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# Lignin Peroxidases, Manganese Peroxidases, and Other Ligninolytic Enzymes Produced by *Phlebia radiata* during Solid-State Fermentation of Wheat Straw

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**The white rot fungus *Phlebia radiata* 79 (ATCC 64658) produces lignin peroxidase (LiP), manganese peroxidase (MnP), glyoxal oxidase (GLOX), and laccase in the commonly used glucose low-nitrogen liquid medium. However, the enzymes which this fungus utilizes for selective removal of lignin during degradation of different lignocellulosic substrates have not been studied before. Multiple forms of LiP, MnP, GLOX, and laccase were purified from *P. radiata* culture extracts obtained after solid-state fermentation of wheat straw. However, the patterns of extracellular lignin-modifying enzymes studied were different from those of the enzymes usually found in liquid cultures of *P. radiata*. Three LiP isoforms were purified. The major LiP isoform from solid-state cultivation was LiP2. LiP3, which has usually been described as the major isoenzyme in liquid cultures, was not expressed during straw fermentation. New MnP isoforms have been detected in addition to the previously reported MnPs. GLOX was secreted in rather high amounts simultaneously with LiP during the first 2 weeks of growth. GLOX purified from *P. radiata* showed multiple forms, with pIs ranging from 4.0 to 4.6 and with a molecular mass of ca. 68 kDa.**

Delignification of lignocellulosic materials by white rot fungi is of great interest and has been investigated to improve the digestibility of wood or straw for animal feed (1, 15, 41, 45) and to reduce costs for the pulp and paper industry (2, 5, 27, 29, 36). Annual plants, such as cereal straw, could serve as alternative types of raw materials for papermaking, and if they were pretreated with selective lignin degraders or enzymes derived from them, the use of such plants could save both forest resources and energy in pulp refining (27). Among 45 strains of fungi screened, *Pleurotus eryngii* was shown to be the most selective species in delignification of wheat straw (40, 41). This species causes effective straw defibration and provides a decrease in refining energy consumption (27). Interestingly, no extracellular peroxidase activity was detected in *P. eryngii* cultures growing on N-limited liquid medium, although such activity is known to promote lignin degradation by *Phanerochaete chrysosporium* (20). Recently, however, a new type of manganese peroxidase (MnP) has been purified from *P. eryngii* grown on C-limited peptone medium (28). Most of the studies to date on the enzymology of lignin degradation by white rot fungi have been carried out with synthetic or organic liquid media (12, 20, 43, 44), and in only a few cases have enzymes produced during solid-state growth been investigated (7, 9, 23, 37, 42). The liquid culture method is well adapted to the common spectrophotometric enzyme assays and conventional enzyme purification procedures. These methods were used to discover two main ligninolytic enzymes, lignin peroxidase (LiP; EC 1.11.1.14) (39) and MnP (EC 1.11.1.13) (21). However, more information about enzymes involved in lignin degradation under conditions closer to natural conditions, that is, during degradation of wood, straw, or grass substrates, is needed. Recent reports show that fungi may utilize mechanisms of lignin depolymerization in wood different from those used in liquid cultures (38). Moreover, in wood substrates, *P. chrysosporium* and *Ceriporiopsis subvermispora* produce MnP isoenzymes which have not previously been described in liquid cultures of these thoroughly studied fungi (7, 23). LiP has been identified in cultures of *P. chrysosporium* grown on mechanical aspen pulp (7), although in much lower titers than those of MnP, and the enzyme has not been purified. *Trametes versicolor* has been found to secrete MnP but not LiP during solid-state fermentation (SSF) of lignocellulosic materials (33, 42), although in liquid cultures, multiple LiP isoforms are produced by this species (14).

Ligninolytic enzymes (LiP, MnP, and laccase) of the white rot fungus *Phlebia radiata* have been thoroughly studied with liquid medium (12, 24–26, 30, 31). We report here the results of enzyme purifications from SSF cultures of *P. radiata*, showing that rather high titers of LiP as well as of MnP and laccase were produced during degradation of wheat straw by this fungus. Also, such enzymes as glyoxal oxidase (GLOX) which have not been purified from *P. radiata* previously (24) were purified and partially characterized.

