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# MOLECULAR CLONING OF LIGNIN PEROXIDASE cDNAs AND GENES FROM A WHITE-ROT BASIDIOMYCETE FUNGUS *PHANEROCHAETE CHRYSOSPORIUM*

BY

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## ABSTRACT

MOLECULAR CLONING OF LIGNIN PEROXIDASE cDNAs AND GENES FROM A WHITE-ROT BASIDIOMYCETE FUNGUS *PHANEROCHAETE CHRYSOSPORIUM*

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*Phanerochaete chrysosporium*, a white-rot basidiomycete fungus, produces extracellular, $H_2O_2$-dependent, glycosylated heme proteins called lignin peroxidases. There has been much recent interest in lignin peroxidases because these enzymes not only play a central role in lignin degradation, but have also been implicated in the detoxification of recalcitrant xenobiotics. Lignin peroxidases are elaborated by the fungus under nitrogen-limited conditions only during secondary metabolism. To better understand the expression, regulation and organization of lignin peroxidase genes in this fungus, molecular cloning procedures have been used to isolate lignin peroxidase cDNAs and genes. Two different types of lignin peroxidase cDNA clones (represented by pCLG4 and pCLG5) were identified in a cDNA library of *P. chrysosporium* using the synthetic oligodeoxynucleotide probes whose sequences were deduced from one lignin peroxidase (H8). Northern hybridization analyses demonstrated that the poly(A) RNA homologous to the above cDNAs was present in 6-day-old (idiophasic) ligninolytic cultures. Immunoassay showed the presence of expressed product of the cDNA clone in *E. coli*. Sequence analyses showed that the cDNA inserts of pCLG4 and pCLG5 (designated CLG4 and CLG5) contain open reading frames encoding lignin peroxidase proteins (designated LG4 and LG5) containing 372 and 371 amino acid residues, respectively. Both mature lignin peroxidases LG4 and LG5 contain 344

amino acid residues with $M_r$ of 36,540 and 36,607, respectively, and are preceded by typical leader sequences for secretion. Although CLG4 and CLG5 did not show cross hybridization, they had relatively high nucleotide homology of 71.5% and amino acid sequence homology of 75%. Six lignin peroxidase genomic fragments (named GLG1 to GLG6) have been isolated from the gene library of P. chrysosporium using CLG4 and CLG5 as probes. Further characterization showed that GLG1 and GLG2 correspond to CLG4 and CLG5, respectively, whereas the other four genomic fragments represent four separate lignin peroxidase genes. The location of the lignin peroxidase gene in each cloned fragment and the transcriptional orientation of each gene have been determined. Sequence analysis of the lignin peroxidase gene in GLG2 showed that this gene has a CAAT box and a typical TATA box sequence. The comparison of the GLG2 sequence with that of CLG5 showed that this gene contained nine small introns whose sizes ranged from 50-62 base pairs. The consensus sequence GTRNGY---YTGAY---YAG is present in all introns.

**To my parents, my wife and my son**

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# INTRODUCTION

Lignin is a complicated, stereochemically complex, aromatic heterogeneous biopolymer and is a major component of vascular tissues in terrestrial plants (103). Lignin biodegradation has been an intensive area of research in the last few decades for several reasons. First of all, lignin is the second most abundant organic polymer in the biosphere, next to cellulose. Worldwide an estimated 25 billion metric tons of lignin are annually biosynthesized by plants. Lignin itself and its degraded products are renewable raw materials potentially useful in various biotechnogical processes. Secondly, lignin is intimately associated with cellulose and hemicellulose in woody plants, limiting the efficiency of bioconversion of these polysaccharides to useful products. Furthermore, since the lignin subunit contains 50% more carbon than cellulose and is recalcitrant to biodegradation, lignin biodegradation plays an important part in the carbon cycle on the earth.

Although the complicated structure of lignin (see Fig. 1 in Literature Review) implies difficulty in biodegradation, a limited number of microorganisms are known to oxidatively degrade lignin to carbon dioxide (16). Among them, one white-rot filamentous fungus, Phanerochaete chrysosporium, is known to degrade lignin more rapidly than most other organisms (see 43 and 44). This organism has become a model organism to study lignin biodegradation because of its great ability to degrade lignin, capacity for rapid growth in both complex and chemically defined media, prolific conidiation, relatively high temperature optimum for growth of about 40°C, and low level of phenol oxidase activity. Lignin degradation in this fungus has been shown to be a secondary metabolic

event and is triggered by nitrogen, carbon or sulfur starvation.

Lignin peroxidase (ligninase), an extracellular, $H_2O_2$-dependent, glycosylated heme protein from P. chrysosporium, has recently been purified and characterized (26,32,106,107). Lignin peroxidase is elaborated by the fungus under nitrogen-limited conditions during secondary metabolism but not during primary growth (19). The enzyme catalyzes oxidative cleavage of a variety of alkyl side chains of lignin-related compounds including $C_\alpha$-$C_\beta$ cleavage of the propyl side chains, a major reaction in fungal depolymerization of lignin (32,107). Lignin peroxidase activity is conveniently measured as veratryl alcohol oxidation to veratraldehyde (107). More recently, at least six proteins (H1, H2, H6, H7, H8 and H10) with lignin peroxidase activity were observed in the extracellular fluid of ligninolytic cultures of P. chrysosporium (60). Proteins H2 and H8 constitute the major lignin peroxidases both in shaken and static cultures of P. chrysosporium (60). Lignin peroxidases have a molecular weights of 39 to 43 kilodaltons, have similar spectral and catalytic properties and antibody raised against lignin peroxidase H8 cross reacts with the other lignin peroxidases demonstrating at least partial homology among these proteins (58,60). However, some differences in peptides produced upon protease digestion have been noted. Multiple forms of lignin peroxidase have been identified by several other investigators (71,85,101). In fact, Leisola et al. (71) identified as many as fifteen lignin peroxidases from the extracellular fluid of lignin-degrading cultures of P. chrysosporium. All these proteins catalyzed $H_2O_2$-dependent oxidation of veratryl alcohol to veratraldehyde (71).

Lignin peroxidases are important enzymes with many potential practical applications. These include upgrading of lignocellulosic biomass, via delignification, for the efficient production of feeds, fuels and chemi-

cals; biobleaching of pulps; increasing efficiency of wood pulping; treatment of industrial wastes; controlled modification of lignins to produce aromatic chemicals; cracking of petroleum; and detoxification of dangerous and recalcitrant environmental pollutants such as dioxins, polybrominated biphenyls, DDT and benzopyrenes.

Numerous efforts have been made to improve the efficiency of lignin biodegradation and the production of lignin peroxidase. These include isolation of lignin peroxidase hyper-producing strains and mutants (7,13,32,60), optimization of culture parameters (7,19,46,60,70,73,99) and use of different kinds of fermentors for large scale production of lignin peroxidases (60,75,76,85). However, the ligninolytic activity and the concentration of the lignin peroxidase enzyme in the extracellular fluid is still quite low even under the best of conditions.

To obtain a better understanding of the nature, organization, expression and regulation of the lignin peroxidase genes and to develop the full bioprocessing potential of these enzymes, initial studies to clone and characterize cDNA and genomic sequences for lignin peroxidase have been done and a primary report on the isolation of cDNA clones has been recently published (114). This dissertation will describe my studies on the isolation and characterization of cDNAs and genes for lignin peroxidase from P. chrysosporium.

# LITERATURE REVIEW

Lignin is the second most abundant component of plant biomass and its biodegradation plays a very important role in the global carbon cycle. Lignin is a complicated, stereochemically complex, heterogeneous aromatic renewable biopolymer. Studies on lignin biodegradation have accelerated greatly in the past ten years, especially after the discoveries that hydrogen peroxide was found to play a central role in lignin degradation (86,95, 96) and the first extracellular $H_2O_2$-dependent enzyme, lignin peroxidase, was purified and characterized from Phanerochaete chrysosporium (26,106). The accumulated knowledge on the microbial degradation of lignin has been summarized in several recent reviews (3,14-16,42-44,54,58,66,69,94,115). This literature review will focus primarily on lignin biodegradation by P. chrysosporium.

## I. GENERAL STRUCTURE OF LIGNIN

The lignin polymer is formed through the peroxidase-mediated dehydrogenative polymerization of three cinnamyl alcohol derivatives: p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Figure 1; 14). The three alcohols are present in different ratios in lignins from different plant species and often in different tissues of the same plants (16,54,103). A variety of linkages of the C-C and C-O-C type are the major linkages in lignin as seen in the schematic formula of a representive portion of the beech lignin (Figure 2; 83). The C-O-C bonds, including $C_\alpha$-O-$C_4$, $C_\beta$-O-$C_5$ and $C_4$-O-$C_5$, account for about 60% of the linkages in spruce lignin and for about 74% of the linkages in birch lignin (54).

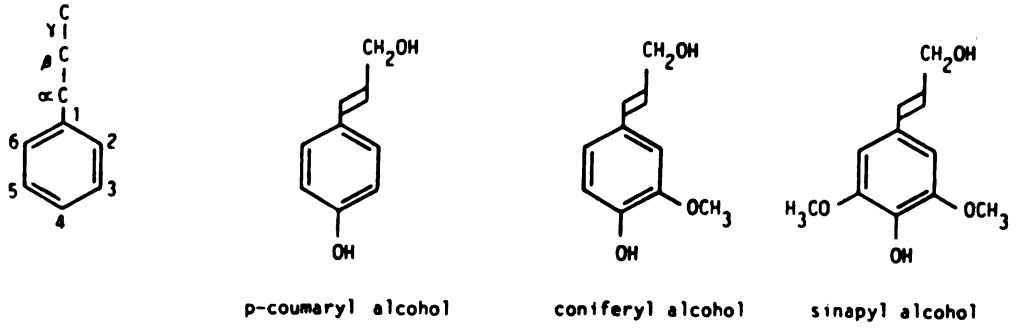


Figure 1. Nomenclature of carbons in lignin monomers and three precursors of lignin, p-coumaryl, coniferyl and sinapyl alcohols (from Buswell and Odier, ref. 14).

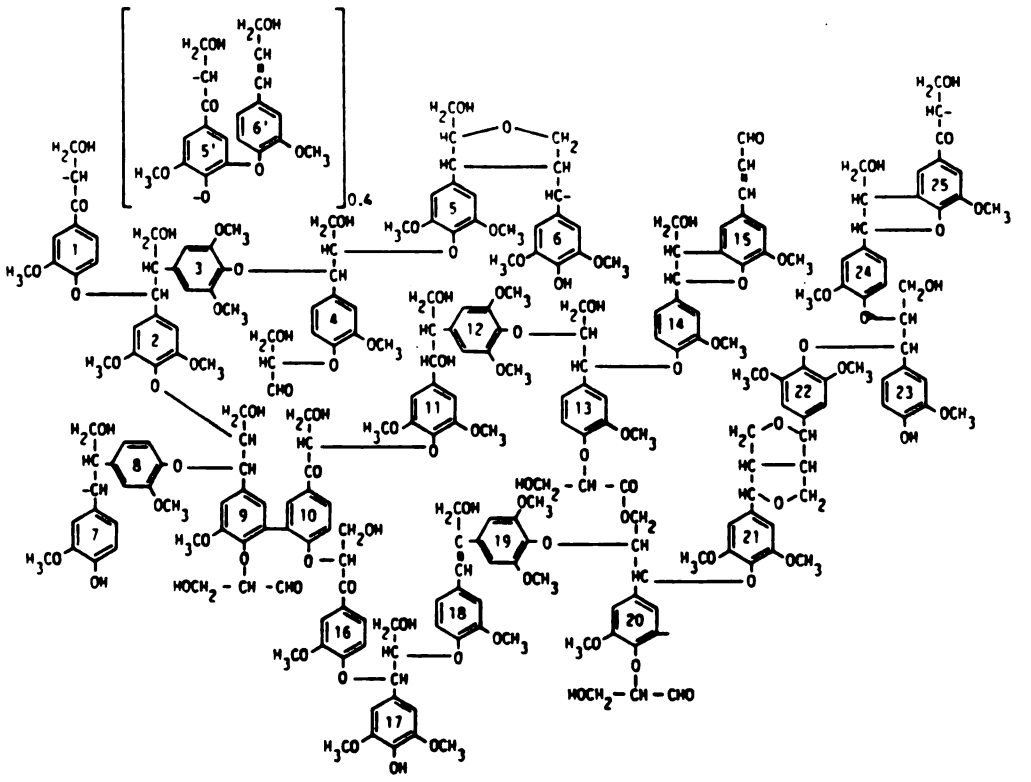

Figure 2. Structure of beech lignin (from Nimz, ref. 83).

## II. MICROBIOLOGY OF LIGNIN BIODEGRADATION

Although lignins are highly complex recalcitrant polymers, a few fungi and bacteria have been reported to degrade lignin to various extents. However, none of the microorganisms have been unequivocally shown to utilize the lignin polymer as a sole carbon or energy source for growth (54,59).

### 1. Fungi

White-rot fungi, including several hundred species of basidiomycetes and a few species of ascomycetes, are believed to be the main organisms responsible for lignin biodegradation in nature (16). White-rot fungi are known to simultaneously degrade all the major components of wood: lignin, cellulose and hemicellulose (16,54,57). One species of white-rot fungus, P. chrysosporium (11,88), is known to degrade synthetic lignin ($^{14}$C-DHPs that are dehydrogenative polymerizates of coniferyl alcohol) and native wood lignins to $CO_2$ more rapidly than most other organisms and has become somewhat of a model organism for studies on lignin biodegradation.

Soft-rot fungi include different ascomycetes and fungi imperfecti and are characterized by softening wood tissue accompanied by significant weight loss. Species of Allescheria, Graphium, Monodictys, Paecilomyces, Papulospora and Thielevia were shown to degrade lignin, although they appear to preferentially attack the wood polysaccharides (17).

Brown-rot fungi include numerous species of basidiomycetes which mainly degrade the polysaccharides in wood but cause only a limited degradation of lignin. The major reaction catalyzed by these fungi is

the demethylation of lignin, resulting in the formation of O-diphenolic units (56) which undergo autooxidation to yield quinone-type chromophores that give the brownish discoloration to the degraded wood. The main difference between brown-rot and white-rot fungi is believed to be the inability of the former to attack aromatic rings or the aliphatic products of aromatic ring cleavage.

### 2. Bacteria

Many genera of eubacteria (Acinetobacter, Aeromonas, Bacillus, Pseudomonoas and Xanthomonas) and actinomycetes (Micromonospora, Nocardia, Streptomyces and Thermonospora) have been reported to degrade different types of extracted lignin and $^{14}C$-labeled DHPs (14,94). However, lignin is not known to be mineralized rapidly or extensively by these bacteria.

## III. PHYSIOLOGY OF LIGNIN BIODEGRADATION

Lignin biodegradation in P. chrysosporium has been shown to be a secondary metabolic event (10,53) and is influenced by a number of factors, such as pH, oxygen, agitation and the concentration of various nutrients in the medium. Some of these factors may directly affect the enzyme activity while the others may play a role in affecting the gene expression.

### 1. Oxygen and agitation

Oxygen is an important requirement for lignin degradation by P. chrysosporium as lignin decomposition is basically an oxidative process. Kirk et al. (61) compared the rates of $^{14}CO_2$ release from $^{14}C$-(ring)-DHP

when P. chrysosporium was grown under different oxygen partial pressures and showed that the cultures grown under 5% $O_2$ released only 1% of the label in $^{14}C$-(ring)-DHP as $^{14}CO_2$ after 35 days of incubation, whereas those grown under 21% and 100% $O_2$ released 47% and 57%, respectively, of the label as $^{14}CO_2$. These results indicate that the lignin-degrading system is functional under low oxygen concentration but at a much lower efficiency. P. chrysosporium grown under 100% oxygen concentration was found to produce more $H_2O_2$ and higher lignin peroxidase activity than that grown in air (19). The highest lignin peroxidase activity was observed in cultures initially (i.e. during primary growth) grown under air and then shifted to pure $O_2$ atmosphere (73). Furthermore, the synthesis of veratryl alcohol, a typical secondary metabolite in P. chrysosporium which is an inducer or mediator of the ligninolytic process, was enhanced by elevated oxygen concentrations (105).

Agitation facilitates oxygen transit from the gas to the liquid phase and would be expected to result in increasing lignin decomposition. However, agitation was shown to completely inhibit lignin degradation in two wild-type strains (BKM-F 1767 and ME 446), but had only a moderate effect on lignin degradation by mutant SC26 (60) derived from BKM-F 1767, and two other wild-type strains (13,32). Leisola and Fiechter (70) later showed that the inhibition of lignin degradation due to agitation by cultures of BKM-F 1767 could be eliminated if veratryl alcohol was added to the growth medium. Addition of detergents, such as Tween-20 or Tween-80, to the cultures also prevented inhibition of ligninolytic activity in agitated cultures (46,99). Lignin peroxidase production in the latter cultures was still dependent on the speed of agitation because too high agitation rate led to complete inhibition of ligninolytic activity (70).

2. Nutrient concentration

Limitation of nitrogen, carbohydrate and sulfur is known to trigger ligninolytic activity in P. chrysosporium (47,53,61). Nitrogen starvation appears to be critical for sustained metabolism of the lignin polymer. This effect of nitrogen limitation on lignin degradation in P. chrysosporium was first observed by Kirk et al. (61) using $^{14}C$-labeled synthetic lignin (ring-$^{14}C$ DHP) and was later confirmed by a number of other workers (12,13,98). Reid (97) also demonstrated onset of ligninolytic activity following N starvation using $^{14}C$-lignin labeled aspen wood lignin as a substrate.

Kirk et al. (61) first found that P. chrysosporium grown in low-nitrogen (0.6 mM $NH_4NO_3$ and 2.4 mM L-asparagine) medium degraded 14±4% and 27±4% of [ring-$^{14}C$]-lignin to $^{14}CO_2$ after 9 and 15 days of incubation, respectively, whereas this fungus grown in high-nitrogen medium (24 mM nitrogen) released only 5±1% and 7±2% of labeled lignin as $^{14}CO_2$ after the same period of incubation. Appreciable differences in lignin degradation were not seen when L-asparagine, ammonium tartrate, urea or sodium nitrate was used as a nitrogen source. They also showed that addition of $NH_4^+$ to cultures immediately prior to the time of appearance of the ligninolytic system delayed its appearance, whereas addition of $NH_4^+$ to ligninolytic cultures resulted in an eventual, temporary decrease of ligninolytic activity (61).

To understand how nitrogen affects the ligninolytic activity, Fenn and Kirk (21) determined intracellular amino acid profiles and the total protein concentrations during onset of ligninolytic activity (measured as degradation of ring-U-$^{14}C$-lignin by P. chrysosporium in low nitrogen medium). It was found that the total amino acid pool increasd to about

580 nmol per culture just before the onset of lignin degradation, then decreased to approximately 400 nmol per culture during and after onset of ligninolysis and the subsequent changes were slight. In both cases, variations in glutamate levels accounted for over half of these changes. Arginine showed a dramatic decrease from 52 to 8 nmol during the same period. In contrast, the changes in intracellular protein concentration followed a manner roughly opposite to that of the concentration of amino acids and protein turnover was rapid (5-7%) during the transition period. This indicates that a number of new enzymes involved in the secondary metabolism are being synthesized. Effects of various nitrogen sources on cell protein concentration and repression of ligninolytic activity in cultures of P. chrysosporium were shown to be different. Glutamate suppressed ligninolytic activity by 83% but protein concentration increased by about 50% in comparison to the control, whereas histidine repressed ligninolytic activity by 76% but no protein increase was observed. Addition of $NH_4^+$ and glutamate showed different effects on the intracellular concentrations of glutamine and arginine, which play pivotal roles in nitrogen uptake and storage. However, both nitrogen compounds produced a similar increase (about 80%) in the intracellular glutamate levels although repression of ligninolytic activity remained in effect even when the glutamate levels returned to values observed in ligninolytic mycelia (22).

Accumulated data indicate that glutamate plays an important role in the regulation of secondary metabolism in P. chrysosporium based on the following observations. A new strain P. chrysosporium, INA-12 (13), produced lignin peroxidase under non-limiting nitrogen conditions (L-asparagine and $NH_4NO_3$ as nitrogen source) and the highest lignin peroxidase enzyme activity was obtained when glycerol was used as the

carbon source. However, when glutamate was used as nitrogen source, no lignin peroxidase activity was detected in the extracellular fluid (13). More recently, the lignin peroxidase enzyme activity in ligninolytic cultures was shown to be strongly repressed after adding $NH_4^+$ or L-glutamate (19,50,95). It has been shown that the onset of ligninolytic activity and idiophasic metabolism (secondary metabolism) in P. chrysosporium was preceded by a 10-fold increase in intracellular cAMP (79). Addition of L-glutamate repressed cAMP levels by 50% within 4 h, and cAMP levels remained low for 12 h in such ligninolytic cultures (80).

Three enzymes involved in glutamate metabolism demonstrated different levels under different nitrogen concentrations (12). Relatively high levels of NADP-glutamate dehydrogenase, which serves a biosynthetic role, and glutamine synthetase activity could be observed under low nitrogen conditions, whereas NAD-glutamate dehydrogenase which probably functions in glutamate catabolism showed low levels under the same conditions. However, the levels of the three enzymes under high nitrogen showed opposite pattern to those observed under low nitrogen conditions (12). At variance with these results, Fenn et al. (22) found that the specific activity of NADP-glutamate dehydrogenase increased at least 2-fold after adding $NH_4^+$ and glutamate. NAD-glutamate dehydrogenase, which was not detected in control cultures, increased with time following the addition of glutamate and $NH_4^+$. These results led them to propose that the various nitrogen repressors acted through biochemical repression of key enzyme(s) catalyzing lignin degradation.

### 3. Induction of the ligninolytic system by lignin and veratryl alcohol

Previous studies showed that the ligninolytic enzyme system is

produced irrespective of the presence or absence of lignin in low-nitrogen cultures of P. chrysosporium (53). However, Ulmer et al. (110) showed that the presence of high concentrations of lignin in the growth medium greatly increased the ligninolytic activity by P. chrysosporium, whereas a low concentration of lignin in the medium did not appreciably increase the ligninolytic activity. Maximal rates of degradation were also directly related to the amount of lignin added at the time of inoculation. This stimulation of ligninolytic activity by lignin did not become apparent until 24 h after the addition of lignin to the ligninolytic cultures previously grown in the absence of the polymer. The large size and the insolubility of lignin preclude its crossing the cytoplasmic membrane to directly react with the regulation system within the cells, suggesting that the inducer(s) may be the product(s) of lignin decomposition rather the lignin polymer itself. Faison and Kirk (19) determined the effect of different lignin monomers, dimers and lignin degradation products on the production of lignin peroxidase (measured as veratryl alcohol oxidation to veratraldehyde) and ligninolytic activity (degradation of $^{14}$C-lignin$\rightarrow$$^{14}CO_2$) and found that some lignin dimers and lignin metabolites gave higher induction than the monomers.

Veratryl alcohol is a typical secondary metabolite synthesized de novo by P. chrysosporium after cessation of primary growth (78,105). Also, it is a substrate for the lignin peroxidase which oxidizes it to veratraldehyde (78). Veratryl alcohol is recently reported to be integrally involved in lignin degradation (40,74). Veratryl alcohol is synthesized via phenylalanine, 3,4-dimethoxycinnamyl alcohol and veratrylglycerol (105). Production of veratryl alcohol, similar to lignin decomposition, was also shown to be suppressed when glutamate

was added to ligninolytic cultures (21). A high concentration of oxygen was shown to increase the synthesis of veratryl alcohol (105).

Veratryl alcohol has been shown to be a better inducer of lignin peroxidase and ligninolytic activity than lignin (20). Lignin peroxidase activity increased about four-fold 8 h after the addition of veratryl alcohol to 5-day ligninolytic cultures; this increase was prevented by adding cycloheximide, an inhibitor of protein synthesis, to ligninolytic cultures (20). Further studies showed that addition of 0.4 mM veratryl alcohol into the medium at the beginning of incubation resulted in an increase in specific activity and total activity of lignin peroxidase in the total heme-containing proteins in the extracellular fluid (60). However, the amount of increase in the synthesis of each lignin peroxidase isozyme was different (see Table 1). These results suggested that veratryl alcohol may act as an inducer of some of the lignin peroxidase genes, however, more direct evidence would be needed to prove this. Two mutants were recently isolated by Liwicki et al. (77), which did not produce veratryl alcohol but still degraded $^{14}C$-lignin-wheat ligninocellulose, suggesting that veratryl alcohol may not be required for lignin degradation as suggested by other investigators (40,74). The fact that lignin peroxidase and ligninolysis do not appear during primary growth (tropophasic cultures) even in the presence of veratryl alcohol suggests that the mere presence of this alcohol is not sufficient to trigger the synthesis of lignin-degrading enzyme complex.

### 4. Metal ions

Metal ions appear to be important for several enzymes involved in ligninolysis. For example, $Mn^{++}$ is absolutely required for the $Mn^{++}$-

dependent peroxidase activity whereas $Fe^{++}$ is required for all heme-containing lignin peroxidases as well as the $Mn^{++}$-dependent peroxidases (see Section V). A significant increase in ligninolytic activity as well as some of the lignin peroxidases was observed upon the addition of trace metal ions to ligninolytic cultures of P. chrysosporium (60). For instance, addition of 6 times the basal level of trace metals to the growth medium produced 2.8- and 2.5-fold increase in lignin peroxidases H1 and H2, respectively, but had little effect on the production of H6, H7 and H8 (60) (see Table 1). To determine which component(s) in the trace metal mixture is the stimulator, individual metal ions were added separately to a medium containing basal level of trace metals. The results showed that either $Cu^{++}$ or $Mn^{++}$ caused an increase in total lignin peroxidase activity equal to that observed with the complete trace metal mixture (60).

The differences in patterns of production of different lignin peroxidases upon addition of veratryl alcohol and metal ions suggest that the expression of lignin peroxidase genes may be regulated differently. The inductive effects of veratryl alcohol and metal ions on different lignin peroxidases are presented in Table 1. Stimulation of production of lignin peroxidases H1 and H2 was much higher than that of the other four lignin peroxidases when increased levels of trace metals were added individually or in combination with veratryl alcohol. Furthermore, the stimulation resulting from the addition of trace metals and veratryl alcohol appeared additive for H1 and H2 but not for others. These results together with the fact that H1 and H2 showed almost identical peptide patterns upon V8 peptidase digestion suggest that H1 and H2 are related proteins and that the expression of genes encoding these proteins might be controlled by the same regulator.

