Characterization of the
Pycnoporus cinnabarinus and *Dichomitus squalens*
Lignin Degrading Systems

Frédéric H. Périé

M.S., Université Technologique de Compiègne, 1986

A dissertation submitted to the faculty of the

Oregon Graduate Institute of Science & Technology

in partial fulfillment of the

requirements for the degree

Doctor of Philosophy

in

Molecular Biology

July 1994

The dissertation "Characterization of the *Pycnoporus cinnabarinus* and *Dichomitus squalens* lignin degrading systems" by Frédéric H. Périé has been examined and approved by the following examination committee:

_____

Michael H. Gold, Thesis Advisor
Professor

_____

Ninian J. Blackburn
Professor

_____

David R. Boone
Professor

_____

V. Renganathan
Associate Professor

ACKNOWLEDGMENTS

I wish to express my deepest and warmest thanks to:

Professor M. H. Gold for his constant help and his inspiring thoughts, and for letting me benefit from his thorough expertise in lignin biodegradation.

Professor N. J. Blackburn for his help and thorough expertise in copper protein chemistry and characterization.

Professor V. Renganathan for helpful discussions and thorough expertise in lignin-degrading enzymes characterization.

Professor D. R. Boone for his consideration and helpful advice.

Dr. M. Alic for her help and advice for DNA characterization and her patient proofreading of my manuscripts.

Dr. H. Wariishi for sharing his expertise in peroxidases characterization and fostering high scientific spirit.

Ms. N. Christie, Ms. T. Hadfield, and Ms. B. Kyler for their extensive assistance in the preparation of this dissertation.

To every person I have interacted with during this dissertation, in particular Professor Gold's team.

I wish to express my deepest thanks to Dr. J. L. Seris, Head of the Department of Biotechnology and Environment at GRL - Elf Aquitaine, for his constant support and understanding. Without him, this work would not have been possible.

To Françoise.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Characterization of the
Pycnoporus cinnabarinus and *Dichomitus squalens*
Lignin Degrading Systems

Frédéric H. Périé Ph.D

Supervising Professor: Michael H. Gold

The degradation of lignin by two white-rot fungi, *Pycnoporus cinnabarinus* and *Dichomitus squalens*, was investigated and their ligninolytic systems were partially characterized.

Lignin degradation and MnP production by *D. squalens* were strictly dependent on the presence of Mn in the culture medium suggesting that MnP is an important component of the *D. squalens* lignin degrading system. In contrast with *Phanerochaete chrysosporium*, MnP production by *D. squalens* is not regulated by medium nitrogen.

Ion exchange chromatography revealed that *D. squalens* produced two MnP isozymes characterized as heme-containing glycoproteins, with M_rs of 46,000 and 48,500. UV-visible spectra of the isozymes in their native form, complexed with cyanide, reduced (ferrous), and reduced and complexed with CO, as well as the spectra of the intermediates involved in the peroxidative cycle, closely resembled those of *P. chrysosporium* MnP. The activities of both isozymes were stimulated by α-hydroxy and dicarboxylic acids. Both isozymes oxidized Mn^{II} to Mn^{III}.

Purification of the laccase produced by *P. cinnabarinus* and *D. squalens* revealed that each enzyme was produced as a series of two or more isozymes. Characterization of the laccases indicated that the enzymes contained four moles of copper per mole of protein. The M_r of the isozymes was in the range of 60,000 to 62,000 for *P. cinnabarinus* isozymes and 64,000 to 66,000 for *D. squalens* isozymes. Spectral characterization suggested that the enzymes contained one type 1 copper, one type 2 copper and two type 3 coppers. Both the *P. cinnabarinus* and *D. squalens* enzymes oxidized phenolic model compounds.

P. cinnabarinus produced a MnP and a laccase when cultured on a lignocellulosic, semi-solid medium, but only a laccase when cultured in a liquid medium. Studies of lignin degradation in liquid and solid cultures of *P. cinnabarinus* indicated that laccase alone did not initiate the mineralization of side-chain and ring-labeled lignin substructures. In contrast, the presence of MnP, in lignocellulosic media or added exogenously to liquid cultures, allowed the depolymerization of lignin. These results provide evidence that MnP is directly involved in lignin depolymerization.

CHAPTER 1
INTRODUCTION

1.1   Structure and Distribution of Lignin

Plant cell walls are complex, heterogeneous structures composed mainly of the polymers cellulose, hemicellulose and lignin. Knowledge of the biodegradation of these polymers is evolving rapidly. Yet important aspects of these processes remain unknown [1]. Deciphering the distribution of lignin within the cell wall and its biogenesis and biodegradation was undertaken more than a century ago [2] when Anselme Payen showed that encrusting matter could be separated from the fibrous matrix by treatment with concentrated nitric acid [3]. Since then, numerous studies have described the lignification of vascular plant cell walls, the structure of the encrusting matter [2,4–13], and the biodegradation of lignin by specific organisms and their enzymes [14–18].

1.1.1   Distribution of lignin in vascular tissues

Lignin is found in the cell walls of all vascular plants and in certain primitive plants, such as ferns and club mosses, but not in true mosses and algae which do not have tracheids [7,18].

Plant tissues are differentiated to serve various functions including photosynthesis and growth, structural integrity, and transport of fluids. Xylem or vascular tissues contain a high level of lignin distributed with hemicellulose in the spaces of intercellulose microfibrils, accounting for 20 to 30% of the dry weight of the plant [7]. Lignin is found within the rigid cell wall of wood fibers in the middle lamellae and in different layers of the primary and secondary walls [9,19,20]. Lignin accumulation begins in the primary wall at the corners of the cells and at the pit borders, and extends to the middle lamellae and toward the lumen through the S1, S2 and S3 layers of the secondary wall [19–21]. The primary wall is lignified from the early phase of S1 cellulose deposition to the early phase of S2 thickening. The secondary wall is lignified separately after S1 cellulose deposition is completed.

The formation of lignin corresponds to a complex series of events, including lignin precursor synthesis in the cytoplasm, transport of monomers as glycosides or in the free state to the maturing cell wall, and polymerization of lignin within the cell walls. Several of the enzymes involved in this process (e.g., transmethylase, hydroxylase and reductase) are present as a series of unique isozymes in angiosperms and gymnosperms. The differences in activity or affinity for the substrates of these enzymes lead to specific reaction products, and are consequently responsible for differences observed in the struture and chemical composition of lignin in angiosperms, gymnosperms, and grass [22].

1.1.2 Structure of lignin

Lignin is a three-dimensional phenylpropanoid polymer linked by a variety of different carbon/carbon and ether linkages between monomeric phenyl-propane units. Its structure was elucidated through organic chemical and UV microscopic analyses. However, a complete understanding of lignin structure became possible only after the production of a pure extract of wood lignin. In 1954, Bjorkman prepared milled wood lignin [23], which is still considered the best lignin preparation. The mechanism of polymer formation was established by Freudenberg and Ertman and involves dehydrogenation reactions of precursors such as coniferyl alcohol, or related compounds such as isogeunol [8,10,13].

Structural analyses of isolated lignin led to studies of its heterogeneity, yielding several classes of lignins identified by their structural elements. The polymer arises from enzyme-initiated oxidation of three phenolic precursors: p-coumaryl, coniferyl, and sinapyl alcohols.

The distribution and specificity of enzymes involved in lignin biosynthesis lead to selective formation of lignin characteristic of gymnosperms, angiosperms, or grass. Softwood lignin, mostly found in gymnosperm, is regarded as compact and polymerized whereas hardwood lignin, found in angiosperms, contains more methoxyl groups and is considered less polymerized. The presence of methoxyls prevents polymerization of precursors by steric hindrance. The structures for spruce and beech lignin have been presented by Freudenberg [24], Adler [2], and Nimz [25]. These depict typical chemical linkages between monomers and their statistical contribution to the whole polymer.

1.2   Biodegradation of Lignin

Most of the carbon/carbon and ether linkages between the phenyl-propanoid monomers of lignin are not readily hydrolyzable, rendering lignin very resistant to microbial degradation. Only a relatively few organisms are capable of partially or completely degrading lignin.

1.2.1   Bacteria

Bacterial lignin degradation has been most extensively studied in actinomycetes, particularly *Streptomyces* spp. Tissues of spruce, maple and other angiosperms are slowly degraded by these bacteria. *Streptomyces viridosporus* and *Streptomyces setonii* caused a 32% to 44% loss of lignin as estimated by chemical analysis of their insoluble residues [26]. Characterization of products isolated from *S. viridosporus*-degraded spruce lignin indicated oxidative alterations similar to those reported for white-rot fungi. Quantitative analyses of lignin degradation by *Streptomyces* using ^{14}C-labeled lignin (DHP) suggested that no more than 20%, often no more than 8%, of the lignin is degraded and converted to $^{14}CO_2$. Other genera of actinomycetes such as *Nocardia* exhibit various abilities to degrade ^{14}C-DHP and grass lignin. After 15 days, 14.1% of labeled methoxyl groups, 9.5% of propyl side chains, and 7.6% of aromatic rings were degraded to $^{14}CO_2$ by *Nocardia autotrophica* [18]. Apparently, bacteria are mostly able to oxidize and mineralize low-molecular-weight compounds, such as dimeric or tetrameric lignin-related compounds, which presumably are able to enter the bacterial cell and to be nonspecifically degraded. Anaerobic degradation of lignin has been reported and is apparently limited by the size of the substrate as well.

1.2.2   Fungi

Several classes of fungi reportedly degrade lignin.

1.2.2.1   Soft-rot fungi

Soft-rot fungi, consisting mostly of ascomycetes and deuteromycetes, cause a softening of wood tissues. Although ^{14}C-DHP mineralization has been observed [27], polysaccharides seems to be the preferred wood polymer metabolized by most of these fungi. The comparisons of *Graphium*, *Monodictys*, *Paecilomyces*, *Papulospora*, and *Allescheria* species for lignin degradation showed that these soft-rot fungi were poor lignin

degraders. *Paecilomyces* and *Allescheria* species were the only soft-rot fungi apparently capable of degrading lignin faster than cellulose [27]. Little is known about mechanisms under which lignin is degraded by these fungi. They apparently demethylate lignin by removal of the methoxyl groups and oxidize rapidly phenolic-related lignin compounds, but they poorly depolymerize synthetic lignin (DHP) [28,29].

1.2.2.2 Brown-rot fungi

Most brown-rot fungi belong to the class of basidiomycetes [30]. These fungi are usually described as wood-rotting fungi that preferentially degrade carbohydrates (cellulose and hemicellulose) and leave a brown residue of modified lignin [31]. This lignin is poorly depolymerized [28,32] and is almost equal in weight to the lignin in sound wood. The main result of brown rot is a decreased methoxyl content [32,33] and an increased phenolic hydroxyl content in the degraded lignin [32,34]. The modified lignin also contains a higher amount of carboxyl and conjugated carbonyl groups, responsible for the dark color [34].

1.2.2.3 White-rot fungi

Fungi belonging to this group are able to efficiently degrade all major components of wood and are considered to be the main agents of lignin decomposition in nature. They apparently degrade lignin more rapidly and more extensively than other microbial groups. Like other filamentous fungi, especially brown-rot fungi, they invade the lumen of wood cells where they secrete enzymes that degrade lignin and other wood components. Colonization of lignified tissues in wood begins with the vessels and ray cells and extends to the fiber. Electron microscopic features of wood decay reveal that lignin is degraded at a distance from the hyphae and is removed progressively from the lumen toward the middle lamella [35,36]. Separation of cells within or adjacent to the middle lamella has also been reported. During its mineralization, lignin undergoes a number of oxidative changes including $C\alpha$-$C\beta$ bond cleavage and, at the $C\alpha$-$C1$ position of the alkyl-phenyl linkage, C-O bond cleavage at β-ether linkages in aryl-ether structures and the aromatic ring opening. Other oxidative reactions, including demethylation, decarboxylation, and $C\alpha$ oxidation, also occur during lignin degradation. Progressive depolymerization releases a wide array of low-molecular-weight fragments, most of them smaller than 1 kDa.

1.2.2.4 *Phanerochaete chrysosporium*

Extensive studies of lignin degradation have been carried out with *Phanerochaete chrysosporium*. Studies by Kirk and coworkers showed that lignin degradation by this fungus was an enzymatic process occurring during the secondary metabolic phase of its growth [37]. In defined media, lignin degradation is triggered by nitrogen, carbon, or sulfur starvation, nitrogen starvation reportedly being most efficient [38]. Growth on spruce milled wood lignin was negligible in the absence of a readily utilizable carbon source such as cellulose or glucose. In addition, lignin degradation was also dependent upon the presence of an alternate carbon source. These results were confirmed with other lignin sources indicating that *P. chrysosporium*, as well as other fungi, would only degrade lignin in the presence of a readily utilizable carbon source. Apparently, an additional carbon source is required to provide sufficient energy to synthesize enzymes and cosubstrates, such as hydrogen peroxide, intermediates or secondary metabolites (e.g., veratryl alcohol) involved in the degradation of lignin. Further studies indicated that oxygen plays a critical role in lignin degradation and that a pure oxygen atmosphere dramatically enhanced lignin degradation by *P. chrysosporium* [38–41]. Direct correlation between oxygen partial pressure and lignin peroxidase production has been reported [42].

1.2.2.5 *Pycnoporus cinnabarinus*

This basidiomycete belongs to the family of *Polyporaceae*. Three different but related species have been described in this genus: *Pycnoporus cinnabarinus*, *Pycnoporus sanguineus*, and *Pycnoporus coccineus*. *P. cinnabarinus* is mostly found in temperate zones, in Europe and North America, whereas *P. coccineus* is found in Asia, and *P. sanguineus* is found in the inter-tropical zone. The species names relate to the abundant production of red pigments that color their fruiting body vermillon, dark red, and brown. All three are white-rot basidiomycetes and degrade lignin under selected conditions [43]. Studies on lignin-degrading enzymes secreted by *P. cinnabarinus* suggest that it produces at least one laccase and possibly a manganese peroxidase, although no direct evidence has been provided to support this result [44]. The strain IFO 6139 of this fungus has been selected for its abundant production of laccase and the apparent absence of peroxidase activity. *P. cinnabarinus* has also been extensively studied for the production of several polysaccharide-hydrolyzing enzymes, among them an α-galactosidase [45,46].