## MATERIALS AND METHODS

**Fungal strain and cultivation conditions.** *P. radiata* 79 (ATCC 64658), previously collected in the neighborhood of Helsinki, Finland, and isolated from decayed wood of an *Alnus* sp. at the Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland (13), was used for the SSF of wheat straw obtained from J. M. Pelayo (SAICA, Zaragoza, Spain). The fungus was cultivated in 2-liter bioreactors containing 100 g of chopped straw (sterilized twice at 121°C for 30 min) and 0.15 g of glucose in 280 ml of deionized $H_2O$ (filter sterilized). The bioreactors were inoculated with 20 ml of homogenized mycelial suspension (13), and stationary cultivations were carried out at 25°C under a constant oxygen flow (86 ml $min^{-1}$). Control bioreactors were not inoculated with fungus but were kept under the same conditions. Fermented straw from one bioreactor and control straw from the parallel bioreactor were harvested weekly starting 1 week after inoculation, so that the cultivation periods were 1, 2, 3, and 4 weeks.

**Enzyme assays.** After it was harvested, straw from a bioreactor was suspended in 1,300 ml of 25 mM sodium acetate buffer (pH 5.5) on a rotary shaker for 1.5 h and subsequently pressed (500 kPa with $N_2$) with simultaneous washing with

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300 ml of buffer to separate extracellular fungal enzymes. Enzyme activities in filtered (Whatman no. 4) liquids were assayed with a Shimadzu 160A UV-visible spectrophotometer as described below, and the liquids were kept at −20°C prior to protein purification.

LiP was measured with veratryl alcohol (vacuum distilled prior to use; Aldrich) (19) at 25°C. The reaction mixture contained 0.1 M sodium tartrate buffer (pH 3.0), 0.4 mM veratryl alcohol, and 1.65 ml of culture filtrate in a total volume of 3 ml. The reaction was started by adding $H_2O_2$ to a final concentration of 0.2 mM, and $A_{310}$ was monitored. However, the standard veratryl alcohol oxidation method previously adopted for assaying LiP activity in colorless fungal cultures was not suitable in the case of SSF, since liquid obtained from the fermented straw had a dark brown color due to the products of straw degradation. As an alternative method, oxidation of the dye Azure B in the presence of $H_2O_2$ at pH 2.5 and 25°C was employed (4). LiP activity was expressed as nanokatals per milliliter. MnP activity was monitored with phenol red (10) at 30°C. Reaction mixtures contained 25 mM lactate, 0.1 mM $MnSO_4$, 1 mg of bovine serum albumin (Sigma) $ml^{-1}$, 0.1 mg of phenol red (Merck) $ml^{-1}$, and 0.5 ml of culture filtrate in 20 mM sodium succinate buffer (pH 4.5) in a total volume of 1 ml. The reaction was started by the addition of $H_2O_2$ to final concentration of 0.1 mM and was stopped after 1 min with 50 μl of 10% NaOH, and $A_{610}$ was measured. Control assays of phenol red oxidation in the absence of $Mn^{2+}$ were carried out by omitting $MnSO_4$ from the reaction mixture. MnP activity was calculated by subtracting the value for phenol red-oxidizing activity in the absence of $Mn^{2+}$ from the value for the activity obtained in the presence of manganese. Activity was expressed as the increase in $A_{610}$ per minute per milliliter. Laccase (EC 1.10.3.2; benzenediol:oxygen oxidoreductase) activity was measured with syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine; EGA-Chemie) (26) at 25°C. The reaction mixture (total volume, 1 ml) contained 0.1 ml of culture filtrate in 0.1 M citrate-phosphate buffer (pH 5.0), and the reaction was initiated by the addition of 0.025 μmol of syringaldazine. The increase in $A_{525}$ was recorded, and the activity was expressed as nanokatals per liter. GLOX activity was determined by a peroxidase-coupled assay, with phenol red being used as the peroxidase substrate (18) at 30°C. The reaction mixture (total volume, 1 ml) contained 50 mM sodium 2,2-dimethylsuccinate (pH 6.0), 10 mM methylglyoxal (Sigma), 0.01% (wt/vol) phenol red, 10 μg of horseradish peroxidase (type II; Sigma), and 300 μl of culture filtrate (to start the reaction). The reaction was stopped by adding 50 μl of 2 N NaOH, and $A_{610}$ was measured. The amount of $H_2O_2$ produced by GLOX in the oxidizing of methylglyoxal was determined from a calibration curve, with concentrations of $H_2O_2$ ranging from 0 to 60 μM. GLOX activity was expressed as nanomoles of $H_2O_2$ produced per minute per milliliter. Cellobiose:quinone oxidoreductase (CBQ)-cellobiose oxidase (CBO) activity was measured with 3-methoxy-5-*tert*-butyl-1,2-benzoquinone (EGA-Chemie) at 25°C (3). The reaction mixture contained 1 μM 3-methoxy-5-*tert*-butyl-1,2-benzoquinone, 2 μM D-cellobiose (Fluka) in 0.1 M sodium acetate buffer (pH 4.5), and 670 μl of culture filtrate in a total volume of 1 ml. The reduction of quinone was monitored at 420 nm, and the activity was expressed as nanokatals per liter. Protease activity was assayed by measuring the increase at $A_{520}$ with Azocoll (Sigma) (8). Protease activity was expressed as units per milliliter. One unit is defined as the amount of enzyme that catalyzed the release of azo dye so that the change in $A_{520}$ was 0.001 optical density $min^{-1}$.