Table 1 Effects of medium additives on production of lignin peroxidases

| Lignin peroxidase[a] | Control[b] | Relative amount: Basal[c] + 0.4 mM veratryl alcohol | Relative amount: Basal + 6 x basal level of metals | Relative amount: Basal + 0.4 mM veratryl alcohol + 6 x basal level of metals |
|---|---|---|---|---|
| H1 | 1.6 | 2.6(1.6) | 4.5(2.8) | 8.6(5.4) |
| H2 | 8.7 | 11.7(1.3) | 21.5(2.5) | 32.9(3.8) |
| H6 | 4.7 | 6.7(1.4) | 5.1(1.1) | 5.5(1.2) |
| H7 | 9.2 | 9.3(1.0) | 11.2(1.2) | 9.4(1.0) |
| H8 | 71.1 | 75.3(1.1) | 77.9(1.1) | 64.9(0.9) |
| H10 | 4.7 | 9.2(2.0) | 6.4(1.4) | 5.7(1.2) |

a Extracllular fluid from the four media was concentrated 20-fold and analyzed by HPLC. Areas under 409 nm absorbing peaks representing heme proteins were integrated and these values are shown above. The number in the parentheses gives the relative areas of each peak from the four samples normalized to the sample designated control.

b The control medium contains a basal medium, 1% glucose, and 10 mM 2,2-dimethylsuccinate pH 4.5.

c The basal medium contained: $1.08 \times 10^{-3}$ M ammonium tartrate, $1.47 \times 10^{-2}$ M $KH_2PO_4$, $2.03 \times 10^{-3}$ M $MgSO_4.7H_2O$, $6.8 \times 10^{-4}$ M $CaCl_2.2H_2O$, $2.97 \times 10^{-6}$ M thiamine.HCl and 10 ml of a trace element solution. The basal trace element solution (called basal level of metals) contained: $7.8 \times 10^{-3}$ M nitriloacetic acid, $1.2 \times 10^{-2}$ M $MgSO_4.7H_2O$, $2.9 \times 10^{-3}$ M $MnSO_4.H_2O$, $1.7 \times 10^{-2}$ M NaCl, $3.59 \times 10^{-4}$ M $FeSO_4.7H_2O$, $7.75 \times 10^{-4}$ M $CoCl_2$, $9.0 \times 10^{-4}$ M $CaCl_2$, $3.48 \times 10^{-4}$ M $ZnSO_4.7H_2O$, $4 \times 10^{-5}$ M $CuSO_4.5H_2O$, $2.1 \times 10^{-5}$ $AlK(SO_4)_2.12H_2O$, $1.6 \times 10^{-4}$ M $H_3BO_3$ and $4.1 \times 10^{-5}$ M $NaMoO_4.2H_2O$.

Data taken from Kirk et al. (60).

## IV. ROLE OF $H_2O_2$ IN LIGNIN DEGRADATION

Hydrogen peroxide is believed to play a central role in lignin degradation (86,95,96). Koenigs (64,65) first found that a number of wood-rotting fungi produced hydrogen peroxide ($H_2O_2$) from glucose in culture or from native substrates in wood and suggested the possible involvement of $H_2O_2$ in lignin degradation by white-rot fungi. Forney et al. (24) later showed that when P. chrysosporium was grown in low nitrogen medium, an increase in the specific activity for $H_2O_2$ production was observed to coincide with the appearance of ligninolytic activity and both activities appeared after the culture entered the stationary phase. The ultrastructural studies of ligninolytic cells of P. chrysosporium demonstrated that hydrogen peroxide production appeared to be localized in periplasmic "microbodies" of cells from ligninolytic cultures grown for 14 days in low nitrogen medium but not in cultures grown for 4 days in the same medium or in 14 day cultures grown in high nitrogen medium (23). This correlation between $H_2O_2$ production and ligninolytic activity was also observed by other groups (8,34). Besides, high oxygen concentration and the presence of lignin in the medium, which are known to stimulate ligninolytic activity in P. chrysosporium, were shown to stimulate the production of hydrogen peroxide (18,19,34). The involvement of hydrogen peroxide in lignin decomposition by P. chrysosporium has been firmly established since lignin and $Mn^{++}$-dependent peroxidases, whose activities are dependent upon the presence of $H_2O_2$, were recently purified from ligninolytic cultures of this fungus (see next section).

## V. ENZYMES INVOLVED IN LIGNIN DEGRADATION

Tien and Kirk (106) and Glenn et al. (26) simultaneously discovered an extracellular $H_2O_2$-dependent enzyme which catalyzed the $C_\alpha$-$C_\beta$ cleavage of lignin model compounds and limited depolymerization of lignin polymers. Since then, different kinds of enzymes which are believed to be involved in lignin biodegradation have been purified and characterized from cell extracts or extracellular fluid of ligninolytic cultures of P. chrysosporium. The data on purified extracellular enzymes from ligninolytic cultures of P. chrysosporium are summarized in Table 2.

### 1. Lignin peroxidases

#### A. Purification procedures

Relatively simple procedures have been developed for purifying lignin peroxidases from P. chrysosporium (32,72,84,107) and these are briefly described here. The extracellular fluid from cultures showing peak lignin peroxidase activity are first separated from the mycelia by filtration of the culture through cheese cloth followed by centrifugation to remove fine particles. The supernatant is then concentrated using either Amicon membrane filtration technique or acetone precipitation (32). The concentrated fluid is dialyzed against appropriate buffer and then purified by HPLC, FPLC or other chromatographic procedures (see Table 2) to separate different enzymes. Purity of the protein is determined by the use of sodium dodecylsulfate polyacryamide gels (SDS-PAGE) and the protein bands are visualized using the silver staining technique.

Table 2 Characteristics of Extracellular Enzymes Purified from Ligninolytic Cultures of Phanerochaete chrysosporium

| Enzyme | Synonym | Strain | purification procedure | M. W. | heme | carbohydrate (%) | cofactor(s) | reference |
|---|---|---|---|---|---|---|---|---|
| Lignin peroxidase | Ligninase | BKM-F1767 | DEAE-Bio-Gel A (Bio-Rad) | 42,000 | + | 13 | $H_2O_2$ | 106,107 |
| | Ligninase H1 H2, H6, H7, H8 and H10 | BKM-F1767 | FPLC Mono Q (Pharmacia) | ND* | + | ND | $H_2O_2$ | 60 |
| | Ligninase-1 | BKM-F1767 | PBE-94 (Pharmacia) | 42,000-43,000 | + | 21 | $H_2O_2$ | 85 |
| | Diarylpropane oxygenase | Gold | DEAE-Sepharose Sephadex G100 (Sigma) | 41,000 | + | ND | $H_2O_2$ | 32 |
| | Diarylpropane oxygenase I | Gold | DEAE-Sepharose Sephadex G100 (Sigma) | 39,000 | + | 6 | $H_2O_2$ | 101 |
| | II | Gold | | 41,000 | + | 6 | $H_2O_2$ | 101 |
| | III | Gold | | 43,000 | + | 6 | $H_2O_2$ | 101 |
| | Diarylpropane oxygenase | ME446 | Blue agarose Sephadex G100 | 41,000 | + | ND | $H_2O_2$ | 26,67 |
| | Lignin peroxidase Fr-II and Fr-III | ME446 | DEAE-Sepharose CL-6B (Pharmacia) | ND | + | ND | $H_2O_2$ | 6 |
| $Mn^{++}$-dependent peroxidase | $Mn^{++}$-dependent, lactate-activated peroxidase | ME446 | DEAE-Sepharose Blue agarose Sephadex G100 | 46,000 | + | ND | $H_2O_2$, $Mn^{++}$ α-hydroxy acids proteins | 26,67 |
| | NADH-peroxidase | ME446 | DEAE-Sepharose CL-6B (Pharmacia) | 46,000 | + | ND | $H_2O_2$, $Mn^{++}$ | 6 |
| | Vanillyacetone oxidase Peroxidase-M2 | BKM-F1767 | DEAE-Bio-Gel A (Bio-Rad) | 45,000-47,000 | + | 17 | $H_2O_2$, $Mn^{++}$ | 85 |

*ND-not determined.

B. Properties

Lignin peroxidase (ligninase) is an $H_2O_2$-dependent, heme-containing, glycosylated extracellular protein which catalyzes the $C_\alpha$-$C_\beta$ cleavage of different lignin model compounds as well as a variety of other aromatic compounds, and depolymerizes lignin polymers. This enzyme contains one protoheme IX as the prosthetic group and has one atom of iron per molecule. The spectral analyses from EA (Electronic Absorption), EPR (Electronic Paramagnetic Resonance) and RR (Resonance Raman) spectroscopy showed that the native enzyme contained high-spin iron and the reduced enzyme contained a high-spin, pentacoordinate ferrous iron (5,100). Although lignin peroxidases purified from different strains appeared very similar to one another, some differences have been noted among them. For example, the absorption spectrum of native lignin peroxidase from the BKM strain used by Kirk and coworkers was not changed upon the addition of dithionite (107) whereas the enzyme from the strain used by Gold and coworkers displayed the red shift (32). The optimal pH of these two enzymes for the oxidation of $\beta$-ether dimers is also different (32,107). The differences between the above two lignin peroxidases may reflect the facts that they were purified from different strains and different conditions were employed for growing these strains. Variation in culture condition is known to have a profound effect on the types and amounts of lignin peroxidase produced (60).

Lignin peroxidases are known to catalyze the following reactions (26,32,36,45,67,72,85,101,106,107):

(1) $C_\alpha$-$C_\beta$ cleavage of $\beta$-1 and $\beta$-O-4 lignin model compounds;

(2) Hydroxylation of some benzylic methylene groups, and $C_\alpha$ and $C_\beta$ of the $C_\alpha$=$C_\beta$ olefinic bond in styryl structures;

(3) Oxidation of phenols, methoxybenzenes and benzyl alcohol;

(4) Intradiol cleavage of phenylglycol;

(5) Partial depolymerization of methylated spruce and birch milled wood lignin as well as $^{14}$C-ring-DHP; and

(6) Ethylene generation from 2-keto-4-thiomethyl butryic acid (KTBA) in the presence of veratryl alcohol.

C. Multiple forms of lignin peroxidase

Renganathan et al. (101) described three lignin peroxidases present in the extracellular fluid of ligninolytic cultures of P. chrysosporium, called forms I, II and III; form II accounted for 85% of the lignin peroxidase activity based on the oxidation of veratryl alcohol to veratraldehyde. Absorption maxima of the native, reduced, and a variety of ligand complexes of three lignin peroxidases were essentially identical and the same products were produced from different lignin model compounds by the three enzymes. However, the specific activities for veratryl alcohol oxidation were different. Later, Kirk et al. (60) identified six lignin peroxidases present in the extracellular fluid of the ligninolytic cultures of P. chrysosporium strains BKM-F 1767 (Figure 3). The HPLC profile of the extracellular fluid from a 5-day flask culture of this fungus showed that there were at least thirteen proteins and ten of them (named from H1 to H10) contained heme (Figure 3). Six of the ten heme proteins (H1, H2, H6, H7, H8, H10) displayed veratryl alcohol oxidation activity. The lignin peroxidases H2 and H8 is the major proteins; H2 and H6 showed higher specific activities than that of H8 but the quantities of these proteins were relatively small. The V8 protease analyses showed that H1 and H2 produced almost identical peptides, H8 produced similar peptide pattern to H1 and H2 but lacked at least two major peptides and H6 and H10 produced very different peptide

patterns from those of H1, H2 and H8, indicating that the lignin peroxidases are related but different proteins. However, polyclonal antibody raised against H8 could react with the other five lignin peroxidases, indicating that there should be amino acid sequence homology present among these lignin peroxidases. The multiple forms of lignin peroxidase were confirmed by other groups as well (6,71). Leisola et al. (71) more recently identified as many as fifteen lignin peroxidases in the extracellular fluid of ligninolytic cultures of P. chrysosporium.

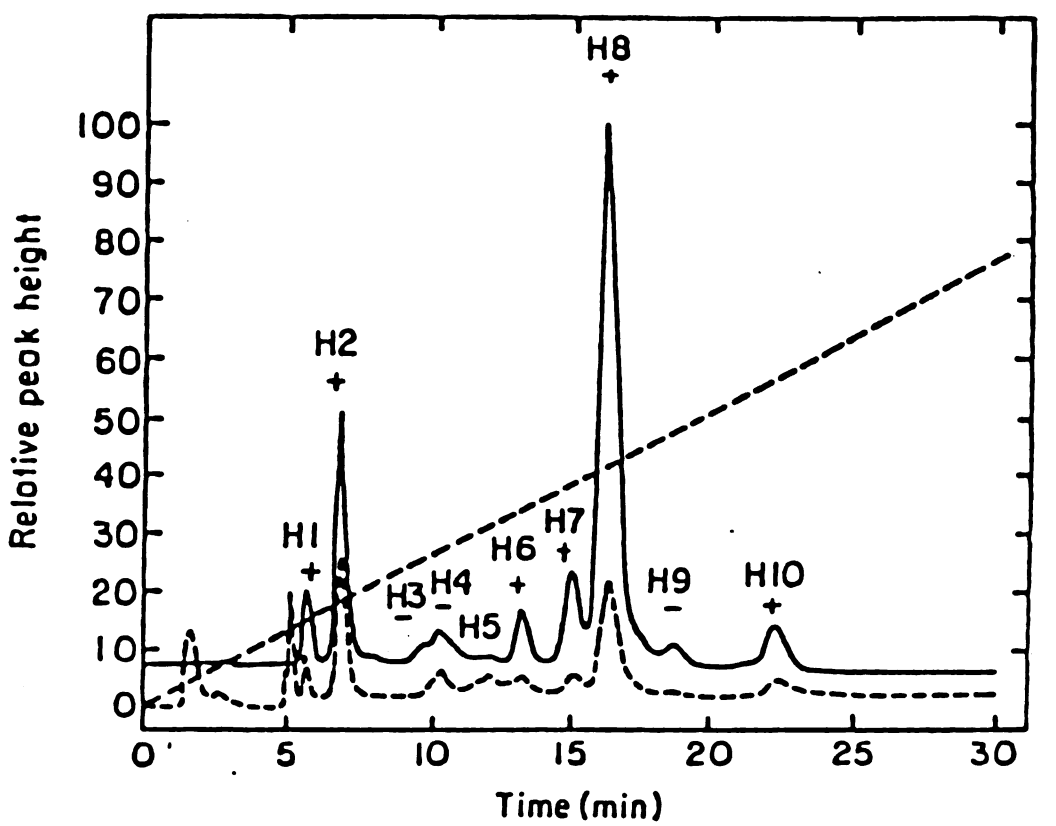


Figure 3. HPLC profile of extracellular fluid from a 5-day ligninolytic culture of P. chrysosporium. The sample of 20-fold concentrated extracellular fluid was used for HPLC analysis. Full line, $A_{409}$; broken curve, $A_{280}$. Lignin peroxidase activity is indicated as positive (+). The sloping line shows the acetate gradient (From Kirk et al., ref. 60)

D. Mechanism of reaction catalyzed by lignin peroxidase

A two single-electron oxidation mechanism has been proposed to explain the $C_\alpha$-$C_\beta$ cleavage of lignin model compounds and other reactions catalyzed by lignin peroxidase (37-39,63,81,104). When diarylpropane is oxidized by lignin peroxidase, the enzyme reacts with $H_2O_2$ to form a two-electron peroxy intermediate compound I (Figure 4). This high potential oxy-ferryl intermediate extracts one electron from the aromatic ring of the substrate (Figure 5), resulting in the fomation of a substrate cationic radical and the one-electron oxidized form of the lignin peroxidase, compound II. The cationic radical cleaves at the $C_\alpha$-$C_\beta$ bond to form a cation from the $\alpha$-carbon moiety and a benzyl free radical in the $\beta$-carbon moiety. The cation immediately deprotonates to produce veratraldehyde as one product. The second radical ($\beta$-carbon moiety) can undergo a variety of reactions including a further one-electron oxidation to yield a cation which subsequently hydrates with water to produce phenylglycerol. The second radical may also react with molecular oxygen to form a peroxy radical. This is consistent with the result of labeled-$O_2$ inserting onto the $\beta$-carbon (102,107). Two molecules of peroxy radicals could interact to yield one molecule of phenylglycerol and one molecule of ketol (39). The compound II of lignin peroxidase could catalyze one-electron oxidation of either the second radical ($\beta$-carbon moeity) or aromatic nucleus of another diarylpropane molecule, and then return to the native state. The presence of different forms of the enzyme and substrate was demonstrated during the oxidation of various lignin model compounds (5,37,39,63,81,86,104).

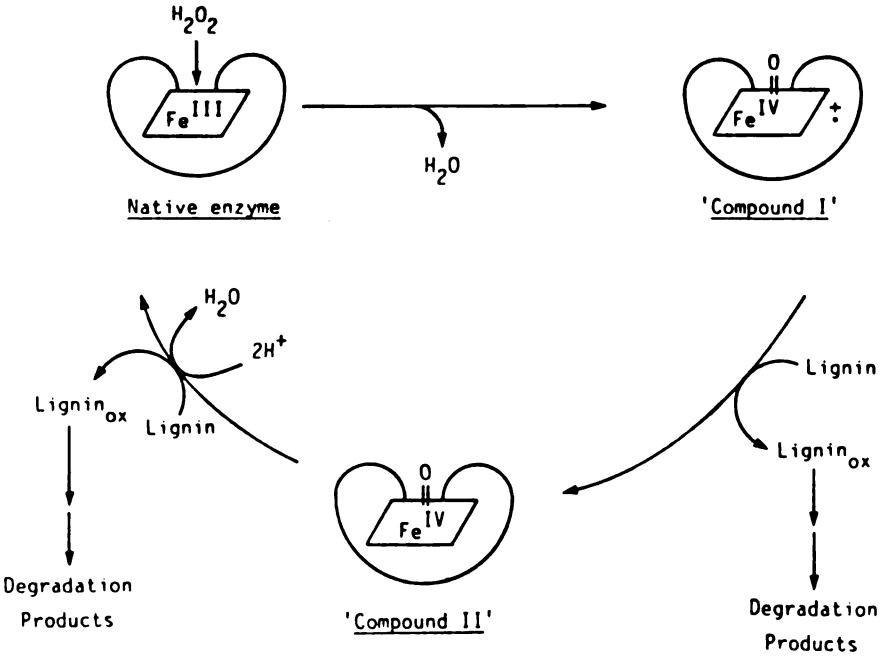


**Figure 4. Catabolic cycle of lignin peroxidase of P. chrysosporium (from Kirk, ref. 55).**

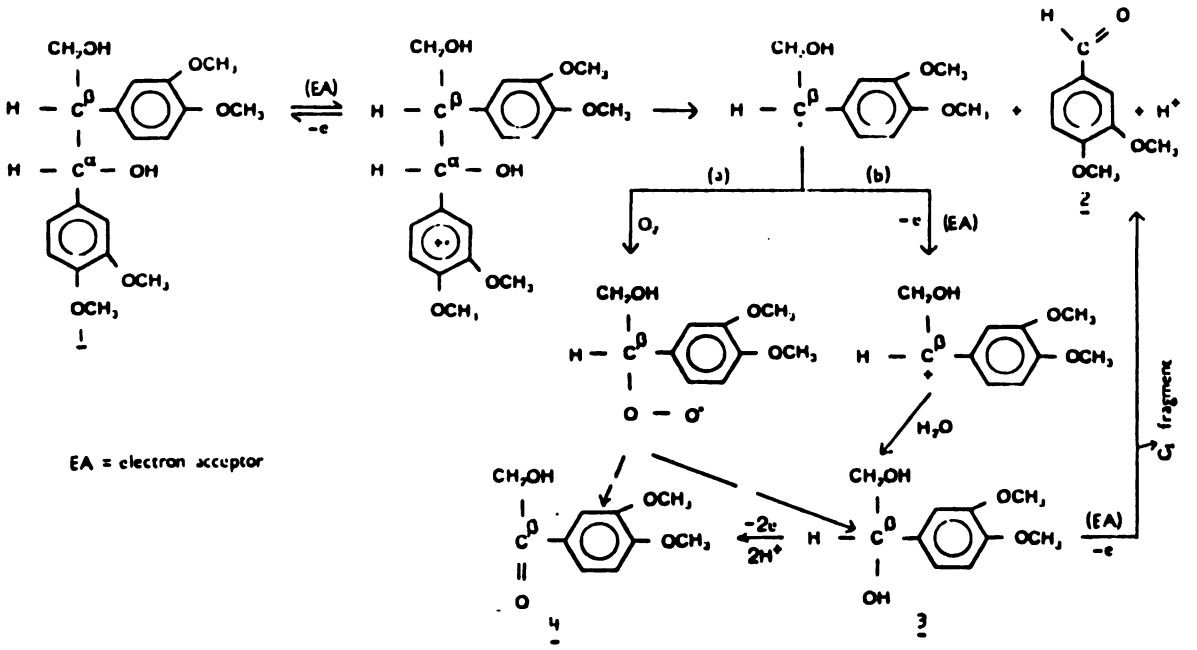


Figure 5. Scheme of $C_\alpha$-$C_\beta$ oxidative cleavage of diarylpropane catalyzed by lignin peroxidase of P. chrysosporium (from Schoemaker et al., ref. 104).

## 2. $Mn^{++}$-Dependent peroxidase

The same procedures as described for lignin peroxidases were used to purify $Mn^{++}$-dependent peroxidase from ligninolytic cultures of P. chrysosporium. This enzyme is also an extracellular, glycosylated heme protein. The enzyme requires not only hydrogen peroxide but also $Mn^{++}$ for its activity to catalyze the oxidation of a variety of phenol derivatives (25) and a number of dyes including Poly B-411 and Poly R-481 (84,85). Unlike lignin peroxidase, this enzyme cannot oxidize veratryl alcohol at pH 5.0 (45). The addition of α-hydroxylic acids and/or exogenous protein (such as egg albumin) to the reaction system stimulated the activity of the $Mn^{++}$-dependent peroxidase from strain ME 446 (67) but not of that from another strain BKM-F 1767 (85). This enzyme also catalyzes the oxidation of NADH or NADPH to produce $H_2O_2$, in the presence of $Mn^{++}$ and $O_2$ (6,85). Unlike lignin peroxidases which are extracellular, $Mn^{++}$-dependent peroxidase appears to be associated closely with the fungal mycelia (85). All the peroxidase activity was lost upon dialysis against 20 mM Na-succinate, suggesting the loose binding of $Mn^{++}$ to the enzyme (25). The enzyme rapidly oxidized $Mn^{++}$ to $Mn^{+++}$ which plays a role in the enzyme mechanism (25). More recently, Leisola et al. (71) showed that six $Mn^{++}$-dependent peroxidases appeared and reached their maximal activity earlier than the lignin peroxidases in ligninolytic cultures of P. chrysosporium. Peptide mapping, amino acid analysis and reaction against specific antibodies showed that all the $Mn^{++}$-dependent peroxidases were probably products of one gene (71).

## 3. $H_2O_2$-Producing enzymes

### A. Glucose oxidase

Fungal glucose oxidase ($\beta$-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of D-glucose to $H_2O_2$ and $\delta$-D-gluconolactone which is nonenzymatically hydrolyzed to D-gluconic acid, in the presence of molecular oxygen. Glucose oxidase appears to play an important role in the production of $H_2O_2$ for lignin degradation in P. chrysosporium according to the following observations (50). Glucose supported the highest level of oxygen-dependent $H_2O_2$ production in cell extracts of ligninolytic cultures compared to a number of other substrates. Glucose-dependent $H_2O_2$ production was exhibited by a single protein band from cell extracts, but not from extracellular fluid, of ligninolytic cultures. This protein band was not seen in cell extracts of non-ligninolytic cultures. Glucose oxidase activity, like ligninolytic activity, was shown to be a secondary metabolic event and was triggered in response to the starvation for nitrogen or carbohydrate. More recently, several glucose oxidase-negative mutants (gox$^-$) were isolated and were shown to have neither the glucose oxidase activity nor the ligninolytic activity, whereas the revertants regained both glucose oxidase and ligninolytic activity (51,90).

Glucose oxidase was purified from the cell extract of ligninolytic cultures of P. chrysosporium ME 446 by the following steps: preparation of cell extracts, DEAE-Sephadex chromotography, Sephacryl chromotography and DEAE-Sephadex chromatography (49). The purified enzyme is a flavoprotein with a native molecular weight of 180,000 and a denatured molecular weight of 80,000, suggesting that the native form may consist of two identical polypeptide chain subunits covalently linked by disulfide bonds. No carbohydrate was found in the enzyme. The enzyme activity has an optimal pH between 4.6 and 5.0. Glucose is believed to be the primary substrate for the enzyme based on the Vmax/Km, whereas

the other carbohydrates, such as L-sorbose, D-xylose and D-maltose, are relatively minor substrates.

B. $Mn^{++}$-dependent peroxidase

$Mn^{++}$-dependent peroxidase described above was also shown to produce $H_2O_2$ by the oxidation of NADH or NADPH (6,25,85). One function of this enzyme may be to produce $H_2O_2$, which is essential for lignin peroxidase activity, from the reduced pyridine nucleotides which were shown to be secreted by P. chrysosporium into the growth medium (68). However, the very low amounts of pyridine nucleotides present in extracellular culture fluid cannot account for the large amounts of $H_2O_2$ produced in ligninolytic cultures.

C. Fatty acyl CoA oxidase

Greene and Gould (35) found that mycelia of lignin-degrading cultures of P. chrysosporium grown in low nitrogen medium consumed $O_2$ and produced extracellular $H_2O_2$ when incubated with fatty acyl-CoA substrates and suggested that peroxisomal fatty acyl oxidase may be an important enzyme for the production of $H_2O_2$ for lignin biodegradation in this fungus. However, the activity of this enzyme was too low to account for the level of $H_2O_2$ produced in lignin-degrading cultures. Also, no correlation between the regulation of production of this enzyme and lignin degradation system has been shown.

D. Glyoxal oxidase

More recently, Kersten and Kirk (52) demonstrated an extracellular $H_2O_2$-producing enzyme, glyoxal oxidase in ligninolytic cultures of P. chrysosporium. This enzyme can oxidize several $\alpha$-hydroxy carbonyl- and dicarbonyl compounds, coupled to the reduction of $O_2$ to $H_2O_2$.

4. Other enzymes

Cellobiose-quinone oxidoreductase (111) may play an indirect role in lignin biodegradation by preventing polymerization of lignin degradation products. The enzyme was purified from the cell extract of primary growth culture of P. chrysosporium and has a molecular weight of 58,000 (112). Cellobiose-quinone oxidoreductase is a flavoprotein with a FAD prosthetic group.

