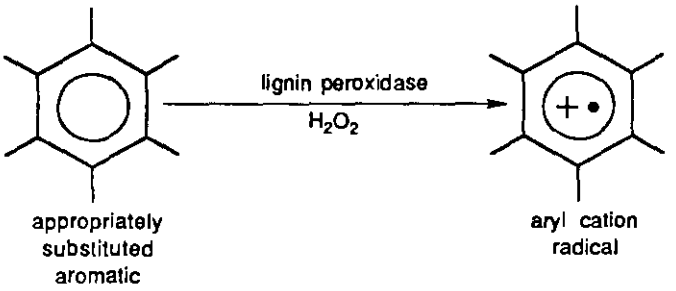
In: Lidstrom, Mary E., ed. Methods in enzymology. Vol. 188. Hydrocarbons and Methylotrophy. San Diego, CA: Academic Press, Inc.; 1990. Chapter 27.

[27] Lignin Peroxidase from Fungi: *Phanerochaete chrysosporium*

By T. KENT KIRK,* MING TIEN, PHILIP J. KERSTEN,* B. KALYANARAMAN, KENNETH E. HAMMEL, and ROBERTA L. FARRELL



Introduction

Lignin peroxidases are unusual oxidizing extracellular peroxidases produced by most of the ligninolytic fungi that cause white rot of wood.[1-4] In the presence of H_2O_2 they catalyze the one-electron oxidation of a wide variety of aromatics to yield, as initial products, aryl cation radicals that subsequently undergo substituent-dependent reactions of both radical and ionic nature.[1-3] These enzymes bring about the oxidative cleavage of lignin model compounds[1-3,5] and are thought to be of key importance in lignin

[1] T. K. Kirk and R. L. Farrell, *Annu. Rev. Microbiol.* **41,** 465 (1987).
[2] J. A. Buswell and E. Odier, *CRC Crit. Rev. Biotechnol.* **6,** 1 (1987).
[3] M. Tien, *CRC Crit. Rev. Microbiol* **15,** 141 (1987).
[4] K. E. Hammel, B. Kalyananman, and T. K. Kirk, *J. Biol. Chem.* **261,** 16948 (1986).
[5] T. Umezawa and T. Higuchi, *FEBS Lett.* **218,** 255 (1987), and papers cited therein.

* This article was written and prepared by T. Kent Kirk and Philip J. Kersten as U.S. Government employees on official time.

1990 by Academic Press, Inc.

biodegradation. They have also been shown to oxidize various polycyclic aromatic hydrocarbons and related structures, including pyrene, anthracene, benzo[*a*]pyrene. dibenzo[*p*]dioxin, and thianthrene.[4,6,7] Certain halogenated aromatics are also oxidized, including 2-chlorodibenzo[*p*]dioxin[4] and 2,4,6-tnchlorophenol.[8] Lignin peroxidases, which are heme glycoproteins, were discovered in the extracellular broth of secondary metabolic cultures of the basidiomycete *Phanerochaete chrysosporium*,[9,10] from which various isoenzyme forms have been purified and studied.[11-14] The major studied lignin peroxidase has a p*I* of 3.5 and has been referred to as H8.[15] A nomenclature scheme has been proposed recently in which H8 is referred to as LiP1.[14] Lignin peroxidase has also been isolated from the lignin-degrading basidiomycetes *Phlebia radiata*[16] and *Trametes versicolor*.[17] The literature has been reviewed recently.[1-3] An earlier description of lignin peroxidase, by Tien and Kirk, is found elsewhere in this series.[18]

Assay Methods

Principles. Three simple and similar assay methods are described below. Although no assays based on oxidation of hydrocarbons have been used, a procedure for demonstrating the oxidation of pyrene is described below after the description of the assays.

The assay most commonly used is based on the oxidation of veratryl

[6] S. D. Haemmerli, M. S. A. Leisola, D. Sanglard and A. Fiechter, *J. Biol. Chem.* **261,** 6900 (1986).

[7] R. P. Schreiner, S. E. Stephens, Jr., and M. Tien, *Appl. Environ. Microbiol.* **54,** 1858 (1988).

[8] K. E. Hammel and P. J. Tardone, *Biochemistry* **27,** 6563 (1988).

[9] M. Tien and T. K. Kirk, *Science* **221,** 661 (1983).

[10] J. K. Glenn, M. A. Morgan, M. B. Mayfield, M. Kuwahara, and M. H. Gold, *Biochem. Biophys. Res. Commun.* **114,** 1077 (1983).