Protein concentration was measured by the Bradford assay (6) with dye reagent (Bio-Rad). For pH determination, 250 mg of straw was suspended in 10 ml of deionized water for 3 h, and the pH was measured (41).

**Protein purification.** Culture liquid plus washings (ca. 1,600 ml from one bioreactor) were kept frozen at −20°C prior to purification. After centrifugation at 12,000 × *g* for 30 min to remove the precipitates, the sample was concentrated to a volume of ca. 300 ml by ultrafiltration in a Minisette system with Omega membrane cassettes (10-kDa cutoff; Filtron). Further concentration and simultaneous dialysis were carried out with an Amicon (Beverly, Mass.) ultrafiltration unit (10-kDa cutoff) in which the sample was concentrated to a volume of 35 ml and dialyzed by washing with 25 mM sodium acetate buffer (pH 5.5). All concentration procedures were carried out at +4°C.

Proteins were fractionated by anion-exchange chromatography on Sepharose-Q Fast Flow medium (Pharmacia) with a fast-performance liquid chromatography apparatus (Pharmacia). The column (1.6 by 20 cm) was equilibrated with 25 mM sodium acetate buffer (pH 5.5), and proteins were eluted at a linear NaCl gradient from 0.05 to 0.3 M. The elution volume was 350 ml, the flow rate was 1.5 ml $min^{-1}$, and fractions of 3.7 ml were collected. $A_{409}$ (heme) and $A_{280}$ (protein) were measured. Enzyme activities (LiP, MnP, laccase, GLOX, and CBQ-CBO) were assayed in eluted fractions. Fractions containing enzyme activities were pooled, concentrated, and desalted in Centricon microconcentrators with cutoffs of 10 kDa, and enzymes were further analyzed by discontinuous gel electrophoresis and isoelectric focusing (IEF).

**Enzyme characterization.** Purified enzymes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), native PAGE (22), and IEF-PAGE. Discontinuous gel electrophoresis was carried out on 10% gels (16 by 20 cm) with a Protean II apparatus (Bio-Rad). Proteins were visualized by silver staining (Bio-Rad kit). SDS-PAGE gels were calibrated with GIBCO/BRL high-range molecular weight standards.

Enzymes were analyzed by IEF with 0.5-mm-thick polyacrylamide slab gels and a Multiphor II apparatus (Pharmacia). Two slightly different pH ranges (pH 5.9 to 3.4 and 4.7 to 3.2) were obtained by mixing ampholytes having a pH range of from 3.5 to 10.0 with ampholytes having a pH range of from 2.5 to 5.0 (Pharmacia). The pH gradient in the gels was measured with a Multiphor surface electrode (Pharmacia). Proteins were stained with silver stain (Bio-Rad).