Huynh and Crawford (45) found that concentrated and unfractionated extracellular fluid of P. chrysosporium BKM-F 1767 could catalyze the conversion of 2-methoxy-3-phenylbenzoic acid (M1) to 2-hydroxy-3-phenylbenzoic acid (aromatic methyl ether demethylase), the conversion of methyl 2-hydroxy-3-phenylbenzoate (M3) to 2-hydroxy-3-phenylbenzoic acid (aromatic methyl ester esterase) and the oxidation of vanillylacetone [4-(4-hydroxy-3-methoxyphenyl)-3-buten-2-one, M2] (vanillylacetone oxidase) in the presence of both $H_2O_2$ and $Mn^{++}$. However, they were able to purify only one of these three enzymes, i.e. vanillylacetone oxidase (45).

## VI. GENETICS AND MOLECULAR BIOLOGY OF LIGNIN BIODEGRADATION

Numerous attempts have been made to improve the ligninolytic activity of P. chrysosporium. These include changing the concentrations of media components (7,60), employing agitation to improve oxygen supply to cells (19,46,70,73), adding different detergents and veratryl alcohol to the media (60,99), isolating different strains including mutants capable of higher lignin peroxidase production (7,13,32,60) and developing different types of fermentors for scaling up growth (60,75,76,85). By these manipulations, lignin peroxidase activity has been increased from about 5 U/L (107) to 1250 U/L (7). However, genetic and molecular

biological studies on P. chrysosporium will be useful not only to obtain better understanding of the nature of lignin degradation but also to greatly improve the ligninolytic activity of P. chrysosporium so that the organism or its products could be used in a variety of biotechnological processes.

1. Genetic studies

Gold and coworkers have developed some basic approaches for the study of the genetics of P. chrysosporium. Since P. chrysosporium produces asexual spores prolifically and has diffuse unrestricted growth on ordinary agar media, Gold and Cheng (27) designed a medium containing L-sorbose and sodium deoxycholate, which induces colonial growth of this fungus. The restricted size and heavy conidial production of colonies permits high plating densities and the use of a replica-plating technique. Gold et al. (30) isolated a number of auxotrophic mutants by using UV and X rays as mutagens. These auxotrophs have been characterized by employing complementation procedures described below (30,82). They spread mycelia from two of different auxotrophs onto solid minimal medium to force complementation (30). The results showed that vigorous growth ensued after a short lag time, suggesting that heterokaryon formation occured in the vegetative mycelial stage. Further studies showed that the vigorous growth was not due to cross feeding but due to complementation between the two different auxotrophs (30). Upon cytological study it was found that the wild-type conidia were 60% mononucleate, whereas the conidia from heterokaryotic mycelia ranged from only 1-10% mononucleate (30). Another approach, protoplast fusion, was also used to demonstrate complementation between auxotrophs (29). The prototrophs from protoplast fusion were found to be readily

homokaryotized and revert to the parent phenotypes, indicating that the prototrophic mycelia were heterokaryotic rather than diploid. Since classical basidiomycete crosses require the development of fruiting bodies, Gold and Cheng (28) demonstrated that fruit body formation in P. chrysosporium was controlled by glucose and nitrogen catabolite repression and that Walseth cellulose was the best source of carbon for the induction of fruit body and consequent basidiospore synthesis in this fungus. Alic and Gold (1) isolated recombinants with different phenotypes, including wild-type and double mutant phenotypes, from various crosses after inducing the formation of fruiting bodies from heterokaryons. The results indicate that the genetic recombination does occur. Cytological studies demonstrated that more than 90% of the basidiospores from wild-type and auxotrophic strains as well as from forced heterokaryons were binucleate. The ratio of recombinants to parental types is about 1:1:1:1, suggesting that the binucleate basidiospores are homokaryotic and that the two nuclei arise from a postmeiotic mitotic event. Further studies showed that the binucleate basidiospores were homokaryotic but not heterokaryotic and that P. chrysosporium had a primary homothallic mating system (2).

More recently, Tien et al. (109) proposed a potential strategy for the lignin peroxidase-dependent selection of lignin-degrading microorganisms. This strategy involves covalently bonding amino acids to lignin model compounds in such a way that lignin peroxidase-catalyzed cleavage of the models produces the amino acids for growth. This procedure may also be used to select mutants which overproduce lignin peroxidase(s) or are defective in production of lignin peroxidase.

Johnsrud and Eriksson (48) used classical mutagenesis and cross-breeding techniques to isolate several cellulase-negative mutants from

P. chrysosporium K-3, one of which was later shown to have higher lignin peroxidase activity than the parent strain (62). Such lignin-degrading, cellulase-deficient mutants may be very useful for biopulping and biobleaching applications in paper industry. A mutant (SC26) from BKM-F 1767 (60) showed the highest total ligninolytic activity, the highest total and specific lignin peroxidase activity compared with the parent strain and other two wild-type strains (ME 446 and K3) as well as three cellulase-negative mutants (62). Furthermore, this mutant degraded lignin under agitation conditions and could adhere well to the plastic discs of a rotating disk fermentor, which is potentially useful for scaling up lignin peroxidase production (60).

Two phenoloxidase-negative mutants (4,31) were isolated and one of them was shown to be pleiotropic lacking lignin degradation and several other idiophasic characteristics as well (33). The revertants from this mutant recovered all lost functions. These results suggested that it may be a regulatory mutant (33) lacking phenoloxidase activity and several other secondary metabolic characteristics. Four classes of phenoloxidase-negative mutants were recently isolated by Liwicki et al. (77). Studies with these mutants indicated that mutations resulting in loss of phenol oxidase and ligninolytic activity were not necessarily pleiotrophic for other idiophasic functions, such as intracellular cAMP levels, sporulation, extracellular glucan production and veratryl alcohol synthesis.

Several glucose oxidase-negative mutants have recently been isolated (51,90). These mutants were shown to be deficient not only in their ability to produce hydrogen peroxide but also in lignin degradation, lignin peroxidase activity, and decolorization of the dye poly-R 481 (51,90). These mutants retained, albeit at a lower level, the capacity

to produce veratryl alcohol (a typical secondary metabolite in P. chrysosporium), and produced conidia at a level comparable to that of the wild type. The revertants recovered all the missing characteristics. These mutants may be another kind of regulatory mutant. Such mutants may be useful for the identification of genetic control elements using molecular cloning procedures.

## 2. Molecular biology

Although there has been some progress in developing classic genetic procedures for P. chrysosporium as described above, the available genetic information for this fungus is meagre compared to other filamentous fungi such as Aspergillus and Neurospora. Use of recombinant DNA procedures is another alternative to obtain a better understanding of the structure, expression, regulation and organization of genes involved in the whole ligninolytic system. A number of molecular biological procedures which have been shown to be successful in cloning and expression of heterologous genes in bacteria, yeasts and other filamentous fungi can be modified and used for cloning and characterizing genes encoding lignin peroxidases and other enzymes in P. chrysosporium.

Reddy and coworkers have developed a number of different procedures suitable for molecular biological studies on P. chrysosporium. These include the isolation of chromsomal DNA and RNA from P. chrysosporium (92,113), preparation of spheroplasts from conidia and DNA transformation for this fungus (91). Rao and Reddy (92,93) constructed a YIp5-$kan^r$ (kanamycin-resistant) vector which is useful for isolating ars (autonomous replication sequence) from yeast and other eukaryotes and isolated an ars sequence from this fungus. By inserting the isolated ars

from P. chrysosporium into plasmid YIp5-kan$^r$, a shuttle vector for E. coli/S. cerevisiae/P. chrysosporium was constructed (91). This vector can transform wild-type strains of P. chrysosporium to G418 resistance (91). Different cDNA clones for lignin peroxidase have been isolated and characterized (114). Broda and coworkers have also developed procedures for isolating mRNA and chromosomal DNA from this fungus (41,89) and showed that its estimated genomic size is about 4-5 X $10^7$ base pairs (88).

A. Development of cloning system

As a first step in the construction of a cloning vector suitable for transformation of P. chrysosporium, an ars sequence was isolated from this fungus. Rao and Reddy (92) constructed a gene library in the S. cerevisiae integration vector YIp5 which contains selection markers for E. coli [Ap$^r$ (ampicillin-resistant) and Tc$^r$ (tetracycline-resistant)] and yeast (URA3) and a replication origin (ori) for E. coli but not for S. cerevisiae. If P. chrysosporium genomic sequences cloned into YIp5 confer on the latter the ability to autonomously replicate in yeast, one should obtain high frequency of transformation of ura$^-$ to ura$^+$. Several ars sequences have been isolated and characterized by using this approach (92). This fungus was later shown to be sensitive to the antibiotic G418 (91), which inhibits protein synthesis in prokaryotes as well as many eukaryotes. This suggested that the kanamycin-resistant gene from bacterial transposon Tn903, that encodes an aminoglycoside phosphotransferase which inactivates both kanamycin and G418, could be used as a selection marker in P. chrysosporium. Randall et al. (91) recently constructed a shuttle vector containing the ars sequence from P. chrysosporium, the kan$^r$ selection marker from Tn903, ori sequence for replication in E. coli, and the URA3 marker for selection in yeast. This

shuttle vector should be very useful for molecular genetic studies of lignin biodegradation and for studying the regulation of secondary metabolism in P. chrysosporium.

Randall et al. (91) have developed a transformation procedure for introducing this shuttle vector into P. chrysosporium. The shuttle vector, designated pRR12, could transform two wild-type strains of P. chrysosporium to G418 resistance with a frequency of about 20 transformants per microgram DNA (91). Although the transformation frequency was relatively low the transformation efficiency was quite high ($3.3 \times 10^{-3}$ to $1.1 \times 10^{-2}$ transformants/regenerated spheroplast). The transforming vector pRR12 present at a low level in the transformants, could be consistently recovered by E. coli transformation. The recovered vector has been shown to be identical to pRR12. The vector is stably maintained in the transformants under selective conditions and can be recovered even after 18 months (91). This is the first demonstration of a transformation system for P. chrysosporium and the first demonstration of a shuttle vector for a filamentous fungus.

B. Molecular cloning of lignin peroxidase cDNAs and genes

Molecular cloning procedures have been well established for E. coli and S. cerevisiae and a number of eukaryotic genes have been cloned by using these two systems. Therefore, either of them can be used to clone lignin peroxidase gene(s) from P. chrysosporium although yeast may be a better molecular cloning system for this fungus (9). Different strategies have been employed to clone genes for lignin peroxidase (87,108,114).

Since some of the genes isolated from other filamentous fungi have already been shown to contain several small introns, the inability to

correctly process introns from foreign genes is a potential problem with the yeast system. Besides, the lack of functional expression of some foreign genes in either yeast or E. coli is another problem due to the differences in promoter sequences. Hence, it might be better to clone lignin peroxidase cDNA(s) first and obtain its expression in a suitable eukaryotic or prokaryotic expression system. These cDNA(s) can also be used as probe to screen the genomic library to isolate lignin peroxidase genes. Since ligninolytic activity in P. chrysosporium (BKM strain) appears after the cessation of primary growth and reaches a peak around the 6th day of incubation, the assumption was made that the messenger RNAs for lignin peroxidase proteins may also be present in 6-day-old cells. Therefore, the strategy to clone lignin peroxidase gene should be: 1) constructing a cDNA library from the ligninolytic cultures of P. chrysosporium into an expression vector (pUC series;114) or phage (λgt11;108); 2) screening the library either with antibody raised against the purified lignin peroxidase (108) or with synthetic oligodeoxynucleotide probes whose sequences were deduced from the partial amino acid sequences of lignin peroxidase (114); 3) characterizing the isolated cDNA clones and sequencing them to make sure that the correct clones were obtained; 4) using the cDNA(s) as probe to screen the genomic library to isolate genomic lignin peroxidase gene(s). Furthermore, the cDNA(s) can be cloned into suitable expression vectors to get maximal production of functional lignin peroxidase, whereas the genomic genes can be used to study the gene structure, regulation of gene expression as well as organization of lignin peroxidase gene(s) on the chromosomes.

By using the above strategy, two different lignin peroxidase cDNA clones have been isolated and sequenced (114 and see chapter 1 and 2 in

this dissertation) and one other cDNA clone which is different from ours was also identified (108). All three cDNA clones showed very high homology in the nucleotide and amino acid sequences (see chapter 2). Northern hybridization showed that only poly(A) RNA from the ligninolytic cultures hybridized with cloned cDNA probes, indicating that the regulation of ligninolytic activity may be at the mRNA level (see chapter 1). Using two cDNA clones as probes, six lignin peroxidase genes have been isolated and characterized and one of them was sequenced (see Chapter 3). The sequence data from the genomic clone showed that this lignin peroxidase gene has typical promoter sequence TATATAA often found in higher eukaryotic genes and contains nine introns. These introns are relatively small (50-62 bp) in comparison with those from higher eukaryotic genes (see Chapter 3).

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# CHAPTER ONE

## CHARACTERIZATION OF LIGNIN PEROXIDASE cDNA CLONES FROM *PHANEROCHAETE CHRYSOSPORIUM*

## ABSTRACT

Characterization of two different classes of lignin peroxidase cDNA clones, pCLG4 and pCLG5, from Phanerochaete chrysosporium is described. The pCLG5 group consists of only one cDNA clone (pCLG5) whereas the pCLG4 group consists of two other clones, pCLG3 and pCLG6. Three oligodeoxynucleotide probes, that correspond to amino acid sequences of tryptic peptides of lignin peroxidase H8, a major component of the multiple lignin peroxidases in P. chrysosporium, showed hybridization with pCLG5 whereas only one of these probes hybridized with the members of the pCLG4 group. Northern hybridization analyses showed that the poly(A) RNA corresponding to the above cDNA clones was detectable in 6-day-old idiophasic cultures grown in low nitrogen medium (i.e. lignin-degrading cultures), but not in 2-day-old primary growth cultures grown in the same medium (i.e. non-ligninolytic cultures). The expression of the lignin peroxidase protein, based on an enzyme-linked immunoblot assay using lignin peroxidase antibody, was obtained with the cDNA insert (named CLG5) in clone pCLG5. These results suggest that CLG5 encodes lignin peroxidase H8. Southern hybridization analyses of restriction enzyme digested total genomic DNA of P. chrysosporium, utilizing labeled cDNA inserts of pCLG4 and pCLG5 as probes, suggested the presence of a lignin peroxidase gene family in this fungus.

# INTRODUCTION

Lignin peroxidase, an extracellular, $H_2O_2$-dependent, glycosylated, heme protein has recently been purified and characterized from a filamentous white-rot fungus P. chrysosporium (6,7,27,28). Lignin peroxidase is elaborated by the fungus under nitrogen-limited conditions during secondary metabolism but not during primary growth (5,16). The enzyme catalyzes oxidative cleavage of a variety of alkyl side chains of lignin-related compounds including $C_\alpha$-$C_\beta$ cleavage of the propyl side chains, a major reaction in fungal depolymerization of lignin (1,7,10,12,28). Lignin peroxidase activity is conveniently measured as veratryl alcohol oxidation to veratraldehyde (28). Kirk et al. (16) recently reported that P. chrysosporium elaborates at least six proteins (H1, H2, H6, H7, H8 and H10) with lignin peroxidase activity. H2 and H8 constitute the major lignin peroxidases both in shaken and static cultures of P. chrysosporium (14,16). Antibody raised against lignin peroxidase H8 cross reacts with the other five lignin peroxidases demonstrating at least partial homology among the lignin peroxidases; however, some differences in peptides produced upon protease digestion have been noted (16). Renganathan et al. (24) independently described three different molecular forms of lignin peroxidases, all of which are glycosylated and range in $M_r$ from 39,000 to 43,000. These results suggest one or both of the following: 1) one gene may code for one lignin peroxidase protein; or 2) product(s) of a single (or multiple) lignin peroxidase gene(s) undergo various post-transcriptional and/or post-translational modifications.

Lignin peroxidases are important enzymes with many potential practical

applications (4,13). These include upgrading lignocellulosic materials, via delignification, for the efficient production of feeds, fuels and chemicals; biobleaching of pulps; increasing efficiency of wood pulping; treatment of industrial wastes; controlled modification of lignins to produce aromatic chemicals; cracking of petroleum; and detoxification of dangerous and recalcitrant environmental pollutants such as dioxins, polybrominated biphenyls, DDT and benzopyrenes (3,9,25). To obtain a better understanding of the nature, organization, expression and regulation of the lignin peroxidase genes and to develop the full bioprocessing potential of these enzymes, I initiated studies to clone and characterize cDNA sequences encoding lignin peroxidase.

Four putative lignin peroxidase cDNA clones have been isolated and a preliminary report was published (34). Detailed characterization of these clones is presented in this chapter.

## MATERIALS AND METHODS

### Isolation of Total RNA and Poly(A) RNA

P. chrysosporium strain BKM-F 1767 (ATCC 24725) was maintained and grown as previously described (15,17) in 50 ml of low nitrogen medium (modified to contain 20 mM NaOAc, pH 4.5, instead of 10 mM 2,2-dimethyl succinate) in 500 ml Erlenmeyer flasks. Flasks were flushed with pure oxygen at the time of inoculation and reflushed every other day. A modified hot phenol extraction procedure was used for RNA isolation (33). Using this procedure, 10 to 20 mg of total RNA was obtained from 1 liter of culture and poly(A) RNA accounted for 1 to 2% of the total RNA (33).

### Construction of cDNA Library

The first strand cDNA was synthesized using AMV (avian myeloblastosis virus) reverse transcriptase and the second strand cDNA was synthesized utilizing RNase H and DNA polymerase I of E. coli (8). Double stranded cDNA was dC-tailed and then annealed with dG-tailed pUC9 (30) which was digested with restriction enzyme PstI. The annealed DNA molecules were transformed into E. coli JM83 (ara lac-pro strA thi $\phi$80dlacZ$\Delta$M15; 30) using the procedure of Hanahan (11). The transformed cells were spread on 2YT plates (1.6% Bacto-tryptone, 1% yeast extract, 0.5% NaCl and 1.5% agar, pH 7.0) supplemented with 100 $\mu$g/ml ampicillin and 40 $\mu$g/ml X-gal (5-bromo-4-chloro-3-indolyl-$\beta$-D-galactoside; 20). Ten thousand white E. coli colonies were picked and individually stored in each well of 96-well microtiter plates.

Differential Hybridization

Lignin peroxidase has been shown to be produced in 6-day-old idiophasic culture of P. chrysosporium grown in low-nitrogen medium; the enzyme was not detectable in 1- or 2-day-old cultures in primary growth (5). Therefore, the differential hybridization technique allows isolation of the cDNA clones specific for the idiophase. It was much easier to screen such a mini-library of idiophasic clones than to screen the total cDNA library for isolating the cloned cDNA of interest. The synthesis of 2- and 6-day cDNA probes from the corresponding poly(A) RNA (2), the preparation of cDNA blots and the cDNA hybridization were previously described (33). Of the 10,000 cDNA clones in the cDNA library, 850 clones were shown to be specific to the 6-day cDNA probe by using this differential hybridization technique.

Hybridization with Oligodeoxynucleotide Probes

The preparations of blots from the 6-day cDNA mini-library and end-labeled oligodeoxynucleotide probes, and the hybridization were carried

out as previously described (33).

Northern Hybridization

The 2-day and 6-day poly(A) RNA prepared as described above were treated with glyoxal (19) and electrophoresed on a 1.2% agarose gel. The RNA was then transferred from agarose gel to nitrocellulose paper (19) and hybridized with nick-translated probes prepared from the lignin peroxidase cDNA clones.

Immunoblotting Assay

The cell extract of each potential cDNA clone was made, diluted with TBS buffer (10 mM Tris.HCl pH 8.0, 150 mM NaCl) and blotted onto nitrocellulose filter paper by the use of Minifold equipment (Schleicher and Schuell, Keene, NH). The filter paper was treated with blocking solution (1% BSA in TBST, which contains TBS plus 0.05% Tween 20) for 30 min and the antibody prepared against lignin peroxidase H8 (1:200 in TBST) was bound to the antigen for 1 h. The filter was washed completely with TBST and then incubated with the anti-IgG alkaline phosphatase conjugate (Promega Biotech, Madison, WI; 1:7,500 in TBST) for 1 h. The filter was then washed with TBST, and was incubated for 15 min in a solution containing 66 $\mu$l nitroblue tetrazolium and 33 $\mu$l 5-bromo-4-chloro-3-indolyl phosphate in 10 ml of alkaline phosphatase buffer (100 mM Tris.HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$). Protein concentration was determined as described by Lowry et al. (18).

Southern Hybridization

P. chrysosporium genomic DNA was isolated as previously described by Rao and Reddy (23) except that incubation with lysis buffer was at room temperature rather than at 65$^{o}$C, and phenol extraction was done only once.

The genomic DNA in TE buffer was digested with different restriction

enzymes (19), the fragments were separated by electrophoresis on a 0.7% agarose gel, and were then transferred onto nitrocellulose paper (19). The cDNA inserts of pCLG4 and pCLG5 were isolated using low melting agarose gel procedure (22), nick-translated (19), and were hybridized with nitrocellulose filter paper containing genomic DNA.

## RESULTS

### Identification of lignin peroxidase cDNA clones

Three oligodeoxynucleotide probes were synthesized as previously described (34) on the basis of two amino acid sequences of selected tryptic peptides. The sequences of the tryptic peptides of lignin peroxidase H8 and those of the corresponding synthetic oligodeoxynucleotide probes used in identifying the cDNA clones are as follows:

| | |
|---|---|
| **Leu-Gln-Lys-Pro-Phe-Val-Gln-Lys** | **Peptide 14** |
| **QUN-CAP-AAP-CCN-UUQ-GUN-CAP-AAP** | **Corresponding mRNA** |
| **GTQ-TTQ-GGN-AAP-CA** | **Probe 14.1** |
| **AAP-CAN-GTQ-TTQ** | **Probe 14.2** |
| **Leu-Val-Phe-His-Asp-Ala** | **Peptide 25** |
| **QUN-GUN-UUQ-CAQ-GAQ-GCN** | **Corresponding mRNA** |
| **CAN-AAP-GTP-CTP-CG** | **Probe 25** |

N = AGCT/U, P = AG, Q = CT/U

Four lignin peroxidase cDNA clones (named pCLG3, pCLG4, pCLG5 and pCLG6) were identified by screening 850 idiophase-specific clones from the cDNA library with probe 14.1 and preliminary restriction analyses showed that pCLG3, pCLG4 and pCLG6 were similar to one another, but pCLG5 was different from the others (34).

To confirm that the four clones contain lignin peroxidase cDNA, recombinant plasmids isolated from these clones were digested with different restriction enzymes and were hybridized with the three oligodeoxynucleotide probes (14.1, 14.2 and 25 shown above). The results (Fig. 1) showed that all three probes hybridized with the cDNA insert in pCLG5 whereas only probe 14.1 hybridized with the cDNA inserts from pCLG3, pCLG4 and pCLG6. Each of the three probes hybridized with only one fragment from a given clone, indicating that the probe is specific for the sequence of the particular cDNA fragment showing the homology. Furthermore, the larger cDNA fragment of pCLG5 hybridized with both probes 14.1 and 14.2, which have overlapping sequences, whereas the smaller cDNA fragment hybridized only with probe 25 (Fig. 1, lane 3 in each panel). These results suggested that these four clones contain lignin peroxidase cDNA sequences and that the pCLG5 cDNA is different from the cDNA inserts in the other three clones and encodes the major lignin peroxidase H8. To prove this point further, recombinant plasmid DNA from each of the four clones was probed with $^{32}$P-labeled cDNA inserts of pCLG4 and pCLG5 individually. The results ( Fig. 2) showed that the pCLG4 cDNA probe did not show detectable hybridization with the cDNA insert from pCLG5, but showed strong hybridization with that from pCLG3, pCLG4 and pCLG6, respectively. Conversely, the pCLG5 cDNA did not show hybridization with that from the other three clones.

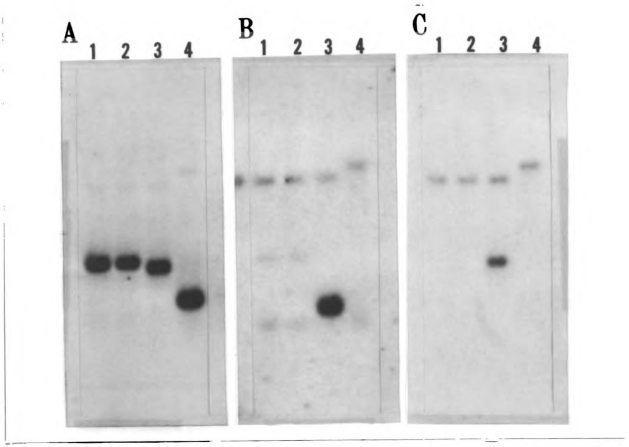


**Figure 1.** Hybridization of lignin peroxidase cDNA clones with three synthetic oligonucleotide probes. Clones pCLG3 and pCLG4 were digested with BamHI and HindIII, pCLG5 was digested with BamHI, HindIII and PstI, and pCLG6 was digested with HindIII and PstI. Southern hybridization of these restriction digests with the three oligonucleotide probes 14.1 (panel A), 25 (panel B) and 14.2 (panel C) is presented. Different lanes contained: pCLG3 (lane 1), pCLG4 (lane 2), pCLG5 (lane 3) and pCLG6 (lane 4).

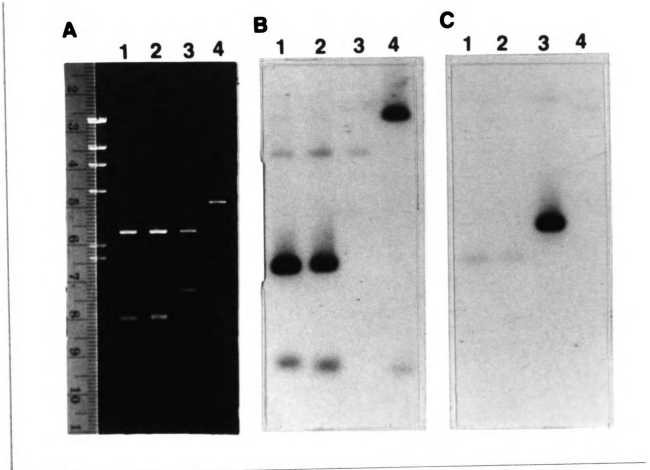


**Figure 2.** Cross-hybridization between the two groups of cDNA clones. All the four cDNA clones were digested with BamHI and HindIII, fractionated on 1% agarose gel (Panel A), transferred onto nitrocellulose filter paper and hybridized with probe made from the pCLG4 cDNA insert (Panel B). After exposure to the X-ray film, the pCLG4 probe was removed and the filter was hybridized with the second probe made from the pCLG5 cDNA insert (Panel C). Lanes 1-4 in each panel contained pCLG3, pCLG4, pCLG5 and pCLG6, respectively. The size marker, HindIII-digested lambda DNA, is shown in Panel A at the extreme left.