[11] M. Tien and T. K. Kirk, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 2280 (1984).

[12] M. H. Gold, M. Kuwahara, A. A. Chiu, and J. K. Glenn, *Arch. Biochem. Biophys.* **234,** 353 (1984).

[13] A. Paszczyynski, V.-B. Huynh, and R. L. Crawford, *Arch. Biochem. Biophys.* **244,** 750 (1986).

[14] R. L. Farrell, K. E. Murtagh, M. Tien, M. D. Mozuch, and T. K. Kirk, *Enzyme Microb. Technol.* **11,** 322 (1989).

[15] T. K. Kirk, S. Croan, M. Tien, K. E. Murtagh, and R. L. Farrell, *Enzyme Microb. Technol.* **8,** 27 (1986).

[16] A. Kantelinen, K. Waldner, M.-L. Niku-Paavola, and M. S. A. Leisola, *Appl. Microbiol. Biotechnol.* **28,** 193 (1988).

[17] L. Jönsson, T. Johansson, K. Sjöström, and P. O. Nyman, *Acta Chem. Scand., Ser. B* **41,** 766 (1987).

[18] M. Tien and T. K. Kirk, this series. Vol. 161, p. 238.

alcohol (3,4-dimethoxybenzyl alcohol) to veratraldehyde in the presence of H_2O_2; the increase in absorbance at 310 nm is monitored.[11] The second method is based on the oxidation, in the presence of H_2O_2, of 1,4-dimethoxybenzene to 1,4-*p*-benzoquinone (monitored at 250 nm).[14,19] The third method is based on the oxidation, in the presence of H_2O_2, of 1,2,4,5-tetramethoxybenzene to the corresponding aryl cation radical, which is relatively stable under the conditions employed; the reaction is followed by monitoring the increase in absorbance at 450 nm.[19] [The course of oxidation of 1,4-dimethoxybenzene and 1,2,4,5-tetramethoxybenzene can also be followed readily by electron spin resonance (ESR) spectroscopy as described below. However, the radical from the 1,4-congener decomposes rapidly, which makes it more difficult to use in kinetic studies.] Final products of the oxidation of the 1,2,4,5-congener are 2,5-dimethoxy-*p*-benzoquinone and 4,5-dimethoxy-*o*-benzoquinone in a 4:1 ratio.[20]

The enzyme concentration for purified preparations may conveniently be determined from the absorbance at 409 nm, using an extinction coefficient of 168 mM^{-1} cm^{-1}.[21] The optimum temperature for the assays is approximately 37°.

Reagents for Method 1

10 m*M* veratryl alcohol (purified by distillation'')
0.25 *M* sodium (+)-tartrate, pH 3.0
10 m*M* H_2O_2 (prepared fresh)

Reagents for Method 2

10 m*M* 1,4-dimethoxybenzene in water (gentle heat aids dissolution)
0.25 *M* sodium (+)-tartrate, pH 3.0
10 m*M* H_2O_2 (prepared fresh)

Reagents for Method 3

10 m*M* 1,2,4,5-tetramethoxybenzene[19] in 50% (v/v) ethanol in water
0.25 *M* sodium (+)-tartrate, pH 3.0
10 m*M* H_2O_2 (prepared fresh)

Procedure for Method 1. Reaction mixtures contain 2 m*M* veratryl alcohol, 0.4 m*M* H_2O_2, 50 m*M* tartrate, and enough lignin peroxidase to give an absorbance change of about 0.2 min^{-1} (1-cm cuvette). Reactions are started by adding the peroxide and are monitored at 310 nm; the molar extinction coefficient of the product, veratraldehyde, is 9300 M^{-1} cm^{-1}.[11]

[19] P. J. Kersten, M. Tien, B. Kalyanaraman, and T. K. Kirk, *J. Biol. Chem.* **260,** 2609 (1985).
[20] P. J. Kersten, B. Kalyanaraman, K. E. Hammel, B. Reinhammar, and T. K. Kirk. *Biochem. J,* in press. (1990).
[21] M. Tien, T. K. Kirk, C. Bull, and J. A. Fee, *J. Biol. Chem.* **261,** 1687 (1986).

Procedure for Method 2. Reaction mixtures contain 0.15 m*M* 1,4-dimethoxybenzene, 0.4 m*M* H_2O_2, 100 m*M* tartrate, and enough lignin peroxidase to give an absorbance change of about 0.2 min^{-1} (1-cm cuvette). Reactions are started by adding the peroxide and are monitored by decrease in absorbance at 286 nm; the molar extinction of the substrate is 1260 M^{-1} cm^{-1}.

