebia brevispora* (34, 43), the secretion of laccase was Mn(II) independent in *Dichomitus squalens* (37). This indicates that the reason for enhancement of laccase production might not be the direct stimulation by higher Mn(II) concentrations but is perhaps connected with the LiP-laccase expression mechanism in the lignin-degrading fungi. Thus, the influence of Mn(II) supplementation on laccase production needs further research.

TABLE 2. Maximal rates of ^{14}C-ring-labelled DHP mineralization by fungi

Fungus	Rate of DHP mineralization (% $^{14}CO_2$ of applied activity evolved/day)[a]
P. radiata 79[b]	2.3 ± 0.3
P. chrysosporium BKM 1767[b]	2.8 ± 0.3
P. tremellosa 79-16[b]	2.5 ± 0.3
P. tremellosa 76-24[b]	2.2 ± 0.1
P. tremellosa 77-51[b]	1.7 ± 0.1
P. tremellosa 77-51[c]	1.2 ± 0.2
P. tremellosa 77-51[d]	0.9 ± 0.1

[a] Mean of four culture replicates ± standard deviation.
[b] Cultivations with basal Mn(II) level; rates were estimated during 8 to 16 days of growth.
[c] Cultivation with 50× Mn(II) plus 5 mM of malonate; growth time, 16 to 24 days.
[d] Cultivation with 50× Mn(II) level; growth time, 16 to 24 days.

The repression of LiP production in *P. tremellosa* by increasing Mn(II) concentrations in this study is in agreement with the previous results for *P. chrysosporium* and some other white rot fungi (5, 34–36). Although, in crude culture liquid, LiP activity was undetectable when assayed with veratryl alcohol, LiP was purified from a 9-day culture supplemented with 50× Mn(II) and malonate. It is possible that the production of LiP under 50-fold Mn(II) concentrations took place in our cultures, but LiP activity could not be assayed because of the interference of Mn ions and chelator in the veratryl alcohol oxidation method, leading to the reduction of the veratryl alcohol radicals to veratryl alcohol (25, 39). On the other hand, Perez and Jeffries (36) reported recently that neither Mn(II) nor Mn(III) in the presence of a chelator inhibits the oxidation of veratryl alcohol by purified LiP.

Efforts to purify MnP from bioreactor cultures of *P. tremellosa* containing both low and high concentrations of Mn(II) (supplemented with the chelator) did not reveal the production of MnP by this species. Moreover, MnP has not been detected in the protein profile of strain 76-24 grown in shake flasks. Other ligninolytic enzymes (e.g., LiP and laccase) capable either of generating Mn(III) from Mn(II) (2, 39) or of oxidizing phenol red directly most probably contributed to the phenol red-oxidizing activity measured in the presence of H_2O_2 and Mn(II), which was observed throughout the study. However, the possibility that MnP activity is not stable in the fungi studied and was absent from culture liquid when harvested on day 9 (Fig. 1e), or was lost in the purification procedure, also exists. MnP activity could also have been expressed in the late stages of cultivation (Fig. 1e and f) in contrast to the pattern found in *P. radiata* 79 (30) and *P. tremellosa* 2845 (17).

In our previous work (17), three LiP isoenzymes were isolated from *P. tremellosa* 2845 (ATCC 48745) grown in shake cultures which were supplemented with veratryl alcohol and Tween 80 and which contained relatively large pellets. MnP-type activity was also found in this strain (5, 17), but the enzyme was not purified. Apparently, LiP production is characteristic in these species, and difficulties in detection of the activity reported previously (3, 5, 42) were probably due to differences in the culture conditions. In this study, at least two major LiP isoenzymes were separated from the extracellular culture liquid of *P. tremellosa* strains. As Table 1 shows, the same LiP isoenzymes were apparently expressed in all three

strains studied here, but they differed in molecular masses and pI values from the LiP isoenzymes of *P. tremellosa* 2845. Also, GLOX, which is an H_2O_2-producing enzyme, and laccase were purified and tentatively characterized in *P. tremellosa* 77-51. With respect to its molecular weight, pI, and pH optimum, GLOX from *P. tremellosa* is similar to the enzyme first described in *P. chrysosporium* (21). The protein peak in the elution profile corresponding to GLOX was not significant in comparison with those of ligninolytic enzymes, but purified GLOX showed high specific activity defined as production of H_2O_2 per milligram of protein per minute (about 2 mM mg^{-1} min^{-1} at pH 6). This is also in agreement with the results of Kersten (20), who proposed on the basis of a high turnover number (k_{cat}) that the amount of GLOX protein required for sufficient production of H_2O_2 in the GLOX-LiP system is much lower than that of LiP.