Restriction Mapping

Detailed restriction maps of the cDNA inserts of pCLG4 and pCLG5 (Fig. 3) clearly show that these cDNA inserts are different from each other. The restriction maps of the cDNA inserts in clones pCLG3 and pCLG6 were identical to that of pCLG4. The estimated sizes of the cDNA inserts in pCLG3, pCLG4, pCLG5 and pCLG6 were 1.35 kb, 1.42 kb, 1.48 kb and 1.17 kb, respectively.

To delineate which cDNA fragment is homologous to the synthetic oligodeoxynucleotide probe 14.1 or 25, pCLG4 and pCLG5 were digested with appropriate restriction enzymes (based on the restriction maps and hybridization results), and the DNA blots were hybridized with the synthetic probes. The results showed that the HindIII-AvaI fragment of pCLG4 cDNA insert and PstI-EcoRI fragment of pCLG5 cDNA insert hybridized to probe 14.1 and the PstI-SstII fragment of pCLG5 hybridized to probe 25 (Fig. 3).

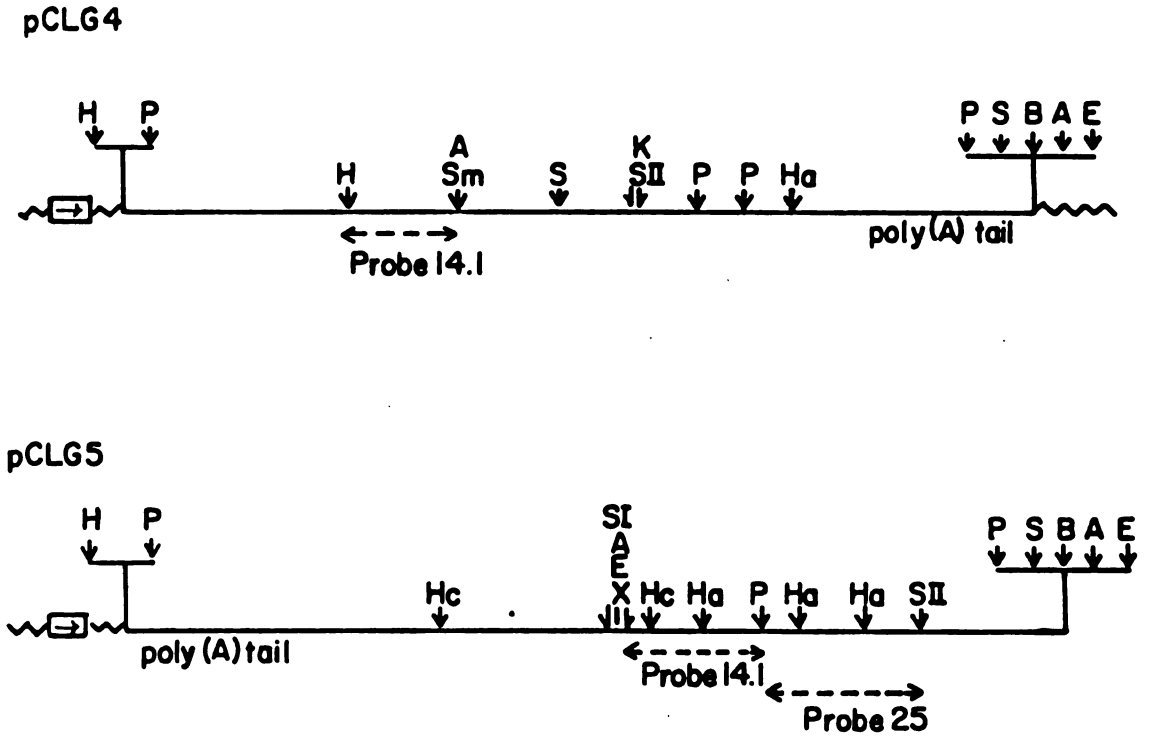


**Figure 3.** Restriction maps of the cDNA inserts in pCLG4 and pCLG5. The wavy lines represent vector sequences and the straight lines represent cDNA inserts. The boxes and arrows represent the promoter of the LacZ gene of E. coli and direction of transcription, respectively. Abbreviations used for the restriction enzymes shown in the figure are: A-AvaI, B-BamHI, E-EcoRI, H-HindIII, Ha-HaeII, Hc-HincII, K-KpnI, P-PstI, S-SalI, SI-SstI, SII-SstII, Sm-SmaI and X-XhoI.

Northern Hybridization Analysis

Previous studies showed that high levels of lignin peroxidase were produced in low nitrogen medium on the 6th day of incubation (i.e. during secondary metabolism), whereas little or no lignin peroxidase was produced on the second day of growth (i.e. during primary growth) in the same medium (5). Therefore, mRNA corresponding to the isolated lignin peroxidase cDNA should not be detectable in 2-day-old P. chrysosporium cultures grown in low nitrogen medium but should be present in otherwise identical 6-day-old cultures. To test this, poly(A) RNA isolated from 2- and 6-day-old cultures of P. chrysosporium grown in low nitrogen medium was probed with the four $^{32}$P-labeled lignin peroxidase cDNAs. Another cDNA clone (p33-B10), which was shown to hybridize with the cDNA probes made from both 2- and 6-day poly(A) RNA in the differential hybridization experiment (see Methods and Materials) was used as a positive control. The results showed that the four cDNA clones hybridized only with mRNA from the 6-day-old cultures (Fig. 4), but not with that from 2-day-old cultures, indicating that lignin peroxidase synthesis is controlled at the level of mRNA production (or perhaps at the level of mRNA stability). The mRNA that hybridized with the plasmid DNA from each clone is very similar in size (~1.6 kb), consistent with the fact that molecular weights of different lignin peroxidases are comparable.

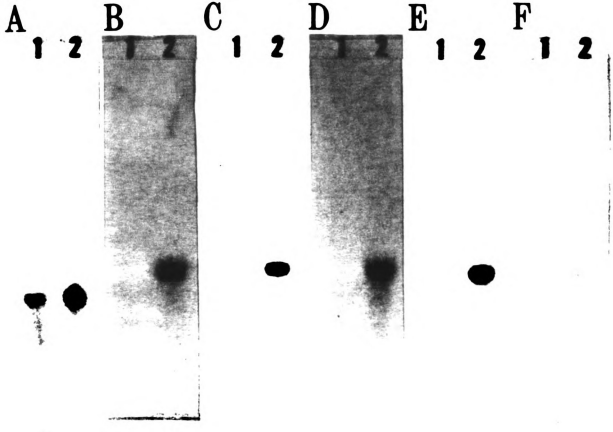


Figure 4. Northern hybridization analysis. Glyoxal-treated 2-day (lanes 1) and 6-day (lanes 2) poly(A) RNA were electrophoresed on a 1.2% agarose gel as described in Materials and Methods. The Northern blots were hybridized with p33-B10 (A), pCLG3 (B), pCLG4 (C), pCLG5 (D), pCLG6 (E), and pUC9 (negative control, F). The cDNA clone p33-B10 was used as a positive control, since it was shown to hybridized with 2-day and 6-day cDNA probes in differential hybridization experiments previously described (33). The 1 kb ladder DNA (BRL, Gaithersburg, MD) was used as the RNA size marker.

Immunoblotting Assay

Since the cDNA was constructed into the E. coli expression vector, pUC9, the possibility existed that lignin peroxidase cDNA might be expressed in E. coli if the insert was in the right orientation and frame. To determine if there is expression of lignin peroxidase in any of the four lignin peroxidase cDNA clones, cell extracts and 50 X concentrated extracellular fluid from each clone were subjected to an enzyme-linked immunoblot assay as described in Materials and Methods. None of the extracts reacted with the lignin peroxidase antibody, indicating that the lignin peroxidase cDNA was not being expressed due to wrong orientation, and/or reading frame or due to some other reasons that are not clear at this time.

To determine the orientation of the cDNA insert in clones pCLG4 and pCLG5, end-labeled oligo$(dT)_{12-18}$ was used to probe the two cDNA clones, which were digested with different restriction enzymes. The results showed that the cDNA insert in pCLG5 was in the opposite orientation to the promoter of the lacZ gene in pUC9, whereas in pCLG4 it was in the correct orientation. To change the orientation of the cDNA fragment in pCLG5, the 1.48 kb BamHI-HindIII cDNA fragment was isolated and recloned into another expression vector pUC18 (21). Extracts of four new pUC18 cDNA clones (pCLG5-1, pCLG5-2, pCLG5-3 and pCLG5-4) were then used in the immunoblotting assay to detect the presence of lignin peroxidase antigen. The results showed that one of the new cDNA clones (pCLG5-1) produced antigen which gave a strong positive reaction with the lignin peroxidase antibody (Fig. 5; row D) whereas the other clones gave weak (pCLG5-2) or no reaction (pCLG5-3 and pCLG5-4). Comparison of the color intensity obtained with different concentrations of cell extract from

the new cDNA clone (pCLG5-1) with that of the purified lignin peroxidase showed that the antigen produced by pCLG5-1 accounted for about 0.1% of the total protein. Identical results were obtained in five separate experiments. The reason for the lack of expression of lignin peroxidase in other clones is not known.

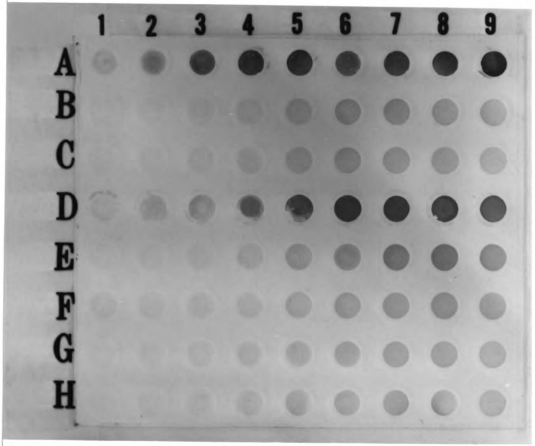


**Figure 5.** Immunoblotting assay for detecting lignin peroxidase production by different cDNA clones. Different lanes contain: purified lignin peroxidase H8 produced by P. chrysosporium (row A); and cell extracts of cDNA clones pUC18 (negative control; row B), pCLG5 (row C), pCLG5-1 to 4, which are the four new clones containing the insert from pCLG5 and pUC18 (21), (rows D to G); and pCLG4 (row H). The purified lignin peroxidase concentrations in each lane of row A are: 1) 0.05 ng; 2) 0.1 ng; 3) 0.2 ng; 4) 0.5 ng; 5) 1 ng; 6) 2 ng; 7) 5 ng; 8) 10 ng; and 9) 20 ng. The protein concentration in cell extracts of different clones was adjusted to contain in each lane: 1) 20 ng; 2) 50 ng; 3) 100 ng; 4) 200 ng; 5) 500 ng; 6) 1,000 ng; 7) 2,000 ng; 8) 5,000 ng; and 9) 10,000 ng.

Southern Hybridization Analysis

The above results lead to the following question: do the poly(A) RNA species, which hybridized with the lignin peroxidase cDNA, represent a single transcript with post-transcriptional modification from one gene or transcripts from different genes? In other words, do the cDNA inserts in pCLG4 and pCLG5 hybridize to different or identical segments of chromosomal DNA? To answer this question, the genomic DNA of P. chrysosporium BKM-F 1767 was isolated and digested with different restriction enzymes, some of which have sites in the cDNA inserts of the clones of interest and some of which have no sites in the inserts. The Southern hybridization of chromosomal DNA of P. chrysosporium with $^{32}$P-labeled cDNA inserts of pCLG4 and pCLG5 showed that the cDNA inserts of pCLG4 and pCLG5 represent different lignin peroxidase genes (Fig. 6).

The Southern hybridization pattern observed with pCLG4 cDNA was consistent with the restriction map of the latter except for the XbaI and XhoI digestion (Fig. 6, panel A), which do not have a site in the pCLG4 cDNA (Fig. 3). It is possible that the gene corresponding to pCLG4 cDNA may have introns or the gene sequences on homologous chromosomes may be different somewhat. The most recent results (see Chapter 3) show that there is an XbaI site in one of the introns of a lignin peroxidase gene that showed strong hybridization with pCLG4 and had many similarities to the restriction map of pCLG4. In contrast to these results, the Southern hybridization patterns shown by the different restriction digests of the chromosomal DNA with the pCLG5 cDNA probe were different from what one would have expected from the bands on the restriction map of pCLG5 (Fig. 6, panel B). For example, there is no BamHI site in the pCLG5 insert, but the BamHI-digested chromosomal DNA

showed four hybridization bands with different intensities. These bands may be due to different lignin peroxidase genes which are homologous to the pCLG5 cDNA insert, or to the existence of introns in the lignin peroxidase gene, or to restriction polymorphism on the two homologs. In addition, there were several weak hybridization bands in each lane (Fig. 6, panel B).

Restriction digests of genomic DNA of P. chrysosporium strain ME 446 (another commonly used strain in lignin biodegradation studies) were also probed as described above. Both pCLG4 and pCLG5 cDNA inserts hybridized to distinct fragments of ME 446 genomic DNA, although once again hybridization patterns observed with the two probes were different, suggesting that pCLG5 and pCLG4 indeed represent different lignin peroxidase genes (Fig. 7). Furthermore, the patterns of hybridization obtained with ME 446 genomic DNA (Fig. 7) were different from those observed with strain BKM-F 1767 (Fig. 6), indicating that there are differences between the genomic DNA of different strains of P. chrysosporium. It is also of interest that ME 446 appears to contain at least two extrachromosomal DNA elements that hybridize strongly with the pCLG4 cDNA insert (Fig. 7, lane 1, panel A). These elements also hybridized strongly with pBR322 (data not shown). These plasmid-like elements are being studied further.

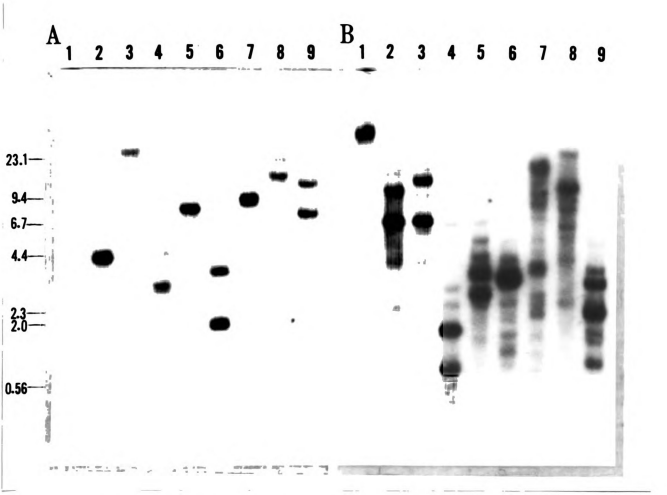


**Figure 6.** Southern hybridization analysis of strain BKM-F 1767. The genomic DNA isolated from 2-day-old cultures of P. chrysosporium BKM-F 1767 was digested with BamHI (lanes A2 and B2), EcoRI (lanes A3 and B3), PstI (lanes A4 and B4), PvuII (lanes A5 and B5), SalI (lanes A6 and B6), SmaI (lanes A7 and B7), XbaI (lanes A8 and B8) and XhoI (lanes A9 and B9), and electrophoresed on a 0.7% agarose gel. The fractionated genomic DNA was transferred onto nitrocellulose paper and probed with the cDNA insert of pCLG4 (panel A). After exposure of the blot to the X-ray film, the probe was washed off and the blot was rehybridized with pCLG5 cDNA insert (panel B). Lanes A1 and B1 contained uncut genomic DNA. The size marker used was HindIII-digested lambda DNA.

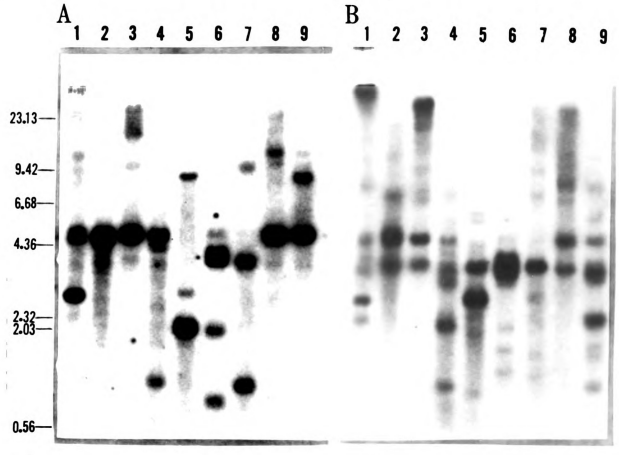


Figure 7. Southern hybridization analysis of strain ME 446. The genomic DNA isolated from 2-day-old cultures of P. chrysosporium ME 446 was digested with BamHI (lanes A2 and B2), EcoRI (lanes A3 and B3), PstI (lanes A4 and B4), PvuII (lanes A5 and B5), SalI (lanes A6 and B6), SmaI (lanes A7 and B7), XbaI (lanes A8 and B8) and XhoI (lanes A9 and B9), and electrophoresed on a 0.7% agarose gel. The fractionated genomic DNA was transferred onto nitrocellulose paper and probed with the cDNA insert of pCLG4 (panel A). After exposure of the blot to the X-ray film, the probe was washed off and the blot was rehybridized with pCLG5 cDNA insert (panel B). Lanes A1 and B1 contained uncut genomic DNA. The size marker used was HindIII-digested lambda DNA.

# DISCUSSION

The results of this study strongly suggest that pCLG5 is a lignin peroxidase cDNA clone and contains the sequence coding for lignin peroxidase H8 based on the following lines of evidence. 1) The pCLG5 cDNA insert showed hybridization with three synthetic oligodeoxynucleotide probes, the sequences of which were deduced from the amino acid sequences of selected tryptic peptides of the lignin peroxidase. Each of the probes used represents a mixture of 32 oligodeoxynucleotides, only one of which has perfect homology with unique sequence of the cloned cDNA; the other oligodeoxynucleotides in the mixture have at least one base mismatched with the cloned cDNA. Under high strigent hybridization conditions, only the oligodeoxynucleotide with perfect homology can hybridize with the lignin peroxidase cDNA. In the Southern hybridization experiments, the washing temperature employed was based on the equation presented by Suggs et al. (26). According to this equation, the estimated $T_d$, which is the temperature at which one-half of the duplexes are dissociated under the experimental conditions, is 36 to 44°C, 38 to 46°C and 28 to 36°C for probes 14.1, 25 and 14.2, respectively. The actual washing temperature used in the experiments was 42°C for probes 14.1 and 25, and 32°C for probe 14.2. Under these conditions, nonspecific hybridization could not exist because the duplexes with one mismatched basepair dissociate at a temperature about 10°C lower than the perfectly matched duplexes (26,31,32). Thus, the hybridization data, using the synthetic probes, strongly support the identification of the cDNA clones. 2) Northern hybridization experiments showed that the cDNA from this clone hybridized only with

the 6-day poly(A) RNA from ligninolytic cultures of P. chrysosporium grown in low nitrogen medium but not with the poly(A) RNA from 2-day cultures (i.e. prior to the onset of lignin degradation) grown in the same medium. This is consistent with previous observations that lignin peroxidase is produced only during secondary metabolism (14,16). This result also suggests that the lignin peroxidase gene is regulated at the level of mRNA production although regulation at the level of mRNA stability cannot be ruled out. 3) The immunoblotting data showed that the product of the cDNA insert in pCLG5 gave a strong reaction with the antibody raised against lignin peroxidase H8 purified from P. chrysosporium BKM-F 1767. 4) The complete nucleotide sequence of pCLG5 cDNA insert has now been determined and the amino acid sequence of the corresponding lignin peroxidase protein (designated LG5) has been deduced (see Chapter 2). Mature lignin peroxidase LG5 contains 344 amino acid residues and is preceded by a leader sequence containing 27 amino acid residues. Amino acid sequences of two tryptic peptides of lignin peroxidase H8 have exactly matching sequences in LG5. The experimentally determined N-terminal sequence of Ala-Thr-Cys-Ser-Asn-Gly-Lys-Val-Val-Pro is found in the deduced N-terminal amino acid sequence of mature LG5 (Dass and Reddy, unpublished data). These results indicate that pCLG5 cDNA encodes lignin peroxidase H8.

On the basis of the hybridization with three synthetic oligodeoxynucleotide probes, restriction analysis, cross-hybridization and chromosomal DNA hybridization, clone pCLG4 represents a second group of lignin peroxidase cDNA clones consisting of pCLG3, pCLG4 and pCLG6. The cDNA inserts of this group of clones strongly hybridized with the synthetic oligodeoxynucleotide probe 14.1, and hybridized only with the 6-day poly(A) RNA from ligninolytic culture of P. chrysosporium grown in

low nitrogen medium, suggesting that pCLG4 may also contain the sequence coding for lignin peroxidase. Nucleotide sequence of pCLG4 cDNA has recently been determined and the amino acid sequence of the corresponding lignin peroxidase protein (designated LG4) has been deduced (Chapter 2). Mature LG4 also contains 344 amino acid residues with an $M_r$ of 36,540, and is preceded by a leader sequence of 28 amino acid residues. The sequences of probes 14.1 and 25, except for one base pair mismatch, are found in pCLG4 cDNA. The experimentally determined N-terminal sequence of Val-Ala-Cys-Pro-Asp-Gly-Val-His-Thr-Ala-Ser-Asn found in lignin peroxidase H2 exactly matches the N-terminal amino acid sequence of mature LG4 (Dass and Reddy, unpublished data). Furthermore, pCLG4 cDNA has a high degree of homology (71.5%) to that of pCLG5 cDNA. These data indicate that pCLG4 cDNA encodes another lignin peroxidase protein.

The hybridization of chromosomal DNA with pCLG4 and pCLG5 definitely shows that multiple lignin peroxidase genes are present in P. chrysosporium and the cDNA inserts in these two clones are from different lignin peroxidase genes (Fig. 6, lane 2 in panel B). The genomic DNA, in addition to the one lignin peroxidase gene corresponding to pCLG5, appears to contain several lignin peroxidase genes whose sequences are homologous to pCLG5 cDNA sequence since different restriction digests of chromosomal DNA showed several bands of hybridization of variable intensities with $^{32}$P-labeled pCLG5 cDNA. This is best illustrated by the hybridization of BamHI-digested genomic DNA with pCLG5 cDNA.

Tien and Tu recently reported on the isolation of another lignin peroxidase cDNA clone (λML1) from a λ-cDNA library of P. chrysosporium (29). In agreement with the results of this study, Tien and Tu concluded

that lignin peroxidase gene expression is regulated at the level of mRNA production. The λML1 cDNA, comparable to pCLG4 and pCLG5 cDNA, encodes a mature lignin peroxidase that contains 345 amino acids and a leader sequence that contains 28 amino acids, which are predominantly hydrophobic. Tien and Tu (29) apparently were able to isolate yet another lignin peroxidase cDNA clone from P. chrysosporium. These data offer further support to our idea that P. chrysosporium contains multiple lignin peroxidase genes.

## ACKNOWLEDGMENTS

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# CHAPTER TWO

## ANALYSIS OF NUCLEOTIDE SEQUENCES OF TWO LIGNIN PEROXIDASE cDNAs FROM *PHANEROCHAETE CHRYSOSPORIUM*

## ABSTRACT

The complete nucleotide sequences of two types of lignin peroxidase cDNAs isolated from the white-rot fungus Phanerochaete chrysosporium, designated CLG4 and CLG5, are presented here. The amino acid sequences of the corresponding lignin peroxidase proteins, designated LG4 and LG5, respectively, have been deduced from the cDNA sequences. Mature lignin peroxidases LG4 and LG5 are preceded by leader sequences containing 28 and 27 amino acids, respectively, and each contains 344 amino acid residues. The estimated molecular weights of mature LG4 and LG5 are 36,540 and 36,607, respectively. Potential N-glycosylation site(s) with the general sequence Asn-X-Thr/Ser are found in both LG4 and LG5. Nucleotide sequence homology between the coding region of CLG4 and CLG5 is 71.5% whereas the amino acid sequence homology between the two lignin peroxidases is 75%. The codon usage of lignin peroxidases is extremely biased in favor of codons rich in cytosine and guanine. Amino acid sequences of two tryptic peptides of lignin peroxidase H8 have exactly matching sequences in lignin peroxidase LG5. Also, the sequences of the oligonucleotide probes, which correspond to the sequences in the tryptic peptides of lignin peroxidase H8 and which were used in isolating the lignin peroxidase clones from the cDNA library, have exactly matching sequences in CLG5. The experimentally determined N-terminal sequences of purified lignin peroxidases H8 and H2 are found in the deduced N-terminal amino acid sequences of mature LG5 and LG4, respectively. These results indicate that CLG5 encodes lignin peroxidase H8 and that CLG4 encodes lignin peroxidase H2.

# INTRODUCTION

Lignin is a major component of woody plants and is the most abundant and widely distributed renewable aromatic polymer on earth (20,23). The wood-decaying basidiomycete, Phanerochaete chrysosporium degrades lignin more rapidly and extensively than most other organisms (12,20). Lignin degradation by this organism is a secondary metabolic event that is triggered in response to nitrogen, carbon, or sulfur starvation (12,20). P. chrysosporiun produces multiple extracellular, glycosylated heme peroxidases named lignin peroxidases that oxidatively cleave lignin and related compounds in an $H_2O_2$-dependent reaction (8,9,13,21,24). It has been suggested that lignin peroxidases also detoxify recalcitrant xenobiotics such as dioxins, DDT and polychlorinated biphenyls (4,6). Lignin peroxidase activity is demonstrable in 6-day-old idiophasic cultures grown in low nitrogen medium but not in 2-day-old nonligninolytic cultures grown in the same medium (7,13). At least six different lignin peroxidases (H1, H2, H6, H7, H8 and H10) have been identified in the extracellular fluid of P. chrysosporium (13). Proteins H2 and H8 constitute the major lignin peroxidases both in static and shaken cultures of this organism (13). The reported molecular weights of lignin peroxidases vary from 39,000 to 43,000 (9,21,24).

A cDNA library has recently been constructed in the PstI site of E. coli vector pUC9 (29), representing 6-day-old lignin-degrading culture of P. chrysosporium. Two different types of lignin peroxidase cDNA clones, pCLG4 and pCLG5, have been identified by probing the cDNA library with a 14-nucleotide-long oligonucleotide probe that corresponds to the sequence of a tryptic peptide of lignin peroxidase H8. Clone

pCLG5, but not pCLG4, also hybridized to probe 14.2 (which has partial overlap with probe 14.1) and probe 25, that corresponds to a sequence in another segment of lignin peroxidase H8 (see Chapter 1). Northern blot analyses showed that the cDNA clones hybridized with the poly(A) RNA extracted from a 6-day-old ligninolytic culture but not with that from a 2-day-old non-ligninolytic culture, suggesting that lignin peroxidase production is regulated at the level of transcription. The two types of clones exhibit little cross-hybridization to each other and have different restriction maps. Furthermore, Southern blots of chromosomal DNA probed with the two types of cDNAs gave very different hybridization patterns. These results suggested that the cDNA inserts in pCLG4 and pCLG5 represent different lignin peroxidase genes and that a family of lignin peroxidase genes is present in P. chrysosporium. In this chapter, the complete nucleotide sequences of these two lignin peroxidase cDNA clones and the predicted amino acid sequences are presented.