Procedure for Method 3. Reaction mixtures contain 0.2 m*M* 1,2,4,5-tetramethoxybenzene, 0.4 m*M* H_2O_2, 50 m*M* tartrate, and enough lignin peroxidase to give an absorbance change of 0.2 min^{-1}. Reactions are started by adding the peroxide and are monitored as increased absorbance at 450 nm; the molar extinction coefficient of the cation radical product is 9800 M^{-1} cm^{-1}.[19] Control reactions are necessary to verify that the rate of cation radical decay is less than the rate of its production.

Comments on Assays

Lignin peroxidase is most active below pH 3, but it is not very stable under these conditions; thus, reaction rates are linear only for about 2 min. For that reason, pH 3 is used here. The assays can be run at pH 4 also, but the reactions are slower. The kinetic data in Table I were determined at pH 4 for three of the substrates.

The enzyme is inactivated by H_2O_2 in the absence of a reducing substrate such as veratryl alcohol. Therefore, care should be taken to minimize the preincubation of enzyme with H_2O_2 in the absence of aromatic substrate. Lignin peroxidase activity shows a high temperature dependence, and therefore for reproducible results the temperature should be held constant; the rate approximately doubles with every 7° increase (M. Tien, unpublished).

We have described three assays. The first two are similar, both measuring two-electron oxidation products. Products in addition to veratraldehyde can be formed on the oxidation of veratryl alcohol, particularly at higher pH values,[22] although under the conditions described here such products are minor. Initial studies indicated that the oxidation of veratryl alcohol proceeds by a direct two-electron oxidation.[21] However, more recent studies by Marquez *et al.* [23] indicate that one-electron-oxidized intermediates are formed. Although the exact chemical mechanism of this oxidation is being investigated, this assay is a convenient and reliable method for measuring activity. In the second assay, *p*-benzoquinone and

[22] S. D. Haemmerli, H. E. Schoemaker, H. W. H. Schmidt, and M. S. A. Leisola, *FEBS Lett.* **220,** 149 (1987).

[23] L. Marquez, H. Wariishi, H. B. Dunford, and M. H. Gold, *J. Biol. Chem.* **263,** 10549 (1988).

methanol are the major products,[19] although other products can be detected. The third assay measures the cation radical directly and is perhaps most representative of the catalytic reaction per se.

Electron Spin Resonance Spectroscopy

Under conditions similar to those of assay Methods 2 and 3 above, the initial products of oxidation—the aryl cation radicals—are stable enough to be detected readily by ESR spectroscopy.[19] The radicals can be detected using a Varian E-9 and E-109 (or equivalent) spectrophotometer operating at 9.5 GHz and employing 100-kHz field modulation. Hyperfine splittings are measured from computer simulations of spectra.[24] The reaction mixtures for direct ESR measurements contain, in a volume of 2 ml, approximately 0.5 μM lignin peroxidase, 0.32 mM H_2O_2. 0.1 M sodium tartrate, pH 2.5, and 1–5 mM of the methoxybenzene substrate. The reactions, at room temperature, are initiated by addition of enzyme, and the mixtures are transferred immediately to an aqueous quartz flat cell positioned in the ESR spectrometer cavity. Figure 1 shows the ESR spectrum of the cation radical of 1,2,4,5-tetramethoxybenzene. Similar incubation conditions are used to detect phenoxy radicals formed from phenolic substrates?' The time course of cation radical formation is monitored at the maximum of the signal intensity with the magnetic field turned off. For certain substrates, the cation radicals or other radical intermediates are not readily detected, but they can be with the use of spin-trapping agents.[24]

Demonstration of Pyrene Oxidation[4]

[This is not described as an assay method.] A 1-ml reaction mixture is prepared in water–*N*,*N*-dimethylformamide (DMF), 8 : 2 by volume, that contains 20 mM sodium tartrate (final), pH 2.5 (solution A). To this solution at room temperature is added pyrene (as a 2 mM stock solution in DMF) to give 20 μM final concentration, and Lip1 (0.5 μM). The reaction is initiated with H_2O_2 (200 μM initially) and followed in repetitive scans between 600 and 220 nm in a double-beam UV–visible spectrophotometer. The reference cell contains solution A, plus DMF to replace the pyrene solution, and H_2O to replace the enzyme and H_2O_2 solutions.