The molecular mass and isoelectric point determined for *P. tremellosa* laccase were comparable to those reported for the other fungal laccases (10, 27, 31, 46, 49). The specific activity of the enzyme measured by using syringaldazine as the substrate is close to the laccase activity described in our previous work with the white rot fungus *Junghuhnia separabilima* (46), but the presence of two nearly symmetrical peaks at the pH optimum is difficult to explain if *P. tremellosa* laccase is a monomer as indicated in the gel electrophoresis analyses (native PAGE and SDS-PAGE). Uncertainty in the conformation of the native laccase from *Agaricus bisporus* has been discussed recently by Perry et al. (38). These authors proposed that native enzyme might be a dimer of identical subunits, which can undergo partial cleavage in the purification process. In addition, we observed that native concentrated laccase isolated from *P. tremellosa* was colorless and its optical absorbance spectrum did not reveal a peak at A_{610} characteristic of the "blue copper" as that, e.g., in *J. separabilima* (46).

Results obtained from DHP mineralization by *P. tremellosa* cultures, supplemented with Mn(II) at various levels, support data published for *P. chrysosporium* (5, 34–36) and *P. brevispora* (34). The addition of the 50-fold Mn(II) concentration to the culture medium caused repression of $^{14}CO_2$ evolution, particularly during the first 2 weeks. Correspondingly, LiP activity measured with veratryl alcohol was also repressed in bioreactors with a 10× and 50× Mn(II) content, pointing to the crucial role of LiP in DHP mineralization by *P. tremellosa*.

The presence or absence of a chelator in the cultures with high Mn(II) concentrations did not affect the pattern of DHP mineralization. This can be explained by the lack of MnP production; that is, Mn(II) was not rapidly oxidized to Mn(III) by MnP. Thus, the precipitation of MnO_2 in the absence of a chelator possibly did not occur in the same way as it did in the case of *P. chrysosporium*, causing derepression of LiP and fast DHP mineralization (35). Although the regulative role of manganese in LiP production and lignin degradation by *P. tremellosa* is obvious, the mechanism of this regulation is not clear. Recently, inhibition of LiP isoenzyme secretion in the presence of a high Mn(II) concentration was reported for *P. chrysosporium* (36). However, in our study, LiP could be purified from the cultures of *P. tremellosa* despite that (i) chelated manganese complexes were present in the medium, (ii) LiP activity was undetectable in crude culture broth by a veratryl alcohol oxidation assay, and (iii) DHP mineralization was repressed under these conditions. We suggest that Mn(II) interfered in the veratryl alcohol assay and prevented detection of actual existing LiP activity in cultures with high Mn(II) concentrations (25, 39). The mechanism of Mn(II) action on the catalytic activity of LiP in DHP mineralization needs further study, and then the role of veratryl alcohol in lignin degradation by LiP should be taken into account (12–14). Our results (16) indicated that a high concentration of Mn(II) and the addition of a chelator could either stimulate ^{14}C-ring-labelled DHP mineralization in many white rot fungi (including *P. tremellosa* 2845), as was found in *D. squalens* (37), have no effect on DHP degradation (e.g., in *P. radiata* 79), or repress DHP mineralization (this study). The influence of Mn(II) on enzymatic activities and lignin degradation may depend on the differences in the ligninolytic systems among the fungi.

In conclusion, we have investigated in more detail the extracellular ligninolytic enzymes of the white rot fungus *P. tremellosa*, which previously has been shown to be a selective lignin degrader suitable for biopulping. Although studies on DHP mineralization pointed to the important role of LiP in lignin degradation by this species, conditions in, e.g., the biopulping process differ from those used in this research. Thus, it is possible that also other ligninolytic enzymes, presumably true MnP, are produced by *P. tremellosa* and are involved in lignin degradation by this fungus. Our results also indicate that the production of MnP in a fungus should be confirmed at least by purifying and tentatively characterizing the enzyme.

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