## MATERIALS AND METHODS

### cDNA sequencing

Lignin peroxidase cDNA clones pCLG4 and pCLG5 were isolated from the cDNA library [cloned into the PstI site of E. coli vector pUC9 (26)], representing poly(A) RNA from a 6-day-old lignin degrading culture of P. chrysosporium strain BKM-F1767 (ATCC 24725), as previously described (29). The lignin peroxidase cDNAs in clones pCLG4 and pCLG5 are designated CLG4 and CLG5, respectively.

The DNA sequence was determined by the dideoxy chain-termination method (22) and the sequencing strategy employed for the two lignin peroxidase cDNAs is shown in Figure 1. Specific fragments were isolated and subcloned in the appropriately digested M13 vectors. The plasmid pCLG5 was cut with PstI-SstI and the resulting fragments (219 bp, 388 bp, and 671 bp) were subcloned in M13mp18 or M13mp19 (18,28). The sequence of the distal parts of the longer clones was determined using several synthetic primers designed on the basis of determined proximal sequences. Based on the preliminary sequence of both clones, several primers were designed and used to sequence the entire complementary strand. The primers bind to pCLG5- and pCLG4-M13 derivatives at intervals of about 200 bases. Thus, sequencing ambiguities in both clones, resulting from compressions due to GC-rich regions, could be resolved by substituting dITP (16) or deoxy-7-deazaguanosine triphosphate in place of dGTP (17).

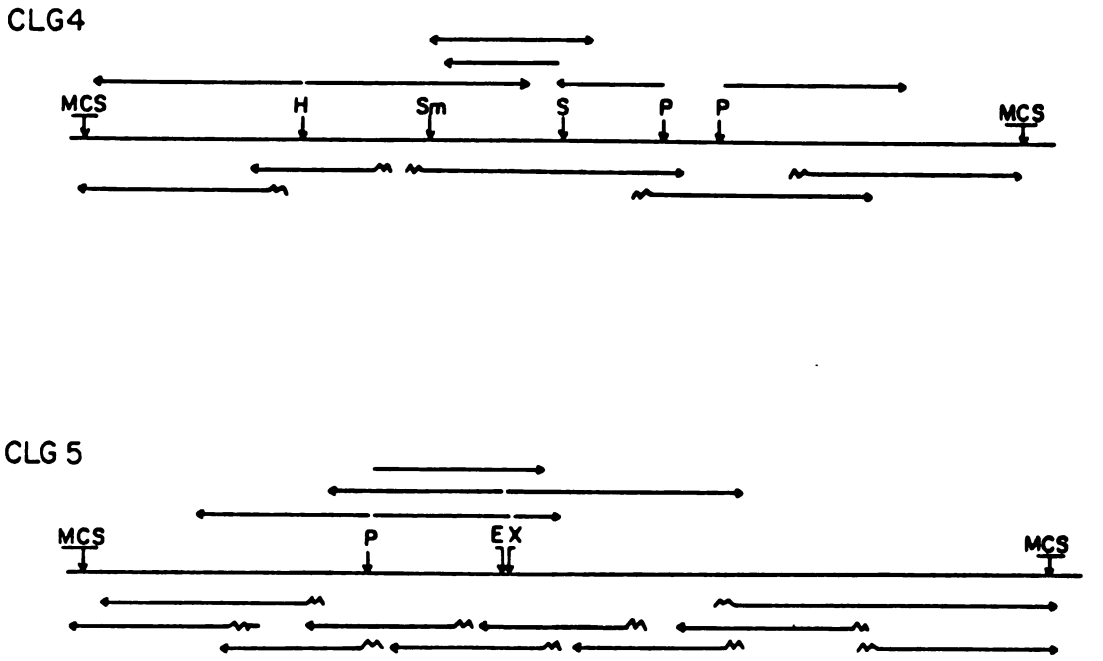


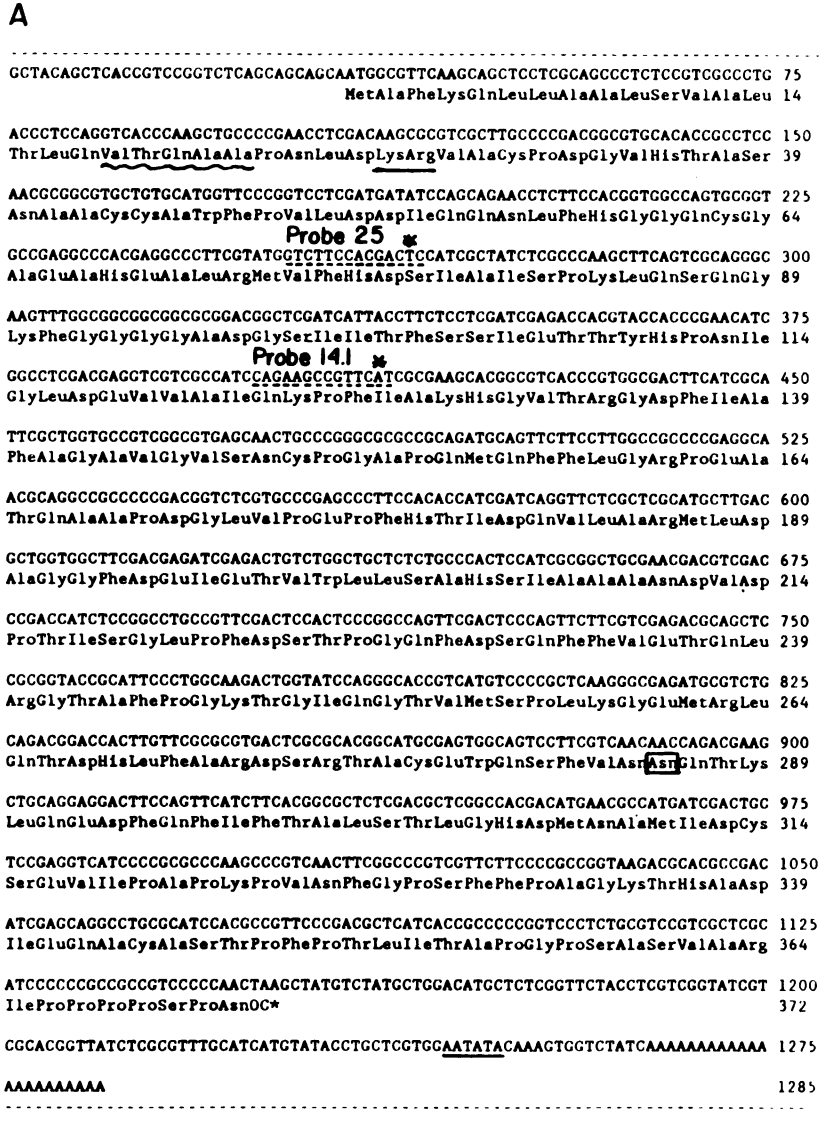

Figure 1. Strategy for determining the sequences of lignin peroxidase cDNAs CLG4 and CLG5. The straight arrows indicate sequencing from restriction sites and the arrows starting with a wavy line indicate sequencing from a synthetic primer. Abbreviations used for the restriction enzymes shown in the figure are: E-EcoRI, H-HindIII, P-PstI, Sm-SmaI, and X-XhoI, MCS, multiple cloning sites.

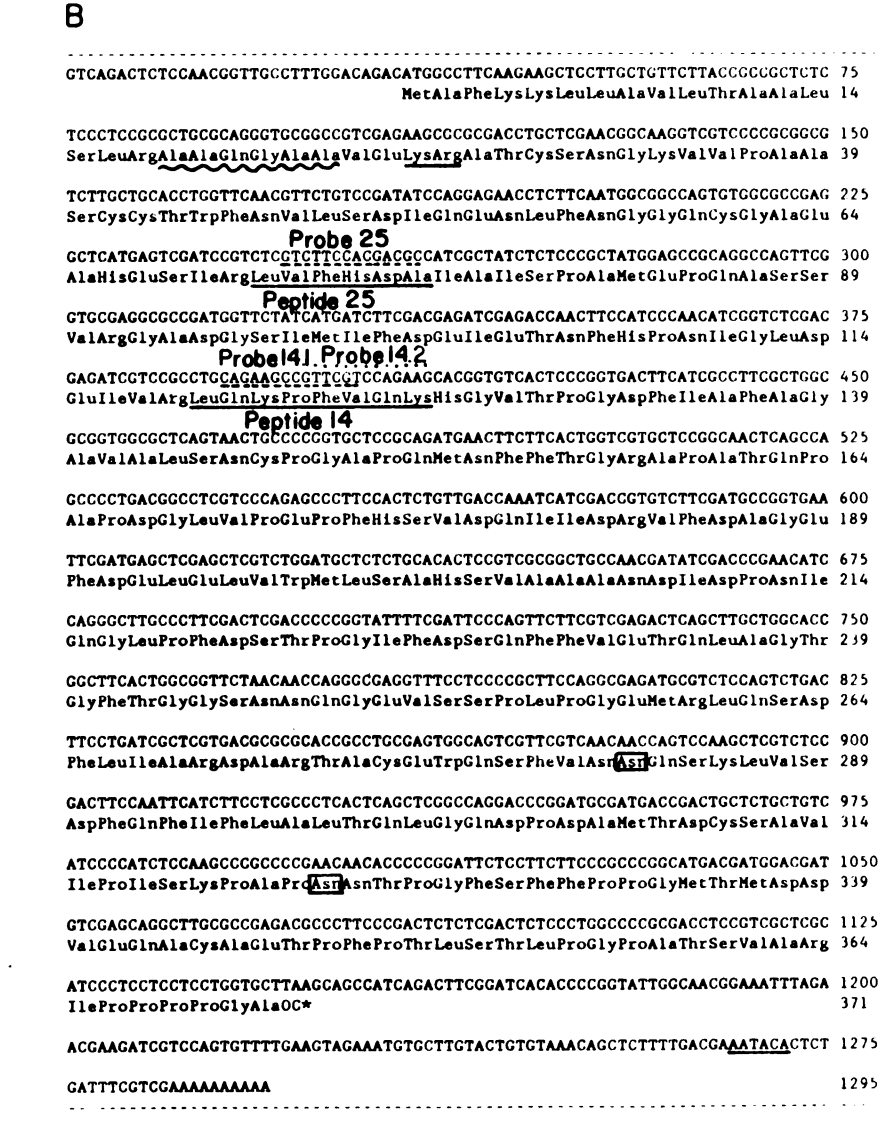
# RESULTS

## Nucleotide Sequences and Deduced Amino Acid Sequences

The complete nucleotide sequences of CLG4 and CLG5 and the predicted amino acid sequences of the gene products (designated LG4 and LG5, respectively) are shown in Fig. 2. CLG4 and CLG5 contain 1263 and 1285 bases, respectively, not including the terminal poly(A) sequences. The coding region of CLG4 is flanked by 33 base pairs (bp) in the 5'-noncoding region and 111 base pairs in the 3'-noncoding regions, whereas CLG5 coding region is flanked by 33 bp and 133 bp in 5'- and 3'-noncoding regions, respectively. Both cDNA sequences contain a relatively high G+C content (60.2% and 65.5% for CLG5 and CLG4, respectively) in the coding region, but a much lower G+C content (44.4% and 48.7% for CLG5 and CLG4, respectively) in the 3'-noncoding regions. The sequence 5'-GACATGG flanking the ATG codon in CLG5 is consistent with the translation initiation sequence of (A/G)NNATGG commonly seen in other eukaryotic genes (1). Genes from filamentous fungi generally have one or more polyadenylation sites. Higher eukaryotic genes possess the polyadenylation signal AATAAA (2) though this is rarely present in yeast genes or genes from filamentous fungi (1). However, the lignin peroxidase cDNA clones from *P. chrysosporium* contain the sequence AATATA in CLG4 and AATACA in CLG5, 14 bp upstream of the poly(A) addition site (Fig. 2).

Figure 2. The complete nucleotide sequences of the lignin peroxidase CLG4 (A) and CLG5 (B). The predicted amino acid sequences which are identical to those of tryptic peptides of lignin peroxidase H8 (29) are underlined (solid lines) while the nucleotide sequences complementary to the oligonucleotide probes 25 and 14.1 are noted with dashed lines. Nucleotide sequence of probe 14.2 is marked with a dotted line on the top. In panel A, the nucleotide sequences of probes 25 and 14.1 are identical to that in pCLG4 except at the positions marked with an asterisk. Wavy line in each panel represents putative signal peptide processing region. The possible glycosylation sites are boxed. The putative polyadenylation sequences near the 3' end are underlined.


A
Probe 25
Probe 14.1


B
Probe 25
Peptide 25
Probe14.1
Probe14.2
Peptide 14

The cDNA sequence of CLG4 contains an open reading frame that codes for a protein of 372 amino acids (M.W. of 39,534) and that of CLG5 codes for a protein containing 371 amino acids (M.W. of 39,417). Alanine is abundant (44 residues, i.e. 12 percent of total amino acid sequences) in both lignin peroxidases. Proline is also abundant in both lignin peroxidases, but tyrosine is absent in LG5. Each lignin peroxidase contains eight cysteine residues. According to the signal hypothesis (11,27), a polypeptide containing 20 to 40 amino acids, that are predominantly hydrophobic, occurs at the amino terminus of a secreted protein. Consistent with this, the amino termini of LG4 and LG5 contain leader sequences (with 28 and 27 predominantly hydrophobic amino acids, respectively), that have the characteristics reported for bacterial and mammalian signal peptides (10,11,19,27). Both signal peptides have a few charged residues at the N-terminus followed by a hydrophobic core and a more polar C-terminal region defining the cleavage site (Fig. 2). A large majority of the eukaryotic signal sequences studied end with alanine or glycine (27). Cleavage of the signal by a presumptive signal peptidase could occur, by analogy with the E. coli system, at one of the several alanine residues in the area around amino acid positions 18-23 in LG5 and 18-22 in LG4. The two basic amino acids, Lys-Arg, found at positions 27-28 in LG4 and 26-27 in LG5 could represent a proteolytic cleavage site, suggesting the existence of a pro-form of lignin peroxidase. A helix breaking residue such as glycine or proline or a large polar residue such as glutamine frequently occurs 4-8 residues before the Lys-Arg cleavage site (27). The presence of proline at positions 20 and 21 is consistent with this. Both mature LG4 and LG5 (i.e. without the leader sequences) contain 344 amino acids with

estimated M.W. of 36,540 and 36,607, respectively. These molecular weights are on the low side of the range of 39,000 to 43,000 reported for lignin peroxidases (21,24). This difference may be accounted for by the glycosylation. In agreement with the fact that lignin peroxidases are glycoproteins, there are two potential N-linked glycosylation sites in LG5 at positions 283 and 323 (Fig. 2B) and one site in LG4 at position 286 (Fig. 2A). Consistent with published results, these glycosylation sites have the general sequence Asn-X-Thr/ser. Besides, mature LG4 and LG5 contain numerous threonine residues (23 and 19, respectively) and serine residues (23 and 26, respectively) which may be potential sites for O-glycosylation.

The sequences of the three synthetic oligonucleotide probes (14.1, 14.2 and 25), which have been used to screen the cDNA library (29), were found in CLG5 (Fig. 2B). Two of these sequences (probes 25 and 14.1) are also found in CLG4, except for one base pair mismatch (Fig. 2A). The amino acid sequences of two tryptic peptides of lignin peroxidase H8 (29) match with the deduced amino acid sequence of CLG5. Most recently we sequenced the first ten residues at the amino terminus of lignin peroxidase H8. This sequence of Ala-Thr-Cys-Ser-Asn-Gly-Lys-Val-Val-Pro is found in the deduced N-terminal amino acid sequence of mature LG5. The first twelve residues at N-terminus of another purified lignin peroxidase H2 from the same fungus have the sequence Val-Ala-Cys-Pro-Asp-Gly-Val-His-Thr-Ala-Ser-Asn which is identical to the N-terminal sequence of the mature LG4.

Hydropathy plots of LG4 and LG5 show a strong hydrophobic N-terminal region, consisting of the leader peptide sequence (Fig. 3). Regions with hydropathy average >1.5 (hydrophobic regions) in various portions of LG4 and LG5 probably represent sequences passing through the interior

of the protein (14). The regions comprising amino acids 221-233 in LG5 and 224-236 in LG4 are strongly hydrophilic, have identical sequences except in one position (see Fig. 4), and contain one proline residue and thus have features of a desirable immunogenic peptide (15) for potentially raising common antibodies against lignin peroxidases. Similarly, another eleven amino acid region, from residues 168 to 178 in LG4 and 165-175 in LG5, has identical sequence, is strongly hydrophilic, and therefore is also likely to be a suitable antigenic peptide for raising lignin peroxidase antisera. A high degree of amino acid sequence homology observed between stretches of CLG4 and CLG5 flanking the above two regions is consistent with this idea.

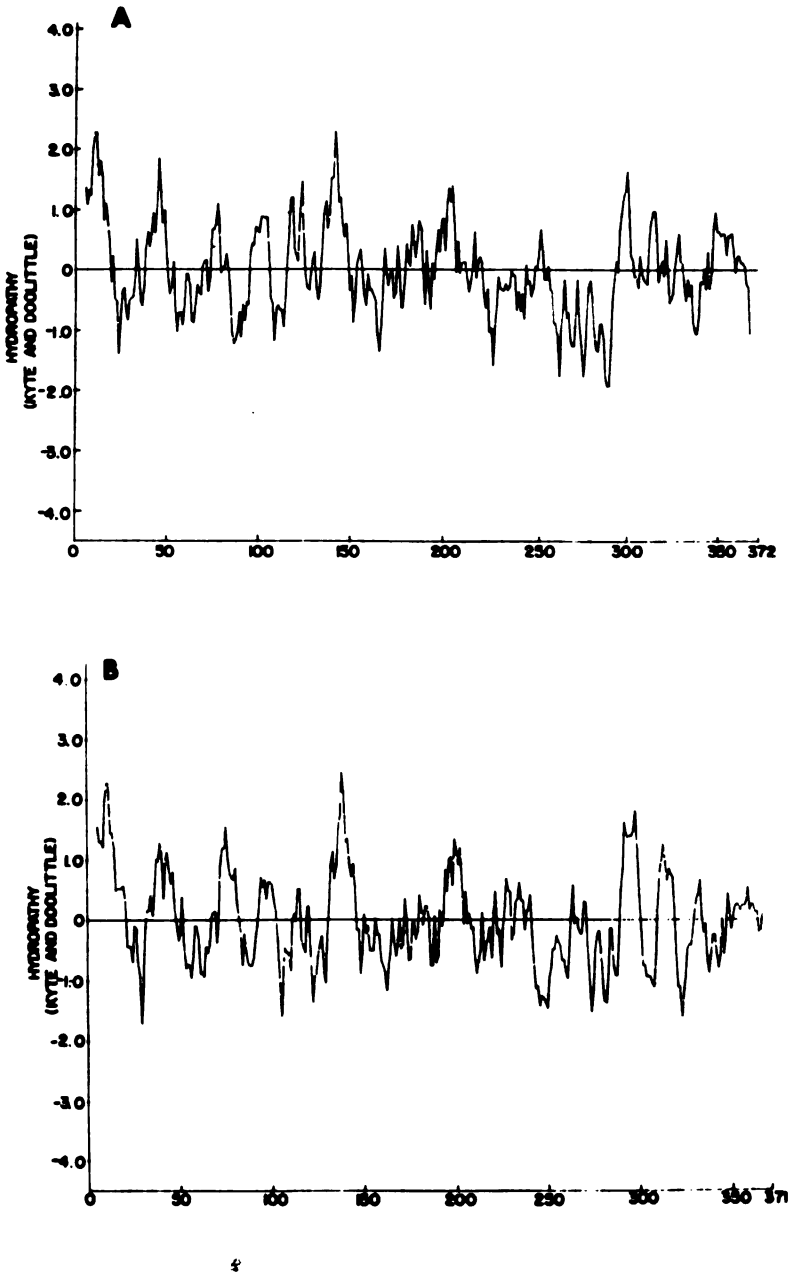



Figure 3. Comparison of the hydropathy plots of two lignin peroxidases LG4 (A) and LG5 (B).

Codon Usage

The codon usage of both lignin peroxidases is extremely biased in favor of codons rich in C and/or G residues (Table 1). The order of preference of the third (wobble) base within each codon family is C > G > T > A in both the lignin peroxidase cDNA clones. This rule is strictly obeyed except for the codons starting with CG and GG (Arg and Gly, respectively). In these two cases CGG and GGG are not used at all. In all codons ending with a pyrimidine, a C-residue is highly preferred, with the exception of AGT (Ser) and GCT (Ala) in LG5. Codons ending in purine preferably use a G- over an A-residue at the third position. Twelve codons, including CGG and GGG, are not used in either lignin peroxidase cDNA. The overall codon usage in CLG4 and CLG5 is not much different from that of the highly expressed genes of another eukaryote, S. cerevisiae (3), in which biased codon usage occurs exclusively in the highly expressed genes. In Neurospora and Aspergillus, there is a tendency to avoid usage of codons ending in A and to prefer using codons ending in pyrimidines (particularly C), especially in highly expressed or constitutive genes (see ref.1). Thus, the highly biased codon useage of CLG4 and CLG5 is likely to reflect their high expression levels as well.

**Table 1. Codon Usage in Lignin Peroxidase cDNAs CLG4 and CLG5**

| AAs | Codons | LG5 | LG4 | AAs | Codons | LG5 | LG4 |
|---|---|---|---|---|---|---|---|
| Phe | TTT | 0 | 1 | Ser | TCT | 8 | 2 |
| | TTC | 28 | 26 | | TCC | 11 | 14 |
| Leu | TTA | 0 | 0 | | TCA | 0 | 0 |
| | TTG | 1 | 1 | | TCG | 6 | 7 |
| | CTT | 4 | 4 | Pro | CCT | 6 | 1 |
| | CTC | 18 | 16 | | CCC | 14 | 15 |
| | CTA | 0 | 0 | | CCA | 3 | 0 |
| | CTG | 3 | 5 | | CCG | 10 | 15 |
| Ile | ATT | 1 | 1 | Thr | ACT | 8 | 3 |
| | ATC | 19 | 20 | | ACC | 10 | 11 |
| | ATA | 0 | 0 | | ACA | 0 | 0 |
| Met | ATG | 9 | 8 | | ACG | 2 | 11 |
| Val | GTT | 4 | 1 | Ala | GCT | 16 | 8 |
| | GTC | 17 | 17 | | GCC | 15 | 17 |
| | GTA | 0 | 0 | | GCA | 2 | 7 |
| | GTG | 2 | 3 | | GCG | 11 | 12 |
| Tyr | TAT | 0 | 0 | Cys | TGT | 1 | 1 |
| | TAC | 1 | 0 | | TGC | 7 | 7 |
| OC | TAA | 1 | 1 | OP | TGA | 0 | 0 |
| AM | TAG | 0 | 0 | Trp | TGG | 3 | 3 |
| His | CAT | 2 | 0 | Arg | CGT | 5 | 4 |
| | CAC | 4 | 11 | | CGC | 5 | 6 |
| Gln | CAA | 2 | 1 | | CGA | 1 | 0 |
| | CAG | 18 | 22 | | CGG | 0 | 0 |
| Asn | AAT | 1 | 0 | Ser | AGT | 2 | 0 |
| | AAC | 15 | 11 | | AGC | 0 | 1 |
| Lys | AAA | 0 | 0 | Arg | AGA | 0 | 0 |
| | AAG | 8 | 11 | | AGG | 0 | 0 |
| Asp | GAT | 8 | 3 | Gly | GGT | 11 | 9 |
| | GAC | 15 | 19 | | GGC | 16 | 22 |
| Glu | GAA | 1 | 0 | | GGA | 1 | 0 |
| | GAG | 17 | 14 | | GGG | 0 | 0 |

Comparison of the Nucleotide and Deduced Amino Acid Sequences

There are a number of similarities between the two lignin peroxidases. Both contain almost the same number of amino acid residues and both are very similar in amino acid composition (Table 2). A comparison of the amino acid sequences of the two lignin peroxidases shows a very high percentage of homology (75%) between the two lignin peroxidases (Fig. 4); the longest stretch of identical sequence between the two lignin peroxidases is a 13 amino acid region from residues from 275 to 287 in LG4 and 272 to 284 in LG5 (Fig. 4). Furthermore, hydropathy plots of the two lignin peroxidases look very similar (Fig. 3). A 71.5% homology was observed between the nucleotide sequences of CLG4 and CLG5 in the coding regions (Fig. 5). The longest stretch of homology spans 20 nucleotides from 724 to 743 in CLG4 and 715 to 734 in CLG5. However, there are some differences between the two lignin peroxidase cDNA clones. For example, CLG4 uses fewer T-residues and more C- and G-residues in the third base of various codons than CLG5 (Fig. 2 and Table 1), resulting in very short stretches of identical nucleotide sequence between the two cDNA fragments. This may explain why no cross-hybridization is detectable between CLG4 and CLG5 when nick-translated probes from the respective cDNA were used. Besides, the degree of homology is low in the 5'- and 3'-noncoding regions in CLG4 (1-33 and 1153-1163, Fig. 2A) and CLG5 (1-33 and 1150-1285, Fig. 2B).

