Physical and enzymatic properties of lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*

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Phanerochaete chrysosporium BKM-1767 secretes multiple lignin peroxidase isoenzymes when grown under nitrogen-limited conditions. Here we report the purification of these heme-containing peroxidases, and their physical and catalytic characterization. Ten hemeproteins, designed H1-H10, were separated by anion exchange HPLC. Six of them, H1, H2, H6, H7, H8, and H10, were lignin peroxidases, oxidizing veratryl alcohol in the presence of H₂O₂. The other four (three peaks were resolved) exhibited manganese-dependent peroxidase activity, oxidizing vanillylacetone in the presence of H₂O₂ and Mn²⁺. The lignin peroxidases have different isoelectric points, between pH 4.7 and 3.3, and molecular weights between 38 and 43 kDa, determined by SDS-PAGE. All are N- and probably O-glycosylated. Three organic substrates and H₂O₂ were used to compare their kinetic properties: the organic substrates were veratryl alcohol, 1,4-dimethoxybenzene, and the lignin model compound 1-(3,4-dimethoxyphenyl)-2-(o-methoxyphenoxy)-propane-1,3-diol. Kₘ and TN values for each of these substrates varied significantly: e.g. for veratryl alcohol Kₘ values were from 86 to 480 µM and TN values were from 1.3 to 8.3 s⁻¹. The ranking of the isoenzyme activities differed with the different substrates, suggesting differences in affinities or in active site accessibilities. The Kₘ for H₂O₂ varied between 13 and 77 µM. Immunological blot analysis and partial proteolytic digestion patterns showed that the isoenzymes have a high degree of homology. The isoenzyme concentrations in extracellular culture fluid were found to vary relatively and absolutely with culture time. A nomenclature scheme for these 10 hemeproteins has been proposed. This scheme should simplify identification of these proteins in the literature as well as be adaptable to others found in *Phanerochaete chrysosporium*.

Keywords: Ligninase; lignin peroxidase; lignin biodegradation; hemeproteins; glycosylation; extracellular enzymes; white-rot; wood decay; peptide analysis

Ligninase, first reported in 1983,¹² appears to play a central role in the initial depolymerization of lignin by the basidiomycete *Phanerochaete chrysosporium*.³⁻⁵ The H₂O₂-activated enzyme functions by oxidizing aromatic nuclei by one electron to aryl cation radicals. They decompose spontaneously via reactions of ionic and radical character.¹⁵

Ligninase, also known as lignin peroxidase, activity is associated with multiple isoenzymes. Leisola et al.⁷ used analytical isoelectric focusing to distinguish 15 lignin peroxidase isoenzymes in the extracellular growth media of *P. chrysosporium* BKM-1767 grown for various times and under various conditions. We had shown earlier that anion exchange high performance liquid chromatography (HPLC) of the concentrated extracellular growth medium of the same strain (grown under optimum ligninolytic conditions) resolves 12 peaks with 280 nm absorbance and at least 10 with 409 nm absorbance. Peaks designated H1, H2, H6, H7, H8, and H10 (Heme 1, Heme 2, etc.) were distinguished by elution at different polarities of sodium acetate, and all had lignin peroxidase (veratryl alcohol-oxidizing) activity. Lignin peroxidase H8 had the same physical and catalytic properties as the original lignin peroxidase isolated and studied by Tien and Kirk.⁵ The isoenzymes were not characterized extensively by either Leisola et al.⁷ or by us.¹ Proper-
ties of one of two lignin peroxidases purified from the same strain used here and by Leisola et al. were determined by Paszczynski et al. It had M = 42–43 kDa and a specific activity of 6–13 U mg⁻¹, depending on time of harvest. It contained 17% neutral carbohydrate and is probably the same as our H8.

Renganathan et al. compared three glycosylated lignin peroxidase isoenzymes from a different strain. They exhibited M = 39, 41, and 43 kDa, and had specific activities of 8, 12, and 19 U mg⁻¹. All three were similar in spectral (heme) properties, and all oxidized several lignin model compounds to the same products.

Molecular genetic approaches have pointed to multiple structural genes for lignin peroxidase. Thus, three different cDNA sequences were reported recently, one of which, presumably H8, corresponds to a genomic sequence also reported recently. The genes show extensive homology.