1.2.2.6 *Dichomitus squalens*

Previously named *Polyporus anceps* (Peck) [47] and *Trametes squalens*, *Dichomitus squalens* (Karst) Reid is a white-rot homobasidiomycete belonging to the family of *Polyporaceae* [47,48]. This fungus has been studied for its ability to degrade the components of wood and straw for fundamental and applied purposes. It has been shown to actively degrade lignin including lignin of aspen [49], oak, spruce, beech [50], and maple woods [51], and straw [50,52] in solid or liquid cultures. Reports indicate that *D. squalens* produces at least one laccase [53], but no lignin peroxidase could be detected [44,54]. A preliminary report of manganese-dependent peroxidase activity has appeared [44]. More in-depth studies have been carried out on the polysaccharide-hydrolyzing enzymes produced by this fungus; several cellulase-related enzymes have been described including 1,4-β-D glucanase, cellobiohydrolase [55], endo-glucanases [56], xylanase [57] and an arabinofuranosidase [58]. No description of the laccase or peroxidases possibly secreted by this fungus have been published.

1.3 Enzymes Involved in Lignin Biodegradation

Since the description of Bavendamm's reaction at the beginning of this century [59], lignin degradation by fungi has been correlated with their ability to oxidize phenols [60], causing the appearance of dark color around cultures of lignin-degrading fungi. Two major groups of oxidative enzymes involved in lignin degradation have been described: polyphenol oxygen oxidoreductase (usually called laccase) and several peroxidases active on phenolic or non-phenolic compounds.

The appearance of extracellular hydrogen peroxide in culture media of *P. chrysosporium* just before the fungus begins to degrade lignin, along with the rate of H_2O_2 production following lignin degradation, suggested that hydrogen peroxide could be involved in the oxidative process and, therefore, that peroxide-dependent enzymes could play a role in lignin degradation [61].

1.3.1 Hydrogen peroxide-generating enzymes

Several hypotheses have been proposed to explain the formation of extracellular hydrogen peroxide during lignin biodegradation. Extracellular manganese peroxidase has been shown to generate H_2O_2 in the presence of reducing agents such as NAD(P)H, glutathione, dithiothreitol, or dihydroxy

fumaric acid [62–64]. It was also reported that *P. chrysosporium* produces extracellular NAD(H) and NADP(H) [65]. However, the suggested mechanism appeared to be very energy-consuming, and the mechanism for NAD(H) or NADP(H) secretion remains to be described. A more convincing candidate for extracellular hydrogen peroxide production is a glyoxal oxidase identified by Kersten and Kirk in ligninolytic cultures of *P. chrysosporium* [61]. This enzyme oxidizes glyoxal and its methyl derivative as well as other α-hydroxy carbonyl and dicarbonyl compounds coupled with the reduction of oxygen to hydrogen peroxide. A third possibility is the veratryl alcohol oxidase found in culture media of the white-rot basidiomycete *Pleurotus sajor-caju* [66]. The enzyme couples the two-electron oxidation of the benzyl alcohol group including veratryl alcohol with the reduction of oxygen to hydrogen peroxide. Similar enzymes have been described in cultures of other basidiomycetes, but their role in lignin degradation and hydrogen production has not been demonstrated [67].

1.3.2 Peroxidases

In 1983, Gold's and Kirk's groups simultaneously announced the identification and the partial characterization of an extracellular hydrogen peroxide-requiring enzyme involved in lignin degradation [68,69]. This enzyme was named ligninase or lignin peroxidase (LiP) after its described function. Its purification to homogeneity revealed that it is a single-chain glycoprotein, with a molecular mass of 41–42 kDa, containing one protoporphyrin IX per molecule [70,71]. The enzyme exists as a series of isozymes with pIs ranging from 3.2 to 4.0 [72–75]. A second peroxidase was described a year later by Gold's group and is now referred to as manganese peroxidase (MnP) [62]. Its purification to electrophoretic homogeneity indicated that MnP is a 46-kDa glycoprotein containing one protoporphyrin IX per molecule [62]. This enzyme also exists as a series of isozymes with pIs ranging from 4.2 to 4.9 [62,63,71]. Subsequently, LiP or MnP enzymes have been identified and characterized in the extracellular medium of other white-rot basidiomycetes. LiP has been found in extracellular culture media of *Coriolus versicolor* [76], *Bjerkandera adusta* [67], *Phlebia radiata* [77–79], and *Panus tigrinus* [80]. MnP has been identified in extracellular media of *Lentines edodes* [81,82], *C. versicolor* [83], *Pleurotus ostreatus* [67], *P. radiata* [77], and *Rigidoporus lignosus* [84].

1.3.2.1 Spectral characterization of LiP and MnP

Table 1.1 Electronic absorption spectral maxima of HRP,
P. chrysosporium LiP, and *P. chrysosporium* MnP

System	Absorption Maxima (nm)			Ref.
Ferric native				
LiP, pH 4.5	408	500	632	70
MnP, pH 4.5	406	502	632	62
HRP, pH 4.5	403	500	641	85
Ferric, low-spin				
CN^--LiP	423	540		70
CN^--MnP	421	546		62
CN^--HRP	422	539		85
N_3^--LiP	418	540	575	70
N_3^--MnP	417	542	580	62
N_3^--HRP	416	534	565	85
Ferrous				
LiP	435	556		70
MnP	433	554		62
HRP	437	556		85
Ferrous, low-spin				
CO-LiP	420	535	568	70
CO-MnP	423	541	570	62
CO-HRP	423	541	575	85

The spectral and structural characterization and the substrate specificity of these enzymes have been carried out most thoroughly with enzymes produced by *P. chrysosporium* (Table 1.1). Both enzymes were shown to be heme proteins containing a single protoporphyrin IX prosthetic group per molecule of protein. It was also shown that they require hydrogen peroxide for their catalytic activities [62,63,71,74]. Identified as true peroxidases, spectral and structural analyses of LiP and MnP were carried out by analogy with well-studied horseradish peroxidase (HRP). Extensive studies on HRP demonstrated that this enzyme is a heme-containing peroxidase, with one protoporphyrin IX per molecule. In the native enzyme, iron, in the ferric state of oxidation [85], is pentocoordinated to four pyrrole nitrogen atoms and one histidine residue

belonging to the protein backbone referred to as the proximal histidine [86]. The sixth coordination remains empty in native HRP [87].

The spectral characterization of *P. chrysosporium* LiP and MnP allows comparison of the spectra of the fungal peroxidases with the spectra of the plant peroxidase. The electronic absorption spectra of native LiP and MnP are characteristic of high-spin ferric hemoproteins, with Soret and visible maxima at 408, 500 and 632 nm [62,70], similar to those of HRP. Like HRP, native LiP and native MnP form typical low-spin hexacoordinate complexes with CN^- and N_3^- [62,70], different from those of cytochrome P_{450} low-spin complexes [88]. The spectra of the reduced enzymes are typical of high-spin, pentacoordinate, ferrous hemes, confirming the similarities of LiP and MnP spectra with those of other plant peroxidases and HRP in particular. The ferrous CO complex spectra confirm that the fifth ligand of the heme iron is not a cysteinate in LiP nor in MnP [88] and, therefore, that these enzymes do not share the same type of Fe ligation with cytochrome P_{450}. EPR spectra of LiP [89] and MnP [90] confirmed that their hemes are high-spin ferric hemes, but indicate that the symmetry of the complex is somewhat higher in LiP and MnP than HRP since the EPR signal corresponds to an axial heme symmetry. The HRP EPR signal is typical of a rhombic signal [91], suggesting a more distorted geometry of the heme.

1.3.2.2 Catalytic cycle of peroxidases

One common feature of the plant peroxidase catalytic cycle is the formation of compound I in the two-electron oxidation of the native enzyme by hydrogen peroxide or organic peroxides [85,92] (Table 1.2). The subsequent reduction of compound I to the native enzyme occurs through formation of an intermediate (compound II) resulting from the one-electron reduction of compound I. It was demonstrated by Dolphin et al. [93] that, with the exception of CCP [94,95], compound I contains a π-cation radical and that the iron is in the Fe^{IV} state. It has been shown that compound II contains Fe^{IV} [96] and, consequently, that the reduction of compound I occurred at the porphyrin ring [85]. The normal peroxidative catalytic cycle is described as follows:

$$HRP + H_2O_2 \rightarrow HRPI + H_2O$$
$$HRPI + AH_2 \rightarrow HRPII + \cdot AH$$
$$HRPII + AH_2 \rightarrow HRP + \cdot AH + H_2O$$
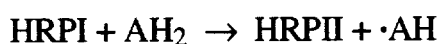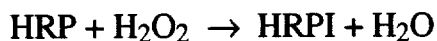
where AH_2 is the reducing substrate and $\cdot AH$ is the free radical product. The catalytic cycle of HRP oxidizes two molecules of substrate per equivalent of peroxide. Depending upon its chemistry, the free radical product may dimerize or attack another species, acting as an intermediate in the process of oxidation.

Table 1.2 Absorption maxima of oxidized intermediates of HRP, *P. chrysosporium* LiP and MnP

System	Absorption Maxima (nm)				Ref.
Native					
HRP	403	500		641	85
LiP	408	500		632	70
MnP	406	502		632	62
Compound I					
HRP	400	557	622	650	92
LiP	408	550	608	650	99
MnP	407	558	617	650	100
Compound II					
HRP	420	527	554		92
LiP	420	525	556		99
MnP	420	528	555		100
Compound III					
HRP	413	546	583		92
LiP	419	543	578		99
MnP	417	545	579		100

The spectral characterizations of the oxidized states of HRP, LiP and MnP also confirm the similarity among LiP, MnP and HRP prosthetic groups.

1.3.2.3 Compound III

Another state of oxidation, HRPIII, was described when HRP was submitted to a large excess of hydrogen peroxide. The oxidation state of HRPIII was found to be 3 equiv above the native state [97,98]. It is now known that compound III can be formed through three reaction paths:

(1) Reaction of compound II with H_2O_2.

(2) Binding of oxygen to the ferrous enzyme.

(3) Binding of the superoxide ion to the native peroxidase.

This oxidation state is not part of the catalytic cycle, but may be involved in the activation of oxygen by the ferrous enzyme and in the formation of superoxide when H_2O_2 reacts with the ferryl enzyme. Its spectral features (shift of the Soret band and additional visible bands) are characteristic of a peroxidase.

1.3.2.4 Substrate specificity

LiP oxidizes non-phenolic, electron-rich aromatic compounds by a single-electron transfer mechanism yielding aryl cation radicals [74,101–105]. The ensuing reactions of aryl cation radicals include $C\alpha$-$C\beta$ cleavage, demethoxylation, β-ether-bond cleavage, hydroxylation of benzylic alcohols, decarboxylation, aromatic ring cleavage, and formation of quinones [14,17,18,106]. The diversity of the reactions may explain the nonspecific action of LiP on lignin model compounds and may indicate the extent of its role in lignin degradation. Until recently, no direct depolymerization of lignin by purified LiP could be demonstrated [107]. However, work by Hammel and Moen in 1991 indicated that crude LiP preparations could catalyze the partial depolymerization of a guaiacyl/syringyl-type lignin [108].

The principal role of MnP is the oxidation of Mn^{II} to Mn^{III} [64]. This highly reactive intermediate is stabilized by chelators such as α-hydroxy-carboxylic and dicarboxylic acids [109] and diffuses from the enzyme active site [64] to attack and oxidize lignin in situ [110]. Mn^{III} is then able to oxidize a variety of organic compounds including phenols [62–64], phenolic lignin model compounds [111], and aromatic amines. Subsequent reactions include $C\alpha$-$C\beta$ bond cleavage of the phenolic $\beta 1$ lignin model dimer and $C\alpha$ oxidation [111]. In the presence of thiol groups, MnP has been shown to catalyze the oxidation of non-phenolic aromatic compounds such as veratryl, anisyl or benzyl alcohols as well as the $C\alpha$-$C\beta$ bond cleavage of a non-phenolic β-aryl-ether lignin dimer through the formation of a thiyl radical [112]. This may broaden the role of MnP in lignin degradation, though it is uncertain that extracellular thiols could play a significant role in the lignin degradation process by white-rot basidiomycetes. Other reactions catalyzed by MnP include the oxidation of NADPH, glutathione, dithiothreitol, and dihydroxymaleic acid. These oxidations occur in the absence of hydrogen peroxide, suggesting that MnP is using molecular oxygen as an oxidizing agent. The reaction results in the production of hydrogen peroxide [63].

1.3.3 Laccases

Benzene-diol oxygen oxido-reductase (EC 1.10.3.2) was first identified at the end of last century in the lacquer tree (*Rhus vernicifera*) [113] and named laccase after the tree. A similar enzyme was subsequently identified in most basidiomycetes and several other classes of fungi. Laccase also is produced by the ascomycetes *Aspergillus nidulans* [114], *Neurospora crassa*, [115] and *Podospora anserina* [116], and by the deuteromycete *Botritys cinerea* [117]. Only a limited number of basidiomycetes such as *P. chrysosporium* do not secrete any detectable laccase.

Characterization studies indicate that laccases are glycoproteins with M_r values ranging from 59,000 (*Pleurotus ostreatus* [118]) to 140,000 (*R. vernicifera* [119]). One of the laccases produced by *P. anserina* has a M_r of 390,000 and is reportedly a tetrameric protein [116]. The carbohydrate contents vary from 10% for most basidiomycete laccases to 45% for *R. vernicifera* laccase [120]. One of the *B. cinerea* laccases reportedly contains more than 80% carbohydrate [121].

