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[23] Lignin Peroxidase of *Phanerochaete chrysosporium*

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Introduction

Ligninase is a generic name for a group of isozymes that catalyze the oxidative depolymerization of lignin. Although undoubtedly produced by other lignin-degrading fungi, these isozymes to data have been isolated only from the basidiomycete *Phanerochaete chrysosporium* Burds.[1,2] These ligninases are extracellular and are produced during secondary metabolism, brought about by nutrient starvation. Nitrogen limitation is usually employed, as described here, but carbon-limited cultures have also been used for ligninase production.[3] The ligninases exhibit a high degree of homology. They are all heme-containing glycoproteins and all cross react with a polyclonal antibody raised to the predominant ligninases.[4] Since they all have overlapping substrate specificities, the exact role of this multiplicity is not yet understood. The number of genes encoding for ligninases is not yet known.

The major isozyme, ligninase H8, has been extensively characterized and is the protein initially isolated by Tien and Kirk.[5] Based on kinetic[6] and spectroscopic data,[7] this ligninase has been characterized as a peroxidase containing one high-spin ferric heme per enzyme molecule.[8] Like horseradish peroxidase, the ligninases are capable of catalyzing a wide range of one- and two-electron oxidations. The substrates of ligninase, however, exhibit much higher reduction potentials. This property, along with its low pH optimum,[6] imparts ligninase with the unique ability to catalyze the oxidative depolymerization of lignin and the oxidation of methoxybenzene-containing lignin-like substrates.[9,10]

[1] M. Tien and T. K. Kirk, *Science* **221,** 661 (1983).
[2] J. K. Glenn, M. A. Morgan, M. B. Mayfield, M. Kuwahara, and M. H. Gold, *Biochem. Biophys. Res. Commun.* **114,** 1077 (1983).
[3] B. D. Faison and T. K. Kirk, *Appl. Environ. Microbiol.* **49,** 299 (1985).
[4] T. K. Kirk, S. C. Croan, M. Tien, K. E. Murtagh, and R. Farrell, *Enzyme Microb. Technol.* **8,** 27 (1985).
[5] M. Tien and T. K. Kirk, *Proc. Natl. Acad. Sci. U.S.A.* **81,** 2280 (1984).
[6] M. Tien, T. K. Kirk, C. Bull, and J. A. Fee, *J. Biol. Chem.* **261,** 1687 (1986).
[7] D. Kuila, M. Tien, J. A. Fee, and M. R. Ondrias, *Biochemistry* **24,** 3394 (1985).
[8] L. A. Anderson, V. Renganathan, A. A. Chiu, T. M. Loehr, and M. H. Gold, *J. Biol. Chem.* **260,** 6080 (1985).
[9] P. Kersten, M. Tien, B. Kalyanaraman, and T. K. Kirk, *J. Biol. Chem.* **260,** 2609 (1985).

Several procedures have been described for growing P. chrysosporium for ligninase production. These procedures differ somewhat in the medium formulation and types of growth vessels: (1) shallow stationary cultures, (2) agitated liquid cultures, and (3) rotating biological contactors (RBCs; disk fermenters). Because the RBCs employ a mutant strain that adheres to the plastic disk[4] and equipment that has to be constructed, their use is not described here. The more recently developed use of agitated culture for production of ligninase permits easier "scale up."[11] Although ligninase can be produced in agitated flask cultures, the reliable use of stirred tank fermenters awaits further development, which is ongoing in several laboratories. In the following we describe the production of ligninase in shallow stationary cultures and in agitated cultures. The stationary cultures give somewhat more reliable and reproducible results than the agitated cultures.