In the study described here, we physically and enzymatically characterized the six lignin peroxidase isoenzymes resolved by HPLC. The study reveals that they are physically similar and that they exhibit a high degree of homology. Perhaps significantly, they exhibit different kinetic parameters. Our study shows further that isoenzyme composition and the relative amount of each isoenzyme in the extracellular culture fluid vary with culture time.

Additionally, we propose a nomenclature scheme for lignin peroxidases and manganese-dependent peroxidases. This system identifies the isoenzyme by its elution on HPLC and its pI. So far undiscovered isoenzymes of the lignin peroxidase and/or manganese-dependent peroxidase family should be easily adapted into this nomenclature scheme.

Materials and methods

Chemicals

The lignin model compound 1-(3,4-dimethoxyphenyl)-2-(o-methoxyphenoxy)-propane-1,3-diol (model I) was prepared earlier. Vanillylacetone was obtained from R. L. Crawford. All other chemicals were purchased reagent grade and were used without further purification, except for veratryl alcohol (Aldrich Chemical Co., Milwaukee, WI), which was vacuum-distilled.

Strain and culture conditions

P. chrysosporium Burds. wild type strain BKM-F-1767 (ATCC No. 24725) was maintained on malt agar slants. For enzyme production, the organism was grown in submerged agitated cultures. The medium in all cases was nitrogen-limited, and contained basal salts and trace elements (BIII medium), 1.0% glucose, 0.5% Tween 80, and 10 mM 2,2-dimethylsuccinate (DMS), pH 4.5. Veratryl alcohol, an apparent inducer of lignin peroxidase activity, as well as substrate, was added at the time of inoculation to the growth medium at a final concentration of 0.4 mM. Cultures were harvested at the time of maximum total lignin peroxidase activity (day 5–6 after inoculation).

Lignin peroxidase assay and reactions

Lignin peroxidase activity in the cultures was measured periodically by determining the rate of oxidation of veratryl alcohol to veratraldehyde. The veratryl alcohol oxidation assay was routinely run with 1–10 µg of enzyme in 2 mM veratryl alcohol, 0.4 mM H₂O₂, and 0.1 mM sodium tartrate, pH 4.0, in a final volume of 0.5 ml. The reactions were started by H₂O₂ addition and were monitored at 310 nm. Oxidation of 1,4-dimethoxybenzene was monitored as decreased absorbance at 286 nm.

Manganese-dependent peroxidase assay

Manganese-dependent peroxidase activity was assayed by the procedure of Paszczynski et al. Protein was determined from the heme absorbance at 409 nm.

HPLC analyses and lignin peroxidase purification

Samples of extracellular culture fluid, concentrated 20-fold using an Amicon (Danvers, MA) Ultrafiltration Unit (YM10 membrane), were dialyzed against 10 mM sodium acetate, pH 6.0, and subsequently filtered through 0.45-µm filters: Gelman Acrodisc Low Protein Binding (Ann Arbor, MI). HPLC analysis was performed on a Gilson (Villiers le Bel, France) system equipped with a UV-vis monitor using a Pharmacia (Piscataway, NJ) FPLC MonoQ anion exchange column. The mobile phase consisted of a gradient from 10 mM to 1 M sodium acetate, pH 6.0, and was applied over a 40-min period at a flow rate of 2 ml min⁻¹ with constant monitoring at 409 and 280 nm.

SDS gel electrophoresis

Lignin peroxidase was analyzed by SDS-polyacrylamide slab-gel electrophoresis (SDS-PAGE) using the modified procedure of Knecht et al. The running gel was 10, 12, or 15% acrylamide, pH 6.0, and the stacking gel was 3.75% acrylamide. Proteins were visualized by Coomassie or silver staining and by immunological (Western) blot analysis. The H8 and H2 antibodies were produced in New Zealand White rabbits. Molecular weight markers were obtained from Sigma (M = 116, 97, 66, 45, 24 kDa).

Isoelectric focusing

The procedures described by Tien and Kirk were used. Proteins were focused over the range of pH 3 to 6, with standards of pI 3.55 (amylglucosidase from Aspergillus oryzae), 4.55 (trypsin inhibitor from soybean), 5.13 (β-lactoglobulin A from bovine milk), and 5.80 (bovine serum albumin) as markers.
5.85 (carbonic anhydrase B from bovine erythrocytes); all were from Sigma.