Ligninolytic enzymes in native PAGE gels and in IEF-PAGE gels were visualized by specific activity stainings. For MnP activity, phenol red in the presence of $H_2O_2$ and $MnSO_4$ was used (34). LiP activity was identified by the same method, but $MnSO_4$ was omitted from the reaction mixture. Laccase activity was visualized with 5 mM guaiacol in 0.1 M citrate-phosphate buffer (pH 5.0) or ABTS (2,2-azinodi-3-ethylbenzothiazoline-6-sulfonic acid; Boehringer-Mannheim) solution in 0.02 M sodium succinate buffer, pH 4.5. For GLOX activity, the following solution was used: 86.2 mM sodium 2,2-dimethylsuccinate buffer (pH 6.0) containing 0.031 mM *o*-dianisidine (3,3′-dimethoxybenzidine; Fluka), 10 mM methylglyoxal, and 17 μg of horseradish peroxidase (type II; Sigma) $ml^{-1}$.

Glycoprotein staining (carbohydrate detection) in SDS-PAGE gels was carried out by the periodic acid-Schiff's reagent method according to the protocol of Pharmacia (35).

TABLE 1. MnP activities in culture extracts and pH values determined during wheat straw degradation by *P. radiata* 79

| Cultivation time (wk) | MnP activity ($\Delta A_{610}$ $ml^{-1}$ $min^{-1}$) | pH[a] |
|---|---|---|
| 1 | 0.068 | 5.08 (5.13) |
| 2 | 0.229 | 4.74 (5.05) |
| 3 | 0.212 | 4.75 (5.16) |
| 4 | 0.087 | 4.75 (5.08) |

[a] The values in parentheses are for the control bioreactors (noninoculated).

## RESULTS

**Enzyme activities in the extracted liquid.** Buffer extractions from both fungus-fermented and unfermented control straw were clear but colored. After 1 week of fermentation, liquids from inoculated and sterile straw had the same rather intensive brown color, while through the later growth stages (2 to 4 weeks), the extract from the fermented straw became less colored, having a light yellow color by week 4 of cultivation. In an effort to remove colored compounds from the liquid extracted from the straw, 0.4% (wt/vol) polyethylenimine (Sigma) was added to the liquid. Although polyethylenimine has been reported to precipitate most of the polyphenols in extracellular culture filtrates of *Lentinula edodes* grown on wood substrate (9), no such effect was obtained with our samples.

While LiP activity in extracts was not detectable by the veratryl alcohol oxidation method, low activity was obtained by the Azure B assay. However, measurements of LiP activity with Azure B in control cultures gave similar values. Assays of other enzymatic activities and protein, in comparison with those of the controls, indicated that the results could not be reliable because of either the presence of inhibitors and color interference or low enzyme titers. Obviously, these factors had less influence on the determination of MnP activity, but their interference in the MnP assay could not be ruled out, as is evident from the comparison of titers of MnP activity measured in culture extracts (Table 1) with those obtained after the purification of enzymes (Fig. 1B and 2) from different growth stages. During fermentation, pH slightly decreased (from 5.08 to 4.74) after the first cultivation week, but then it did not change up to week 4 (Table 1).

**Enzyme purification.** Although colored compounds were partially removed by the concentration procedure (ultrafiltration), the concentrated samples had an intensive brown color. LiP activity in concentrates was undetectable by both the veratryl alcohol and Azure B assay methods, but high MnP activity was found. Also, moderate levels of laccase, GLOX, and CBQ-CBO activities were detected. Protease activity was very low in crude concentrates. During the purification procedure, most of the colored compounds were tightly bound to the

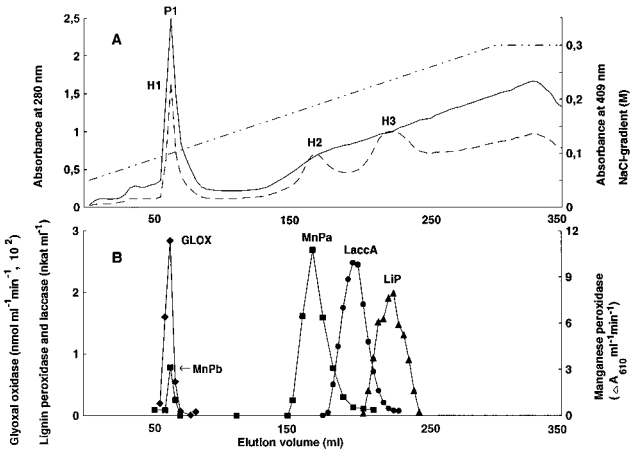


FIG. 1. Purification of extracellular proteins from a 1-week-old culture of *P. radiata* grown on wheat straw. (A) ——, $A_{280}$; — — , $A_{409}$; —··—, NaCl gradient. (B) Enzyme activities.