**Table 2. Comparison of Amino Acid Composition in LG4 and LG5**

| AAs | LG4 | | LG5 | |
|---|---|---|---|---|
| | Number | Percentage | Number | Percentage |
| Ala | 44 | 11.83 | 44 | 11.86 |
| Arg | 10 | 2.69 | 11 | 2.96 |
| Asn | 11 | 2.96 | 16 | 4.31 |
| Asp | 22 | 5.91 | 23 | 6.20 |
| Cys | 8 | 2.15 | 8 | 2.16 |
| Gln | 23 | 6.18 | 20 | 5.39 |
| Glu | 14 | 3.76 | 18 | 4.85 |
| Gly | 31 | 8.33 | 28 | 7.55 |
| His | 11 | 2.96 | 6 | 1.62 |
| Ile | 21 | 5.65 | 20 | 5.39 |
| Leu | 26 | 6.99 | 26 | 7.01 |
| Lys | 11 | 2.96 | 8 | 2.16 |
| Met | 8 | 2.15 | 9 | 2.43 |
| Phe | 27 | 7.26 | 28 | 7.55 |
| Pro | 31 | 8.33 | 33 | 8.89 |
| Ser | 24 | 6.45 | 27 | 7.28 |
| Thr | 25 | 6.72 | 20 | 5.39 |
| Trp | 3 | 0.81 | 3 | 0.81 |
| Tyr | 1 | 0.27 | 0 | 0.00 |
| Val | 21 | 5.65 | 23 | 6.20 |
| Total | 372 | | 371 | |

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LG5 MAFKKLLAVLTAALSLRAAQGAA--VEKRATCSNGKVVP---AASCCTWFNVLSDIQENLFNGGQ 60
LG4 MAFKQLLAALSVALTLQVTQ-AAPNLDKRVACPDG-VHTASNAA-CCAWFPVLDDIQQNLFHGGQ 61

LG5 CGAEAHESIRLVFHDAIAISPAMEPQASSVR-GADGSIMIFDEIETNFHPNIGLDEIVRLQKPFV 124
LG4 CGAEAHEALRMVFHDSIAISPKLQSQGKFGGGGADGSIITFSSIETTYHPNIGLDEVVAIQKPFI 127

LG5 QKHGVTPGDFIAFAGAVALSNCPGAPQMNFFTGRAPATQPAPDGLVPEPFHSVDQIIDRVFDAGE 189
LG4 AKHGVTRGDFIAFAGAVGVSNCPGAPQMQFFLGRPEATQAAPDGLVPEPFHTIDQVLARMLDAGG 192

LG5 FDELELVWMLSAHSVAAANDIDPNIQGLPFDSTPGIFDSQFFVETQLAGTGFTGGSNNQGEVSSP 254
LG4 FDEIETVWLLSAHSIAAANDVDPTISGLPFDSTPGQFDSQFFVETQLRGTAFPGKTGIQGTVMSP 257

LG5 LPGEMRLQSDFLIARDARTACEWQSFVNNQSKLVSDFQFIFLALTQLGQDPDAMTDCSAVIPISK 319
LG4 LKGEMRLQTDHLFARDSRTACEWQSFVNNQTKLQEDFQFIFTALSTLGHDMNAMIDCSEVIPAPK 323

LG5 PAPNNTPGFSFFPPGMTMDDVEQACAETPFPTLSTLPGPATSVARIPPPPGA              371
LG4 PV-NFGPSF--FPAGKTHADIEQACASTPFPTLITAPGPSASVARIPPPPSPN              372
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Figure 4. Comparison of amino acid sequences of LG4 and LG5. The colons and periods between two lignin peroxidase protein sequences represent identical and conserved amino acid residues, respectively. Sequences are aligned to maximize homology with a minimal number of gaps which are represented by dotted lines. The longest stretch of identical amino acid sequence is underlined. The single letter code representing amino acids was previously described (5).

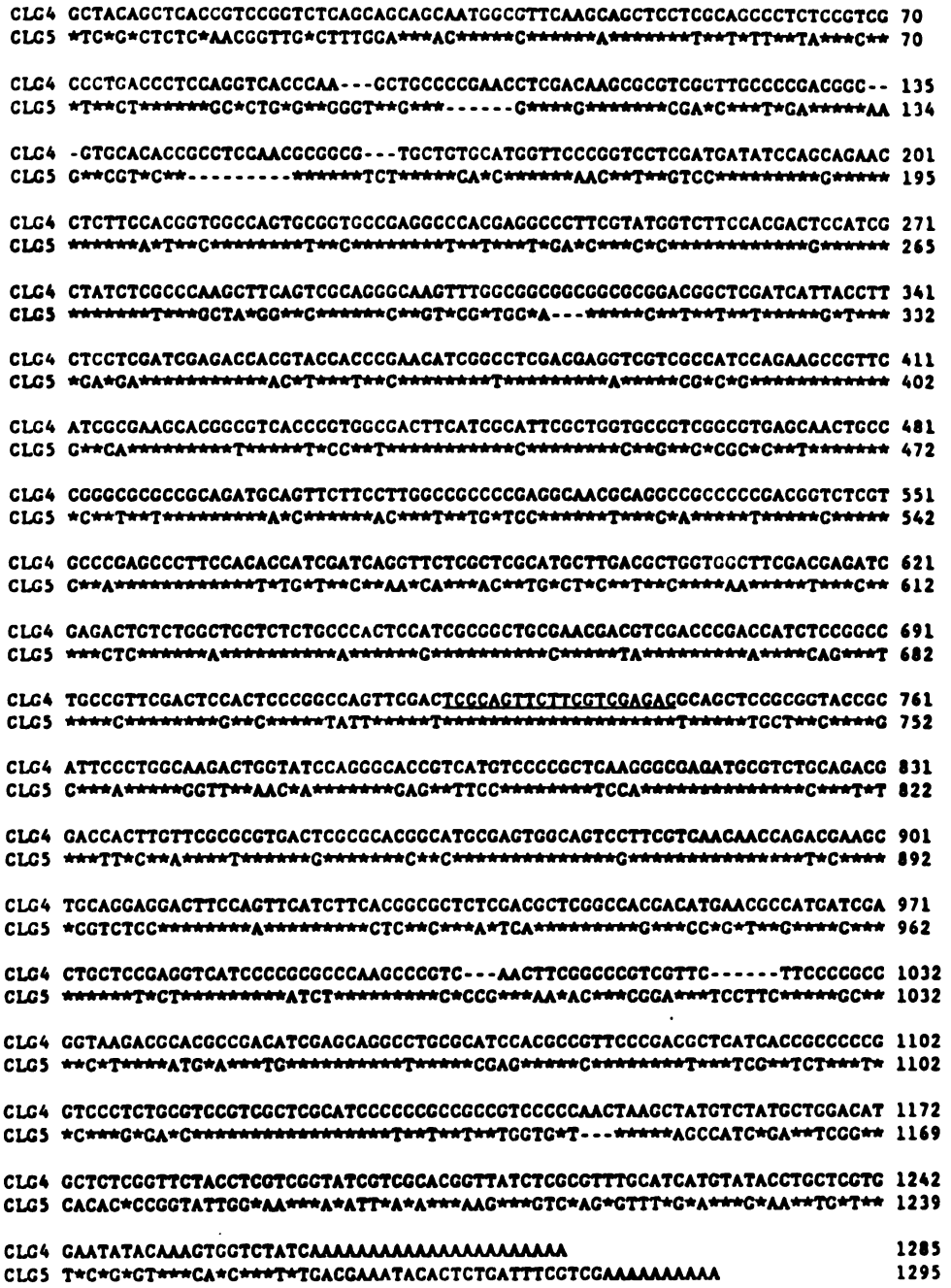


Figure 5. Comparison of nucleotide sequences of CLG4 and CLG5. Only the variant nucleotides are presented in CLG5. The asterisks represent the identical nucleotides. Sequences are aligned to maximize homology with a minimal number of gaps which are represented by dotted lines. The longest stretch of identical nucleotides between CLG4 and CLG5 is underlined.

## DISCUSSION

Previous studies (Chapter 1) suggested that the cDNA insert in CLG5 encodes lignin peroxidase H8, the predominant lignin peroxidase in P. chrysosporium grown in low nitrogen medium under static conditions (13). The results of this study show that the amino acid sequence deduced from the complete cDNA sequence of CLG5 matches the amino acid sequence of two tryptic peptides (#14 and #25B) obtained from purified lignin peroxidase H8 (29). The experimentally determined N-terminal sequence of lignin peroxidase H8 (Ala-Thr-Cys-Ser-Asn-Gly-Lys-Val-Val-Pro) is found in the deduced amino acid sequence of LG5. Immunoblotting data showed that the protein expressed by the lignin peroxidase cDNA clone in E. coli is reactive with the antibody raised against lignin peroxidase H8. These data unequivocally demonstrate that CLG5 represents one of the lignin peroxidase genes. Furthermore, the signal sequences and putative glycosylation sites in the deduced amino acid sequences of both LG4 and LG5 are consistent with the fact that lignin peroxidases are extracellular, glycosylated proteins.

The high degree of homology in the nucleotide sequences of CLG4 and CLG5 and the remarkable similarities in the deduced amino acid sequences of these clones indicate that CLG4 and CLG5 represent two separate members of a lignin peroxidase gene family. Furthermore, the N-teminal amino acid sequence of another purified lignin peroxidase (H2) from the same fungus exactly matched the N-terminal amino acid sequence of mature LG4 deduced from CLG4 sequence. These results provide additional support to the idea that CLG4 represents another lignin peroxidase gene.

A report on the isolation and sequencing of cDNA (λML1) from P.

chrysosporium was recently published (25). A comparison of λML1 with CLG4 and CLG5 shows that these represent related but different genes. The amino acid and nucleotide sequences of λML1 showed a high degree of homology to those of above two cDNA clones. The homology of the nucleotide sequence between CLG4 and λML1 and CLG5 and λML1 is 74.1% and 81.2%, respectively, whereas the amino acid sequence homology is 78.5% and 85%, respectively. The mature lignin peroxidase of λML1, similar to LG4 and LG5, is preceded by a leader sequence; contains a Lys-Arg cleavage site, N-glycosylation and polyadenylation sites, and is regulated at the level of mRNA production. The estimated molecular weight of 37,072 for the λML1 lignin peroxidase is comparable to the molecular weights of 36,540 and 36,607, respectively, for LG4 and LG5.

The similarities in nucleotide and amino acid sequences suggest that the genes encoding LG4 and LG5 originated from one ancestral lignin peroxidase gene. Different Southern hybridization patterns were seen when the chromosomal DNA of P. chrysosporium was probed with $^{32}$P-labeled CLG4 and CLG5 (see Chapter 1). For example, four bands appeared when BamHI-digested chromosomal DNA was hybridized with labeled CLG5, whereas no BamHI site is found in CLG5 itself. Restriction digestion of chromosomal DNA with other restriction endonucleases produced similar results. On the other hand, Southern hybridization of the chromosomal DNA using CLG4 as the probe resulted in banding patterns which are in accordance with the restriction pattern observed with CLG4. These results suggest that after the ancestral lignin peroxidase gene was duplicated, mutations continously appeared in lignin peroxidase genes corresponding to CLG4 and CLG5 and these became different members of the same gene family.

## ACKNOWLEDGEMENTS

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# CHAPTER THREE

## MOLECULAR CLONING OF A FAMILY OF LIGNIN PEROXIDASE GENES FROM *PHANEROCHAETE CHRYSOSPORIUM* AND SEQUENCE ANALYSIS OF A GENE ENCODING THE MAJOR LIGNIN PEROXIDASE

## ABSTRACT

Eleven lignin peroxidase genomic clones have been identified by probing a Phanerochaete chrysosporium (strain BKM-F 1767) BamHI genomic library with lignin peroxidase cDNAs CLG4 and CLG5. These clones fall into six groups, designated pGLG1, pGLG2, pGLG3, pGLG4, pGLG5 and pGLG6, based largely on the size and restriction map of each genomic fragment (designated, respectively, GLG1, GLG2, GLG3, GLG4, GLG5 and GLG6). The location of the lignin peroxidase gene in each cloned fragment and the orientation of transcription in each gene have been determined. Restriction maps of lignin peroxidase genes in GLG1 and GLG2 are similar to those of CLG4 and CLG5. The other four clones contain sequences homologous to CLG5. Sequence analysis of GLG2 showed that it encodes a mature lignin peroxidase with an $M_r$ of 36,607 which is preceded by a leader sequence of 27 amino acid residues. A sequence similar to the TATA box and a possible CAAT box are located 45 and 90 bp upstream of the startpoint of CLG5. By comparing the sequence of this gene with that of cDNA clone CLG5, nine small introns whose sizes range from 50 to 62 bp have been identified. These introns are randomly distributed in the gene and all have the consensus sequence GTRNGY---YTGAY---YAG. A possible polyadenylation signal sequence AATACA is located 14 bp upstream of the termination point of cDNA.

# INTRODUCTION

Phanerochaete chrysosporium, a white-rot basidiomycete, is known to produce during idiophase an extracellular, glycosylated, heme-containing protein called lignin peroxidase that obligatorily requires $H_2O_2$ for activity (14,15,39,40). This enzyme catalyzes oxidative cleavage of lignin and a variety of lignin model compounds (1,14,15,17,19,39,40). Lignin peroxidase has also been implicated in the detoxification of recalcitrant xenobiotics such as dioxins, polychlorinated biphenyls and benzopyrenes (8,9,16,36). The activity of lignin peroxidase is routinely assayed by measuring $H_2O_2$-dependent veratryl alcohol oxidation to veratraldehyde (40).

Several recent reports indicate that P. chrysosporium elaborates not one but a family of related lignin peroxidases (2,22,26,34), the number of which varies depending on the strains, culture conditions and other factors that have not been well defined (12,20,22,34). Kirk et al. (22) reported that P. chrysosporium produced at least six heme-containing proteins (H1, H2, H6, H7, H8 and H10) with veratryl alcohol oxidation activity. Lignin peroxidases H2 and H8 are reported to be the major proteins in the extracellular fluid of ligninolytic cultures of P. chrysosporium strain BKM-F 1767. Polyclonal antibody raised against lignin peroxidase H8 was shown to cross react with the other lignin peroxidases, indicating some degree of homology among these proteins. Analysis of protease digestion patterns revealed that H1 and H2 produced identical peptides, whereas H8 produced a pattern that was similar but lacked at least two major peptides present in H1 and H2 (22). The peptide cleavage patterns of H6 and H10 were very different from the H1,

H2 and H8 patterns. These results indicate varying degree of relatedness among different lignin peroxidases. Renganathan et al. (34) independently described three different molecular forms of lignin peroxidases from another strain of P. chrysosporium, all of which are glycosylated and range in $M_r$ from 39,000 to 43,000. Homology among multiple lignin peroxidases of P. chrysosporium was also reported by Leisola et al. (26). These results raise the question whether the different lignin peroxidase proteins are encoded by different genes, or are encoded by the same gene but are the results of different post-transcriptional and/or post-translational modifications.

We recently isolated two different lignin peroxidase cDNA clones (CLG4 and CLG5) from the cDNA library of P. chrysosporium BKM-F 1767 and presented the complete sequences of these cDNAs (44, Chapter 1 and 2). These two clones did not show cross-hybridization but had a very high degree of homology in nucleotide sequences (71.5%) in the coding regions and in amino acid sequences (75%) of the predicted lignin peroxidases. The two mature lignin peroxidase proteins contain 344 amino acid residues, are preceded by typical signal sequences, and have similar hydropathy (Chapter 2). However, the two cDNA clones have very different sequences in both the 5'- and 3'-noncoding regions, suggesting that the poly(A) RNAs corresponding to the two cDNAs are from different genes rather than from the same gene that undergoes post-transcriptional modifications. A third lignin peroxidase cDNA isolated from P. chrysosporium, also showed high degree of homology in nucleotide and amino acid sequences to those of CLG4 and CLG5 but had low homology with those two cDNA clones in the noncoding regions (41, Chapter 2). Furthermore, CLG4 and CLG5 showed very different hybridization patterns with the chromosomal DNA of P. chrysosporium, indicating that the

sequences in CLG4 and CLG5 correspond to different lignin peroxidase genes (Chapter 1). Probing of restriction enzyme digested chromosomal DNA with CLG4 showed a hybridization pattern very similar to the CLG4 restriction map, suggesting that there may be only one lignin peroxidase gene corresponding to CLG4. However, when CLG5 was used as probe in a parallel experiment, several different bands of hybridization of varying intensity were obtained, suggesting that, in addition to the lignin peroxidase gene corresponding to CLG5, there might be several other lignin peroxidase genes whose sequences may be partially homologous to CLG5.

In this chapter, I describe the isolation and characterization of six lignin peroxidase genes including their restriction maps, gene boundaries and the transcriptional orientation of each gene. The DNA sequence analysis of one lignin peroxidase gene corresponding to the CLG5, is also presented.

## MATERIALS AND METHODS

Plasmids, Strains and Media The shuttle vector YRp12 (37) was used for construction of the genomic library of P. chrysosporium BKM-F 1767 (ATCC 24725). This vector contains the entire pBR322 sequence and TRP1, URA3 and ars sequences from Saccharomyces cerevisiae. Vector pUC19 (30,42) was used for subcloning and characterizing the cloned genomic fragments containing lignin peroxidase gene. Vectors M13mp18 and M13mp19 (30,42) were used for DNA sequence analysis. Escherichia coli DH5α (BRL, Gaithersburg, MD) [$F^-$ endA1 hsdR17($r_k^-$ $m_k^+$) supE44 thi-1 recA1 gyrA96 relA1 (argF-laczya)U169 ϕ80dlacZΔM15 $\lambda^-$] was used as the host strain for library construction and for subcloning. E. coli JM107 [endA1, gyrA96, thi, hsdR17, supE44, relA1, $\lambda^-$, (lac-proAB)/F', traD36, proAB, $lacI^qZ$

ΔM15] (42) was used as the host strain for sequencing. Malt extract medium (21) was used for growing P. chrysosporium and LB (27) and YT media (29) were used for growing E. coli strains DH5α and JM107, respectively.

DNA Isolation Plasmid DNA and M13 RF DNA were isolated by using rapid alkaline procedure (27). Isolation of chromosomal DNA from P. chrysosporium was described previously (33). Different cDNA or genomic DNA fragments were isolated using low-melting temperature agarose gel (32).

Construction of Genomic Library A BamHI site is not present in either CLG4 or CLG5 and BamHI genomic DNA digests exhibit five bands of different sizes that hybridize with CLG4 and CLG5. Hence, we made the assumption that each BamHI genomic fragment that shows hybridization with CLG4 and CLG5 probably contains one entire lignin peroxidase gene. Vector YRp12 was digested with BamHI, dephosphorylated with calf intestine alkaline phosphatase and then ligated with completely BamHI-digested chromosomal DNA of P. chrysosporium. The ligated DNA was transformed into E. coli DH5α using the procedure described by Hanahan (18). The transformed cells were spread on LB plates supplemented with ampicillin (100 μg/ml), incubated at 37°C for 18 h, and all the ampicillin resistant transformants ($Ap^r$) on the plates were pooled if the ratio of tetracycline sensitive transformants ($Tc^s$) to $Ap^r$ was higher than 50% and stored at -20°C until use. This genomic library contains about 19,000 recombinant clones.

Identification of Lignin Peroxidase Clones from Genomic Library Colony hybridization was used for screening lignin peroxidase clones from the genomic library (27). Nitrocellulose filters harboring 4,000 genomic clones each were prepared as described previously (43) and were

first hybridized with the cDNA probe CLG4. Then, the probe was washed off and the same filters were hybridized with the cDNA probe CLG5. The positive clones were streaked on LB plates for purification. The purified colonies from each plate were picked and spotted on a LB master plate and another LB plate on which nitrocellulose filter was placed. Both plates were incubated at 37°C for 14 h. The colonies on the filters were lysed in situ (43) and then hybridized with the respective probe as described above.

DNA Blotting and Hybridization To determine the location of lignin peroxidase gene in each isolated clone and their transcriptional orientation, the plasmid DNA from each recombinant clone was digested with different restriction enzymes, the fragments were separated on 1% agarose gel, transferred onto nitrocellulose filters (27), and then probed with various $^{32}$P-labeled fragments of the two cDNAs, CLG4 and CLG5.

DNA Sequencing Different restriction enzymes were used to produce various fragments for subcloning into the appropriately digested sequencing vectors M13mp18 or M13mp19. The sequencing strategy employed is shown in Figure 1. The dideoxy chain-termination procedure (35) and $^{35}$S-dATP were used to sequence lignin peroxidase gene in GLG2.

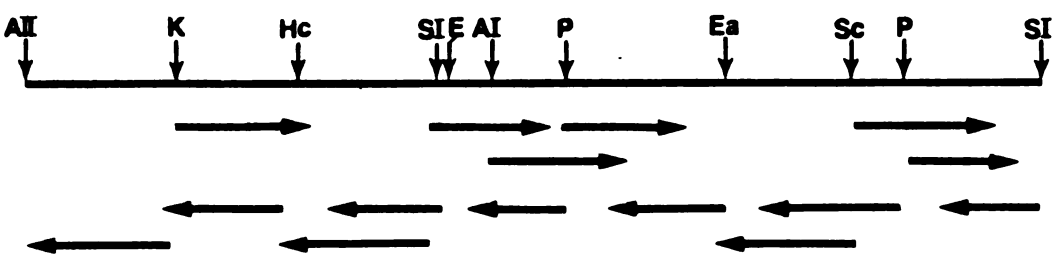



Figure 1. Sequencing strategy of lignin peroxidase gene in GLG2. Abbreviations for restriction enzymes are: AI-AvaI, AII-AvaII, E-EcoRI, Ea-EagI, Hc-HincII, K-KpnI, P-PstI, SI-SstI and Sc-ScaI.

## RESULTS

### Isolation and Identification of Lignin Peroxidase Genomic Clones

The YRp12 genomic library of P. chrysosporium was probed with CLG4 and CLG5 to identify the lignin peroxidase genomic clones. One clone that showed hybridization with CLG4 was designated pGLG1. Ten clones that hybridized with probe CLG5 were digested with BamHI, EcoRI and XhoI and the restriction patterns and the size of each cloned fragment were compared. Based on these data, the 10 positive genomic clones could be divided into 5 groups and these were designated pGLG2, pGLG3, pGLG4, pGLG5 and pGLG6. All clones except pGLG3 contained a BamHI site on either side of the genomic inserts, whereas only one BamHI site could be recovered in pGLG3; the other site was apparently lost during the library construction.

To reconfirm that the six types of clones contain sequences homologous to CLG4 or CLG5, the genomic inserts (named GLG1, GLG2, GLG3, GLG4, GLG5 and GLG6) from these clones were isolated, purified and recloned into pUC19 and these new clones were used in the rest of the work described here. All the new clones were digested with BamHI (or BamHI-HindIII for GLG3 in new clone), fractionated on agarose gel and then probed with $^{32}$P-labeled CLG4 and CLG5. The results showed that only GLG1 hybridized with CLG4 (Fig. 2B), consistent with the Southern hybridization analyses previously reported (Chapter 1). The other five cloned fragments showed hybridization with the cDNA probe CLG5 but the intensity of hybridization varied (Fig. 2C). The strongest hybridization was shown by GLG2, implying that this genomic clone may correspond to CLG5, whereas the other four clones showed less intense hybridization bands, suggesting that the sequences of these clones have partial homology to CLG5.

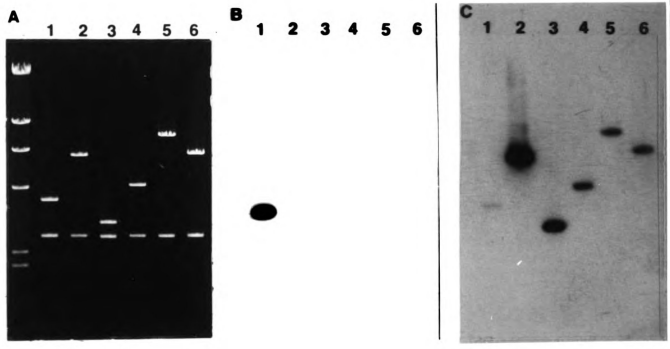



Figure 2. Identification of lignin peroxidase genomic clones using cDNAs CLG4 and CLG5 as probes. Panel A, agarose gel picture; Panel B and C, hybridization of the genomic clones with CLG4 and CLG5, respectively. In each panel, lanes 1 to 6 contained GLG1, GLG2, GLG3, GLG4, GLG5 and GLG6 in pUC19, respectively. The size marker, HindIII-digested lambda DNA, is shown in panel A.

Characterization of Lignin Peroxidase Clones

Consistent with the above suggestions, the restriction maps of middle regions of GLG1 and GLG2 were closely comparable to those of CLG4 and CLG5, respectively. The restriction maps of the other four lignin peroxidase genomic clones were different from that of GLG2, suggesting that these represent different lignin peroxidase genes (Fig. 3). The estimated sizes of GLG1 to GLG6 are 3.82 kb, 6.49 kb, 3.03 kb, 4.81 kb, 7.76 kb and 6.55 kb, respectively.

The cDNA sequence data (Chapter 2) showed that the two cDNA fragments, including the coding region and the 5'- and 3'-flanking sequences, are smaller than any of the cloned genomic fragments. To define the limits of lignin peroxidase gene, each clone was digested with one or more restriction enzymes, fractionated on agarose gels and then hybridized with the entire $^{32}$P-labeled cDNA CLG4 or CLG5. The heavy lines in Fig. 3 show the possible coding region in each gene. All the cloned fragments except GLG4 are believed to contain intact lignin peroxidase genes because these regions showing hybridization are either in the middle of the cloned fragments, such as GLG1, GLG2 and GLG3, or far away from the cloning site, such as GLG5 and GLG6. The BamHI site of GLG4 is very close to the 3'-end of the coding region.

Since the transcriptional orientation of CLG4 and CLG5 are known, different segments of each cDNA were used as probes to determine the transcriptional direction of each lignin peroxidase gene. The same filters which had been hybridized with the entire cDNA fragment were also used for successive hybridization with the 3' and/or 5' fragments of cDNAs and the orientation of each lignin peroxidase gene is shown in Fig. 3.