In situ heme extinction coefficients

The extinction coefficient of isoenzyme H8 was determined earlier, based on its protoporphyrin IX pyridine hemochromogen complex.12 Extinction coefficients of the hemos were calculated based on absorbance of the native enzymes at 409 nm and of their pyridine hemochromogen complexes in comparison to H8.

Determination of TN and K_M

These parameters were determined as described by Tien et al.21 at pH 4.0.

Peptide digest (V8 protease) analyses

Ten micrograms of purified lignin peroxidase isoenzyme were mixed with 40 µl of a 10 µg ml⁻¹ solution of V8 protease (Sigma type XVII, Sigma, St. Louis, MO) in 0.1 M Tris-HCl, pH 7.0. The samples, in 25% glycerol containing bromophenol blue, were loaded onto 15% SDS-PAGE gels with 3.75% stacker and electrophoresed until the blue dye reached the gel interface. At that time, electrophoresis was stopped for 30 min to allow proteolytic digestion, and then continued. The peptides were visualized by silver staining.22 Molecular size standards for SDS-PAGE were from Sigma (M_r = 14–70 kDa).

Periodic acid staining (PAS)

The lignin peroxidases were stained for the presence of carbohydrates by reaction with periodic acid followed by Schiffs Reagent.24 Purified lignin peroxidase isoenzymes were electrophoresed on a 10% SDS-polyacrylamide gel which was subsequently incubated with 3.75% stacker and electrophoresed until the blue dye reached the gel interface. At that time, electrophoresis was stopped for 30 min to allow proteolytic digestion, and then continued. The peptides were visualized by silver staining.22 Molecular size standards for SDS-PAGE were from Sigma (M_r = 14–70 kDa).

Concanavalin A–Sepharose binding

Concentrated P. chrysosporium culture fluid was loaded onto a concanavalin A-Sepharose (Sigma) column in 10 mM sodium acetate, pH 6.0, 2.0 mM NaCl, with a gradient of methyl α-β-mannopyranoside from 0 to 0.2 M. Enzymes that eluted from the column were identified by MonoQ HPLC analysis.

N-Glycanase digestion

N-Glycanase (Genzyme, Cambridge, MA) was used to digest asparagine-linked carbohydrates from the lignin peroxidases.25 For complete digestion, the glycoprotein sample (2.5 µg) was first denatured by boiling for 3 min in the presence of 0.5% SDS and 0.1 M β-mercaptoethanol. The sample was diluted into 0.2 M sodium phosphate, pH 8.6, 5 mM EDTA, 1.25% NP-40 [Nonidet P40 (octylphenoxypoly-ethoxyethanol)], and 0.26 U of N-glycanase; total sample volume was 50 µl. The reaction mixture was incubated for 19 h at 30°C, and the sample was then subjected to SDS-PAGE analysis. M_r standards (Sigma) were 116, 97, 66,45, and 24 kDa.

For determining the effect of N-glycanase digestion on lignin peroxidase activity, the lignin peroxidases were not denatured. The lignin peroxidase sample was diluted into the sodium phosphate-EDTA buffer and 43 U of N-glycanase ml⁻¹ of sample was added. The reaction mixture was incubated overnight at 30°C, after which the deglycosylated lignin peroxidase was tested for activity.

Results and discussion

Ten hemeproteins (409 nm-absorbing) were apparent when concentrated culture fluids were subjected to anion exchange HPLC. These were designated H1–H10. H1, H2, H6, H7, H8, and H10 exhibited lignin peroxidase activity, oxidizing veratryl alcohol to veratraldehyde in the presence of H_2O_2. They were eluted in typical preparations at sodium acetate polarities of 0.16, 0.18, 0.34, 0.40, 0.43, and 0.58, respectively. Lignin peroxidase H8 was the dominant isoenzyme in 5–6-day cultures and corresponds to the one first isolated by Tien and Kirk by DEAE column chromatography. For the studies here, each of the lignin peroxidases was further purified by one or two additional elutions from the HPLC column.