The starting material exhibits peaks at 260, 272, 304, 318, and 334 nm, which decrease in intensity as the reaction progresses. The accumulation of products is accompanied by a general increase in absorbance from 350 to

[24] K. E. Hammel, M. Tien, B. Kalyanaraman, and T. K. Kirk, *J. Biol. Chem.* **260,** 8348 (1985).

[25] E. Odier, M. D. Mozuch, B. Kalyanaraman, and T. K. Kirk, *Biochimie* **70,** 847 (1988).

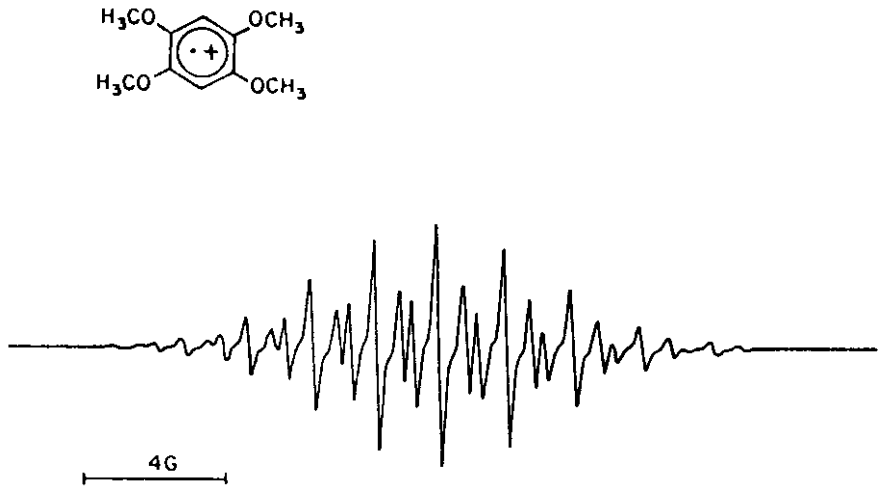


FIG. 1. Electron spin resonance spectrum of the cation radical of 1,2,4,5-tetramethoxybenzene.

550 nm. Near isosbestic points occur at 246, 256, 276, 298, and 340 nm. The two major products (~ 85% of the total) are pyrene-1,6-quinone and pyrene- 1,8-quinone.[4]

Enzyme Production

Methods for lignin peroxidase production in early studies used culture conditions optimized for lignin degradation in shallow stationary flasks.[9,15] Advantages with this type of experimental setup include the small scale and the ease with which the samples can be taken for analyses. These considerations remain important in studies of xenobiotic or radiolabeled substrate degradation studies. However, scale-up of enzyme production with shallow stationary cultures is labor intensive and becomes impractical. Consequently, a number of methods for lignin peroxidase production have been devised, including growing the fungus in rotating biological contactors,[15] in rotating drums,[13] and immobilized in gels.[26] For most studies, however, and for routine production of enzyme, the fungus is grown as pellets in agitated 2-liter flasks. Conditions required for lignin peroxidase production under nutrient nitrogen starvation (to achieve the necessary secondary metabolic state) are presented below with emphasis on key parameters required for stationary and agitated Erlenmeyer flask cultures. *Phanerochaete chrysosporium* (strain BKM-F-1767; ATCC 24725)

[26] Y.-Y. Linko, M. Leisola, N. Lindholm, J. Troller, P. Linko, and A. Fiechter, *J. Biotechnol.* **4,** 283 (1986).

has been investigated extensively under both conditions and produces the highest level of lignin peroxidase activity of the wild-type strains studied.[27]

The composition of agar slants for maintenance and spore production is 10 g glucose, 10 g malt extract, 2 g peptone, 2 g yeast extract, 1 g L-asparagine, 2 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 1 mg thiamin-HCl, and 20 g agar per liter. After several days (preferably> 7) of incubation at 39°, dense conidiation is apparent over the agar surface; the slants are then stored in a refrigerator (5°) until needed but are used within 8 weeks. Spores are suspended in sterile water by gently rubbing the surface of the flooded agar with a sterile inoculation loop or glass rod. The spore suspension is passed through sterile glass wool to remove mycelia. and the spore concentration is determined at 650 nm (an absorbance of 1 cm^{-1} is approximately 5×10^6 spores/ml).