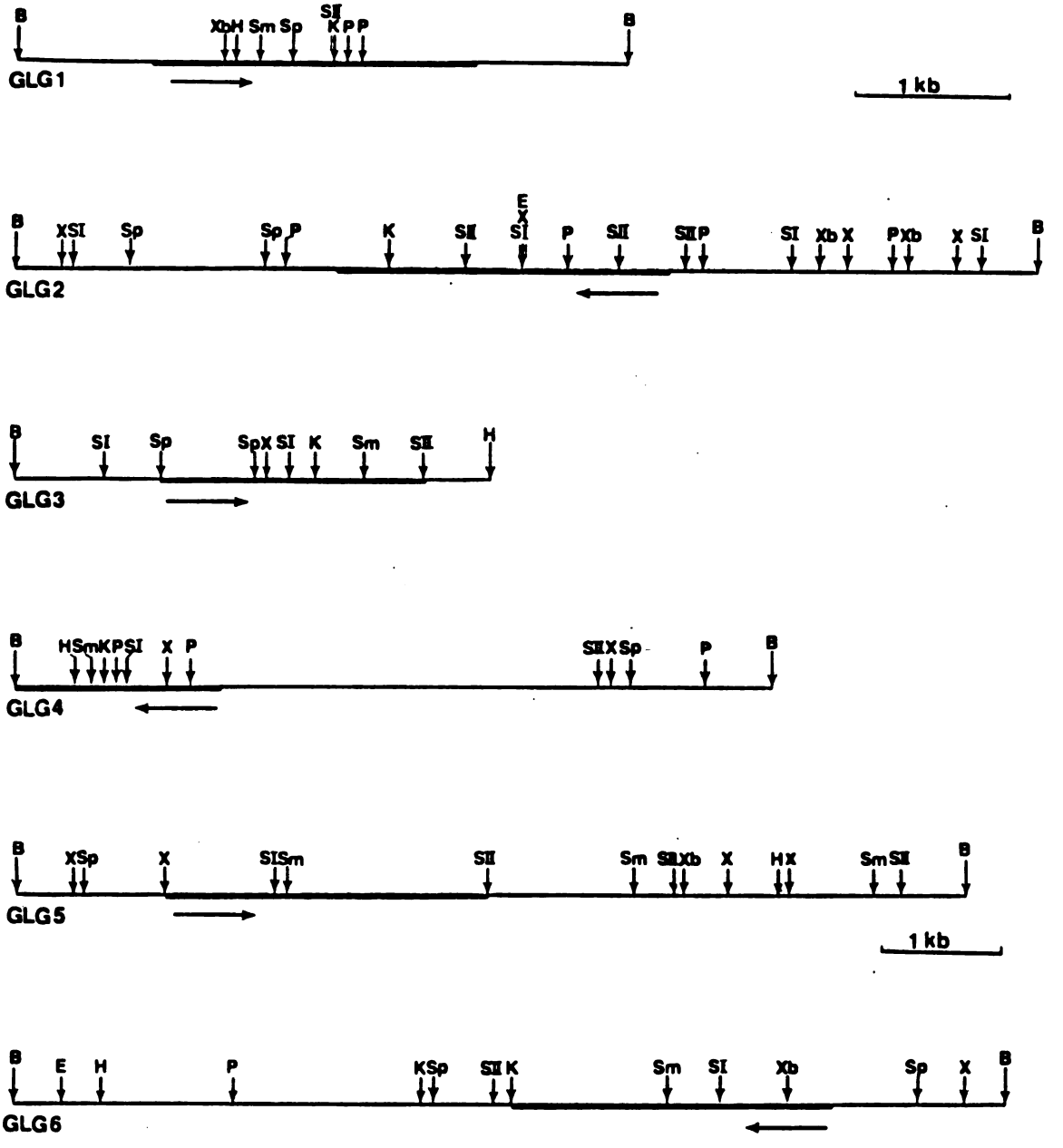



Figure 3. Restriction maps of six lignin peroxidase genomic clones. The heavy lines represent the gene locations and the arrow with long line underneath each restriction map represents the transcriptional direction. The abbreivations for restriction enzymes are: B, BamHI; E, EcoRI; H, HindIII; K, KpnI; P, PstI; Sm, SmaI; Sp, SphI; SI, SstI; SII, SstII; X, XhoI and Xb, XbaI.

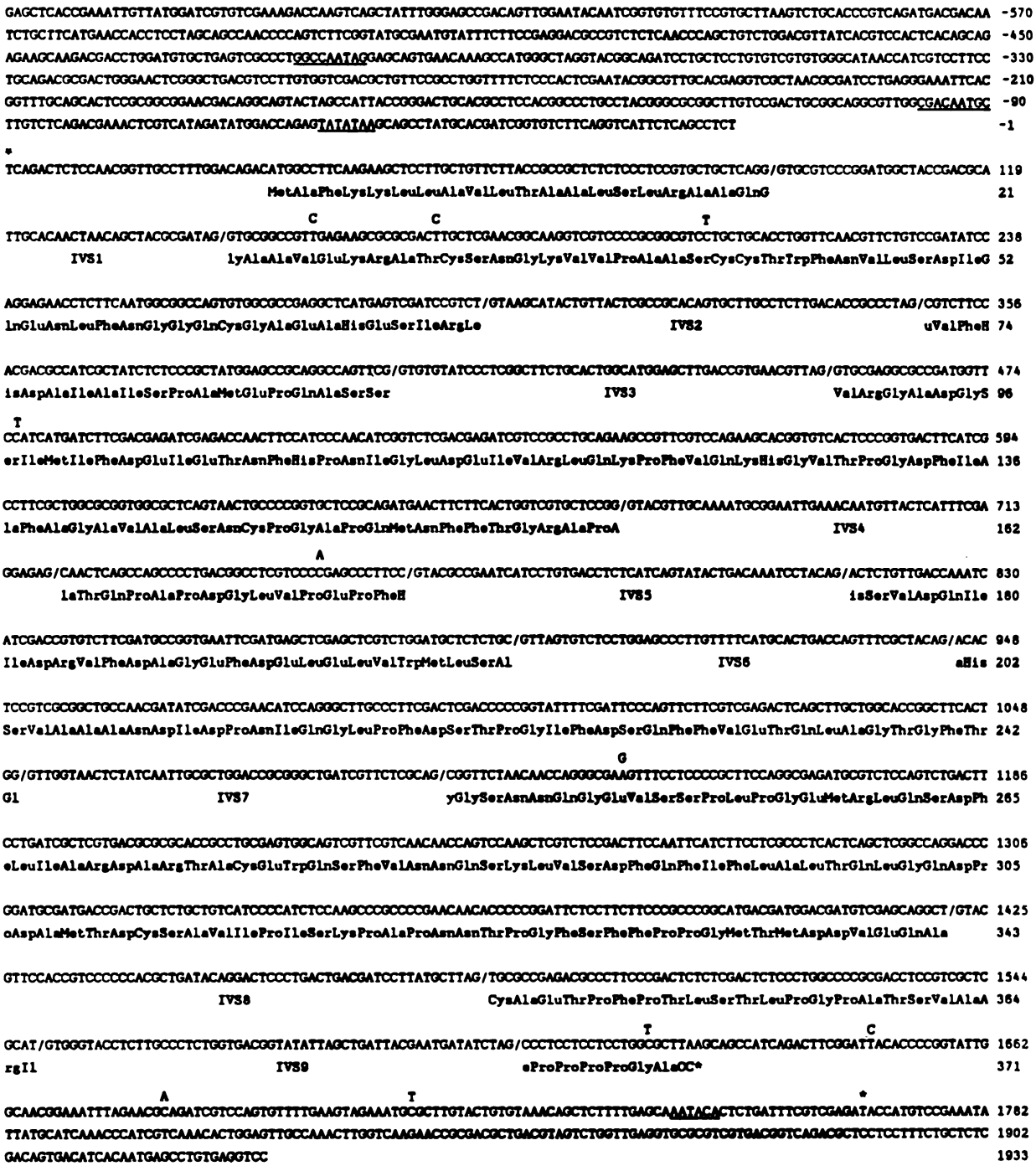
DNA Sequence Analysis of GLG2

Clone GLG2 appears to correspond to CLG5 based on the intensity of hybridization of the former with CLG5 and the many similarities in restriction maps of CLG5 and GLG2. Since CLG5 encodes the major lignin peroxidase (H8), the 2.6 kb SstI-AvaII fragment in the middle of GLG2 (see Fig. 3) was sequenced and the amino acid sequence was deduced (Fig. 4). These data show that the lignin peroxidase gene in GLG2 codes for a protein containing 371 amino acid residues with an $M_r$ of 39,417, and the mature protein ($M_r$=36,607) is preceded by a leader sequence of 27 amino acids, which is believed to be important for the secretion of this enzyme. The leader sequence has the characteristics of typical bacterial and mammalian signal peptides, i.e. have a few charged residues at the N-terminus followed by a hydrophobic core and then a more polar C-terminal region defining the cleavage site. The two basic residues, Lys-Arg at position 26-27, may represent a proteolytic cleavage site, suggesting the existence of a pro-form of lignin peroxidase. The mature lignin peroxidase has two potential N-glycosylation sites at positions 283 and 323 with the sequence Asn-Gln-Ser and Asn-Asn-Thr, which are consistent with the published consensus sequence of Asn-X-Thr/Ser. These results are consistent with the fact that lignin peroxidase purified from ligninlolytic cultures of P. chrysosporium is an extracellular, glycosylated protein.

The sequence data showed that the promoter sequence TATATAA is 45 bp upstream and a possible CAAT box sequence (CGACAATGC) is 90 bp upstream of the 5' startpoint of CLG5. Nine small introns are identified in the lignin peroxidase gene in GLG2 by comparing the genomic and cDNA sequences. The size of the introns ranges from 50 to 62 bp. Each intron

has GT and AG at the 5'- and 3'-end, respectively. A possible polyadenylation signal sequence AATACA is located 14 bp upstream of the poly (A) addition site. Comparison of the sequences between lignin peroxidase genomic and cDNA clones showed that there were several minor substitutions in both the coding and 3'-noncoding regions (see Fig. 4). All substitutions in the coding region are located at the third nucleotide of each codon, with the result the same amino acid sequence is seen in spite of these substitutions.

Figure 4. Nucleotide sequence of lignin peroxidase gene in GLG2 and the predicted amino acid sequence. The start and termination points of the corresponding cDNA (CLG5) are indicated by asterisks (*) over the DNA sequence. Sequences homologous to CAAT box, TATA box, and the potential hexanucleotide polyadenylation signal (AATACA) are underlined. IVS1 to IVS9 represent the nine intervening sequences (introns) identified in the lignin peroxidase gene. The boundary of each intron is marked by slashes. Bases in CLG5 cDNA that are different from those in GLG2 are presented immediately above the corresponding genomic bases.



# DISCUSSION

## Lignin Peroxidase Gene Family

Six types of lignin peroxidase genomic clones have been isolated by screening the BamHI-library of P. chrysosproium BKM-F 1767 with the previously isolated lignin peroxidase cDNAs, CLG4 and CLG5. Five of them represented by pGLG2 through pGLG6 showed hybridization with CLG5 under high stringent conditions. These data are consistent with earlier results (Chapter 1) showing that BamHI-digested chromosomal DNA of P. chrysosporium gives four bands of hybridization with CLG5. The sizes of the five lignin peroxidase clones represented here (7.76 kb, 6.55 and 6.49 kb, 4.81 kb and 3.03 kb) are comparable to those of the BamHI bands. Since lignin peroxidase gene in GLG2 showed the strongest hybridization with CLG5 and has similar restriction map to that of CLG5, it appeared that this clone contained the genetic analogue of CLG5. A comparison of the sequence data of GLG2 with that of CLG5 clearly shows this to be true. Clones pGLG3, pGLG4, pGLG5 and pGLG6 displayed strong hybridization with CLG5 but had different restriction patterns compared to that of CLG5 or GLG2, suggesting that these are partially homologous to GLG2, but represent different lignin peroxidase genes. Partial sequencing of GLG4 has shown that these two (GLG2 and GLG4) have much higher nucleotide homology than that seen between CLG4 and CLG5 (data not shown). One genomic clone (pGLG1) showed strong hybridization with CLG4 (see Fig. 2B, lane 1) and its restriction map was similar to that of CLG4 (Fig. 3), suggesting that the lignin peroxidase gene in this genomic clone corresponds to CLG4. Consistent with this idea, the previous results (Chapter 1) showed that $^{32}$P-labeled CLG4 gave a single

band of hybridization with BamHI-digested chromosomal DNA. Restriction enzyme XbaI cuts GLG1 into two fragments, but does not have any site in CLG4, suggesting that this site may be located in an intron of this lignin peroxidase gene. Comparison of detail restriction digestions of CLG4 and GLG1 showed that XbaI site is located in one intron (data not shown). These results indicate that the genomic clones represent a family of six lignin peroxidase genes.

Tien and Tu (41) isolated and sequenced another cDNA clone (λML1) from P. chrysosporium, which has a high degree of nucleotide and amino acid sequence homology with CLG4 and CLG5 (41, Chapter 2). None of the six lignin peroxidase clones described here has restriction map similar to that of λML1, implying that P. chrysosporium may have one more lignin peroxidase gene corresponding to this cDNA.

The results clearly show that six members of the lignin peroxidase gene family in P. chrysosporium BKM-F 1767 have been isolated. HPLC profile showed that there were six primary lignin peroxidase proteins in the extracellular fluid of P. chrysosporium grown in low nitrogen medium (22). It remains to be proven that each isolated lignin peroxidase gene encodes one of these six lignin peroxidase proteins. The sequence data of CLG5 and genomic clone GLG2 provide very strong evidence that GLG2 codes for lignin peroxidase H8, a major extracellular protein produced by P. chrysosporium in ligninolytic cultures. We recently determined amino acid sequence at the N-terminus of another purified lignin peroxidase, H2, and showed that this sequence (Val-Ala-Cys-Pro-Asp-Gly-Val-His-Thr-Ala-Ser-Asn) exactly matches with the first twelve amino acid residues of the mature lignin peroxidase deduced from cDNA CLG4. These data indicate that GLG1 encodes another major lignin peroxidase H2. The sequencing of the other lignin peroxidase proteins and genes is

now getting under way.

General Structure of Lignin Peroxidase Gene in GLG2

In high eukaryotic genes, two consensus sequences have been identified to be important for initiation of transcription: the CAAT box 70 to 90 bases and the TATA box 20 to 40 bases upstream of the major start point (5,25). In yeast, most genes have the TATA box 40-120 bp upstream of the mRNA initiation sites (11,38), whereas the CAAT box is generally either clearly absent or partially disguised (11). In filamentous fungi, some genes possess the similar canonical TATA sequence at approximately the expected position, while others simply have an AT-rich region 30-100 bp upstream of the 5′ start point of mRNA (3). Sequences related to the CAAT consensus have also been described in some fungal genes, but this is often not very clear (3). The lignin peroxidase gene of P. chrysosporium contains a typical TATATAA sequence which lies 45 bp upstream of the start point of cDNA CLG5. There is a sequence CGACAATGC, similar to the CAAT box, 90 bp upstream of the startpoint of cDNA, even though it does not contain the typical GGC/GCAATCT sequence found in higher eukaryotic genes. There are two other CAAT box-like sequences far upstream of the cDNA start point, at positions -415 (GGCCAATAG) and -575 (CGACAATCT), respectively, and both have more conserved sequences with the typical CAAT box than the first CAAT box (-99 bp) in this gene. In both yeast and fungal genes, the mRNA start site is characterized by a preceding pyrimidine-rich sense strand (3,6,10) and this feature is present in lignin peroxidase gene GLG2, also. The ratio of CT to the total sequence between TATA box and start point of cDNA is 61.4% (27/44).

The sequence surrounding the initiator codon ATG in GLG2 is CAGACATGG,

in which 7 of 9 nucleotides match with the eukaryotic translation initiation site consensus sequence CCA/GCCATGG (25). Using point mutations flanking the initiator codon AUG, Kozak (24) more recently identified ACCATGG as optimal sequence for translation initiation by eukaryotic ribosome and a purine in position -3 is critical for efficient translation. In GLG2, there is a G located at -3 position of ATG. Similar to other filamentous fungal genes (3,7,31), GLG2 contains several small introns (see below for detail discussion). The codon usage in GLG2 is very biased in favor of codons ending with C- and/or G-residues. Comparison of codon usage of lignin peroxidase cDNAs and gene with that of different filamentous fungal and yeast genes is shown in Table 1. The comparison shows that the codon usage in P. chrysosporium is less biased than that in yeast but more similar to that in Neurospora than to that in Aspergillus (Table 1). Some codons in S. cerevisiae are extremely preferred but are not or very poorly used in lignin peroxidase gene. For example, the preferred codon for Arg in yeast is AGA (90%) while this codon is not used at all in P. chrysosporium. The same situation is found in Cys, Gln, Glu, Leu and Pro in which yeast prefers codons ending with A or T (Leu is exception) whereas in P. chrysosporium codons ending with C or G are preferred (Table 1). These data suggest that the translation efficiency for the lignin peroxidase cDNAs in yeast cells may be very low.

Sequencing of many higher eukaryotic genes has revealed that AATAAA, which usually lies 10-30 bp upstream of the poly (A) addition site, is by far the predominant sequence directing cleavage and polyadenylation of pre-mRNA although minor nucleotide substitutions, such as AATTAA, AATACA and AATATA, are known to occur (6). However, the consensus sequence AATAAA is rarely present in either yeast or filamentous fungal

genes (3). In contrast to this, at the 3'-end of the lignin peroxidase gene GLG2, AATACA sequence is present 14 bp upstream of the polyadenylation site, although this sequence is believed to be relatively inefficient for polyadenylation in higher eukaryotic genes (6). A similar sequence AATATA was found in the same position in cDNA CLG4. However, the cDNA λML1 showed a different sequence AAATAT 13 bp upstream of the poly(A) addition site (41). The consensus G/T cluster sequence found downstream of the poly(A) addition site in other eukaryotic genes is absent in GLG2 (6).

Table 1. Comparison of Codon Usage in P. chrysosporium[a] (P), Aspergillus (A), Neurospora (N) and Yeast[b] (Y).

| AAs | Codons | P | A | N | Y | AAs | Codons | P | A | N | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Phe | TTT | 1 | 26 | 16 | 14 | Ser | TCT | 16 | 20 | 19 | 49 |
| | TTC | 99 | 74 | 84 | 86 | | TCC | 48 | 29 | 43 | 45 |
| Leu | TTA | 0 | 3 | 0 | 7 | | TCA | 0 | 9 | 3 | 3 |
| | TTG | 4 | 16 | 9 | 87 | | TCG | 30 | 14 | 13 | 1 |
| | CTT | 16 | 20 | 26 | 2 | | AGT | 3 | 10 | 5 | 2 |
| | CTC | 66 | 28 | 47 | 1 | | AGC | 3 | 18 | 17 | 1 |
| | CTA | 0 | 6 | 2 | 4 | Pro | CCT | 17 | 29 | 23 | 16 |
| | CTG | 14 | 27 | 16 | 0 | | CCC | 42 | 40 | 57 | 1 |
| Ile | ATT | 8 | 42 | 28 | 51 | | CCA | 5 | 13 | 9 | 83 |
| | ATC | 92 | 53 | 70 | 48 | | CCG | 36 | 18 | 11 | 0 |
| | ATA | 0 | 5 | 2 | 1 | Thr | ACT | 27 | 31 | 22 | 46 |
| Met | ATG | 100 | 100 | 100 | 100 | | ACC | 50 | 43 | 57 | 46 |
| Val | GTT | 12 | 29 | 22 | 55 | | ACA | 0 | 16 | 12 | 5 |
| | GTC | 78 | 44 | 63 | 44 | | ACG | 23 | 10 | 9 | 3 |
| | GTA | 0 | 6 | 3 | 1 | Ala | GCT | 28 | 35 | 28 | 72 |
| | GTG | 10 | 21 | 12 | 1 | | GCC | 32 | 35 | 62 | 26 |
| Tyr | TAT | 0 | 33 | 17 | 12 | | GCA | 12 | 15 | 3 | 1 |
| | TAC | 100 | 67 | 83 | 88 | | GCG | 28 | 15 | 7 | 1 |
| His | CAT | 8 | 21 | 25 | 16 | Cys | TGT | 21 | 36 | 15 | 96 |
| | CAC | 92 | 79 | 75 | 84 | | TGC | 79 | 64 | 85 | 4 |
| Gln | CAA | 8 | 33 | 16 | 94 | Trp | TGG | 100 | 100 | 100 | 100 |
| | CAG | 92 | 67 | 84 | 6 | Arg | CGT | 45 | 27 | 36 | 7 |
| Asn | AAT | 2 | 24 | 9 | 5 | | CGC | 52 | 32 | 38 | 0 |
| | AAC | 98 | 76 | 91 | 95 | | CGA | 3 | 13 | 5 | 0 |
| Lys | AAA | 0 | 19 | 4 | 8 | | CGG | 0 | 13 | 6 | 0 |
| | AAG | 100 | 81 | 96 | 92 | | AGA | 0 | 7 | 4 | 90 |
| Asp | GAT | 27 | 49 | 35 | 32 | | AGG | 0 | 8 | 11 | 3 |
| | GAC | 73 | 51 | 65 | 68 | Gly | GGT | 35 | 34 | 45 | 95 |
| Glu | GAA | 6 | 36 | 10 | 96 | | GGC | 63 | 36 | 45 | 3 |
| | GAG | 94 | 64 | 90 | 4 | | GGA | 2 | 21 | 7 | 1 |
| | | | | | | | GGG | 0 | 9 | 3 | 1 |

a Codon usage information for P. chrysosporium is based on the data obtained with the three lignin peroxidase cDNAs and one gene (41, Chapter 2 and 3).

b Codon usage information for Aspergillus (3), Neurospora (3), and yeast (4) is based on data obtained with eight, eleven and ten genes, respectively.

Arrangement and Structure of Introns

Nine introns have been identified in lignin peroxidase gene in GLG2 by comparing its sequence with that of the corresponding cDNA CLG5. The position, size and phase of each intron in this gene is shown in Table 2. All the introns are very small (50-62 bp) and are randomly distributed in the coding region. Thus, the number of introns of lignin peroxidase gene of P. chrysosporium is much higher than that found in yeast genes. For example, in S. cerevisiae, only seventeen of several hundred sequenced genes were found to contain one or two introns (13) and the intron in each gene is located at the extreme 5'-end. The size of the introns in lignin peroxidase gene is very similar to that of genes from other filamentous fungi (7,23,28,31). For example, the glucoamylase gene of Aspergillus niger contains five introns, four of them are 55 to 75 bp in length (7). The $\beta$-tubulin gene of Neurospora crassa contains six introns and five of them are 57-74 bp in size (31). The phase of intron does not show any specificity (see Table 2).

The comparison of exon/intron junctions with the consensus eukaryotic splice junctions (3) is shown in Fig. 5. All introns in lignin peroxidase gene in GLG2 seem to have the consensus sequence GTRNGY...YTGAY...YAG, similar to the introns of genes from other lower and higher eukaryotic organisms.

Our results show that introns are probably a common occurence in lignin peroxidase genes. Preliminary sequencing data with GLG1 and GLG4 have shown that introns are present in these two genes also (data not shown).

Table 2. Comparison of the Positions, Phases and the Lengths of Nine Introns of Lignin Peroxidase Gene in GLG2.

| Intron | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Position | 21 | 71 | 89/90 | 161 | 175 | 201 | 243 | 343/344 | 365 |
| Size | 52 | 51 | 52 | 50 | 52 | 52 | 51 | 62 | 58 |
| Phase | I | II | III | I | I | II | II | III | II |

The positions are given in amino acid residue numbers in the lignin peroxidase protein. The sizes of introns are presented as base pairs (bp). Phases I, II and III mean that the introns follow the first, second and third nucleotide of a codon, respectively.

```
-----------------------------------------------------------------------
IVS1 AGG/GTGCGTCCCGGATGGCTACCGACGCATTGCACAA-----CTAAC-------AGCTACGCGATAG/GT
IVS2 TCT/GTAAGCATACTGTTACTCGCCGCACAGTGCTTGCCTC--TTGAC-----------ACGCCCTAG/CG
IVS3 CGG/GTGTGTATCCCTCGGCTTCTGCACTGGCATGGAGC----TTGAC--------CGTGAACGTTAG/TG
IVS4 CGG/GTACGTTGCAAAATGCGGAATTGAAACAATGTTA-----CTCAT---------TTCGAGGAGAG/CA
IVS5 TCC/GTACGCCGAATCATCCTGTGACCTCTCATCAGTATA---CTGAC---------AAATCCTACAG/AC
IVS6 TGC/GTTAGTGTCTCCTGGAGCCCTTGTTTTCATGCA------CTGAC------CAGTTTCGCTACAG/AC
IVS7 TGG/GTTGCGTAACTCTATCAATTGCGCTGGACCGCGGG----CTGAT---------CGTTCTCGCAG/CG
IVS8 GCT/GTACGTTCCACCGTCCCCCCACGCTGATACAGGACTCC-CTGAC-TGACGATCCTTATGCTTAG/TT
IVS9 CAT/GTGGGTACCTCTTGCCCTCTGGTGACGGTATATTAG---CTGAT---TACGAATGGATATCTAG/CC
-----------------------------------------------------------------------
```

| | | | | | |
|---|---|---|---|---|---|
| P. chrysosporium | SK/GTRNGY | 32-37 | YTGAY | 9-19 | YAG/N |
| High eukaryotes | CAG/GTAAGT | | CTAAT | $(T/C)_n$ | NCAG/G |
| Yeast | N/GTATGT | | TACTAAC | | YYNYAG/N |
| Filamentous fungi | g/GTAYGTT | | TGCTAAC | | ACAG/g |

Figure 5. Conservation of intron/exon junction and internal sequences in lignin peroxidase gene of P. chrysosporium. The conserved intron/exon junction and internal sequences of genes from high eukaryotes (28), yeast (23) and Filamentous fungi (3) are also presented for comparison. The boundaries of introns and exons are marked by slashes. Letters in upper case represent conservation in >70% of introns and lower case represents 50-70% for all filamentous fungi, including P. chrysosporium. Abbreviations for single letter codes are: K—G or T, N—A or C or G or T, R—A or G, S—C or G and Y—C or T.

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# APPENDICS

# APPENDIX A

## IDENTIFICATION OF cDNA CLONES FOR LIGNINASE FROM PHANEROCHAETE CHRYSOSPORIUM USING SYNTHETIC OLIGONUCLEOTIDE PROBES

By

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# IDENTIFICATION OF cDNA CLONES FOR LIGNINASE FROM *Phanerochaete chrysosporium* USING SYNTHETIC OLIGONUCLEOTIDE PROBES[+]

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Four cDNA clones for ligninase were isolated from the cDNA library (constructed into the PstI site of *E. coli* vector pUC9) representing 6 day-old lignin degrading culture of *Phanerochaete chrysosporium* by the use of three synthetic oligonucleotide probes corresponding to partial amino acid sequences of tryptic peptides of the ligninase. Each of the three probes, 14.1, 14.2 and 25, represents a mixture of 32 12- or 14-base long oligonucleotides. Three cDNA clones hybridized with probe 14.1 but not with probe 25 or 14.2, but one cDNA clone hybridized with all of the three probes. Differential hybridization studies showed that these clones are unique to 6-day poly(A) RNA, but not to 2-day poly(A) RNA. 

Lignin is a highly complex, amorphous, aromatic polymer, which is a major component of woody plants, and is the second most abundant renewable organic resource on the earth (1). Research has intensified worldwide on ligninolytic microorganisms and their enzymes because of their possible industrial potential in biopulping and in the conversion of lignin and lignocellulosic materials to useful chemicals (1). *Phanerochaete chrysosporium*, a white-rot basidiomycete, is a rapid lignin degrader and is most widely studied from both the basic and applied standpoints. Lignin degradation in this organism is a secondary metabolic event and is triggered in response to N, C or S starvation (1-3). Two enzymes, glucose oxidase and ligninase, which are believed to be involved in lignin degradation, have been purified and characterized from

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ligninolytic cultures of this fungus (4-6). Ligninase is a $H_2O_2$-dependent, extracellular heme protein with a molecular weight of 41,000-42,000, and catalyzes $C_\alpha$-$C_\beta$ cleavage of the propyl side chains of lignin, a major reaction in fungal depolymerization of lignin (5,6). Ligninase is an extremely important enzyme because of its possible application in pulping wood, biobleaching of pulp, treating wastes, conversion of byproduct lignins to useful chemicals, and detoxification of xenobiotics such as dioxins, DDT and polychlorinated biphenyls (1, 5-7).