Physical properties

All of the lignin peroxidase isoenzymes have similar molecular weights (38–43 kDa) as determined by SDS-PAGE and have similar in situ extinction coefficients at 409 nm (Table 1). Molecular weight determinations are only approximate because glycoproteins do not bind SDS quantitatively and therefore migration in polyacrylamide gels is not linearly related to the log of molecular weight. Similar molecular weights have been reported in general for P. chrysosporium lignin peroxidases.12,21–12,9,11 The cDNA sequences for three lignin peroxidase isoenzymes point to a nonglycosylated molecular weight of approximately 37 kDa.12,5

Although lignin peroxidase H6 eluted as a single peak from the HPLC system, two Coomassie blue-(or silver-) staining bands were observed by SDS-PAGE. When electrophoresed in a polyacrylamide gel system without the denaturants β-mercaptoethanol and SDS,

Table 1 Physical properties of ligninase isoenzymes

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>H1</th>
<th>H2</th>
<th>H6</th>
<th>H7</th>
<th>H8</th>
<th>H10</th>
</tr>
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<tbody>
<tr>
<td>e (nm)</td>
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<td>165</td>
<td>162</td>
<td>177</td>
<td>168</td>
<td>182</td>
</tr>
<tr>
<td>Molecular mass (kDa)</td>
<td>38</td>
<td>38</td>
<td>40.42</td>
<td>42</td>
<td>42</td>
<td>43</td>
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</table>
Figure 1. SDS-PAGE analysis of the lignin peroxidase isoenzymes before and after digestion with N-glycanase. Lanes: 1, M, marker proteins; 2 and 3, 10 μg each of ovalbumin with and without N-glycanase treatment; 4–15, lignin peroxidase isoenzymes with and without N-glycanase treatment. Amounts of lignin peroxidase per lane (μg): H1, 3; H2, 8; H6, 7; H7, 12; H8, 10; H10, 10.

however, H6 gave a single band (data not shown), migrating slightly more slowly than lignin peroxidase H8. Resolution into two bands with the denaturants suggests that H6 was a mixture of two closely similar proteins. Data on glycosylation, discussed below, indicate approximately the same amount of N-linked and O-linked glycosylation for the two bands of H6 observed by SDS-PAGE.

The six isoenzymes isolated here exhibited different isoelectric points (pl)s, determination of which allowed a comparison to be made with the lignin peroxidases separated by Leisola et al. Our isoenzymes have the following pl:s: H1, 4.7; H2, 4.4; H6, 3.7; H8, 3.5; and H10, 3.3. These values are in the same range as those reported by Leisola et al. from nitrogen-limited cultures. Their lignin peroxidase proteins designated 3 (pi 4.4), 6 (pl 3.8), 7 (pl 3.7), 9 (pl 3.5), and 10 (pl 3.2) correspond closely to our isoenzymes H2, H6, H7, H8, and H10, respectively. Our H1 (pl 4.7) corresponds to their lignin peroxidase protein 1 (pl 4.7) from carbon-limited cultures, but not to any from their nitrogen-limited cultures. Their lignin peroxidase protein 8 (pl 3.6) from nitrogen-starved cultures perhaps corresponds to one of the two component proteins in our protein H6, which occasionally was resolved into two close bands.

The six isoenzymes were examined qualitatively for glycosylation by three procedures: digestion with N-glycanase followed by SDS-PAGE analysis, periodic acid staining (PAS), and binding to concanavalin A. By all three criteria, all six isoenzymes are glycoproteins.

The enzyme N-glycanase (peptide: N-glycosidase F) hydrolyses asparagine-N-linked carbohydrates from glycoproteins. Proteins that contain N-linked carbohydrate exhibit increased mobility on SDS-PAGE. The new nobilities reflect the size of the protein, which, however, still might contain oxygen-(O-) linked carbohydrate. All six lignin peroxidase isoenzymes exhibited increased SDS-PAGE mobility after N-glycanase digestion (Figure 1), indicating that all contain carbohydrate. From the changes in apparent molecular weight following N-glycanase digestion, the sizes of the removed carbohydrate can be estimated roughly to be 2.5–3 kDa.

Periodic acid reacts with glycoproteins by cleaving between vicinal hydroxyl groups in the carbohydrate moieties. The resulting aldehyde groups react with Schiff's Reagent to give a pink color. The isolated isoenzymes were subjected to electrophoresis on SDS-PAGE and the gels subjected to the PAS procedure; all isoenzymes were stained, as were positive control glycoproteins ovalbumin and IgG; negative control proteins, protein A and Escherichia coli cell lysate gave no coloration. Notably, all of the N-glycanase treated lignin peroxidases still gave positive PAS reactions, indicating that they very likely contain O-linked carbohydrate.