1.3.3.1 Spectral characterization of laccase

This blue copper-containing enzyme has been extensively studied by various spectroscopic techniques including UV-visible and circular dichroism spectroscopy [122–124], EPR [125], NMR [126] and X-ray absorption, and has been compared to other simpler copper-containing blue proteins such as azurin or plastocyanin. Results of these investigations indicate that laccase contains four atoms of copper per molecule. They are spectroscopically different and are referred to as type 1, type 2 and type 3 copper. Laccase typically contains one type 1, one type 2 and two type 3 copper atoms.

1.3.3.2 Type 1 copper

UV-visible spectroscopy shows a strong band at 600 nm responsible for the blue color of the protein. The molar absorption coefficient per type 1 copper is 4000 M^{-1} cm^{-1} to 6000 M^{-1} cm^{-1}. Weaker bands at 450 nm and 800 nm, not always resolved in laccases, also originate from type 1 copper and are more readily detectable in simpler copper proteins such as azurin or plastocyanin [120]. The three bands are associated with charge-transfer and d-d transitions in the near-infrared region. The EPR spectrum of type 1 copper is characterized by g values similar to those of simple copper (II) chelates. The A value of the main hyperfine splitting is approximately one-half of that found for the simple complexes [127]. EPR spectroscopy suggests that type 1 copper is

coordinated in a site with less than axial symmetry as three g values are resolved on spectra recorded at 35 GHz. The X-ray crystal structure of oxidized plastocyanin [128] and azurin [129] show the copper ion coordinated to a cysteine sulfur and to two histidyl imidazole ligands, and also to a distant methionine sulfur atom, in a highly distorted tetrahedral complex. Similarities of laccase spectroscopic properties with these proteins suggest that type 1 copper in laccase might have the same ligands as described for azurin and plastocyanin [120].

1.3.3.3 Type 2 copper

Type 2 copper has no detectable absorption band in the visible wavelength region and EPR parameters ($g_\parallel = 2.24$, $g_\perp = 2.05$, and $A_\parallel = 19.5$) typical for copper coordinated to nitrogen and oxygen ligands in tetragonal complexes [120].

1.3.3.4 Type 3 copper

All laccases contain two copper ions which are EPR non-detectable under most conditions. The pair of antiferromagnetically coupled copper ions show a strong band in the 330-nm wavelength region of the near-UV absorption spectrum with a molar absorption coefficient of 3000 M^{-1} cm^{-1}. Another band at 740–745 nm is also generated by type 3 copper.

Ligand field energy suggests that type 3, as well as type 2, copper has tetragonal geometries and that the two type 3 coppers in laccase are not equivalent [127]. EPR spectroscopy of fluoride- and azide-bound laccase indicates that fluoride binds to type 2 copper, but strongly perturbs the electronic feature of type 3 copper, suggesting that fluoride interacts with type 2 and type 3 copper [127]. Furthermore, competitive binding of azide and fluoride to type 2 and type 3 copper in native and type 2-depleted laccase suggests that azide forms a bridge between one type 3 and the type 2 copper, further suggesting that the oxygen binding site, previously believed to be type 3 copper, is in fact a trinuclear cluster formed by type 2 and type 3 copper [130]. In this structure, as demonstrated by low-temperature, magnetic, circular dichroism studies, azide or molecular oxygen would bridge the type 2 and one of the type 3 coppers [131]. Reduction of the bound oxygen molecule would occur through intramolecular electron transfer, originating from the type 1 copper site. The latter site is reduced by the organic substrate and would, subsequently, reduce type 3 and type 2 copper [132,133].

1.3.3.5 Catalytic cycle of laccase

It is now accepted that the type 1 copper site interacts with the reducing substrate [120] and that the type 2 and type 3 sites are involved in oxygen binding. Many kinetic studies helped elucidate the laccase catalytic cycle [120,132]. The catalytic cycle of laccase includes four one-electron oxidations of the organic substrate and the reduction of one molecule of oxygen to water. In the proposed mechanisms [134–137], type 2 copper participates in the transfer of an electron from type 1 to type 3 copper.

1.3.3.6 The role of laccase in lignin biodegradation

Laccase has a broad substrate specificity. It preferentially oxidizes p-diphenol, but was shown to oxidize a variety of diphenols, triphenols, aromatic amines and phenolic lignin model compounds [31,59,138] to form the corresponding phenoxy radical. However, its role in lignin biodegradation remains unclear and is still debated.

Evidence against its contribution to lignin degradation was provided through specific inhibition studies, indicating that lignin degradation is not prevented when specific laccase inhibitors were added to the fungal culture medium [139]. Work on lignin model compound oxidation by purified laccase suggests that a condensation reaction is favored over depolymerization [140,141].

It is commonly accepted that purified laccase cannot catalyze the oxidation of non-phenolic compounds. Yet, purified laccase can catalyze the oxidative $C\alpha$-$C\beta$ cleavage and ring opening of selected phenolic lignin model compounds [142–144]. Laccase is also responsible for demethylation of methoxy groups [138,145], and decarboxylation of acidic lignin model compounds [146,147], leading to formation of the corresponding quinones, potentially reduced to generate another site for laccase or dioxygenase attack.

Several studies on the degradation of milled wood lignin by purified laccase indicate that laccase catalyzes the molecular weight reduction of the polymer and facilitates its solubilization, rendering it more accessible to the fungus [138,148].

Recent results suggest that laccase oxidizes non-phenolic compounds under selected conditions, involving the presence of a non-natural, radical-forming intermediate [149].

Finally, another study suggests that laccase could mimic MnP activity and generate Mn^{III} chelates [150] shown to be involved in lignin biodegradation in cultures of MnP-producing fungi [62,64].

1.4   Summary of Research

Biochemical and physiological studies on *P. chrysosporium* have demonstrated that two extracellular peroxidases, LiP and MnP, along with an H_2O_2-generating system, appear to be the major components of this organism's lignin degradative system. Yet the individual role of each of the peroxidases has not been described. At the beginning of this work, it was commonly accepted that the major enzyme in lignin biodegradation was LiP, and that phenol oxidases would play a secondary role in this process.

The present work describes the characterization of two lignin degradative systems, different from *P. chrysosporium*'s system, and presents some lignin degradation studies *in vivo* allowing partial characterization of the role of MnP and laccase *in vivo*.

The presence of manganese in the culture medium of *D. squalens* induces the expression of MnP activity. The regulation of MnP expression and lignin degradation under several growth conditions are presented in Chapter 2 (see also ref 151). The expression of MnP appears to be closely correlated with the presence of manganese in the medium. Nitrogen starvation, necessary for *P. chrysosporium* MnP expression, is apparently not necessary for the expression of *D. squalens* MnP. The lignin degradation dependency on manganese also is demonstrated, and a correlation betweeen lignin degradation and MnP expression is demonstrated.

Chapter 3 presents the results of natural and synthetic lignin degradation by *P. cinnabarinus*. This organism was selected because of its high level of laccase production and apparent absence of peroxidase expression. Cultures of *P. cinnabarinus* under various conditions allowed assay of the role of laccase in the biodegradation of lignin *in vivo*.

Purification and partial characterization of two MnPs produced by *D. squalens* are presented in Chapter 4. The comparison of *D. squalens* MnPs and *P. chrysosporium* MnPs suggests that the *D. squalens* enzymes are closely related to *P. chrysosporium* 's MnPs.

The laccases produced by *D. squalens* and *P. cinnabarinus* were purified to homogeneity and partially characterized. The results, presented in Chapter 5, indicate that they share many properties with other fungal laccases.

In summary, this research characterized lignin-degrading systems from two different fungi, and compared their properties with the extensively studied system of *P. chrysosporium*. It suggests that MnP plays a central role in the degradation of lignin. This work also suggests that laccase alone cannot catalyze the complete degradation of lignin. Finally, this work indicates that MnPs produced by *D. squalens* share many properties with *P. chrysosporium* MnP.

CHAPTER 2
MANGANESE REGULATION OF MANGANESE PEROXIDASE EXPRESSION AND LIGNIN DEGRADATION BY THE WHITE-ROT FUNGI *DICHOMITUS SQUALENS*

2.1 Introduction

White-rot basidiomycetes are primarily responsible for the initiation of lignin decomposition in wood [14,17,18]. When cultured under ligninolytic conditions, *Phanerochaete chrysosporium*, the best-studied white-rot basidiomycete, produces two extracellular heme peroxidases—manganese peroxidase (MnP) and lignin peroxidase (LiP)—which, along with an H_2O_2-generating system, constitute the major components of its lignin-degrading system [14,18,61,71]. Other white-rot fungi apparently secrete unique combinations of peroxidases and oxidases. *Coriolus versicolor* and *Phlebia radiata* each produce one or more laccases in addition to LiP and MnP [76,77,83,152,153]. *Pleurotus sajor-caju* secretes an aryl alcohol oxidase [66], a laccase, and several peroxidases [154]. *Bjerkanda adusta* secretes an aryl alcohol oxidase [155], and *Rigidoporus lignosus* apparently secretes a laccase and a MnP [156]. Previous reports demonstrated that *Dichomitus squalens* (*Polyporus anceps*), under various conditions, efficiently degrades natural lignin from birch [35], aspen [49], spruce [157], and from wheat, barley, and grape straw [50]. To broaden our understanding of the ligninolytic mechanims elaborated by various white-rot fungi, we have undertaken to identify and characterize the extracellular oxidative enzymes of *D. squalens* and their regulation. We examined the ability of *D. squalens* to degrade synthetic lignins under various conditions. In addition, we demonstrated that under ligninolytic conditions this organism expresses a laccase [53] and a MnP, and that the expression of MnP is regulated by Mn. In contrast, neither extracellular LiP nor veratryl alcohol oxidase activity was detected in cultures grown under a wide variety of conditions.

2.2 Material and Methods

2.2.1 Organism

Dichomitus squalens (Karst) Reid (*Polyporus anceps* Peck) (CBS 432.34) was obtained from F. Zadrazil and maintained on potato dextrose/yeast extract slants. Mycelial clamp connections were readily observed, indicating that the isolate was maintained in the dikaryotic state [54,158].

2.2.2 Culture conditions

The organism was grown at 28°C in 250-ml Erlenmeyer flasks in either 100-ml shaking cultures (150 rpm, 2.5 cm radius) or in 20-ml stationary cultures where indicated. Cultures were incubated under air for 6 days, after which they were purged every 3 days with 100% oxygen. A variety of growth media was used in our attempts to identify the extracellular peroxidases and oxidases produced by *D. squalens*. The organism was routinely grown in shaking cultures in a high-carbon, 2% glucose, low-nitrogen, 1.2 mM ammonium tartrate medium (HCLN) containing 3 mM manganese as previously described [38]; or in the following synthetic medium (per liter): 20 g glucose, 2.5 g L-asparagine, 0.15 g L-phenylalanine, 0.03 g adenine, 0.1 mg thiamine, 1 g KH_2PO_4, 0.1 g Na_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 5 mg $CuSO_4 \cdot 5H_2O$, 16 mg $MnSO_4 \cdot H_2O$, and 0.01% Tween 80.

In specific experiments, the final Mn concentration of the above media was adjusted to 0, 1, 2, 5, 10 and 100 mM. In addition, veratryl alcohol (6 mM), benzyl alcohol (6 mM), or guaiacyl lignin (dehydropolymerizate) DHP (2 mg) were added to the above media containing 10 mM Mn as potential inducers of LiP [72].

Additional media were used in stationary cultures in attempts to detect LiP activity. Several different inorganic salt and vitamin recipes [38,39,76] were used in conjunction with the following media: high carbon, low nitrogen (HCLN), 2% glucose and 1.2 mM ammonium tartrate; high carbon, high nitrogen (HCHN), 2% glucose and 12 mM ammonium tartrate; low carbon, high nitrogen (LCHN), 0.2% glucose and 12 mM ammonium tartrate; low carbon, low nitrogen (LCLN), 0.2% glucose and 1.2 mM ammonium tartrate.

The organism was cultured in lignocellulosic media under stationary conditions on either birch or spruce sawdust, or ground wheat straw saturated with water. This medium was not supplemented with any additional nutrients.

Two sets of stationary cultures were prepared with each medium (HCLN, HCHN, LCHN, LCLN containing the described salts [38]) as well as the birch sawdust medium. The first set was as described and the second set contained veratryl alcohol (6 mM).

2.2.3 Enzyme assays

Unless otherwise indicated, enzyme assays were conducted in 1-ml reaction mixtures at 30°C using the extracellular medium of fungal cultures as an enzyme source.

Laccase activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol at 469 nm. Reaction mixtures contained 2,6-dimethoxyphenol (0.2 mM) and enzyme in 20 mM succinate buffer (pH 3.0).

MnP activity was measured by monitoring the formation of a Mn^{III}-malonate complex at 270 nm [112]. Reaction mixtures contained $MnSO_4$ (0.2 mM), H_2O_2 (0.1 mM), and enzyme in 50 mM Na-malonate (pH 4.5).

Cell envelope-bound MnP was assayed by monitoring the oxidation of 2,6-dimethoxyphenol at 469 nm. Reaction mixtures contained 2,6-dimethoxyphenol (0.2 mM), H_2O_2 (0.1 mM), $MnSO_4$ (0.2 mM), and cell envelope enzyme fraction (10 mg) in 50 mM Na-malonate (pH 4.5). The reaction was incubated for 1 h at 28°C, after which the cell debris was removed by centrifugation at $3,000 \times g$ and the absorbance of the supernatant was measured at 469 nm.

Intact cell-bound MnP was measured by suspending a mycelial pellet in a reaction mixture containing 2,6-dimethoxyphenol (0.2 mM), H_2O_2 (0.1 mM), and $MnSO_4$ (0.2 mM) in 50 mM Na-malonate (pH 4.5). The reaction was incubated for 15 min, after which the mycelium was removed by filtration and the absorbance of the filtrate was measured at 469 nm.