The culture media for both stationary and agitated cultures contain, per liter of culture: 2 g KH_2PO_4 (from a 40× stock solution), 0.5 g $MgSO_4 \cdot 7H_2O$ (40× stock), 0.1 g $CaCl_2 \cdot 2H_2O$ (40× stock), 1% glucose (10× stock), 10 m*M* pH 4.2 *trans*-aconitate or 2,2-dimethyl succinate (10× stock), 1 mg thiamin-HCl (1000× stock), 0.2 g ammonium tartrate (40× stock), 100 ml spore suspension inoculum (made fresh; OD_{650nm} 0.5), and 10 ml of trace element solution (100× stock, see below). Sterile distilled water is added to make I liter. The thiamin and KH_2PO_4 stock solutions are filter sterilized; the others are autoclaved as separate solutions.

The 100× stock solution of trace elements is made by first dissolving 1.5 g of nitrilotriacetic acid in 800 ml water with adjustment of the pH to approximately 6.5 with KOH. The following are then added with dissolution: 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CoSO_4$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$, 10 mg $A1K(SO_4)_2 \cdot 12H_2O$, 10 mg H_3BO_3, and 10 mg $Na_2MoO_4 \cdot 2H_2O$. Water is added to 1 liter.

For shallow stationary cultures, 10 ml of culture fluid is used in rubber-stoppered 125-ml Erlenmeyer flasks at 30–39°. Cultures are flushed with 100% O_2 2 days after inoculation. Enzyme production can be stimulated by including 0.4 m*M* veratryl alcohol final concentration and trace elements (7 times the above concentration) at the time of inoculation.[15] A stock solution of veratryl alcohol is filter-sterilized through a 2.0-μm filter.

For agitated cultures, 750 ml of culture fluid is used in 2-liter flasks on a rotary shaker at 120 rpm (2.5-cm-diameter cycle) at 30–39°. In this case, 0.05% Tween 20 or Tween 80 (final concentration) is included in the medium at the time of inoculation. Cultures are flushed with 100% O_2 after

[27] T. K. Kirk, M. Tien, S. C. Johnsrud, and K.-E. Eriksson, *Enzyme Microb. Technol.* **8,** 75 (1986).

2 days and daily thereafter. Under both stationary and agitated conditions, maximal lignin peroxidase activity is observed between days 4 and 7, depending on temperature. Evidence suggests that enzyme production is highest near 30°. but that maximum titer takes longer to develop.[28] Enzyme production can be stimulated by increasing the concentration of trace elements 7-fold.

Enzyme Purification

The lignin peroxidase-containing extracellular fluid can be harvested by centrifugation (10,000 g for 10 min) at 4° or by passage through cheesecloth. The fluid is then concentrated by ultrafiltration in a Millipore Minitan unit with a 10-kDa cutoff membrane. Alternatively, the isoenzymes can be precipitated with 66% acetone at - 10°, then dissolved in column buffer.[12] Four liters of culture supernatant are typically concentrated to about 50 ml. The concentrate is filtered (0.45-μm pore size) to remove precipitated mycelial polysaccharide and further concentrated (Amicon YM10, Danvers, MA, 10-kDa cutoff) to a final volume less than 15 ml. The preparation is then dialyzed overnight at 4° against 4 liters of either 10 m*M* sodium acetate, pH 6, for anion-exchange fast protein liquid chromatography (FPLC), or 5 m*M* sodium succinate, pH 5.5 for chromatography on DEAE-BioGel A. Approximately 90% recovery of enzymatic activity is typically obtained at this point.

The lignin peroxidase isoenzymes are purified by anion-exchange chromatography on DEAE-BioGel A, or on a larger scale by Q-Sepharose Fast Flow (Pharmacia, Piscataway, NJ) column chromatography, or by FPLC with a Mono Q column or Fast Flow Q column (Pharmacia).

For chromatography on DEAE-BioGel A,[18] all steps are performed at 4°. The column (1 × 16 cm) is equilibrated with 5 m*M* sodium succinate, pH 5.5. The sample is loaded and eluted from the column with a NaCl gradient (0.14 *M*, total volume of 600 ml). A typical elution profile is shown in Fig. 2. The predominant peak (fraction 240) is isoenzyme LiP1 (H8), and is over 95% pure as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or isoelectric focusing. The isolated lignin peroxidase isoenzymes are dialyzed against 5 m*M* potassium phosphate buffer, pH 6.5, for storage at −20°.Recovery of total lignin peroxidase activity in the culture supernatant in this purification is about 75%.