Sepharose-Q, but with the increase of the salt gradient over 0.16 M, some of the color was eluted from the column. These eluted compounds interfered with the monitoring of $A_{280}$ for protein and, to a lesser extent, $A_{409}$ for heme (Fig. 1A). Protein profiles obtained from 1-, 2-, 3-, and 4-week-old cultures were rather similar, containing a big protein peak, P1, with a corresponding hemeprotein peak, H1, and two hemeprotein peaks, H2 and H3 (Fig. 1A). Profiles of ligninolytic enzymes detected at different growth stages are shown in Fig. 1B and 2. In 1-week-old cultures (Fig. 1B), the enzyme profile was very similar to the pattern usually obtained with liquid cultures of *P. radiata* 79 grown on low-nitrogen liquid medium supplemented with 56 mM glucose, 1.0 mM veratryl alcohol, and 0.05% (wt/vol) Tween 80 (30). GLOX and MnP (designated MnPb) were found in the protein peak, P1 (Fig. 1B). The major MnP peak (designated MnPa) corresponds to H2 in Fig. 1A. Further in the elution profile, laccase, designated LaccA, and LiP (the corresponding peak in Fig. 1A is H3) were found. GLOX, MnP, laccase, and LiP titers were high (Fig. 1B) and comparable to those obtained with liquid cultures. As Fig. 1B and 2 show, the aging of the culture caused changes in the patterns of the extracellular ligninolytic enzymes produced by *P. radiata* 79 on straw. In the first enzyme peak, corresponding to P1 (Fig. 1A), a new laccase activity appeared (designated LaccB in Fig. 2), while previously described enzyme activities (GLOX, MnPa, LaccA, and LiP in Fig. 1B) diminished and became negligible by week 4 of growth. Increasing titers of MnPb activity were detected over time in P1 (Fig. 2).

CBQ-CBO activity was also detected, but its occurrence was rather sporadic in the elution profiles.

**Enzyme characterization.** Purified enzymes were analyzed by several gel techniques and characterized by their molecular masses and pIs (Table 2). For purposes of comparison, data for enzymes previously isolated from liquid cultures of *P. radiata* 79 are shown in Table 2. These results will be discussed later. In SSF of wheat straw, all ligninolytic enzymes of *P. radiata* were expressed in multiple forms, that is, as sets of enzymes with similar pI values and proximate molecular masses. By IEF-PAGE, LiP was separated into a major isoform (pI 4.0) and two minor isoforms which appeared as a double band with pI values of 3.85 and 3.90 (Table 2). After SDS-PAGE and glycoprotein staining, two distinct bands with molecular masses of 45 to 46 (major) and 44 kDa (minor) could be observed. However, since LiP was separated into three protein bands by native PAGE, we suppose that three LiP isoforms were produced on straw by *P. radiata* and that two of them had the same molecular masses. The patterns of LiP isoforms did not change during different growth periods.

MnPb, found in the protein peak P1 (Fig. 1A) after the first cultivation week, consisted of a major set of enzyme isoforms with an acidic pI (3.9 to 3.3) and traces of a minor group of MnP proteins with a more basic (5.3 to 4.9) pI (Fig. 3, lane 4). The latter group of MnP isoforms was regularly found in enzyme purifications from 1-week-old cultures, but only in relatively low amounts. These MnPs can be seen as weak bands in the upper part of lane 4 in Fig. 3. IEF-PAGE analysis of MnPb isoforms eluted at later growth stages (2 to 4 weeks) showed that MnPs with basic pI values disappeared, but increasing amounts of MnPs with acidic pIs were secreted after the second growth week (Fig. 3, lane 3), and this enzyme pattern did not change up to week 4 (Fig. 3, lanes 2 and 3).