LiP activity was measured by monitoring the oxidation of veratryl alcohol at 310 nm [18,26]. Extracellular LiP activity was also measured by monitoring the oxidation of a diarylpropane at 310 nm as previously described [71]. Reaction mixtures contained veratryl alcohol (2.5 mM), H_2O_2 (0.1 mM), and enzyme in 20 mM Na-succinate buffer (pH 3.0).

Aryl alcohol oxidase (AAO) activity was measured by monitoring the oxidation of veratryl or anisyl alcohol at 310 or 290 nm, respectively, as described [66,155]. Reaction mixtures contained veratryl or anisyl alcohol (2.5 mM) in 20 mM Na-succinate (pH 3–5.5) or 20 mM Na-phosphate (pH 6–7).

2.2.4 Cell envelope enzyme preparation

Cultures were filtered through miracloth and the cells were washed with ice-cold 0.05% NaCl. The cells (0.5 g) were frozen in liquid nitrogen and ground with sand in a mortar and pestle. One mL of 10 mM Na-phosphate (pH 7.0) containing glycerol (10%) and phenylmethylsulfonyl-fluoride (0.5 mM) (buffer A) was added and the extract was stirred for 5 min at 4°C. After the sand was removed by sedimentation, the extract was centrifuged at 3,000 × g for 10 min; the pellet was washed in buffer A and recentrifuged at 3,000 × g. Finally, the pellet was resuspended in 50 mM Na-malonate (pH 4.5). Microscopic examination indicated that the cells were uniformly broken.

2.2.5 ^{14}C-DHP degradation

2-^{14}C-side chain-, ^{14}C-methoxy-, and ^{14}C-U-ring-labeled guaiacyl dehydropolymerizates (DHPs) were prepared from labeled coniferyl alcohol precursors as previously described [159]. The specific activity of each substrate was adjusted to 3×10^5 cpm/mg with unlabeled guaiacyl DHP. Radiolabeled substrates (50,000 cpm/flask in 25 mL DMF) were added to each agitated culture on the third day of growth. Evolved ^{14}CO$_2$ was measured every 3 days as described previously [38,159].

2.2.6 Ammonium ion, amino acid, and glucose determinations

Prior to analysis, samples of the extracellular fluid were ultrafiltered through an Amicon Diaflo YC05 membrane (500 MW cutoff). The concentration of ammonium ion in HCLN cultures was monitored by the indophenol blue method [160]. The total concentration of amino acids in the medium of Asn-Phe cultures was monitored by the ninhydrin method [161]. The individual concentrations of Asn and Phe were measured by HPLC on a C$_8$ reverse-phase column (Beckmann Ultrasphere 5 mm) after derivatization with o-phthalaldehyde [162] using isocratic elution with methanol:water (7:3). Free glucose in the extracellular fluid was determined using the phenol-sulfuric acid method [163].

2.3 Results

A time course for DHP degradation in HCLN shaking cultures of *D. squalens* is shown in Figure 2.1a. Under these conditions, *D. squalens*

mineralized 40–50% of each substrate. The residual ammonium concentration and the growth curve for the organism under these conditions are also shown in Figure 2.1a. The dry weight of the mycelium in the culture increased until day 20 after which it decreased significantly, probably due to autolysis. The free ammonium concentration in HCLN cultures decreased rapidly over the first 7 days and was not detectable after day 10.

A time course for DHP degradation in Asn-Phe cultures is shown in Figure 2.1b. Comparison of the results from the two culture conditions demonstrates that *D. squalens* was able to degrade DHP in either medium, but that ring- and side chain-labeled DHPs were degraded more extensively in the HCLN medium. In contrast, the Asn-Phe medium supported a considerably faster rate and extent of growth. The maximum mycelium dry weight achieved in the Asn-Phe medium was approximately 150% of that in the HCLN medium. *D. squalens* also degraded side chain-labeled DHP in HCHN cultures (data not shown). The extent of side chain-labeled DHP degradation in HCHN cultures was 33% and 50% of that achieved in HCLN and Asn-Phe cultures, respectively.

A time course (day 3 through day 30) for extracellular MnP and laccase enzymatic activity in HCLN shaking cultures containing 3 mM Mn is shown in Figure 2.2a. Cultures grown in the absence of Mn had no detectable MnP activity throughout the course of the experiment whereas, in cultures containing Mn, soluble extracellular MnP activity was first detectable on day 5, reached a maximum on day 18, and persisted throughout the course of the experiment. In contrast, extracellular laccase activity appeared on day 3 and reached a maximum on day 9 after which it slowly declined. Furthermore, the expression of laccase activity was not dependent on the presence of Mn. Because soluble extracellular MnP activity was first detectable only after 5 days of growth in HCLN cultures, cell envelope-bound MnP activity was also assayed. As shown in Figure 2.2a, cell envelope-bound MnP activity was detectable on day 3 and increased significantly beyond day 10. The appearance of this cell envelope-bound activity also was completely dependent on the presence of Mn in the medium (data now shown). The appearance of cell envelope-bound activity correlated well with the MnP activity measured in the intact cell assay.

A time course for extracellular MnP and laccase activity in Asn-Phe cultures grown in the presence or absence of 50 mM Mn is shown in Figure 2.2b. Again, cultures grown in the absence of Mn exhibited no detectable extracellular MnP activity throughout the course of the experiment.
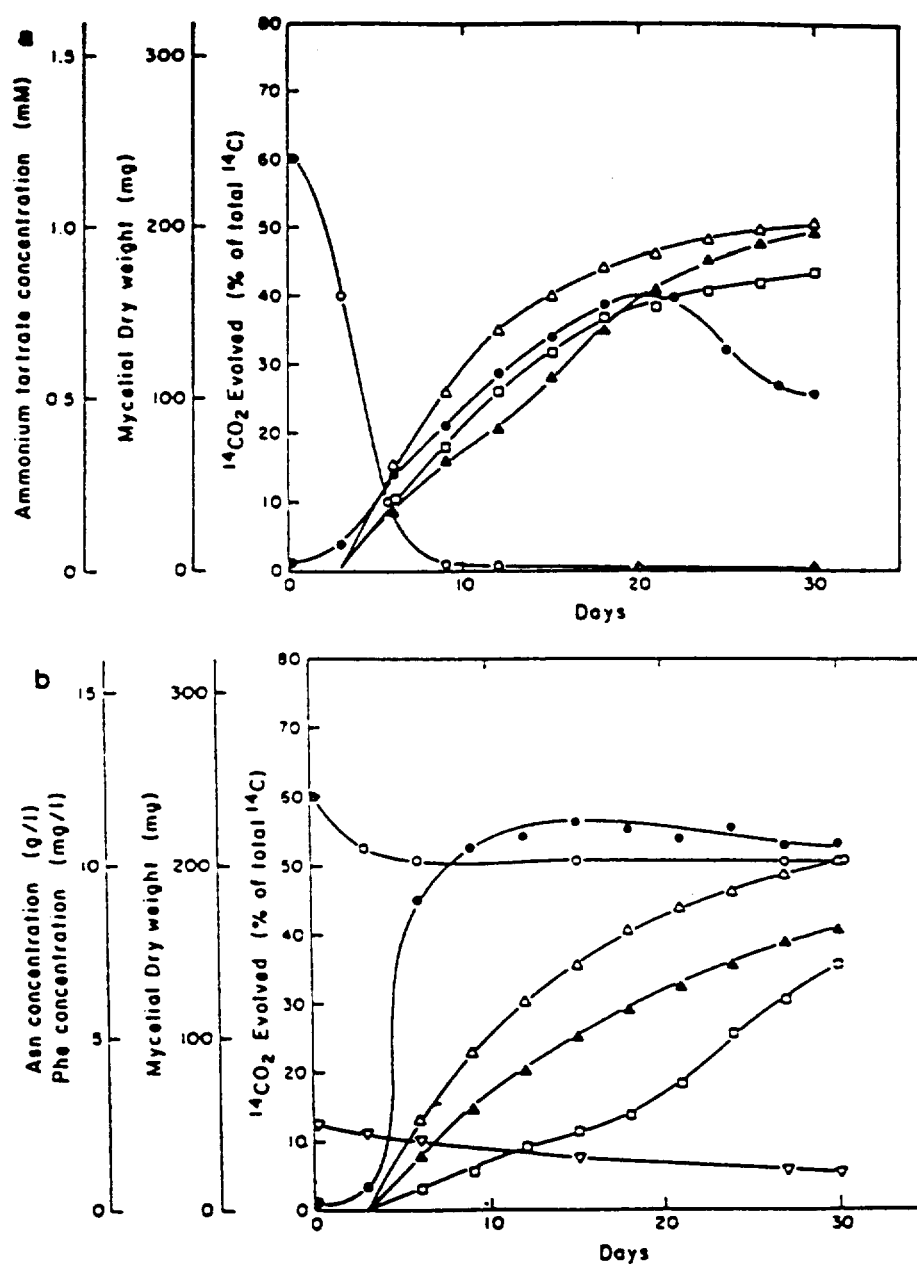
Figure 2.1 Mineralization of ^{14}C-DHPs by *D. squalens*.
(a) Time course for the evolution of ^{14}CO$_2$ from methoxy- ($\triangle$), side chain- ($\square$), and ring-labeled ($\blacktriangle$) DHP in HCLN cultures. The time dependence of mycelium dry weight ($\bullet$) and nitrogen depletion ($\circ$) is also shown.
(b) Time course for the evolution of ^{14}CO$_2$ from methoxy- ($\triangle$), side chain- ($\square$), and ring-labeled ($\blacktriangle$) DHP in Asn-Phe cultures. The time dependence of mycelium dry weight ($\bullet$) Asn ($\triangledown$) and Phe ($\circ$) concentrations is also shown. Shaking cultures were purged periodically with 100% O$_2$ and evolved ^{14}CO$_2$ was measured as described in the text.
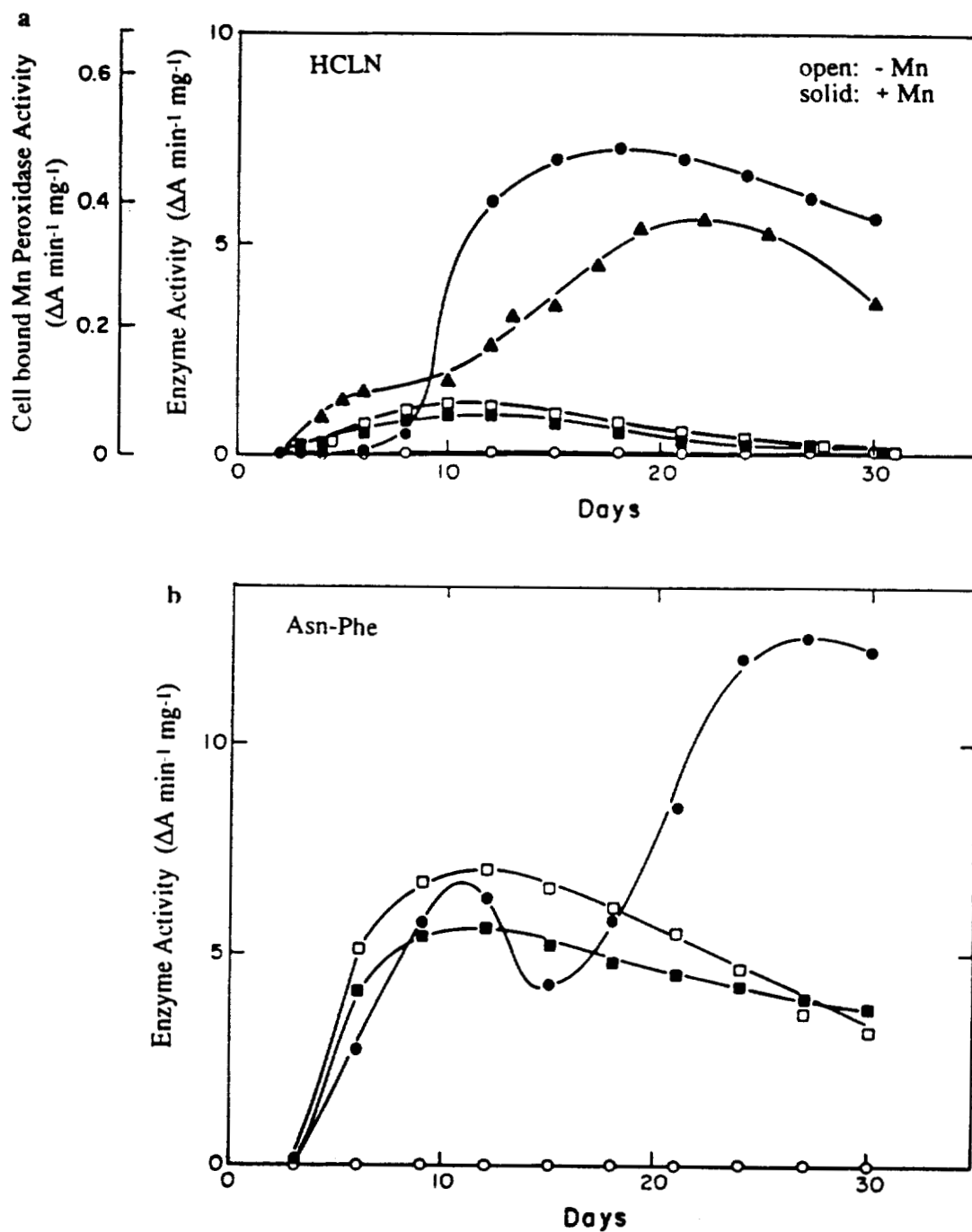
Figure 2.2 Effect of Mn on the expression of extracellular MnP and laccase activity. Soluble activity from cultures grown in the presence (●) and absence (○) of Mn, soluble laccase activity from cultures grown in the presence (■) and absence (□) of Mn, and cell envelope-bound MnP activity from cultures grown in the presence (▲) of Mn were determined as described in the text. Shaking cultures were grown in HCLN (a) and in Asn-Phe (b) media.