For purification by FPLC, the concentrated dialyzed extracellular fluid, free of polysaccharide, is used. The mobile phase consists of a gradient

[28] M. Asther, C. Capdevila, and G. Corrieu, *Appl. Environ. Microbiol.* **54,** 3194 (1988)

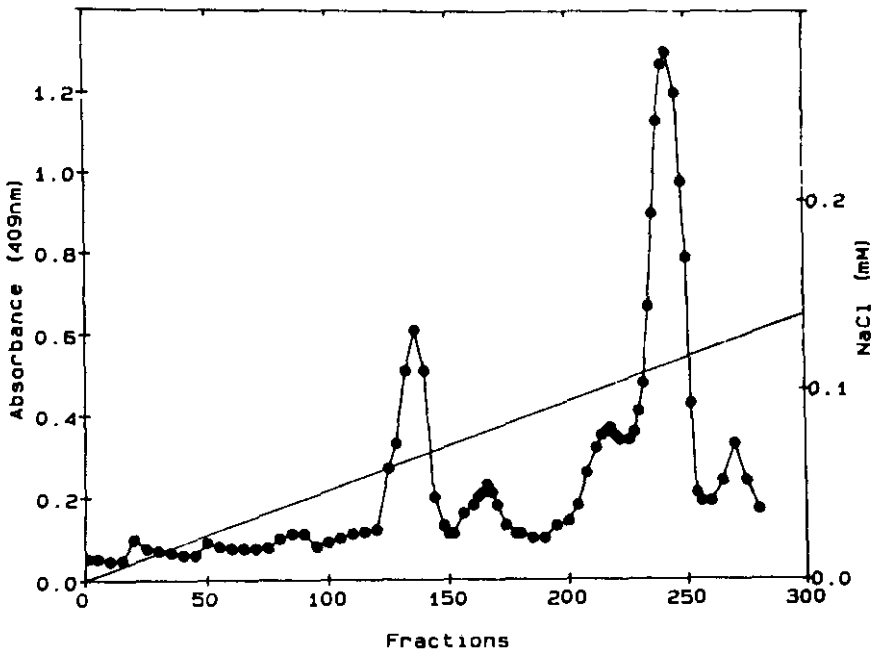


FIG. 2. DEAE-BioGel A elution profile of 409 nm-absorbing proteins in the extracellular fluid from 5-day flask cultures. The sloping line show the NaCl gradient. Fractions of 2 ml were collected. The major peak is LiP1 .

from 10 m*M* to 1 *M* sodium acetate, pH 6.0, applied over a 40-min period at a flow rate of 2 ml/min with monitoring at 409 nm (heme) and at 280 nm (protein). (Monitoring at 409 nm is far more sensitive than 280 nm.) A typical profile is shown in Fig. 3. The peaks designated MnP are associated with manganese peroxidase activity.[13,15,29] In most preparations the eluant containing each isoenzyme is about 85% pure; they can be purified further by repeated passages. The isoenzymes elute at the following sodium acetate molarities: LiP1, 0.43 *M*; LiP2, 0.18 *M*; LiP3, 0.16 *M*; LiP4, 0.34*M*; LiP5, 0.40*M*; and LiP6, 0.58 *M*. Total recovery of activity in this procedure is about 50% after purification of the isoenzymes to greater than 98% by repeated passages. Specific activities and other properties of the isoenzymes are given by Farrell *et al.*[14]

The extracellular culture fluid is prepared to Q-Sepharose Fast Flow column chromatography by the same procedure as for FPLC. The column is equilibrated with 10 m*M* sodium acetate buffer, pH 6.0 (NA buffer), at 4°. Concentrated culture fluid, dialyzed against NA buffer, is loaded onto the column, which is then washed with NA buffer. A gradient from 10 m*M*

[29] M. Kuwahara, J. K. Glenn, M. A. Morgan, and M. H. Gold, *FEBS Lett.* **169,** 241 (1984).