MnPa isoforms corresponding to the H2 peak (Fig. 1A) and eluting later in the enzyme profile had acidic pI values (3.9 to 3.4) (Fig. 4B, lanes 2 to 4). As Fig. 4B (lanes 2 to 4) shows, the enzyme patterns of MnPa expressed in 1-, 2-, and 3-week-old cultures (Fig. 1B and 2A and B) are similar and did not notably differ from the enzyme pattern found with MnPb (Fig. 4B, lane 1). Moreover, MnPa and MnPb showed the same patterns when analyzed by SDS-PAGE and native PAGE. By SDS-PAGE, these enzymes separated in several close protein bands, with a predominant MnP protein with a size of 50 kDa (Table 2). This major band was observed also by native PAGE.

During later cultivation stages (weeks 2 to 4) (Fig. 2), titers of MnPb eluting in the beginning of the profile increased while MnPa activity diminished in the second part of the enzyme profile (Fig. 2C). Nevertheless, enzyme patterns, pI values, and

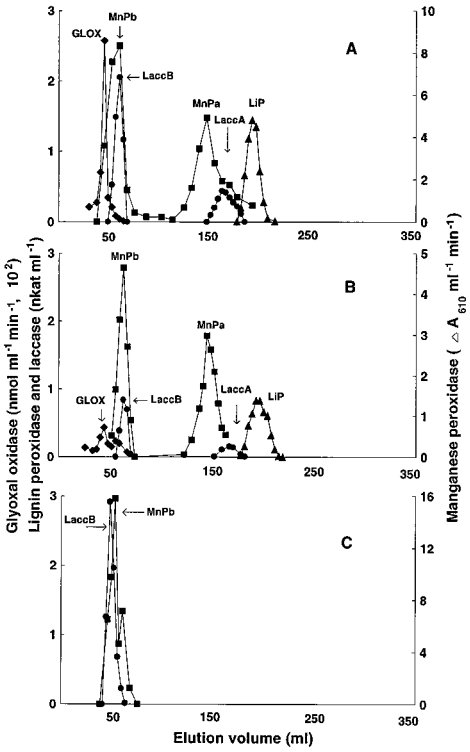


FIG. 2. Enzymes separated from 2- (A), 3- (B), and 4-week-old (C) cultures of *P. radiata* grown on wheat straw.

TABLE 2. Comparison of enzymes isolated from *P. radiata* 79 grown on wheat straw and those produced in liquid cultures

| Enzyme | | Molecular mass (kDa) | | pI | |
|---|---|---|---|---|---|
| Wheat straw | Liquid culture[a] | Wheat straw | Liquid culture | Wheat straw | Liquid culture |
| LiP | LiP2 (24, 25, 30, 31) | 45–46 | 45 | 4.0 | 3.9 |
| | NR[b] | 44 | | 3.8–3.9 | |
| MnPa | MnP (16, 24, 25, 30, 31) | 50 | 48 | 3.9–3.4 | 3.7 |
| MnPb | MnPx (24, 25, 30) | ND[c] | 47 | 5.3–4.9 | 4.7 |
| | NR | 50 | | 3.9–3.3 | |
| GLOX | GLOX (24) | 68 | 68 | 4.6–4.0 | NR |
| LaccA | Laccase (30, 31) | 64 | 64 | 3.5–3.4 | 3.5 |
| LaccB | NR | 64 | | 3.5–3.4 | |

[a] Numbers in parentheses indicate references.
[b] NR, not reported or not found in liquid cultures.
[c] ND, not determined.

molecular masses of MnPa and MnPb were similar. Results with laccase showed the same tendency. Laccase secreted by the 1-week-old culture (LaccA in Fig. 1B) had the same pI and molecular mass as the laccase that appeared in the first part of the enzyme profile from aged cultures (LaccB in Fig. 2). In both cases, the laccase was found to consist of two isoforms by IEF analysis but showed one protein band by SDS-PAGE and native PAGE.

Enzyme patterns of GLOX from the 1- and 2-week-old cultures were similar and revealed multiple forms when analyzed by IEF, with pI values ranging from 4.0 to 4.6 (Fig. 4A). By SDS-PAGE, one broad protein band with a size of 68 kDa which showed GLOX activity by native PAGE analysis was observed after activity staining.