In contrast, in cultures grown in the presence of Mn, extracellular MnP activity was first detectable on day 3 and reached a first maximum at day 12. After a brief decline, MnP activity then increased steadily until approximately day 24, after which it remained constant through day 30. Again, the expression of laccase activity was not dependent on the presence of Mn in Asn-Phe cultures. Mn-independent laccase activity reached a maximum on approximately day 10 and then slowly declined. The level of laccase activity attained in Asn-Phe cultures (Figure 2.2b) was consistently at least 5- to 6-fold greater than that attained in HCLN cultures (Figure 2.2a).

In contrast to the above results, neither LiP nor aryl alcohol oxidase activity was detected in the extracellular medium of HCLN or Asn-Phe shaking or stationary cultures incubated in the presence of 0, 1, 5, 10, or 100 mM $MnSO_4$. Furthermore, neither extracellular LiP nor aryl alcohol oxidase activity was detectable in shaking or stationary cultures containing HCHN, LCHN, LCLN or the lignocellulose medium with any of the putative inducers described in the Materials and Methods section.

The effect of the presence of Mn on the degradation of ^{14}C-labeled DHP is shown in Figure 2.3. A time course for the degradation of ^{14}C ring-, side chain-, and methoxy-labeled DHP in HCLN cultures containing 3 mM Mn is shown in Figure 2.3a. All three labeled DHPs were degraded extensively over the 30 days of the experiment in Mn-containing cultures. The sum of the $^{14}CO_2$ evolved after 30 days was 40–50% of that introduced for each of the substrates. In contrast, in cultures grown in the absence of Mn, all three labeled DHPs were degraded very slowly with less than 10% of the labeled DHPs mineralized during the 30-day experiment.

A time course for the evolution of $^{14}CO_2$ from labeled DHPs in Asn-Phe cultures is shown in Figure 2.3b. In the Asn-Phe medium, the degradation of both ^{14}C ring- and side chain-labeled DHP also was dependent on the presence of Mn in the medium. For these two DHPs, the extent of $^{14}CO_2$ evolved in the absence of Mn was less than 15% of that evolved in the presence of Mn. In contrast, significant amounts of $^{14}CO_2$ were evolved in Asn-Phe cultures incubated with methoxy-labeled DHP in the absence of Mn. After 30 days, cultures incubated with methoxy-labeled DHP in the absence of Mn evolved approximately 70% as much CO_2 as that evolved in the presence of Mn.
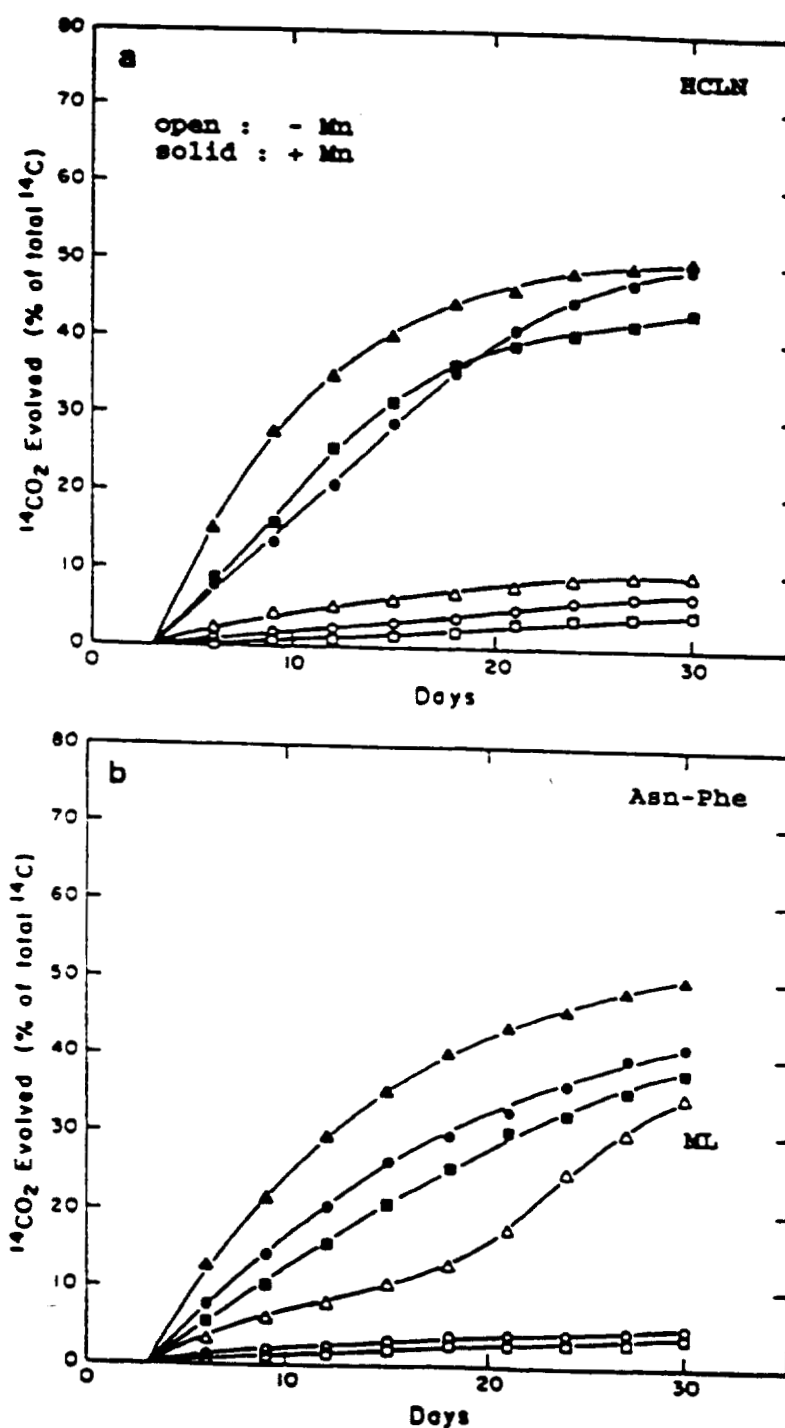
Figure 2.3 Effect of Mn on the mineralization of ^{14}C-labeled DHPs.
The evolution of $^{14}CO_2$ from ring-labeled DHP in the presence (●) and absence
(○) of Mn is shown, as are the evolution of $^{14}CO_2$ from side chain-labeled DHP
in the presence (■) and absence (□) of Mn, and the evolution of methoxy-
labeled DHP in the presence (▲) and absence (△) of Mn. Shaking cultures
were grown in HCLN (a) and Asn-Phe (b) media.

2.4 Discussion

LiP and MnP were first isolated from the extracellular medium of
P. chrysosporium [14,18,71] and the enzymes from this organism have been
extensively characterized. Both enzymes are extracellular heme-containing
glycoproteins which exist as a series of isozymes. Considerable chemical and
physiological evidence reviewed previously [7,14,17,18,71,106] suggests that
these enzymes constitute the major components of the lignin degradative system
of *P. chrysosporium*. LiP catalyzes the oxidative cleavage of non-phenolic
lignin model compounds by a mechanism involving the formation of a substrate
aryl cation radical [7,14,18,106]. A recent report also demonstrated that crude
lignin peroxidase preparations catalyze at least the partial depolymerization of
synthetic angiosperm lignin [108]. MnP catalyzes the oxidation of Mn^{II} to Mn^{III}
and the latter, complexed with an organic acid, readily oxidizes phenolic lignin
model compounds [62–64,100,111]. Recently, we demonstrated that
homogeneous MnP also catalyzes the partial depolymerization of synthetic
angiosperm and gymnosperm lignin [164]. In addition, it has recently been
demonstrated in *P. chrysosporium* that Mn, the primary substrate of MnP,
regulates the expression of the enzyme [165,166] by activating the transcription
of the MnP gene [166].

The results shown in Figure 2.1 demonstrate that, unlike
P. chrysosporium [14,18], *D. squalens* degrades lignin under nitrogen-sufficient
as well as nitrogen-limiting conditions. As shown in Figure 2.1b, after an initial
lag, lignin degradation proceeds efficiently in Asn-Phe and in HCLN cultures.
Furthermore, *D. squalens* degrades side chain-labeled DHP in the HCHN medium
to approximately 33% of the extent of degradation in the HCLN medium (data
not shown).

MnP activity is readily detected in the medium from Asn-Phe cultures and
the time courses for both extracellular MnP and laccase activities correlate well
with that for DHP degradation (Figures 2.1b and 2.2b). In contrast, in HCLN
Mn-containing cultures, soluble MnP activity is detectable only after 5 days and
reaches a maximum on day 18, whereas lignin degradation proceeds rapidly
starting from day 6 (Figures 2.1a and 2.2b). However, under HCLN conditions,
significant levels of cell envelope-bound MnP activity are detectable on day 4
and this activity probably accounts for the early onset of lignin degradation.
Cell envelope-bound MnP activity has also been detected in *P. chrysosporium*
[63]. Presumably both cell envelope-bound and soluble extracellular MnP are

capable of generating freely diffusible Mn^{III} which, in turn, oxidizes the terminal phenolic substrate, lignin. We have verified that cell envelope-bound MnP is capable of oxidizing 2,6-dimethoxyphenol in both cell-free and intact cell assay systems.

The results shown in Figure 2.2 demonstrate that, under both high and low nitrogen conditions, *D. squalens* secretes both laccase and MnP. However, in contrast to *P. chrysosporium*, no LiP activity is detectable in *D. squalens* cultures grown under a variety of conditions including various concentrations of carbon, nitrogen, and Mn in both agitated and stationary cultures. Likewise, LiP activity is not detected in *D. squalens* cultures treated with various putative inducers of *P. chrysosporium* LiP activity [72]. In addition, no extracellular aryl alcohol oxidase is detectable in this organism. These results, along with the previous reports described above [155,156], suggest that LiP may not be an essential component of the lignin degradation system of every white-rot fungus and that alternative oxidative enzymes such as MnP are capable of degrading lignin.

In contrast to the results with nutrient nitrogen, the presence of Mn in *D. squalens* cultures controls the expression of MnP and the extent of lignin degradation. The results in Figure 2.2 demonstrate that *D. squalens* produces an extracellular laccase in HCLN and Asn-Phe media in the presence and absence of Mn. In contrast, the appearance of both extracellular soluble and cell envelope-bound MnP activity is completely dependent on the presence of Mn in the medium. In HCLN cultures, the extent of DHP degradation in the absence of Mn is less than 15% of that in the presence of Mn. The small amount of $^{14}CO_2$ evolution observed may be a result of lignin degradation by laccase which is expressed in the absence of Mn or by another enzyme. It is likely that the Mn effect on lignin degradation is due to Mn induction of MnP expression, as has been shown for *P. chrysosporium* [166], and because Mn serves as the substrate for MnP. However, at this time the less-likely possibility that another Mn-dependent enzyme is involved in lignin degradation cannot be ruled out. Enzyme assays demonstrate that *D. squalens* MnP enzymatic activity is dependent on Mn and the results in Figure 2.2 show that Mn regulates MnP expression in *D. squalens*.

The results in Figure 2.3b demonstrate that methoxy-labeled lignin is degraded in Asn-Phe, but not in the HCLN, medium in the absence of Mn, correlating with the expression of laccase. Laccase expression in this organism

is independent of Mn and is activated by growth under high nitrogen conditions. These results suggest that extracellular laccase is capable of demethoxylating guaicyl DHP *in vivo*. The oxidation of dimeric lignin model compounds by laccase has been described [7].

In conclusion, these results suggest that MnP and probably laccase play important roles in the degradation of lignin by at least some white-rot fungi, and that LiP may not be an indispensible component of the lignin degradative system of every white-rot fungus. The results also suggest that a variety of oxidative enzymes may be utilized by white-rot fungi for lignin degradation.

CHAPTER 3
LIGNIN DEGRADATION IN LIQUID AND SEMISOLID CULTURES BY THE WHITE-ROT BASIDIOMYCETE
PYCNOPORUS CINNABARINUS

3.1   Introduction

The study of lignin degradation by white-rot basidiomycetes has focused primarily on *P. chrysosporium* [17,18] because of its high rate of lignin degradation [167]. Under ligninolytic conditions, this fungus produces two extracellular heme peroxidases—manganese peroxidase (MnP) and lignin peroxidase (LiP)—which, along with an H_2O_2-producing system, constitute the major components of its lignin degrading systems. Yet other white-rot fungi degrade lignin [39,168–170] without necessarily producing LiP under ligninolytic conditions [44,67,84,151]. *P. cinnabarinus* [39,40,43,171,172] has been reported to degrade lignin under selected conditions. The rate of lignin degradation appears to vary among different strains [39]. Results suggest that *P. cinnabarinus* possibly produces laccase and an undefined peroxidase [44]. In this chapter, the degradation of different lignin substructures by the white-rot fungus *P. cinnabarinus* (IFO 6139) is investigated under different culture conditions. The production of MnP and laccase are examined under various conditions. Furthermore, the effect of MnP and laccase from *P. cinnabarinus* on the degradation of lignin are examined *in vivo*.

3.2   Material and Methods

3.2.1   Organism

A culture of *P. cinnabarinus* (Jacq. ex Fr) Karst (IFO 6139) was obtained from H. Tanaka (Kinki University, Nara, Japan) and maintained on Vogel's medium supplemented with malt extract and yeast extract agar [173,174].

29

3.2.2 Culture conditions

The organism was grown at 28°C in 250-ml Erlenmeyer flasks in 100-ml shaking cultures (150 rpm, 2.5 cm radius), in 20-ml stationary cultures on a medium containing 1.8% agar or on lignocellulose. The cultures were purged with 100% oxygen every 3 days after the third day of growth. Two synthetic growth media were used [38,151]. A nitrogen-rich medium containing 2% glucose and 35 mM nitrogen provided by asparagine and phenylalanine (Asn-Phe medium) was used to obtain high laccase activity. Kirk's nitrogen-limited medium (2% glucose, 2.4 mM nitrogen: HCLN) was used to optimize lignin degradation. In both media, Mn^{II} was adjusted to 30 µM. In specific experiments, veratryl alcohol (6 mM) or benzyl alcohol (6 mM) was added to the media in attempts to induce LiP activity.