Maintenance of Fungus and Preparation of Spore Inoculum

Cultures of *P. chrysosporium* (strain BKM-F-1767; ATCC 24725) are maintained on supplemented malt agar slants; the medium is described below. Of the strains that have been studied, strain BKM-F-1767 produces highest ligninase activity, although activity is produced by all examined wild-type strains.[12]

Composition of agar for maintenance and spore production (per liter):

Glucose, 10 g
Malt extract, 10 g
Peptone, 2 g
Yeast extract, 2 g
Asparagine, 1 g
KH_2PO_4, 2 g
$MgSO_4 \cdot 7H_2O$, 1 g
Thiamin-HCl, 1 mg
Agar, 20 g

Spore production in the slants usually requires 2 to 5 days of growth at 39°, Spores (conidia) are prepared by suspension in sterile water followed by passage through sterile glass wool to free it of contaminating mycelia. Spore concentration is determined by measuring absorbance at 650 nm (an absorbance of 1.0 cm^{-1} is approximately 5×10^6 spores/ml).

[10] K. E. Hammel, M. Tien, B. Kalyanaraman, and T. K. Kirk, *J. Biol. Chem.* **260,** 8348 (1985).
[11] A. Jäger, S. Croan, and T. K. Kirk, *Enzyme Microb. Technol. Appl. Environ. Microbiol.* **50,** 1274 (1985).
[12] R. K. Kirk, M. Tien, S. C. Johnsrud, and K.-E. Eriksson, *Enzyme Microb, Technol.* **8,** 75 (1986).

Culture Media

Stock Reagents

1. Basal III medium (per liter):

KH_2PO_4, 20 g
$MgSO_4$, 5 g
$CaCl_2$, 1 g
Trace elements solution (see below), 100 ml

2. Trace element solution (per liter):

$MgSO_4$, 3 g
$MnSO_4$, 0.5 g
NaCl, 1.0 g
$FeSO_4 \cdot 7H_2O$, 0.1 g
$COCl_2$, 0.1 g
$ZnSO_4 \cdot 7H_2O$, 0.1 g
$CuSO_4$, 0.1 g
$AlK(SO_4)_2 \cdot 12H_2O$, 10 mg
H_3BO_3, 10 mg
$Na_2MoO_4 \cdot 2H_2O$, 10 mg
Nitrilotriacetate,[13] 1.5 g

Culture Composition (Shallow Stationary Cultures)

The following items are added per liter of shallow stationary cultures:

Basal III medium (filter sterilized), 100 ml
10% glucose (autoclaved), 100 ml
0.1 *M* 2,2-dimethylsuccinate, pH 4.2 (autoclaved), 100 ml
Thiamin (100 mg/liter stock, filter sterilized), 10 ml
Ammonium tartrate (8 g/liter stock, autoclaved), 25 ml
Spores (absorbance at 650 nm = 0.5), 100 ml
Veratryl alcohol (4 m*M* stock, filter sterilized), 100 ml
Trace elements (filter sterilized), 60 ml

Culture Composition (Agitated Cultures)

The medium for agitated cultures has the same composition as that for stationary cultures except that 0.05% Tween 20 or Tween 80 is added, and the fungus is introduced as a mycelial suspension instead of a spore suspen-

[13] Dissolve nitrilotriacetate in 800 ml H_2O, adjust pH to ~ 6.5 with 1 *N* KOH, add each component, and then bring the volume to 1 liter.

sion. The detergent is solubilized and sterilized by autoclaving a 1% solution in distilled water; 50 ml of this solution is added to the above medium. The mycelial inoculum is prepared by growing the fungus from spore suspension in stationary 2.8-liter Fernbach flasks containing 50 ml of the above medium (without detergent). After 48 hr at 39°, the mycelium plus medium is blended for 1 min in a blender (100 ml; 45 mg dry wt). The resulting suspension is substituted for the spores in the above culture formulation.

Growth and Harvest

Shallow stationary cultures (10 ml) are grown in rubber-stoppered, 125-ml Erlenmyer flasks at 39° under 100% oxygen. They are flushed with oxygen at the time of inoculation and again on day 3. Preparations typically utilize 400 flask cultures yielding about 3.8 liters of ligninase-containing culture supernatant. Care is taken not to perturb the cultures after the mycelial mats have formed, which takes about 24 hr. Attempts to scale up production via a proportional increase in both culture volume and flask size or with the use of shallow pans resulted in lower activity.