According to carbohydrate staining, all the lignin-modifying enzymes described were glycoproteins. In addition to the GLOX, MnP, and laccase enzymes, a number of other proteins were found in peak P1 by IEF and SDS-PAGE. These proteins could potentially play a role in straw lignin degradation by *P. radiata*, and their characterization is under way in our laboratory.

## DISCUSSION

Our results show that *P. radiata* 79 produced high titers of LiP, MnP, laccase, and GLOX during SSF of wheat straw. MnP and LiP activities have been found in concentrated culture extracts of *P. chrysosporium* grown for 3 days on mechanical aspen pulp (7). Subsequently, LiP, MnP, and GLOX proteins have been detected by Western blot (immunoblot) analysis after fractionation of crude extract on concanavalin A-Sepharose, but in contrast to the case with *P. radiata*, LiP has not been obtained by protein purification by anion-exchange chromatography (7). The white rot fungus *T. versicolor*, which produces LiP, MnP, and laccase in liquid culture (14), was found to secrete MnP and laccase during bleaching, demethylation, and delignification of pulp (33) and SSF of wheat straw (42), but no LiP activity was reported. MnP activity was demonstrated in 12 fungal species grown on complex solid medium supplemented with oak sawdust (32). An enzyme-inhibiting substance in the sawdust extracts was purported to prevent assays of LiP activity, although Western blots revealed the presence of LiP-like proteins in four of the fungi studied. In MnP-producing fungi, such as *C. subvermispora* (23), *Pleurotus ostreatus* (28, 42), *P. eryngii* (27), and *L. edodes* (9), LiP has not been detected either in liquid cultures or during solid-state degradation of lignocellulosic substrates. Therefore, to date, MnP, expressed during delignification of natural substrates by white rot fungi, has been studied in more detail than, e.g., LiP. However, it is possible that the other LiP-producing lignin degraders in addition to *P. radiata* utilize LiP in SSF, but LiP is not detectable, since enzyme activity assays are not compatible with the solid substrate conditions used, as has been demonstrated in this paper. Difficulties in the detection of LiP in the protein purification profiles can also be due to the

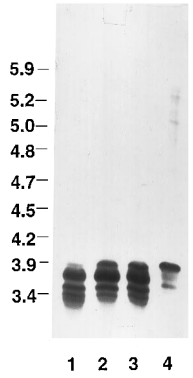


FIG. 3. IEF analysis of MnP separated from *P. radiata* during SSF of wheat straw. The isoenzyme patterns of MnPb at different growth times are shown. Lanes: 1, 4 weeks; 2, 3 weeks; 3, 2 weeks, 4, 1 week. MnP activity staining was done with phenol red. The pH gradient is indicated at the left.

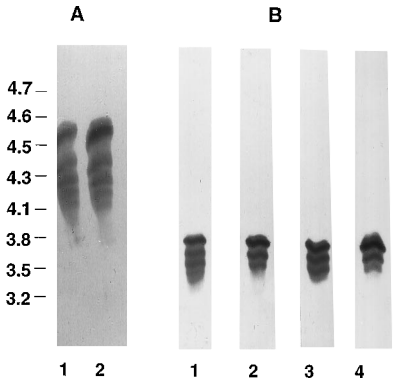


FIG. 4. IEF of GLOX and MnP separated from *P. radiata* during SSF of wheat straw. (A) Activity staining of GLOX (see Materials and Methods). Lanes 1 and 2, GLOX activities from 1- and 2-week-old cultures, respectively. (B) Comparison of MnP isoenzyme patterns. Lane 1, MnPb isolated from 4-week-old culture; lanes 2, 3, and 4, MnPa isolated from 3-, 2-, and 1-week-old cultures, respectively. MnP activity staining was done with phenol red. The pH gradient is indicated at the left.

colored aromatic compounds derived from solid substrates. This suggests that new methods need to be developed for the assaying and purification of LiP during SSF.