The organism was cultured in lignocellulose-containing media under stationary conditions at 28°C on unsupplemented ground wheat or rye grass straw. Semisolid conditions were obtained by saturating the ground straw with water. Approximately 3 g wheat or rye grass straw were added to each 200-ml flask.

3.2.3 Enzyme assays

Unless otherwise indicated, enzyme assays were conducted in 1 mL of reaction mixture at 30°C using the extracellular medium from a fungal culture.

Laccase activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol (2,6-DMP) at 469 nm. Reaction mixtures contained 2,6-DMP (0.2 mM) and enzyme in 20 mM Na-succinate, pH 3.5.

MnP activity was measured by monitoring the formation of a Mn^{III}-malonate complex at 270 nm [112]. Reaction mixtures contained $MnSO_4$ (0.2 mM), H_2O_2 (0.1 mM), and enzyme in 50 mM Na-malonate, pH 4.5.

LiP activity was measured by monitoring the oxidation of veratryl alcohol at 310 nm [14,18], and by measuring the oxidation of 1-(3',4'-diethoxyphenyl)-1,3-dihydroxy (4''-methoxyphenyl) propane, a diarylpropane lignin model compound, as previously described [71]. Reaction mixtures contained veratryl alcohol (2.5 mM), H_2O_2 (0.1 mM), and enzyme in 20 mM Na-succinate, pH 3.0.

Aryl alcohol oxidase activity was measured by monitoring the oxidation of veratryl alcohol at 310 nm as previously described [66]. Reaction mixtures contained veratryl alcohol (2.5 mM) in 20 mM Na-succinate, pH 3.0–5.5, or 20 mM Na-phosphate, pH 6.0 and 7.0.

Enzymes activities in agar media were determined in the supernatant after the cultures were centrifuged at $10,000 \times g$ for 20 min.

3.2.4 HPLC analysis

Veratryl alcohol was determined by HPLC on a C_8 reverse-phase column Beckmann Ultrasphere Octyl 5 μm (4.6 mm × 25 cm) by using an isocratic elution with methanol:water (3:7).

3.2.5 ^{14}C-DHP degradation

2-^{14}C-side chain- and ^{14}C-methoxy-guaiacyl DHPs were prepared as previously described [159]. The specific activity of each substrate was adjusted to 3×10^5 cpm/mg with unlabeled guaiacyl DHP. Radiolabeled substrates (25,000 cpm per flask in 10 μl of dimethylformamide (DMF)) were added to each culture on the third day of growth. Evolved ^{14}CO$_2$ was measured every 3 days as previously described [38,159].

3.2.6 Exogenous enzymes

Cultures of *P. cinnabarinus* were grown under stationary conditions in Kirk's nitrogen-limited medium (HCLN) at 28°C. After 3 days of growth, 25,000 cpm of side chain-labeled DHP were added to each culture. Where indicated, MnP and laccase purified from the extracellular medium of *D. squalens* (Chapters 4 and 5) were added to each culture. Each flask received 250 nkat laccase or 250 nkat MnP, or 250 nkat laccase and 250 nkat MnP (measured for 2,6-DMP oxidation with $\varepsilon_{469} = 49.6$ mM^{-1}cm^{-1}). Controls contained 500 nkat laccase per flask. H$_2$O$_2$ was generated by the glucose oxidase (50 U/culture)–glucose system. The degradation of side-chain DHP was followed as described above. The enzymes in solution in 10 mM Na-2,2-dimethylsuccinate, pH 4.5, were added every 3 days with a curved needle, through the rubber stopper, under the mycelial mat. Two sets of cultures were also grown to follow the enzyme activities. They were not supplemented with radioactive DHP. One set of cultures was used to follow the activity of enzymes produced by *P. cinnabarinus*. The second set was supplemented with MnP and laccase to obtain a final activity of 250 nkat/culture for each enzyme. 50 U glucose oxidase were added every time laccase or MnP were added to the cultures. The amount of enzyme added to the DHP-supplemented cultures was calculated according to the activity measured in the control set to bring the total laccase or

MnP activity to 250 nkat. The cultures were purged with 100% O_2, 2 h after the addition of the enzymes.

3.2.7 *In vitro* reactions

In vitro oxidations were carried out at 28°C under 100% O_2 in 10 mM Na-succinate, pH 3.0 (1 mL), containing laccase (5 μg) and DHP (2.5×10^4 cpm) in 10 μl DMF. Upon completion of the reactions, radioactivity in aliquots was determined in Ecolite (ICN Biochemicals) before and after centrifugation (5,000 *g*, 10 min) to determine the amount of reaction-generated insoluble material [164]. Subsequently, 0.5 mL of supernatant was applied to a Sephadex LH 20 column (1.2 cm × 40 cm) equilibrated with a 0.1 mM solution of $LiCl_2$ in DMF. The column was eluted with the same solvent and 1-ml fractions were collected. The column was calibrated with ^{14}C-methanol. Fractions corresponding to the unreacted lignin and to methanol were counted in Ecolite (1 mL reaction mixture/4 mL Ecolite).

3.2.8 Methanol formation

0.5 mg unlabeled syringyl- and guaiacyl-type DHPs were reacted with laccase as described above. Aliquots of the reaction mixtures were collected after 20 min, 6 h, and 24 h, and the methanol content was analyzed after distillation [175] by using chromotropic acid assay [176].

3.3 Results

Time courses for the production of extracellular laccase and MnP in HCLN, Asn-Phe, and lignocellulosic media are shown in Figures 3.1a and 3.1b. The highest laccase activities were obtained in the nitrogen-rich medium under stationary conditions. The maximum laccase activity in Asn-Phe was 8-fold higher than in HCLN and 2-fold higher than in the lignocellulosic medium. The decline of laccase activity after reaching a maximum was more rapid in liquid cultures than in lignocellulose-containing cultures in which the laccase activity persisted throughout the course of the experiment. The laccase activity was inducible by various aromatic compounds, such as benzyl alcohol, veratryl alcohol, guaiacol, and the diarylpropane compound, causing the activity to increase by a factor of 2 to 5. No induction was observed with 2,5-xylidine. 0.01% Tween 80 caused complete inhibition of laccase production. No MnP
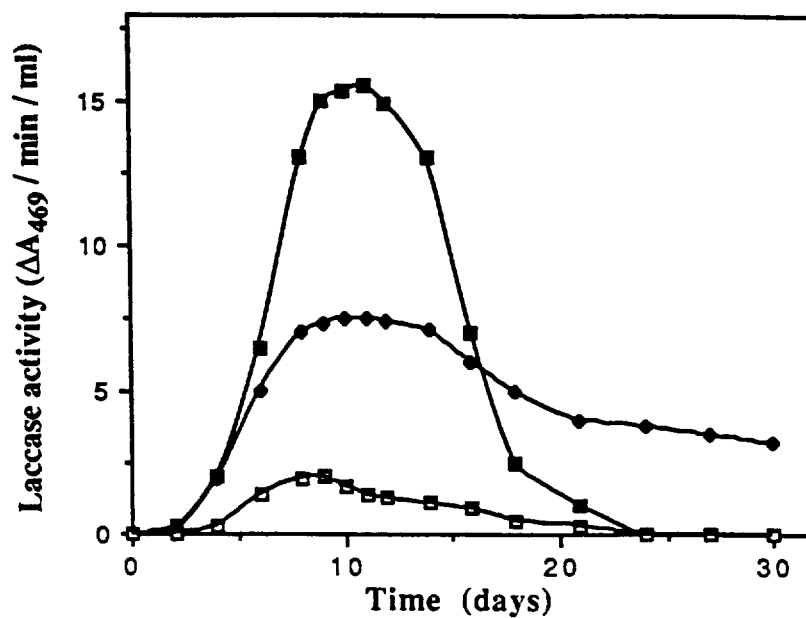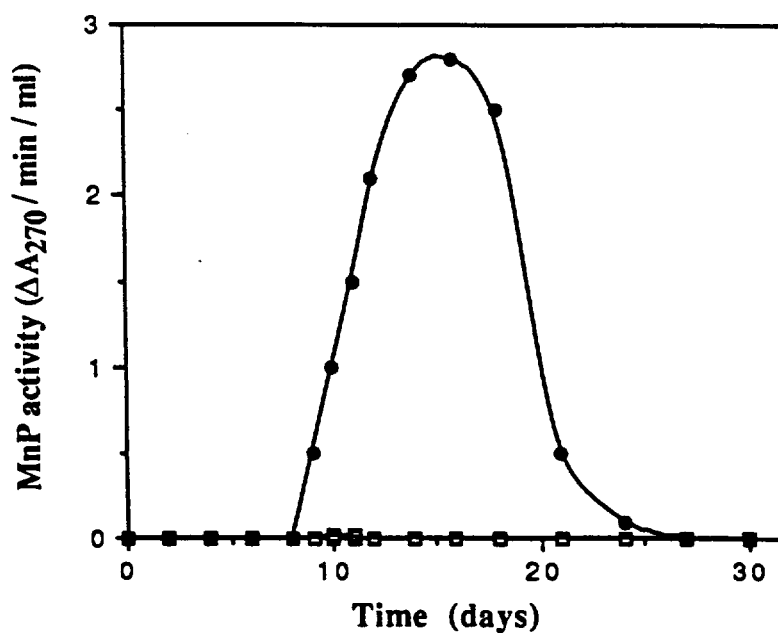
Figure 3.1 Production of extracellular laccase and MnP by *P. cinnabarinus*.
(a) Production of laccase in Asn-Phe liquid media, under stationary conditions
(■), in HCLN liquid media, under stationary conditions (□), and in cellulose
containing media (♦).
(b) Production of MnP in HCLN (□) and Asn-Phe (■) liquid media, under
stationary conditions, and in lignocellulose containing media (●).

activity could be detected in the extracellular medium of either nitrogen-rich or nitrogen-limited liquid cultures under a variety of conditions (Figure 3.1b). In contrast, a low level of MnP activity was detected in the lignocellulose-containing media. The onset of MnP production appeared after day 10. The activity reached a maximum on day 15 and then declined, becoming undetectable after day 22. Enzyme activities were followed in agar-containing cultures, and in nitrogen-rich and nitrogen-limited media from day 10 to day 15. No MnP activity was detected under these conditions. Laccase activity was similar to that found in liquid stationary cultures.

Each culture condition described (agitated or stationary liquid, agar or lignocellulosic) was assayed for LiP activity. No LiP activity was found in *P. cinnabarinus* cultures. In particular, veratryl alcohol addition to the media did not induce the production of LiP, and no veratryl alcohol production was detected.

Time courses for ^{14}C-methoxyl-labeled DHP mineralization by *P. cinnabarinus* are shown in Figures 3.2a and 3.2b. In nitrogen-rich media, the fungus metabolized almost 65% more substrate in 30 days (28% of the total ^{14}C) than in the nitrogen-limited media (17% of the total ^{14}C). In both high- and low-nitrogen media, the agar substrate appeared to provide the best conditions for lignin degradation.

Figure 3.3 shows the mineralization of ^{14}C-side chain-labeled DHP in HCLN and Asn-Phe media under stationary and solid conditions. The agar-containing, nitrogen-limited medium provided the most favorable conditions for lignin degradation (8% after 30 days). No degradation was observed during the first 6 days of incubation, after which the rate of evolution of ^{14}CO$_2$ gradually increased indicating the highest rate between day 10 and day 18 after which the rate of mineralization slowed down with no further ^{14}CO$_2$ release after day 24. In contrast, liquid conditions did not allow any significant lignin mineralization. A negligible amount (less than 3%) of lignin was mineralized in the agar-containing Asn-Phe medium.