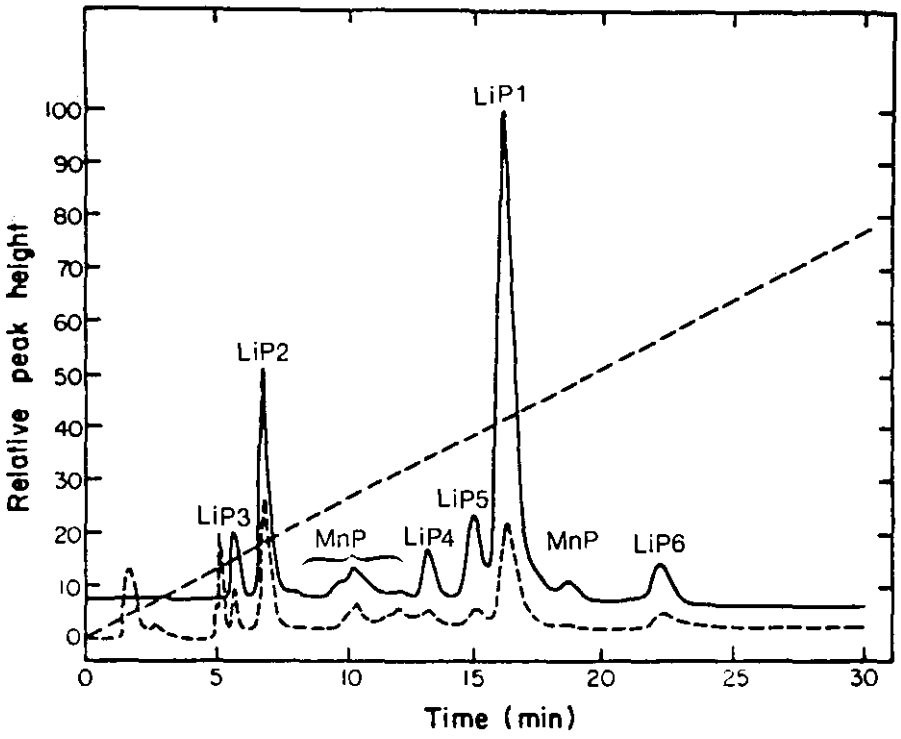


FIG. 3. Mono Q FPLC profile of a culture fluid sample. Full and dashed curves show absorbance at 409 and 280 nm, respectively. The lignin peroxidase isoenzymes are designated LiP1–6. The MnP designations are for peaks with manganese peroxidase activity. The sloping line shows the acetate gradient. Adapted from Kirk *et al.* [15]

to 1 *M* sodium acetate, pH 6.0, is then applied to the column run throughout at a flow rate of 8 ml/min. Fractions are collected and absorbance at 409 nm is monitored. Elution of the isoenzymes from the column is similar to that from Mono Q.

Purification of the isoenzymes by preparative isoelectric gel electrophoresis has also been described.[30]

Enzyme Properties

Up to 15 isoenzymes of the lignin peroxidase of *P. chrysosporium* BKM-F-1767 have been separated by isoelectric focusing from cultures grown under various conditions and for various culture times.[30] In our

[30] M. S. A. Leisola, B. Kozulic, F. Meussdoerffer, and A. Fiechter, *J. Biol. Chem.* **262,** 419 (1987).

work with this strain under nitrogen-limited growth, we have isolated and studied six isoenzymes using the fungal growth conditions and the FPLC purification scheme presented here.[14,15] These six isoenzymes have the following p*I* values and molecular weights (MW in parentheses): LiP1, 3.5 (42K); LiP2, 4.4 (38K); LiP3, 4.7 (38K); LiP4, 3.7 (40K, 42K); LiP5, approximately 3.6 (42K); and LiP6, 3.3 (43K). In earlier work. the designations were HI (LiP3), H2 (LiP2), H6 (LiP4), H7 (LiP5), H8 (LiP1), and H10 (LiP6).[14] The relative abundances of these isoenzymes differ with culture time, but LiP1 is usually the major one in nitrogen-limited cultures.

At least four structural genes have been described which encode the lignin peroxidase isoenzymes in *P. chrysosporium.* Both cDNA and structural gene sequences have been published.[31-37]

Substrate Specificity. Although lignin peroxidase is readily oxidized by H_2O_2, it is not affected by other peroxides including fer(-butyl hydroperoxide and cumene hydroperoxide. It is oxidized by *m*-chloroperbenzoic acid and *p*-(nitroperoxy)benzoic acid, however.[38] Specificity for the aromatic substrate, although it has not been studied per se, is apparently determined largely by the oxidation potential.[4,20] The enzyme oxidizes structurally diverse aromatic compounds; many different lignin-related structures have been studied.[1-3,5] Lignins are oxidized by lignin peroxidase, resulting in spectral changes and in both partial polymerization and partial depolymerization.[25,39,40]

Spectral Properties Lignin peroxidase has absorbance maxima of 409 and 502 nm.[11,23,41] Spectral changes that accompany oxidation to Compound I, reduction of Compound I to Compound II, and reduction of Compound II to resting enzyme have been reported.[11,21,23] (Compound I is the two-electron oxidized state of peroxidases, and Compound II the one-