Glycoproteins containing α-D-mannose or α-D-glucose bind to the lectin concanavalin A. We found that the six isoenzymes were all bound by a concanavalin A-Sepharose column. All six were eluted together from the column by a gradient of methyl α-D-mannopyranoside (0–0.2 M).

Polyclonal antibodies to ligninases H2 and H8 were made independently in rabbits. The antibodies were reacted with the lignin peroxidases in immunological (Western) blot experiments. H2 and H8 antibodies both showed cross-reactivity to all the other isoenzymes, indicating some degree of homology. We had shown earlier that polyclonal antibody to H8 cross-reacts with all of the isoenzymes.

Considerable, but incomplete, homology among the six lignin peroxidase isoenzymes was also disclosed by analyzing the peptides produced on V8 proteolysis. This protease cleaves proteins at glutamic acid residues and less frequently at aspartic acid residues. The experimental conditions were such that partial proteolytic digestion was achieved so that a peptide digestion pattern could be observed. The sizes of the peptides are given in Table 2. The peptides from H1 and H2 were essentially identical, and the peptides from H6, H7, and H8 were identical. H1 and H2 gave three peptides (28.9, 15.9, and 14.9 kDa) not seen with H6, H7, or H8. The latter three had one unique peptide of 17.2 kDa not seen with H1 and H2. The pattern of H10 suggests that it is more susceptible to V8 protease digestion: there was an absence of residual undigested protein and of higher molecular size peptides. H10 did, however, have four peptides in common with H1, H2, H6, H7, and H8; it also had one unique peptide of 18.4 kDa. The V8 peptide results show, therefore, that all of the lignin peroxidase isoenzymes are related to each other because of the four shared peptide fragments. Additionally, it appears that H1 and H2 form one class; H6, H7, and H8 belong to a second class; and H10 belongs to a third class. Whether these groups reflect different structural genes or different post-translational modifications is now being investigated through gene cloning experiments.
Catalytic properties

The specific activities of the isoenzymes with vemtryl alcohol as substrate varied over a twofold range (U mg\(^{-1}\)): H2, 13.9; H6, 12.9; H1, 11.1; H7, 8.7; H10, 8.4; and H8, 7.9. The value for H8 is the same as reported earlier.9 and our values are similar to or somewhat lower than those reported by others for isolated lignin peroxidases from \(P.\ chrysosporium\).7,10,11,19

The two major lignin peroxidases from 6-day cul-
tures, H2 and H8, were tested to determine whether partial N-deglycosylation using the enzyme N-glycanase affects catalytic activity against veratryl alcohol. The results of two independent experiments with each isoenzyme showed that activity actually increased slightly after treatment. It should be noted that the N-glycanase treatment of the nondenatured enzymes would not affect buried (inaccessible) N-linked carbohydrates. Maione et al.28 have also found that the activity of a recombinant lignin peroxidase H8 was essentially equivalent to that of \(P.\ chrysosporium\) H8. This recombinant protein, reconstituted after being expressed in Escherichia coli, was nonglycosylated. Therefore, glycosylation does not appear important for enzymatic activity. This finding is similar to the observation that the degree of glycosylation does not greatly affect the specific activity of fungal endoglucanases.29

Three aromatic substrates and \(H_2O_2\) were used to assess the kinetic properties of the isoenzymes: the aromatic substrates were veratryl alcohol, 1,4-dimethoxybenzene, and the lignin model compound 1-(3,4-dimethoxyphenyl)-2(o-methoxyphenox)-propane-1,3-diol (model I). The principal product from veratryl alcohol and Model I is veratraldehyde9 and from 1,4-dimethoxybenzene, \(p\)-benzoquinone.25 Results of \(K_m\) and TN determinations are given in Table 3. \(K_m\) values for the aromatic substrates varied significantly; e.g. for veratryl alcohol, \(K_m\) values were from 86 to 480 \(\mu\)M. Similarly, TN values varied two- to sixfold; for veratryl alcohol the isoenzymes varied from 1.3 to 8.3 \(s^{-1}\). The ranking order of the isoenzymes differed with the different substrates, suggesting differences in affinity or in active site accessibility. The \(K_m\) for \(H_2O_2\) varied between 13 and 77 \(\mu\)M.