Figure 3.4 shows the time course for mineralization of ^{14}C-side chain-labeled DHP in wheat and rye grass straw media. The fungus degraded approximately 10–12% of the lignin after a month of growth, indicating that the rate of degradation is 25 to 50% faster in lignocellulosic media as compared with agar-containing synthetic media. Most of the degradation occurred from day 10 to day 18 after which the formation of ^{14}CO$_2$ declined significantly. The

a.

b.
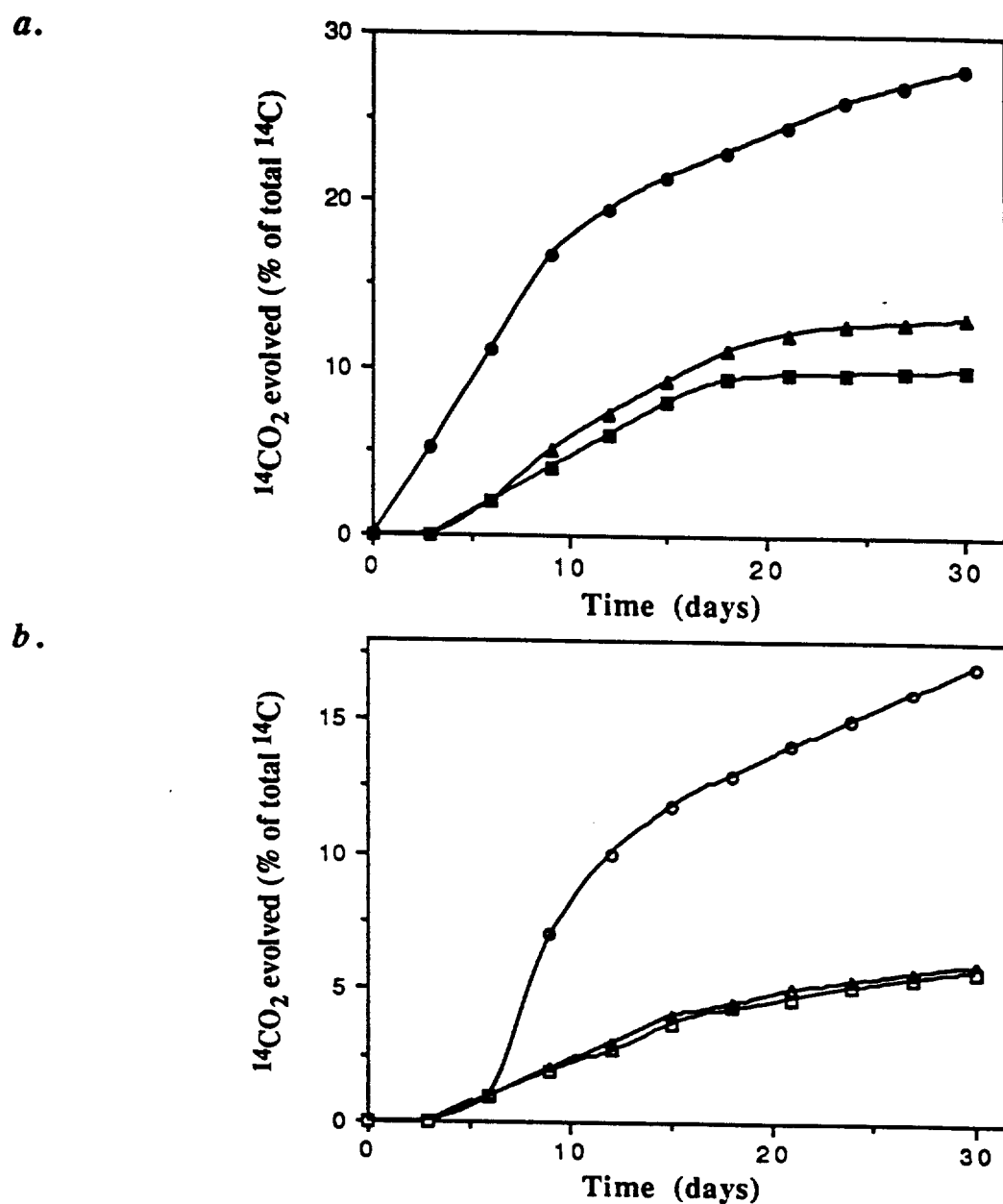
Figure 3.2 Mineralization of ^{14}C-labeled DHPs by *P. cinnabarinus*.
(a) $^{14}CO_2$ evolution from ^{14}C-methoxy-labeled guaiacyl DHP incubated in
P. cinnabarinus cultures in agar-containing Asn-Phe media (●), in Asn Phe
liquid media under stationary conditions (▲), and in Asn Phe liquid media with
agitation (■).
(b) $^{14}CO_2$ evolution from ^{14}C-methoxy-labeled guaiacyl DHP incubated in *P.
cinnabarinus* cultures in agar-containing HCLN media (○), in HCLN liquid
media under stationary conditions (Δ), and in HCLN liquid media with agitation
(□).
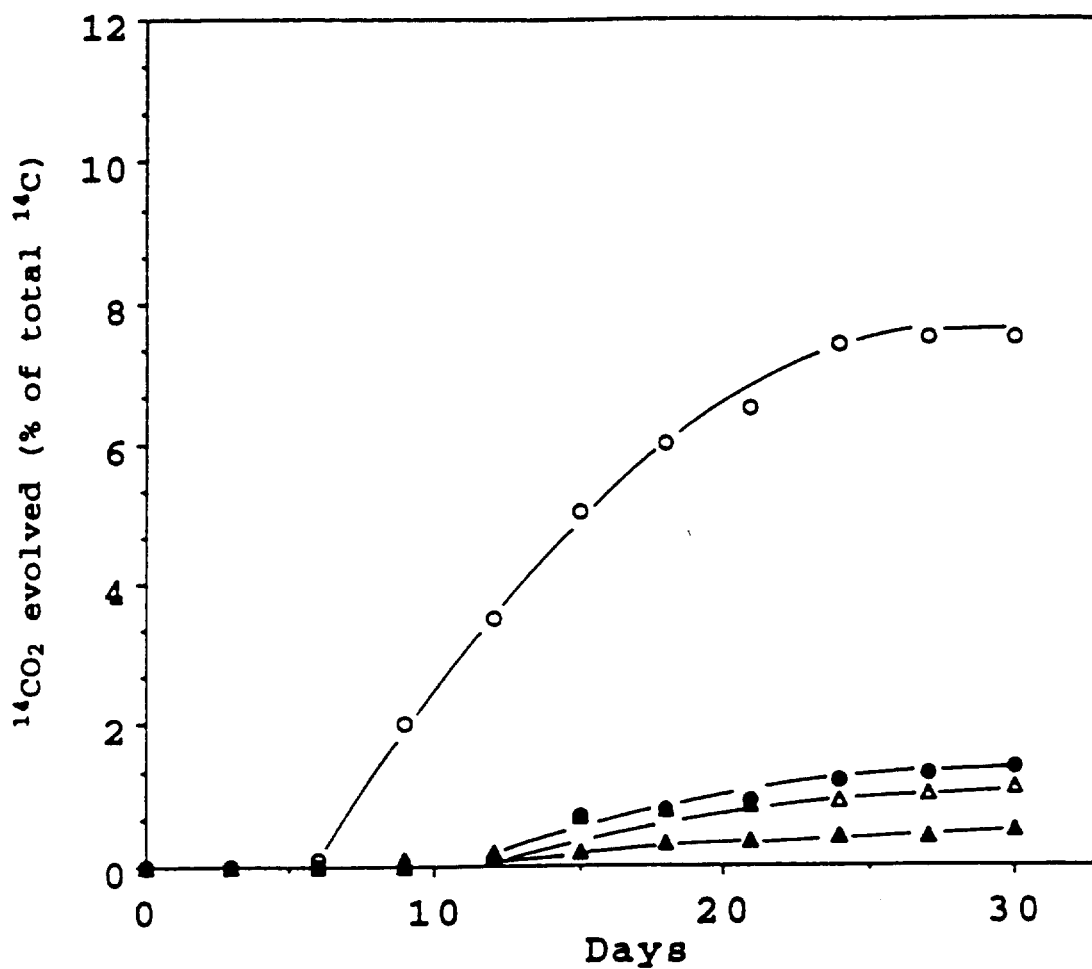
Figure 3.3 $^{14}CO_2$ **evolution from** ^{14}C**-side chain-labeled guaiacyl DHP incubated in** *P. cinnabarinus* **cultures in agar-containing media.**
In Asn-Phe media (●), in Asn Phe liquid media under stationary conditions (▲), in agar-containing HCLN media (o), and in HCLN liquid media under stationary conditions (Δ).
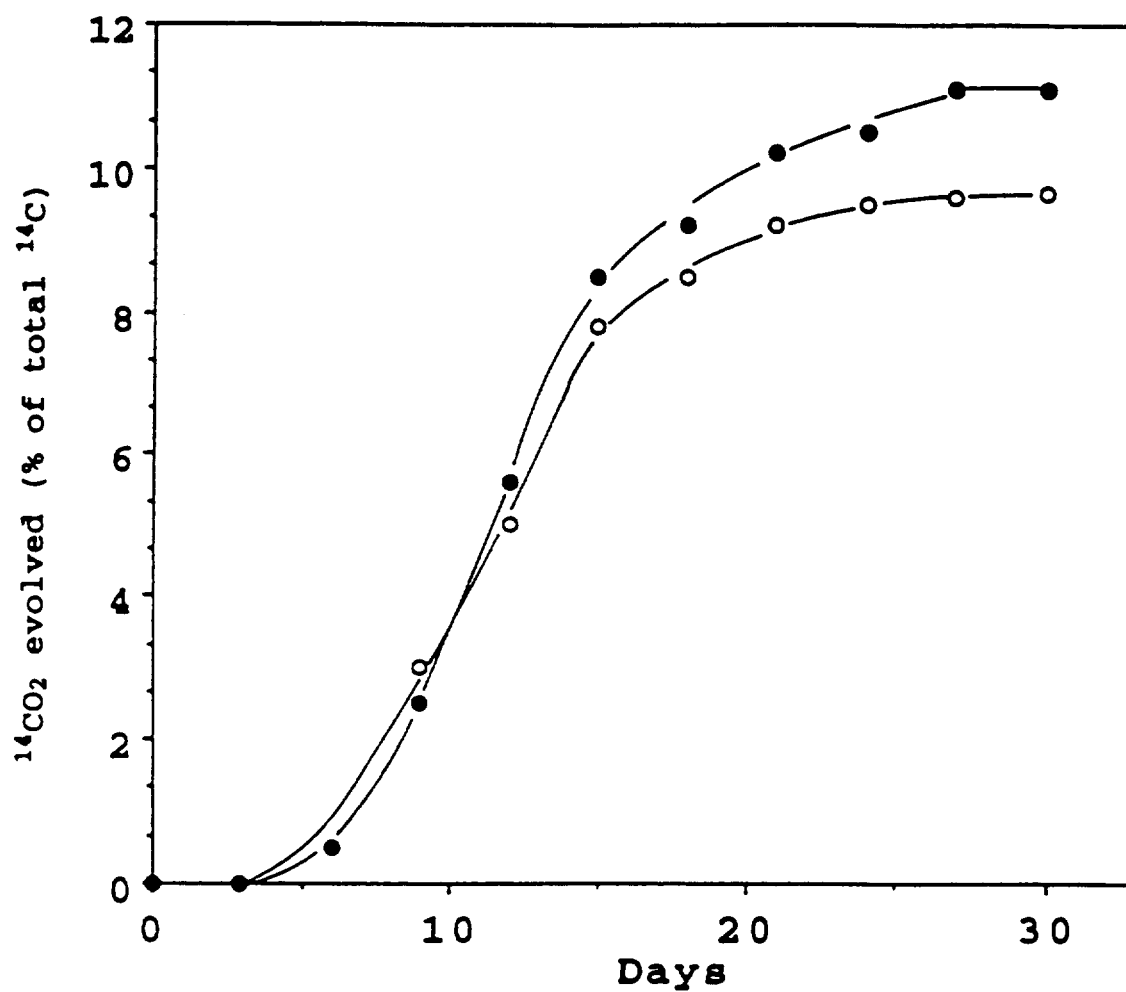
Figure 3.4 $^{14}CO_2$ evolution from ^{14}C-side chain-labeled guaiacyl DHP
incubated in *P. cinnabarinus* cultures in lignocellulosic media.
In rye grass straw (o) and in wheat straw (●).

degradation rate difference between wheat straw and rye grass straw media were not significant.

Figure 3.5a shows time courses of ^{14}C-side chain-labeled DHP mineralization in HCLN liquid cultures supplemented with purified MnP and laccase. The addition of laccase alone did not result in appreciable DHP depolymerization. Less than 3% of the initial substrate was mineralized after 17 days and approximately 3% when glucose oxidase and laccase were added to the cultures. In contrast, the addition of MnP to the culture medium produced a sharp increase in lignin mineralization. Cultures were purged with 100% O_2 just before the addition of the enzymes and 2 h after addition. The time course for $^{14}CO_2$ evolution indicates that approximately 25% of the evolved CO_2 is produced during the first 2 h following the addition of MnP. The rate of degradation under these conditions was approximately 70% faster than the rate of degradation observed in HCLN agar-containing cultures. The rate of mineralization was approximately 30% slower in cultures supplemented with laccase and MnP than in cultures containing MnP alone. Figure 3.5b shows the laccase and MnP activities in the control set of cultures. Laccase remained stable throughout the experiment whereas most of the MnP activity was lost within 5 h following addition.