Agitated cultures, 45 or 750 ml, are grown in either 125-ml Erlenmeyer flasks, or 2-liter Erlenmeyer flasks, respectively. The cultures are grown at 39° on a rotary shaker with a 2.5-cm-diameter cycle, the small flasks at about 200 rpm, and the larger ones at about 125 rpm. The rubber-stoppered culture flasks are flushed with 100% O_2 at the time of inoculation, and daily thereafter. Enough cultures are grown to yield approximately 3.8 liters of culture supernatant (about 4.2 liters of cultures).

Mycelial growth under the nitrogen-limited conditions stops by day 2 and ligninase activity appears in the extracellular fluid on day 4, coinciding with development of a brown coloration on the mycelia (which is only observed with the excess trace elements solution). Under both stationary and shaken incubation, activity reaches a maximum on days 5 and 6. When the maximum is reached, the supernatant is obtained by centrifugation at 10,000 g for 5 min at 4°. The yellow supernatant (3.3 liters, Table I), which contains all of the activity (0.075 U/ml), is then concentrated by ultrafiltration (Millipore Minitan unit) using a 10-kDa cut-off membrane. After concentration to approximately 40 ml, the preparation is filtered (0.45-μm pore size), which removes precipitated mycelial slime, then further concentrated (Amicon, 10-kDa cut-off) to a final volume of 13.5 ml. The sample is then dialyzed overnight against 4 liters of either 10 m*M* sodium acetate, pH 6, for Mono-Q chromatography, or 5 m*M* sodium succinate, pH 5.5, for chromatography on DEAE-BioGel A (see below). As shown in Table I, the concentration and dialysis step results in very little

TABLE I
PURIFICATION OF LIGNINASE ISOZYMES[a]

Sample	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Total activity (U)	Specific activity (U/mg)	Recovery (%)
ECF[b]	3300	0.076	0.013	251	5.71	100
ECF						
Concentrated (Minitan/Amicon)	13.5	16.9	1.23	229	13.8	89
Pre-FPLC (dialyzed/filtered)	17	12.95	0.7	220	18.5	88
FPLC purified[c]						
H1	8.7	0.52	0.07	4.5	7.24	1.8
H2	17.7	2.1	0.13	36.3	16.4	14
H6	13.1	0.33	0.06	4.36	5.5	1.7
H7	4.3	0.33	0.1	1.4	3.28	0.5
H8	31.8	1.56	0.21	49.6	7.6	20
H10	25.7	0.25	0.09	6.35	2.1	2.5

[a] Ligninolytic cultures of BKM were grown and harvested as described by Kirk *et al.*[4]
[b] ECF, Extracellular fluid. Please note that the specific activity increased after concentration due to loss of low-molecular-weight components which contributed background in the protein assay.
[c] Peaks From repeated injections were pooled, dialyzed against 5 m*M* sodium tartrate, pH 4.5, and assayed for protein activity.[4]

loss in total activity. Total percentage recovery is usually in the high eighties (Table I). We typically concentrate on the day of harvest and then dialyze overnight.

Assay Method

Principle

Ligninase catalyzes the oxidation of veratryl alcohol by H_2O_2[6] to veratraldehyde. The alcohol exhibits no absorbance at 3 10 nm whereas the aldehyde absorbs strongly (molar extinction coefficient = 9300 M^{-1} cm^{-1}). Use is made of this property in a continuous spectrophotometric assay.

Reagents

10 m*M* veratryl alcohol
0.25 *M* *d*-tartaric acid, pH 2.5
5 m*M* H_2O_2 (prepared daily)

Procedure

Reaction mixtures contain 2 m*M* veratryl alcohol (K_m = 60 μM), 0.4 m*M* H_2O_2 (K_m = 80 μM), 50 m*M* tartaric acid, and enough ligninase to give an absorbance change of 0.2/min.