Preliminary examination of the four nonlignin peroxidase hemeproteins

In isolating the lignin peroxidase isoenzymes, we also observed four nonlignin peroxidase hemeproteins: H3 and H9 were essentially pure by HPLC analysis, and H4 and H5 were obtained as a mixture. None of these hemeproteins exhibited veratryl alcohol-oxidizing activity, but all oxidized vanillylacetone in tartrate buffer in a reaction dependent on the presence of Mn\(^{2+}\) and \(H_2O_2\). These enzymes, therefore, are “manganese-dependent peroxidases,” which have been isolated from ligninolytic cultures of \(P.\ chrysosporium\) and partially characterized.30-32 Leisola et al.7 reported separation of a total of six manganese-dependent peroxidases by analytical isoelectric focusing from the extracellular broth of \(P.\ chrysosporium\) grown for various times under carbon- and nitrogen-limiting conditions.

Effect of culture time on isoenzyme pattern

The quantities and relative proportions of the isoenzymes varied with culture age, as shown by analysis of the extracellular culture supernatants on days 2, 4, 5, 6, 8, and 10 after inoculation. The concentrates were normalized to represent equal volumes of culture supernatant for chromatography. Figure 2 shows the HPLC profiles monitored at 409 nm. On day 2 there was no detectable 409 nm-absorbing material; by day 4 such material was present, and by day 5 there were at least 10 peaks with 409 nm absorbance. Most of the 280 nm-absorbing material corresponded to the 409 rim-absorbing material, indicating that hemeproteins predominated.7 Peak numbers for the hemeproteins were assigned earlier on the basis of the day 5 profile.8

Table 4 gives the integrated areas of the 409 nm-absorbing proteins from each day of culture. H8, the most abundant isoenzyme under these culture conditions, was present in approximately the same amounts from each day. The quantities of H1 and H2 increased with culture age, while the H6 and H7 amounts decreased.

\[\text{Table 2 Peptides of lignin peroxidases from partial V* proteo-
ysis: peptide sizes (kDa)}\]

<table>
<thead>
<tr>
<th>Lignin Peroxidase</th>
<th>H1</th>
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\(\text{Table 3 Catalytic properties of lignin peroxidases with three substrates)}\]

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>(H_2O_2)</th>
<th>Veratryl alcohol</th>
<th>Model I</th>
<th>1,4-Dimethoxybenzene</th>
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<tr>
<td>H1</td>
<td>(K_m)</td>
<td>(K_m)</td>
<td>TN(^2)</td>
<td>(K_m)</td>
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<td>H8</td>
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<td>89</td>
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<td>H10</td>
<td>24</td>
<td>190</td>
<td>444</td>
<td>0.43</td>
</tr>
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</table>

\(\text{Table 3 Catalytic properties of lignin peroxidases with three substrates)}\]

1 \(K_m\) units are micromolar
2 \(\text{TN} = \text{Turnover number per second}\)
Characterization of lignin peroxidase isoenzymes: R. L. Farrell et al.

on days 5, 6, 8, and 10. H2 increased from day 4 to day 6 and then decreased by almost two-thirds by day 10. H6 and H7 were each present in approximately equal amounts on days 5 and 6, and then they, too, decreased markedly by days 8 and 10. In contrast to the other isoenzymes, H10 increased from day 5 to day 10; the increase was over sevenfold. It is possible that H10 is derived from the other isoenzymes. These results show that these proteins are continually being synthesized and degraded. The total amount of hemeprotein varied at most by 20% from day 5 to day 10. Tonon and Odier\(^\dagger\) showed that enzyme activity is lost by both turnover (presumably proteolysis) and destruction by \(H_2O_2\). Our earlier work showed that growth medium composition and method of cultivation affect the composition of the isoenzyme mixture. Leisola et al.\(^\dagger\) reported qualitative variations in isoenzymes with culture age and medium composition.