As a first step in obtaining a better understanding of lignin degradation by P. chrysosporium at the molecular level and to develop the full bioprocessing potential of this organism, we initiated studies to isolate and characterize the ligninase cDNA clones. We describe here for the first time the isolation of cDNA clones for ligninase by screening the cDNA library of P. chrysosporium using three synthetic probes, corresponding to amino acid sequences of different portions of the ligninase in this organism.

**MATERIALS AND METHODS**

Poly(A) RNA extraction. P. chrysosporium strain BKM-F 1767 (ATCC 24725) was maintained and grown as previously described (4) except that 20 mM sodium acetate (pH 4.5) replaced 2,2-dimethylsuccinate in the medium. RNA was extracted using a modification of the hot phenol extraction procedure (9). Oligo(dT)-cellulose was used to purify poly(A) RNA which usually accounted for 1-2% of the total cellular RNA.

Construction of cDNA library. Double stranded cDNA, synthesized from poly(A) RNA from 6-day cultures as previously described (10), was dC-tailed and then annealed with dG-tailed pUC9 (11) which was digested with restriction enzyme PstI. The annealed DNA molecules were used to transform E. coli JM83 (ara Δlac-pro strA thi ø80dlacZ ΔM15) using the procedure of Hanahan (12). The transformed E. coli cells were spread on 2 YT plates (13) supplemented with 100 μg/ml ampicillin and 40 μg/ml X-gal (13; 5-bromo-4-chloro-3-indolyl-β-D-galactoside). Ten thousand white, potential cDNA clones were picked and were individually stored in each well of 96-well microtiter plates.

Differential hybridization. The HATF filter containing cloned cDNA was prepared as previously described (9). The 2-day and 6-day cDNA probes were made from 2-day and 6-day poly(A) RNA, respectively, as described (14). The filter papers were prehybridized and hybridized with 2-day probe at 42°C for 36 h. After exposure to the X-ray film, the 2-day probe was washed off and the filters were then hybridized with the 6-day probe.

Ligninase peptide sequencing and oligonucleotide probe construction. Tryptic peptides of ligninase (6) were prepared as described by Giegel et al. (15). Peptides were manually sequenced by the Edman degradation procedure as described by Tarr (16). Amino acids were identified as their phenylthiohydantoin derivatives using an HPLC detection system as described by Swenson et al. (17). Oligonucleotides were synthesized using an Applied Biosystems 380A DNA Synthesizer utilizing the method of Caruthers (18).

Hybridization with oligonucleotide probes. The HATF filter paper containing cloned cDNA prepared as above or nitrocellulose paper harboring fragments of recombinant plasmids was prehybridized at 37°C overnight (9), and hybridized with the synthetic probe at 25°C for 1 h. The paper was then washed once in 6 X SSC/0.05% sodium pyrophosphate for 30 min at 25°C and once in the same solution for 10 min at 42°C or 30°C (for probe 14.2 only). The oligonucleotide probes were end-labeled with T4 polynucleotide kinase.

Other procedures. Plasmid isolation was carried out using the rapid alkaline procedure (19). The digestion of plasmid DNA, the electrophoresis of DNA on agarose gel and the transfer of DNA fragments from agarose gel to nitrocellulose paper were carried out as described by Maniatis et al. (9).

**RESULTS AND DISCUSSION**

Synthesis of oligonucleotide probes. Several tryptic peptide fractions of the main ligninase protein H8 (22) were sequenced to find a consecutive series of low-redundancy amino acids suitable for probe construction. Two such peptide fractions, 14 and 25B, were found (see below) and three oligonucleotide probes (14.1, 14.2 and 25) deduced from the amino acid sequences in these peptides were constructed. Due to the redundancy of the genetic code each probe consists of a mixture of 32 different oligonucleotide sequences.

**Peptide Fraction 14:**

| | |
|---|---|
| Leu-Gln-Lys-Pro-Phe-Val-Gln-Lys | Amino Acid Sequence |
| QUN-CAP-AAP-CCN-UUQ-GUN-CAP-AAP | Corresponding mRNA |
| GTQ-TTQ-GGN-AAP-CA* | Probe 14.1 Sequence |
| AAP-CAN-GTQ-TTQ | Probe 14.2 Sequence |

**Peptide Fraction 25B:**

| | |
|---|---|
| Leu-Val-Phe-His-Asp-Ala | Amino Acid Sequence |
| QUN-GUN-UUQ-CAQ-GAQ-GCN | Corresponding mRNA |
| CAN-AAP-GTP-CTP-CG | Probe 25 Sequence |

Differential hybridization. It has been known that ligninase activity is not detectable in 2-day cultures, but relatively high levels of activity are found in 6-day cultures of P. chrysosporium grown in low N medium (6-7). Thus, differential hybridization of the cDNA library with the 2-day and 6-day probes (see Materials and Methods) should allow us to isolate cDNA clone(s) specific

*N = AGCT/U, P = AG, Q = CT/U


Figure 1. Differential hybridization and identification of cDNA clone for ligninase. Hybridization of cDNA clones with 2-day probe (A), 6-day probe (B), or synthetic probe 14.1 (C) was performed as described in Materials and Methods. A representative cDNA clone for ligninase in each hybridization is indicated by arrow.

for the enzymes involved in secondary metabolism such as ligninase. Therefore, all clones of the cDNA library were hybridized with 2-day and 6-day cDNA probes and about 850 clones specific for 6-day mRNA were isolated.

Identification of cDNA clones for ligninase. Four ligninase cDNA clones, designated pCLG3, pCLG4, pCLG5 and pCLG6, were identified after screening the above mentioned 850 clones with the oligonucleotide probe 14.1. These four clones were confirmed to be unique to 6-day mRNA by doing a second differential hybridization. One representative clone is shown in Fig. 1. Recombinant plasmid DNA from each of the clones was digested individually with restriction enzymes PstI or BamHI-HindIII. The fragments were electrophoretically separated on 1% agarose gel, transferred to nitrocellulose paper and were then hybridized with the synthetic probes 14.1 and 25. Ethidium bromide stained agarose gels and the results of hybridization (Fig. 2) showed that there was a PstI site in the cDNA insert of each of the four clones and that each of the recombinant plasmids, except pCLG6, contained

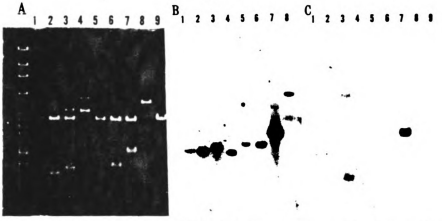


Figure 2. Hybridization of plasmid DNA from ligninase cDNA clones with the synthetic oligonucleotide probes. DNA from each clone was digested with PstI (lanes 1-4) and BamHI-HindIII (lanes 5-8), respectively, subjected to electrophoresis on 1% agarose gel, stained with ethidium bromide (A), transferred onto nitrocullulose paper and hybridized with probe 14.1 (B) or probe 25 (C) as described in Materials and Methods. Different lanes contained: pCLG3 (lanes 1, 5), pCLG4 (lanes 2, 6), pCLG5 (lanes 3, 7) and pCLG6 (lanes 4, 8). Vector pUC9, digested with HindIII, was loaded on lane 9 as a negative control.

three PstI sites. Plasmid pCLG6 appears to have lost the PstI site at the junction of the vector with the cDNA insert and, therefore, yields only two PstI fragments (Fig. 2A). Note that none of the cDNA clones were completely digested by restriction enzyme PstI even when 5-fold excess of enzyme was added. This incomplete digestion may be due to dC-dG tailing used in cDNA library construction. The PstI digestion resulted in the generation of one cDNA fragment from each clone which hybridized with probe 14.1 (Fig. 2B). None of the four clones contained BamHI site in their respective cDNA inserts. Cloned cDNA insert in pCLG3, pCLG4 and pCLG6 each contained one HindIII site, whereas that in pCLG5 lacked this site (Fig. 2A). Plasmid pCLG6 gave only two fragments on BamHI-HindIII digestion (the smaller fragment is not visible in Fig. 2A), suggesting that this plasmid has lost not only a PstI site (see above) but also the BamHI site. Thus, BamHI-HindIII digestion resulted in the generation of three fragments from clones pCLG3 and pCLG4 and two fragments from pCLG5 and pCLG6. One of the BamHI-HindIII fragments from each clone hybridized with probe 14.1. Only one of the four cDNA clones, pCLG5, hybridized with probe 25 (Fig. 2, C). A comparison of the hybridization patterns of pCLG5 with probes 14.1 and 25 showed that the small fragment of

the cDNA insert from PstI digestion hybridized with probe 25, whereas the larger one hybridized with probe 14.1 (lane 3 in Fig. 2B and C, respectively). Probe 14.2 showed the same hybridization pattern as probe 14.1 for pCLG5 but showed no hybridization with the other three clones (data not shown). These hybridization results indicate that the ligninase cDNA clone, pCLG5, corresponds to the main ligninase protein H8 described above.

Synthetic oligodeoxyribonucleotides are widely employed as hybridization probes for the isolation of desired cloned DNA sequences (20). Oligonucleotides are known to hybridize at specific sites in cloned DNA and mixtures of oligonucleotides are used to screen for the desired clone with greater confidence (20). In this study, we have shown that specific synthetic oligonucleotide probes, corresponding to partial amino acid sequences of ligninase, hybridized strongly to different cDNA clones. The washing temperatures of 42°C (for probe 14.1 and 25) and 30°C (for probe 14.2) used for washing hybridized filters fall within the range of washing temperatures predicted by the equation of Suggs et al. (21). These results together with the other data presented above lead us to conclude that pCLG3, 4, 5 and 6 are indeed ligninase cDNA clones.

It has recently been shown that there are several proteins showing ligninase activity in the extracellular culture fluid of P. chrysosporium (22). Antibody raised against the main ligninase protein, H8, was shown to react with the other ligninase species as well (22). These data suggest that these ligninase species might either be the products of the same gene, but have undergone different post-transcriptional/post-translational modifications, or are products of different genes with shared homologous sequences which are responsible for the ligninase activity and the antigen-antibody reaction. The size of the cDNA insert in each cDNA clone is comparable: 1.35 kb, 1.42 kb, 1.48 kb and 1.2 kb in pCLG3, pCLG4, pCLG5 and pCLG6, respectively. However, their restriction enzyme digestion patterns are different. From our preliminary results (data not shown), pCLG5 is different from other three clones based on the restriction map and hybridization analysis. For

example, each of the restriction enzymes EcoRI, XhoI and SstI has a recognition site in the pCLG5 insert, but not in the inserts in the other three clones. On the other hand, HindIII and SalI have one site each in the inserts of pCLG3, pCLG4 and pCLG6 but not in that of pCLG5. Nick-translated pCLG3 insert did not hybridize with pCLG5 but did hybridize with the other three cDNA clones. Also, very different hybridization patterns were observed when nick-translated pCLG3 and pCLG5 inserts were hybridized with the different restriction enzyme digests of P. chrysosporium chromosomal DNA (data not shown). These results indicate that the cDNA sequences in pCLG3, pCLG4 and pCLG6 are very similar if not identical and that pCLG3 and pCLG5 represent different genes. Further characterization of the latter two clones, including sequencing, is in progress.

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# APPENDIX B

# USE OF SYNTHETIC OLIGONUCLEOTIDE PROBES FOR IDENTIFYING LIGNINASE cDNA CLONES

By

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# APPENDIX B

## USE OF SYNTHETIC OLIGONUCLEOTIDE PROBES FOR IDENTIFYING LIGNINASE cDNA CLONES

Ligninase, an extracellular, $H_2O_2$-dependent, glycosylated heme protein, has recently been purified from a white-rot basidiomycete, Phanerochaete chrysosporium (1,2). This enzyme is synthesized under nitrogen-limited conditions during secondary metabolism. This enzyme has many potential applications, such as upgrading lignocellulosic materials via delignification for the efficient production of fuels, feeds and chemicals; biobleaching of pupls; treatment of industrial wastes; controlled modification of lignins to produce aromatic chemicals and cracking of petroleum. To better understand the nature, organization, expression and regulation of the ligninase genes and to develop the full bioprocessing potential of this enzyme, we initiated studies to isolate and characterize the cDNA clones for ligninase.

### Principle

Synthetic oligodeoxyribonucleotides are useful as specific probes for the detection and isolation of cloned cDNA or gene sequences of interest (3-5). As a general approach, a chemically synthesized mixture of oligonucleotides whose sequences represent all possible codon combinations, predicted from a partial peptide sequence within a protein, are employed. Therefore, one of the oligonucleotides in the mixture must be complementary to a region of DNA coding for the protein.

Since probes which form duplexes with a single base pair mismatch have significantly less thermal stability than their perfectly matched counterpart, appropriate choice of hybridization temperature or filter wash temperature would virtually elimilate the formation of mismatched duplexes without affecting the formation of perfectly matched ones. Hence, the use of stringent hybridization criteria would allow the selection of the single correct sequence from the mixture.

The basic steps involved in cloning ligninase cDNA from P. chrysosporium are as follows (6): 1) construction of cDNA library using poly(A) RNA from a 6-day-old lignin degrading culture; 2) isolation of cDNA clones specific for 6-day culture using differential hybridization; 3) synthesis of oligonucleotide probes, deduced from partial amino acid sequences of ligninase; 4) use of these probes to screen, isolate and identify the ligninase clones from the 6-day specific cDNA mini-library.

**Isolation of poly(A) RNA**

P. chrysosporium strain BKM-F 1767 (ATCC 24725) is grown in 50 ml of low nitrogen medium (modified to contain 20 mM NaOAc, pH 4.5, instead of 10 mM 2,2-dimethyl succinate) in 500 ml Erlenmeyer flasks (6). Flasks are flushed with pure oxygen at the time of inoculation and reflushed every other day. A modified hot phenol extraction procedure is used for RNA isolation (7). In this procedure, mycelia from 1 liter of a 6-day-old culture are harvested by centrifugation, washed with 50 mM NaOAc (pH 5.2) and suspended in 20 ml of extraction buffer (0.15 M NaOAc pH 5.2, 5% SDS and 2 mM EDTA). The mycelial suspension is then mixed with 10 ml phenol and 25 g glass beads (0.45 mm in size) and blended in an Omni-Mixer (Sorvall) for 20 min. Ten ml of chloroform:isoamyl alcohol (24:1) is added to this mixture and the blending is carried out for an

additional 10 min. The mixture is then heated at 60°C for 15 min while gently shaking and is chilled on ice. After centrifugation (10,400 x g for 20 min at 4°C), the upper phase is extracted with phenol-chloroform (1:1), the RNA is precipitated with ethanol and the pellet is dissolved in 10 ml of 2 mM EDTA. After heating at 65°C for 10 min, the RNA solution is chilled on ice for 10 min and 10 ml of 2 X loading buffer (1 M NaCl, 20 mM Tris.HCl pH 7.5, 2 mM EDTA and 1% SDS) is added. The RNA solution obtained is generally too viscous to pass through the oligo(dT)-cellulose column, hence, it is mixed with oligo(dT)-cellulose powder (BRL, Gaitherburg, MD) and the mixture is gently shaken for 30 min at room temperature. The oligo(dT)-cellulose with the bound poly(A) RNA is then spun down, washed with loading buffer and then packed in a small glass column (10 x 1 cm). The column is washed with loading buffer and the poly(A) RNA is eluted with TES buffer (10 mM Tris.HCl pH 7.5, 1 mM EDTA and 0.2% SDS). For isolating 2-day poly(A) RNA, the same procedure is used except that the RNA solution is directly loaded on the oligo(dT)-cellulose column. Using this procedure, 10 to 20 mg of total RNA is obtained from 1 liter of culture and poly(A) RNA accounts for 1 to 2% of the total RNA.

**Construction of cDNA Library**

The first strand cDNA is synthesized using AMV (avian myeloblastosis virus) reverse transcriptase and the second strand cDNA is synthesized utilizing RNase H and DNA polymerase I of *E. coli* (8). Double stranded cDNA is dC-tailed and then annealed with dG-tailed pUC9 (9) which is digested with restriction enzyme *Pst*I. The annealed DNA molecules are transformed into *E. coli* JM83 (*ara lac-pro strA thi* 80d*lacZ* M15) using the procedure of Hanahan (10). The transformed cells are spread on 2YT

plates (1.6% Bacto-tryptone, 1% yeast extract, 0.5% NaCl and 1.5% agar, pH 7.0) (11) supplemented with 100 μg/ml ampicillin and 40 μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 11). Ten thousand white E. coli colonies that are potential cDNA clones are picked and individually stored in each well of 96-well microtiter plates.

**Differential Hybridization**

Rationale Ligninase has been shown to be produced in 6-day-old idiophasic culture of P. chrysosporium grown in low-nitrogen medium; the enzyme is not detectable in 1- or 2-day-old cultures in primary growth. Therefore, differential hybridization technique allows isolation of the cDNA clones specific for the idiophase. It is much easier to screen such a mini-library of idiophasic clones than to screen the total cDNA library for isolating cloned cDNA of interest.

Procedure Each cDNA clone is replicaplated onto 137 mm HATF filter (Millipore, Bedford, MA) which is placed on LB plates (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl and 1.5% Bacto-agar, pH 7.2; 100 μg/ml ampicillin is added just before pouring plates) and then is grown at 37°C for 14 h. The filter paper is peeled off and dried on 3 MM Whatman chromatography paper for 20 min. One circular 3 MM paper (about 137 mm diameter) is placed in each of four Petri dishes (150 x 10 mm) labeled 1, 2, 3 and 4. The 3 MM papers are saturated with 10% SDS, denaturation solution (1.5 M NaCl and 0.5 M NaOH), neutralizing solution (1.5 M NaCl and 0.5 M Tris.HCl pH 8.0) and 2 x SSPE (0.36 M NaCl, 20 mM $NaH_2PO_4$ and 2 mM EDTA, pH 7.4) in Petri dishes 1, 2, 3 and 4, respectively. The cDNA clones on the HATF filters are lyzed, denatured and neutralized in dishes 1, 2 and 3, respectively, by placing the filter in each of the plates for 5 min (7). The filter is then placed in

dish 4 for 5 min. The cDNA blots are then baked at 80°C for 4 h, wetted with 6 x SSC (7) and then washed in prewashing solution (3 x SSC and 0.1% SDS) at 65°C for 10 h with several changes of the same solution to completely remove all cell debris. After briefly blotting on a 3 MM paper, every eight blots are put in one hybridization bag and 16 ml of hybridization solution (50% formamide, 5 x Denhardt's solution, 1 M NaCl, 10 mM Tris.HCl pH 8.0, 1 mM EDTA, 50 mM $NaH_2PO_4$ pH 6.8, 10 μg/ml carrier DNA) is added. The blots are prehybridized at 42°C overnight, the synthetic 2-day cDNA probe (see below) is added at a final concentration of 1 x $10^6$ cpm/ml and the hybridization is carried out at 42°C for 36 h. The hybridized blots are washed in high stringent solution (10 mM Tris.HCl pH 7.5, 1 mM EDTA, 0.1% SDS, 0.1% $Na_4P_2O_7$ and 50 mM NaCl) three times at room temperature and thrice at 65°C (each wash is for 15 min). The blots are then exposed to X-ray film for a suitable length of time and the film is developed (7). The probe is then washed off at 65°C for 2 h in distilled water and the bolts are hybridized with the 6-day cDNA probe (see below).

The 2-day and 6-day cDNA probes are synthesized, respectively, from poly(A) RNA isolated from 2- and 6-day-old cultures using a modification of the procedure of Berlin and Yanofsky (12). The reaction mixture used for probe synthesis contains in 50 μl: 50 mM Tris.HCl pH 8.3, 8 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 1 mM dATP, dTTP and dGTP, 1 μg oligo-$(dT)_{12-18}$, 1 μg poly(A) RNA, 50 U RNasin and 200 μCi α-$^{32}$P dCTP (~600 Ci/mmole). The reaction is started by adding 300 U M-MLV (Moloney murine leuemia virus) reverse transcriptase (BRL, Gaitherburg, MD). The reaction is carried out at 43°C for 1 h and then 2 μl of 0.5 M EDTA, 10 μl of 10 mg/ml carried DNA and 7 μl of 20% SDS are added. The unincorporated nucleotides are separated from the labeled cDNA by

passing the reaction mixture through a Sephadex-G50 column. The fractions showing the high radioactivity are combined and to this cDNA elute 1/10 volume of 2 M NaOH is added and the mixture is heated at 65°C for 5 min. After chilling on ice for 10 min, 2 M HCl (equal to 1/10 volume of the cDNA elute) is added to neutralize the probe.

Of the 10,000 cDNA clones in our cDNA library, 850 clones are shown to be specific to the 6-day cDNA probe using the differential hybridization technique described above. A representative differential hybridization blot is shown in Fig. 1.

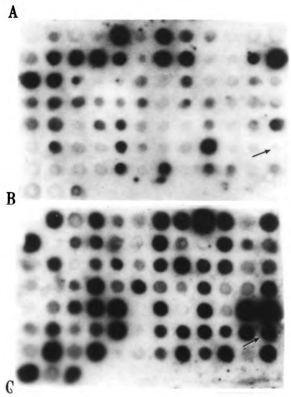


Figure 1. Differential hybridization and identification of a ligninase cDNA clone. The cDNA clones in the library are hybridized with the 2-day cDNA probe (A), 6-day cDNA probe (B), or synthetic oligonucleotide probe 14.1 (C). Note that the ligninase cDNA clone indicated by arrow in each panel shows hybridization with the 6-day cDNA probe but not with the 2-day cDNA probe. From: Y. Z. Zhang, G. J. zylstra, R. H. Olson, and C. A. Reddy, Biochem. Biophys. Res. Commun. 137, 649 (1986).

**Identification of Ligninase cDNA Clones**

Oligonucleotide probes Three oligonucleotide probes deduced from the amino acid sequences of selected tryptic peptides of ligninase H8 (13) are used for screening the mini-cDNA library. The sequences of these probes are shown below:

| | |
|---|---|
| GTQ-TTQ-GGN-AAP-CA | PROBE 14.1 |
| AAP-CAN-GTQ-TTQ | PROBE 14.2 |
| CAN-AAP-GTP-CTP-CG | PROBE 25 |

N = AGCT/U, P = AG, Q = CT/U

All probes are mixture of 32 oligonucleotides because of the redundancy of the genetic code. The oligonucleotides are end-labeled with polynucleotide kinase in a 50 $\mu$l total reaction mixture containing kinase buffer (50 mM Tris.HCl pH 8.0, 10 mM $MgCl_2$ and 15 mM DTT), 500 ng synthetic oligonucleotide (dissolved in water), 100 $\mu$Ci $\gamma$-$^{32}$P ATP (4500 Ci/mmole, ICN, Irvine, CA) and 10 units T4 polynucleotide kinase (IBI, New Haven, CT). The mixture is incubated at 37°C for 30 min and then cooled on ice until use.

Preparation of cDNA blots The cDNA blots are prepared as described above using the 6-day specific cDNA clones.

Hybridization of cDNA blots with synthetic probes The cDNA blots are prehybridized at 37°C for 4 h in a solution containing 6 x SSC, 1 x Denhardt's solution, 0.5% SDS, 0.05% $Na_4P_2O_7$ and 10 $\mu$g/ml carried DNA. The prehybridization solution is drained out and hybridization solution (6 x SSC, 1 x Denhardt's solution, 0.05% $Na_4P_2O_7$ and 20 $\mu$g/ml tRNA), along with probe 14.1, is added. The hybridization is carried out at room temperature for 1 h. The hybridized blots are washed once at room temperature in a solution containing 6 x SSC and 0.05% $Na_4P_2O_7$ for 30

min and once at 42°C in the same solution for 10 min.

Four cDNA clones show strong hybridization with probe 14.1. A representative clone is shown in Fig. 1C.

To further identify these clones, the plasmid DNA isolated from these clones is digested with different restriction enzymes in such a way that each clone gives three unequal fragments, one from the vector and two from the cDNA insert. The fragments are transferred onto nitrocellulose paper using Southern blotting (7) and the DNA blots are hybridized with the above three synthetic probes as described above. The temperature for the second washing for probe 14.2 is at 32°C. The results show that only the cDNA insert in clone pCLG5 hybridizes with all the three probes, whereas the other three clones (pCLG3, pCLG4 and pCLG6) show detectable hybridization with probe 14.1 only (Fig. 2). Furthermore, each of the three probes hybridizes with only one cDNA fragment from a given clone, indicating that the probes are specific for unique sequences in the cDNA. Additional studies show that the product expressed by the cDNA insert of pCLG5 is immunoreactive with the ligninase (H8) antibody, indicating that the cloned insert in pCLG5 is ligninase cDNA. The sequence analyses show that the probe sequences used for identifying ligninase cDNA clones are present in both pCLG4 and pCLG5.

The procedure described above for isolating the ligninase cDNA of P. chrysosporium is potentially applicable for isolating the ligninase genes from other organisms. Besides, the procedure described can be used to isolate genes for other secondary metabolic enzymes such as glucose oxidase which has recently been purified from P. chrysosporium (14).

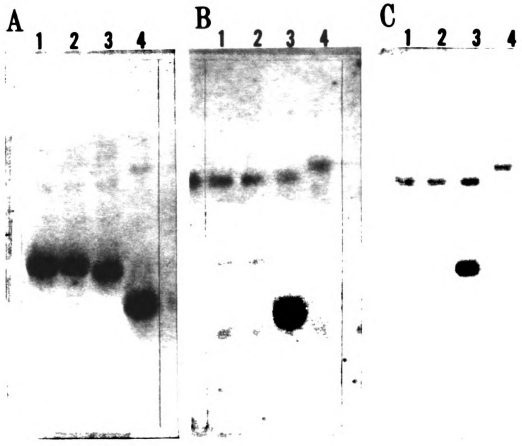


Figure 2. Hybridization of ligninase cDNA clones with three oligonucleotide probes. Clones pCLG3 and pCLG4 are digested with BamHI and HindIII, pCLG5 is digested with BamHI, HindIII and PstI, and pCLG6 is digested with HindIII and PstI. The DNA blots are hybridized with probe 14.1 (panel A), 14.2 (panel B) and 25 (panel C). Different lanes contain: pCLG3 (lane 1), pCLG4 (lane 2), pCLG5 (lane 3) or pCLG6 (lane 4).

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