Comments on Assay

Although the ligninase is most active at pH below 3, it is not very stable; thus reaction rates are linear only for about 2 min. The ligninase is also inactivated by H_2O_2 in the absence of a reducing substrate, such as veratryl alcohol. Consequently, care should be taken to minimize the preincubation of ligninase with buffers of low pH (pH < 3.0) or with H_2O_2 in the absence of veratryl alcohol. For reproducible results, the temperature should be held constant because the ligninase shows a high temperature dependence; the rate approximately doubles with every 7° increase.[6]

Reagents for enzyme activity are commercially available and, except for veratryl alcohol, do not require further purification. Prior to use, veratryl alcohol is vacuum distilled to free it of the trace contaminant methyl-3-methoxy-4-hydroxybenozate, which is a better ligninase substrate than veratryl alcohol.[6] This contaminant probably can also be removed by extracting a solution in ether or dichloromethane with aqueous alkali. Unless removed, this phenolic contaminant causes distinct lag periods in the initial rate, most noticeable at low enzyme activity.

Purification of Ligninase (s)

Multiple ligninases of *P. chrysosporium* can be separated by either ordinary column chromatography using DEAE-BioGel A or by FPLC (or HPLC) with the Mono-Q anion-exchange column of Pharamacia. For chromatography on DEAE-BioGel A, the column (1 × 16 cm) is equilibrated with 5 m*M* sodium succinate buffer, pH 5.5; the sample is loaded and then eluted with a NaCl gradient (0–0.14 *M*, total volume of 600 ml). All steps are performed at 4° .

Mono-Q is the method of choice due to its superior resolution. The results of the Mono-Q separation are summarized in Table I. Following dialysis, the sample is again filtered through a 0.45-μm filter (Gilson Co., Madison, WI, low protein-binding filter). The Mono-Q column capacity is 25 mg total protein or 5 mg/peak. The 10-ml preparation described above can be purified by five injections of 2 ml. The sample is loaded with 10 m*M* sodium acetate, pH 6.0, and eluted with a gradient from 10 m*M* to 1 *M* sodium acetate, pH 6.0, over a 40-min period at 2 ml/min. We use

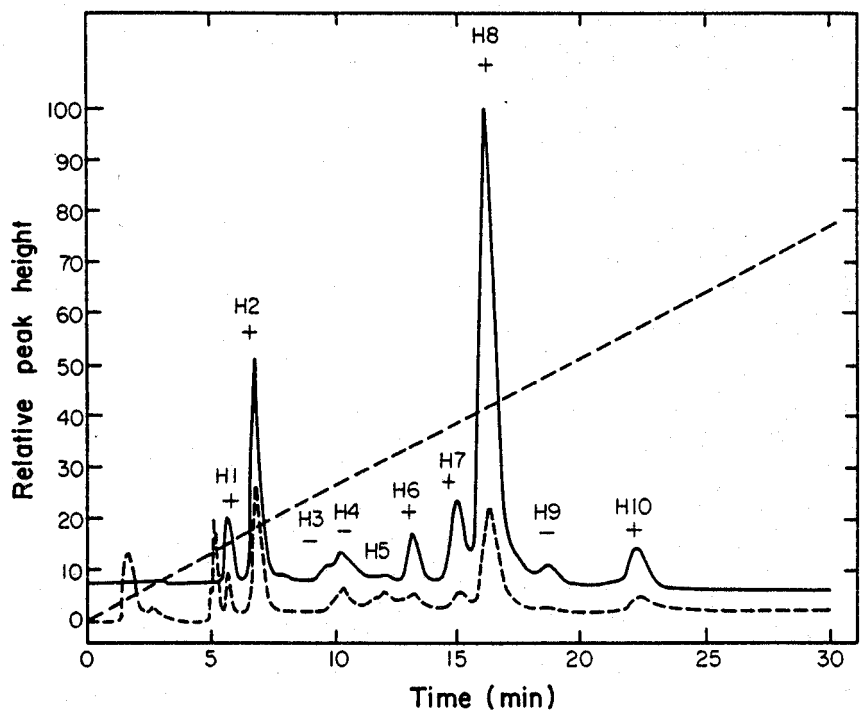

FIG. 1. FPLC profile of extracellular fluid from 5-day flask cultures. Full and dashed lines show absorbance at 409 and 280 nm, respectively. Veratryl alcohol-oxidizing activity is indicated qualitatively as positive (+) or negative (–). The sloping line shows the acetate gradient. Reproduced from Kirk *et al.* [4]

this method at room temperature, but return the protein to 4° after elution.