Conclusions

Our results are in good accord with—and extend—those from other laboratories.\(^7,10,11\) We suspected that the large number of lignin peroxidase proteins reported by Leisola et al., \(^7\) and perhaps some of the multiplicity reported here, is due in part to post-translational modifications reflecting differences in age and culture conditions. The total number of structural genes is not yet known, although three have been described. Therefore, the molecular basis for multiplicity is as yet unclear. Also unclear is the significance of lignin peroxidase multiplicity to lignin degradation. Tonon and Odier\(^\dagger\) showed that veratryl alcohol, which is secreted by cultures, protects only one of four isoenzymes in strain INRA-12 from inactivation by \(H_2O_2\). Thus the isoenzymes differ in stability, and, as our work shows, in catalytic properties and quantity. It therefore seems likely that certain isoenzymes might be more important than others in lignin degradation. It is also possible that the isoenzymes act synergistically.

These questions, and the question of the basis for multiplicity, are being pursued in part by molecular genetics approaches.

Since the discovery of lignin peroxidase in 1983, this family of isoenzymes has been referred to by several names, e.g. ligninase, lignin peroxidase, diarylpropane oxygenase. The individual isoenzymes have been identified either by their HPLC elution profile, e.g. H1 through H10 as used in this paper, or by their isoelectric points.

We now suggest a nomenclature for the lignin peroxidase and manganese-dependent peroxidase isoenzymes. We suggest the first isoenzyme isolated and characterized, previously called H8, with a pI of 3.5, should be called LiP1. This is the most abundant

![Figure 2 Anion exchange HPLC profile (A<sub>400nm</sub>) of concentrated extracellular broth from cultures of the indicated ages](image)

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>Day 4 % of total lignin peroxidase</th>
<th>Day 5 % of total lignin peroxidase</th>
<th>Day 6 % of total lignin peroxidase</th>
<th>Day 8 % of total lignin peroxidase</th>
<th>Day 10 % of total lignin peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>20,300 11</td>
<td>58,890 6</td>
<td>80,100 7</td>
<td>44,540 5</td>
<td>17,940 2</td>
</tr>
<tr>
<td>H2</td>
<td>79,900 42</td>
<td>244,820 24</td>
<td>300,190 27</td>
<td>173,160 19</td>
<td>120,190 12</td>
</tr>
<tr>
<td>H6</td>
<td>21,590 11</td>
<td>119,910 11</td>
<td>117,700 11</td>
<td>45,020 5</td>
<td>43,100 4</td>
</tr>
<tr>
<td>H7</td>
<td>5,540 3</td>
<td>66,620 6</td>
<td>77,180 7</td>
<td>70,040 8</td>
<td>40,760 4</td>
</tr>
<tr>
<td>H8</td>
<td>61,420 33</td>
<td>490,820 48</td>
<td>440,660 39</td>
<td>357,150 44</td>
<td>412,820 42</td>
</tr>
<tr>
<td>H10</td>
<td>NI</td>
<td>49,160 5</td>
<td>101,610 9</td>
<td>166,180 19</td>
<td>352,950 36</td>
</tr>
</tbody>
</table>

NI, not integrated

Table 4 Quantities of lignin peroxidase isoenzyme from the extracellular culture fluid of P. chrysosporium

isoenzyme present under nitrogen limitation, even after 10 days of culture, as revealed in our study. The second most abundant lignin peroxidase, herein referred to as H2 with a pI of 4.4, should be designated LiP2.

The nomenclature scheme for the lignin peroxidases and manganese-dependent peroxidases mentioned in our study is given in Table 5. Other isoenzymes in the lignin peroxidase family or in the manganese-dependent peroxidase family, not mentioned in this study, can be given sequential numeration in the LiP or the MnP series.

We suggest that this scheme also be used to identify genes encoding these isoenzymes. For example, the gene encoding the protein LiP1 should be referred to as the LiP1 gene rather than the H8 gene. We suggest that if two isoenzymes have different pIs and were found to be transcribed from the same gene, therefore differing in post-translational modification(s), that they be given the same LiP number. To signify differences in post-translational modification(s), a dash (-) can be used and the two isoenzymes differentiated by a second numeration. For example, if there are two lignin peroxidase isoenzymes with different isoelectric points but which are found to be encoded by the same gene, and they differ in their glycosylation, then they would be called LiPN-1 and LiPN-2 where N is their number in the LiP nomenclature.

We hope that this nomenclature scheme will simplify enzymological and molecular biological studies of the lignin peroxidase and manganese-dependent peroxidase isoenzyme families.

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References