As has been reported previously, *P. chrysosporium* (7) and *C. subvermispora* (23) grown on aspen pulp and pine wood chips, respectively, produced MnP isoforms different from those expressed by the same fungi in liquid cultures. Regarding LiP and MnP isoforms expressed during the degradation of straw by *P. radiata*, we found differences between those and the isoforms produced by the fungus in liquid cultures. By liquid cultivation with basal medium both alone (24, 25, 30) and with 0.6% (wt/vol) lignocellulose additions (30), LiP3 is the most abundant isoform of LiP detected. However, as Table 2 shows, during SSF of wheat straw, *P. radiata* apparently secreted LiP2 as the major LiP isoform (24, 25, 30, 31) in addition to two minor unknown LiP isoforms. No LiP3 was detected. Interestingly, addition of insoluble lignocellulose substrates prevented assay of LiP activity in the liquid cultures of *P. radiata*, but LiP isoforms have been previously isolated by protein purification and SDS-PAGE (30).

In liquid cultures, the major *P. radiata* MnP isoform was reported to have a pI of 3.7 and a molecular mass of 48 kDa (16, 24, 25, 30, 31), while an additional isoform, MnPx (pI 4.7 and a molecular mass of 47 kDa), has been described previously (24, 25, 30). In contrast, several different sets of MnP isoforms were expressed during SSF of straw. According to the protein elution profiles, the set of MnP isoforms designated here as MnPa corresponds to the MnP described previously (Table 2). MnPb consists of two sets of MnP isoforms, among which those with basic pI values (5.3 to 4.9) obviously correspond to the MnPx found earlier (Table 2). The laccase isoform pattern was also similar to that reported previously (30, 31), except that the newly isolated enzyme, LaccB, has not been purified from the liquid cultures of *P. radiata*. GLOX has previously been found in the liquid cultures of *P. radiata* (24), but it has not been studied in detail before.

The harvesting of the extracellular enzymes during different stages of growth in our experiments showed that the patterns of the ligninolytic enzymes produced also changed in the process of straw lignin degradation by *P. radiata*. After 1 week of growth, the enzyme profile resembled that of liquid cultures of *P. radiata* (16, 24, 25, 30, 31) in terms of the protein peaks monitored by the elution profile, except that LiP and MnP were found to have different isoform patterns than those reported for liquid cultures. At the later stages of SSF, peaks showing MnP and laccase activities were located at the beginning of the elution profile. This phenomenon has not been previously described in the enzyme purifications from liquid cultures (30). Peaks of LiP and GLOX activities decreased through weeks 2 to 4 of cultivation, while a new MnP and laccase were secreted in increasing amounts. Simultaneous expression and further decrease in LiP and GLOX activities during SSF of wheat straw by *P. radiata* might point to the synergistic ligninolytic action of these enzymes, as has been reported for *P. chrysosporium* (11, 17).

Unexpectedly, the newly described laccase and MnP isoforms that appeared in protein peak P1 at the late growth stages had the same patterns of enzyme isoforms, pI values, molecular masses (by SDS-PAGE), and electrophoretic mobilities by native PAGE as laccase and MnP isoenzymes dominating the earlier growth stages. These newly described enzymes possess enzymatic activity, so the possibility that they are denatured forms of the previously found laccase and MnP must be ruled out. The anomalous behavior of MnP and laccase (designated MnPb and LaccA) observed by anion-exchange chromatography and IEF analysis may suggest that the surface charge of these enzymes has been changed because of the binding of some compounds derived from straw, which may lead to the decrease in the binding capacity of the enzymes to Sepharose-Q medium during protein purification. By subsequent IEF analysis, the contaminating compounds have been separated from the proteins, which allowed the correct pI values to be obtained. Thus, MnPa and MnPb may represent the same MnP isoenzyme. This also accounts for LaccA and LaccB. However, similar behavior has not been detected in the other enzymes studied, such as LiP or GLOX.

Recent reports indicate that isoenzymes produced by white rot fungi during solid substrate degradation might be coded for by different genes than those enzymes produced in liquid culture (7, 23). The N-terminal sequences of the ligninolytic enzymes studied will reveal if this is the case during straw degradation by *P. radiata*, i.e., if the multiple enzyme isoforms of MnP and laccase detected are products of different genes (are true isoenzymes) or if they are posttranslational protein modifications.

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