Figure 1 shows the profile from the FPLC column showing the absorbance at 409 nm (heme absorbance) and 280 nm (total protein). Figure 2 shows the profile from the DEAE-BioGel A column (showing only the 409-nm absorbance). As clearly demonstrated by the elution profiles, the resolution is much better on the Mono-Q than the DEAE-BioGel A column. Both profiles indicate the presence of numerous proteins. Over 13 proteins can be detected from the Mono-Q column; most of them are baseline resolved. The peaks designated H1, H2, H6, H7, H8, and H10 all have veratryl alcohol-oxidizing activity in addition to activity toward various dimeric models of lignin! These enzymes are the ligninases. The other peaks (H3, H4, H5, and H9) are the Mn-dependent peroxidases characterized by Glenn and Gold[14] and Paszczyhski *et al.*[15] The recovery of each of the ligninase isozymes from the Mono-Q column is given in Table I. The

[14] J. K. Glenn and M. H. Gold, Arch. *Biochem. Biophys.* **242,** 329 (1985).
[15] A. Pasznynski, V.-B. Huynh, and R. Crawford, *Arch. Biochem. Biophys.* **244,** 750 (1986).

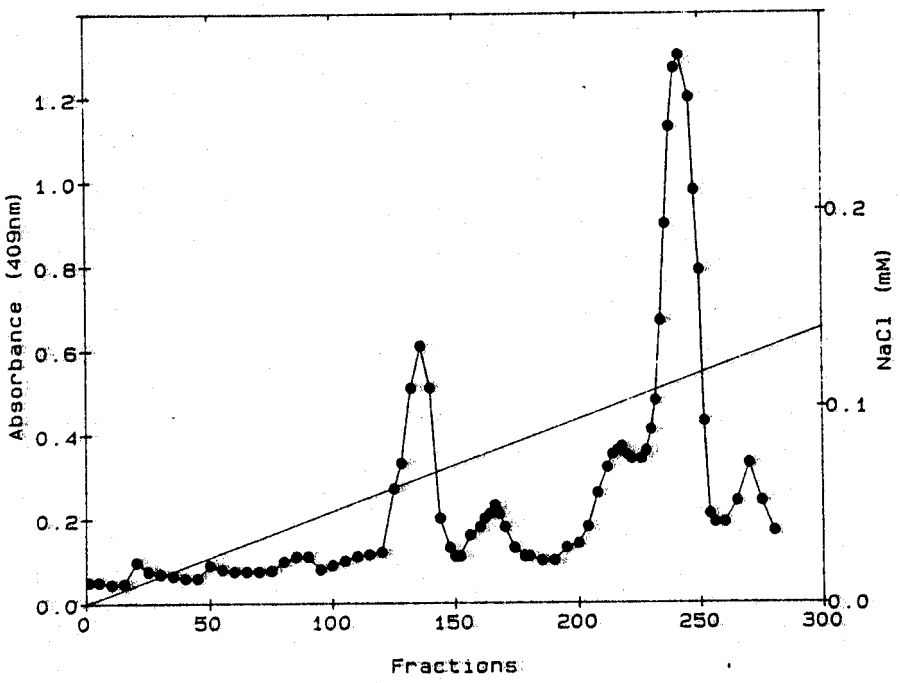

FIG. 2. DEAE-BioGel A profile of sample similar to that shown in Fig. 1. Only absorb ance at 409 nm is shown. Sloping line shows NaCl gradient. Fractions of 2 ml were collected.

total activity (accounted for by the six isozymes listed above) recovered from the Mono-Q is usually 50% of the original activity (Table I). The relative amount of each isozyme varies depending on culture additives such as vetatryl alcohol and the trace elements solution. 4 Differences are also seen between the stationary and agitated cultures at different harvest times. Consequently, a range of 20 to 40% of the total veratryl alcohol-oxidizing activity can be recovered in the purified H8 fraction.