[31] M. Tien and C.-P. D. Tu, *Nature (London)* **328,** 742 (1987).
[32] H. A. deBoer, Y. Z. Zhang, C. Collins, and C. A. Reddy, *Gene* **60,** 93 (1987).
[33] R. L. Farrell, P. Gelep, A. Anilionis, K. Javahenian, and T. E. Maione, Eur. Patent Appl. 87,810,516.2 (1987).
[34] T. L. Smith, H. Schalch, J. Gaskell, S. Covert, and D. Cullen, *Nucleic Acids Res.* **16,** 1219 (1988).
[35] H. Schalch, J. Gaskell, T. L. Smith, and D. Cullen. *Mol. Cell. Biol.* **9,** 2743 (1989)
[36] Y. Asada, Y. Kimura, M. Kuwahara, A. Tsukamoto, K. Koide, A. Oka, and M. Takanami, *Appl. Microbiol. Biotechnol.* **29,** 469 (1988).
[37] I. Walther, M. Kalin, J. Reiser, F. Suter, B. Fritsche, M. Saloheimo, M. Leisola, T. Teeri, J. K. C. Knowles, and A. Fiechter, *Gene* **70,** 127 (1988).
[38] V. Renganathan and M. H. Gold, *Biochemistry* **25,** 1626 (1986).
[39] T. E. Maione, K. Javahenian, M. A. Belew, L. E. Gomez, and R. L. Farrell, *in* "Lignin Enzymic and Microbial Degradation" (E. Odier, ed.), pp. 177–183. INRA Publications, Paris, 1987
[40] S. D. Haemmerli, M. S. A. Leisola, and A. Fiechter, *FEMS Lett.* **35,** 33 (1986).

TABLE I
KINETIC PARAMETERS OF VARIOUS SUBSTRATES

Kinetic parameter	Values for various substrates[a]				
	H_2O_2	Veratryl alcohol	1,4-Dimethoxy-benzene	1,2,4,5-Tetra-methoxybenzene	Model I
K_m (μM)	17	89	73	90	111
k_{cat} (sec^{-1}	—	1.3	8.6	11	0.5
k_{cat}/K_m (M^{-1} sec^{-1})	—	1.48×10^4	11.7×10^4	12×10^4	0.45×10^4

[a] Kinetic determinations for 1,2,4,5-tetramethoxybenzene were done in 50 m*M* sodium tartrate, pH 3.0;[20] those for the other substrates were determined in 100 m*M* sodium tartrate, pH 4.0.[14]

electron oxidized state.) Raman resonance spectroscopic properties have been described.[4]

pH Optimum. Lignin peroxidase exhibits a pH optimum of approximately pH 2.5–3.0.[11,21,23] Marquez *et al.* [23] have shown that conversion of Compound I to Compound II, and of the latter back to resting enzyme, in the presence of substrate, exhibits a pH optima below 3.0. 4s previously described, the enzyme is rapidly inactivated at pH values below about pH 3.0.[21]

Kinetic Properties of LiP1. Kinetic parameters for lignin peroxidase isoenzyme LiP1 are given in Table I for hydrogen peroxide and for the aromatic substrates veratryl alcohol, 1,4-dimethoxybenzene, 1,2,4,5-tetramethoxybenzene. and the lignin substructure model compound 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)propane-1,3-diol (Model I).

Summary

Lignin peroxidase of *Phanerochaete chrysosporium* can be produced and purified relatively easily. Described here is production and separation of six isoenzymes, using a commonly studied wild-type strain. The fungus is grown on a chemically defined nitrogen-limiting medium in stationary or agitated flasks, the extracellular broth containing the lignin peroxidases is concentrated, and the isoenzymes are separated and purified by anion-exchange chromatography. The isoenzymes have similar physical and catalytic properties but somewhat different kinetic properties. They are encoded at least in part by different structural genes. Lignin peroxidases, in

[41] L. A. Andenson, V. Renganathan, A. A. Chiu, T. M. Loehr, and M. H. Gold, *J. Biol. Chem.* **260,** 6080 (1985).

the presence of H_2O_2, oxidize their aromatic substrates by one electron to cation radicals, which undergo diverse reactions of ionic and radical nature. Purification and characterization of lignin peroxidases from other lignin-degrading fungi have also been reported.

Acknowledgments

The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture of any product or service to the exclusion of others which may be suitable.

Printed on recycled paper