Under the growth conditions described above, H8 is the predominant ligninase. This isozyme is the enzyme previously purified by Tien and Kirk[5] and most likely the same as that characterized by Godd et al.[16] This isozyme is the most extensively studied and characterized. Collecting the H8 peak manually from the Mono-Q column provides a highly purified H8 fraction. Reinjecting the Purified peak into the Mono-Q indicates that it is over 98% pure (Fig. 3). [17] Subjecting the purfied H8 to SDS-polyacrylamide gel electrophoresis also indicates that the preparation is homogeneous (Fig. 3).

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

[17] U. K. Laemmli, *Nature (London)* **227,** 680 (1970).

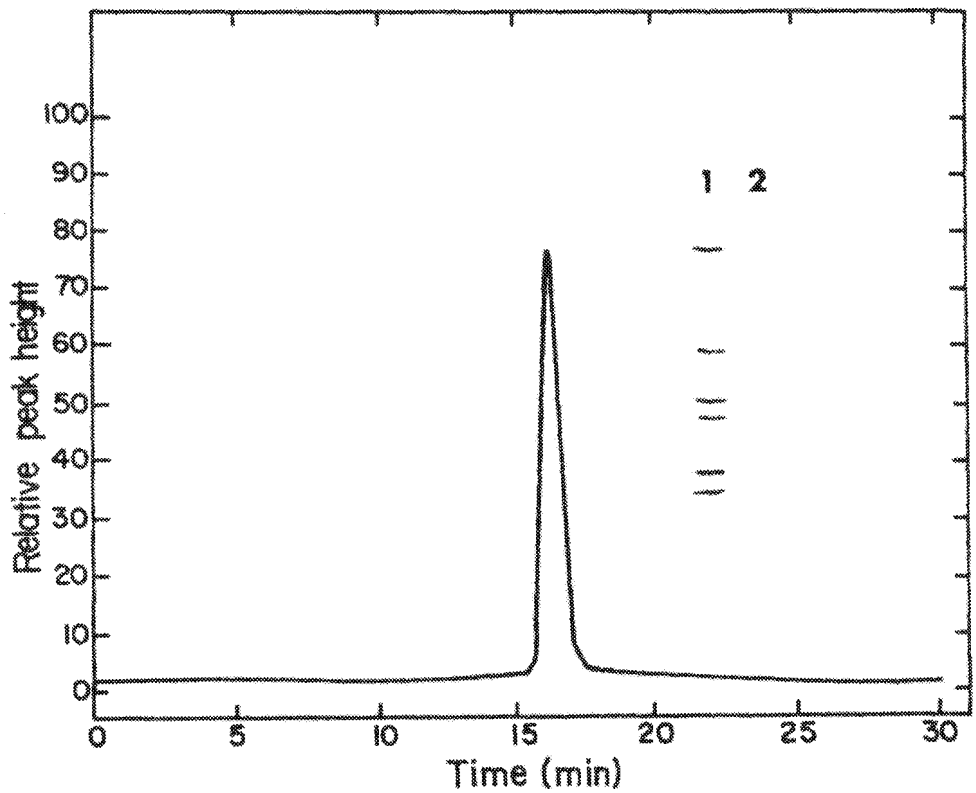

FIG. 3. Purity of H8 as determined by two techniques. A sample of H8, collected from an elution shown in Fig. 1, was rechromatographed on Mono-Q and also subjected to SDS-polyacrylamide gel electrophoresis. Chromatography on Mono-Q was as described in the legend of Fig. 1 except that absorbance at 280 nm was monitored. Electrophoresis was performed with 10% acrylamide by the method of Laemmli.[17] Lane 2 contains 5 μg of ligninase H8. Lane 1 contains molecular weight markers (from top): 66K, 45K, 36K, 29K, 24K, and 14.2K.

Storage

The purified ligninase (H8) from either DEAE-BipGel A or Mono-Q chromatography is then concentrated and dialyzed against 5 m*M* potassium phosphate buffer, pH 6.5. Rapid freezing with liquid nitrogen and storage at $-20°$ or below yields a preparation stable for months.

Properties of the Ligninase Isozymes

The ligninase isozymes are similar in structure and function. Some of their physical properties are summarized in Table 11. The molecular weight of the isozymes, as determined by SDS-polyacrylamide gel electrophoresis, vary from 38,000 for H1 and H2 to 46,000 for H10 (Table 11). The molecular weight must be considered an upper estimation because the ligninases are all glycoproteins, as demonstrated by their ability to bind to

TABLE II
PHYSICAL PROPERTIES OF LIGNINASE ISOZYMES

Isozyme	Molecular weight[a]	Carbohydrate[b]	ε_{409} ($mM^{-1}\,cm^{-1}$)[b]	Peptide homology[c]
H1	38,000	+	169	H2(++);H7,H8(+)
H2	38,000	+	165	H1(++);H7,H8(+)
H6	43,000	+	162	H10(+);H7,H8(+)
H7	42,000	+	177	H8(++);H1,H2(+)
H8	42,000	+	168	H7(++);H1,H2(+)
H10	46,000	+	182	H6(+);H7,H8(+)

[a] M. Tien, unpublished.
[b] From Farrell *et al.* [18]
[c] From Kirk *et al.* [4]

conconavalin A-Sepharose.[18] The absorption spectrum of the ligninases [exhibiting 409 nm (Soret) absorbance] suggests that they are all heme proteins. This was verified with formation of a diagnostic pyridine hemochromogen complex.[18] The extinction coefficients of the various ligninases, as determined by quantitation of the heme content with the pyridine hemochromogen method, range from 162 $mM^{-1}\,cm^{-1}$ for H6 to 182 $mM^{-1}\,cm^{-1}$ for H10.[18]

The isoenzymes are fairly homologous in primary and tertiary structure. Polyclonal antibodies preparedagainst H8 cross react with ligninase H2, H10, and H8 (to itself), indicating homology between these different enzymes." Analysis of the peptides produced after protease (V8) digestion by electrophoresis on SDS–PAGE gels indicated that H1 and H2 are almost identical.[4] Peptides from H8 are similar to H1 and H2, but lack at least two major peptides.[4] The peptides producedfrom H6 and H10 are most similar to H7 and H8.[4]

The ligninase isozymes are also similar in their catalytic properties. Resulsts from Farrell *et al.*[18] indicate that the K_m and V_{max} exhibited by the isozymes for H_2O_2 with various aromatic substrates are not significantly different. Based on the lack of significant kinetic properties, it is difficult to ascertain thephysiological significance of the multiple ligninases.

Physical and Kinetic Properties of Ligninase H8

Ligninase H8 contains one protophorphyrin IX-derived heme per enzyme molecule and is composed of 15% by weight carbohydrate.[6] The electron absorption spectrum of ligninase H8 is typical of most heme

[18] R. A. Farrell, K. E. Murtagh, M. Tien, M. D. Mozuch, and T. K. Kirk, *J. Biol. Chem.*, submitted.

proteins, showing a Soret peak at 409 nm and visible absorption bands at 498 and 630 nm.[8] The ferric (resting) ligninase forms complexes with cyanide and azide.[8] The ligninase can be reduced with dithionite or deazaflavin; the artificially reduced enzyme complexes with CO, NO and O_2.[8] The reduced enzyme is not involved in catalysis since CO is not an inhibitor.[6]

The ESR spectrum ofthe ferriligninase shows g values at 5.83 and 1.99, indictive of a high-spin ferric heme.[8] Resonance Raman results by Kuila *et al.*[7] describes the heme as most similar to those of peroxidases. Kuila and co-workers based their results on resonance Raman spectrum at the low-frequency range, which showed striking similarities between the ferroligninase and ferro-horseradish peroxidase. Kinetic results[6] are in close accord with the results of Kuila *et al.*,[7] indicating a mechanism similar to otherperoxidase.

The kinetics of ligninase catalysis have been studied by both steady state and transient state techniques.[6] The steady state studies of veratryl alcohol oxidation indicate that the mechanism of catalysis is Ping-Pong.[6] Ping-Pong kinetics are consistent with the mechanism of other peroxidases.[19] The initial step in catalysis is the reaction of ligninase with H_2O_2, resulting in formation of an oxidized enzyme intermediate. This intermediate returns to the resting state by oxidizing its aromatic substrates. The productive binding rate for ligninase with H_2O_2 (V/K) is $1.0 \times 10^5\, M^{-1}\,\mathrm{sec}^{-1}$.[6] This rate constant is approximately 100 times lower than that observed with most other peroxidases.

Transient state kinetic studies of the ligninase show that two intermediate states ofthe enzyme are formed during catalysis. These two states are similar to those formed by other peroxidases; they are the classical intermediates compounds I and II characterized by Chance.[20] Formation of ligninase compounds I and II were detected by stopped flow rapid-scan spectral analysis of the reaction between ligninase and H_2O_2.[6] The initial step in catalysis is the reaction ofligninase with H_2O_2 to form compound I, which is two-electron oxidized. This reaction procedes with a second-order rate constant of $5.8 \times 10^5\, M^{-1}\,\mathrm{sec}^{-1}$, which is in close agreement with the productive binding rate obtained from steady state kinetics. Compound I then reacts with a substrate molecule to form product and the compound II intermediate of ligninase, which is one-electron oxidized. Compound II returns to resting enzyme by reacting with another molecule of substrate.

The catalytic cycle described above, where two molecules offree radical products are formed per turnover, is common for all peroxidases. Formation of free radical products during ligninase catalysis has been demon-

[19] G. L. Kedderis and P. F. Hollenberg, *J. Biol. Chem.* **258,** 12413 (1983).
[20] B. Chance, *Arch. Biochem. Biophys.* **41,** 416 (1952).

strated by ESR spectroscopy. Kersten *et al.* [9] detected the cation radicals of methoxybenzenes by EST spectroscopy; Hammel *et al.* [10] detected radicals from dimeric model compounds of lignin through ESR spin-trapping techniques. Largely through the results of these two studies, a generalized mechanism for lignin degradation can be formulated. This generalized mechanism involves a central role for substrate aryl cation free radicals. Cation radicals can undergo a wide range of reactions; the type of reactions can be affected by the ring substituents. Substrates with a -hydroxy-containing propyl side chains (prominent in lignin) preferentially undergo carbon–carbon bond cleavage. [10] Methoxybenzenes cation radicals tend to hydrate and demethylate for form methanol and benzoquinones. [9] Because the cation radicals are stable enough to diffuse away from the active site into the "bulk phase," thrie fate can also be dependent on the components of the bulk phase. Thus the pH, concentration of dioxygen, and concentration of other radicals can affect the addition of H_2O, addition of dioxygen, and diverization with other radicals (reactions all observed with lignin models).

Much of our understanding of the chemisttry of cation radicals has been provided by Snook and Hamilton. [21] These workers studied the formation and degradation of aryl cation radicals in chemical systems. These studies have provided a model for ligninase catalysis; they have indicated that mechanistically, the chemistry of cation radicals accounts for most if not all of the prominent reactions observed in lignin biodegradation. Carbon-carbon bond cleavage of propyl side chains, loss of methoxyls, oxidation of benzylic hydroxyls, and ring opening and mechanically consistent with a free radical mechanism. It is thus apparent that the future utilization of ligninase will require not only an understanding of ligninase catalysis, but also the chemistry of cation radicals.

[21] M. E. Snook and G. A. Hamilton, *J. Am. Chem. Soc.* **96,** 860 (1974).