The elution patterns for ^{14}C-side chain- (Figure 3.6a) and ^{14}C-methoxy-labeled DHP incubated with pure laccase are shown in Figure 3.6b. Approximately 22% of ^{14}C-side chain-labeled DHP was precipitated by centrifugation after 1 h incubation with 5 μg of purified laccase. No low-molecular-weight fraction was detected. Approximately 35% of the ^{14}C-methoxy-labeled DHP was modified after 40 min incubation with laccase. 25% was precipitated by centrifugation and 8% was detected in the peak corresponding to methanol. After 8 h of incubation, approximately 43% of the substrate was modified and 13% of the radioactivity was detected in the methanol peak. After 20 h, approximately 65% of the substrate was modified and 16.5% of the radioactivity was detected in the methanol fraction. These results are confirmed by the experiments presented in Figure 3.7. Unlabeled guaiacyl- and syringyl-type DHP (0.5 mg) were incubated with pure laccase (0.5 μg). Methanol concentrations evolved from both DHPs increased during the first hour of the reaction. Approximately 1.6 μg of the guaiacyl-type DHP was converted to methanol (0.3% w/w), whereas 4.6 μg of methanol evolved from the syringyl-type DHP (0.9% w:w).
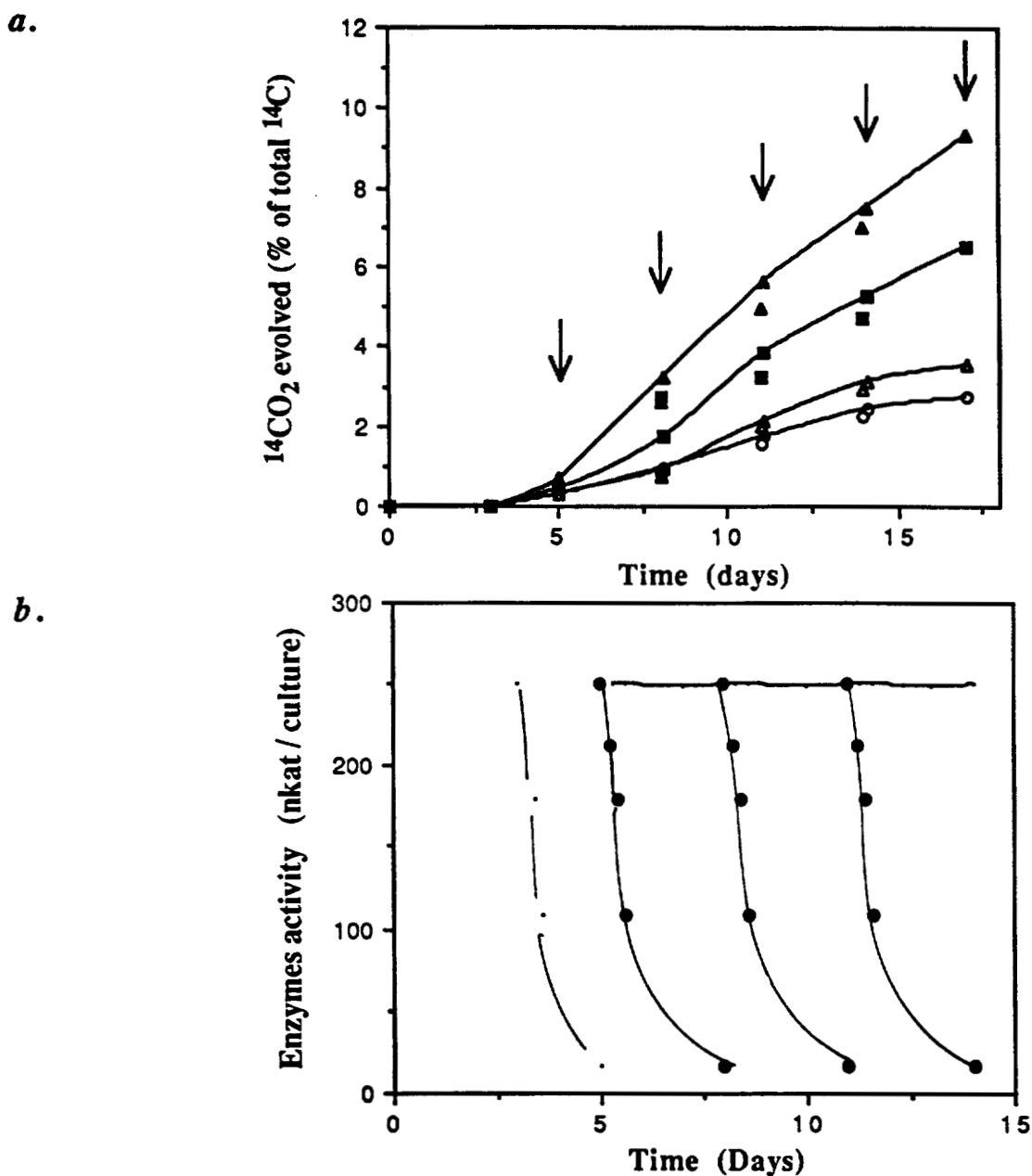
Figure 3.5 Effect of exogenous enzyme addition on *in vivo* DHP mineralization by *P. cinnabarinus*.

(a) $^{14}CO_2$ evolution from ^{14}C-side chain-labeled guaiacyl DHP in *P. cinnabarinus* cultures supplemented with laccase (250 nkat) (o); with laccase (250 nkat) and glucose oxidase (50 U) (Δ); with MnP (250 nkat) and glucose oxidase (50 U) ($\blacktriangle$); and with laccase (250 nkat), MnP (250 nkat), and glucose oxidase (50 U) ($\blacksquare$). Arrows indicate the addition of enzymes.

(b) Time course of enzyme activity in a control culture of *P. cinnabarinus* in a HCLN liquid medium under stationary conditions. Glucose oxidase (50 U), MnP, and laccase were added after each O_2 purge to adjust the activity to 250 nkat for each enzyme. Laccase activity (nkat/culture) ($\cdot$); MnP activity (nkat/culture) ($\bullet$).
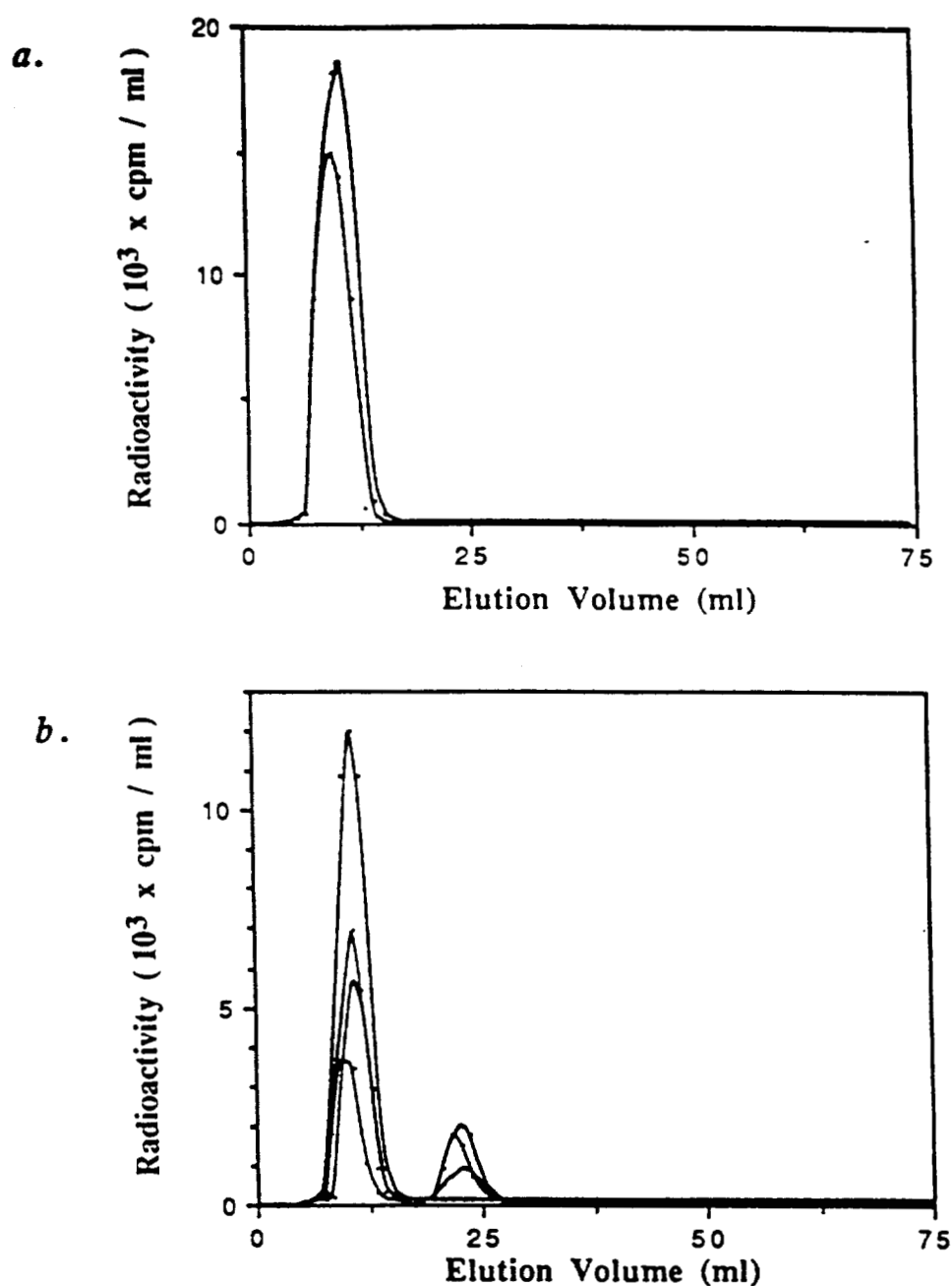
Figure 3.6 DHP depolymerization by purified laccase: Gel permeation profiles.
(a) ^{14}C-side chain-labeled guaiacyl DHP. Upper curve: control; lower curve: after incubation with laccase for 60 min.
(b) ^{14}C-methoxy-labeled guaiacyl DHP. Highest peak: control; decreasing peaks: after incubation with laccase for 40 min, 6 h, and 20 h.
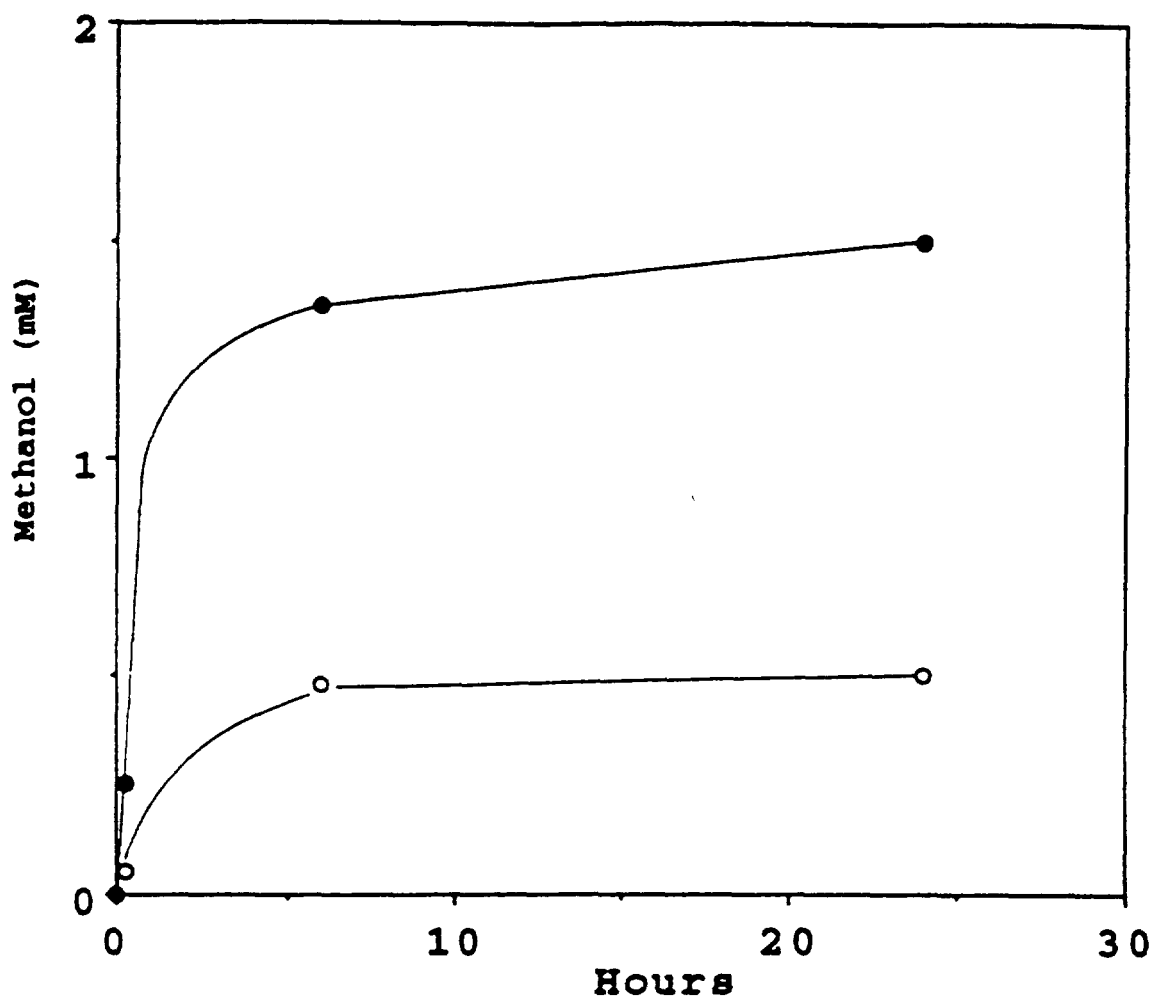
Figure 3.7 DHP depolymerization by purified laccase. Methanol production from guaiacyl- and syringyl-type DHP incubated in laccase for 20 min, 6 h, and 24 h. The reaction mixtures contained 0.5 mg lignin in 1 mL 10 mM Na-succinate, pH 3.5, and 5 μg laccase.

3.4   Discussion

Culture conditions known to promote lignin degradation by *P. chrysosporium* have been used to investigate lignin degradation and laccase production by *P. cinnabarinus*. Considerable chemical and physiological evidence reviewed previously suggests that LiP and MnP constitute major components of the lignin-degradative system of *P. chrysosporium* [17,18,106,177]. LiP catalyzes the oxidative cleavage of non-phenolic lignin model compounds by a mechanism involving the formation of a substrate aryl cation radical [7,18,106,177], and a crude preparation of LiP catalyzes the partial depolymerization of syringyl/guaiacyl-type lignin [108]. MnP catalyzes the oxidation of Mn^{II} to Mn^{III}, and the latter, complexed with an organic acid, oxidizes phenolic lignin model compounds [62–64,100,111]. Homogeneous preparations of MnP catalyze the partial depolymerization of guaiacyl and guaiacyl/syringyl-type lignin [164]. Laccase, commonly found in many wood-rotting fungi, also has been implicated in lignin degradation [5,178,179]. This enzyme preferentially oxidizes substituted phenolic compounds. One of the major roles attributed to laccase in lignin degradation is the demethylation of the ring methoxy groups commonly found in lignin [145,180,181]. There is also evidence that laccase can degrade the side chain of selected phenolic lignin-dimer model compounds (b-0-4 syringyl type [142,143], b-1 dimer [143,144]), open the ring of select lignin model dimers [144], and degrade natural lignin [141,148]. Laccase cooperation with other enzymes [84,182] and possible radical mediators [149,181] has been suggested.

The results shown in Figures 3.1a and 3.1b indicate that laccase is the major lignin-demethylating enzyme found in *P. cinnabarinus* liquid cultures and that, unlike *P. chrysosporium* ligninolytic enzymes, is produced under high nitrogen conditions. The lower laccase activity measured in HCLN cultures may be explained by a less suitable medium composition for laccase production and fungal growth. In contrast, MnP was found only in lignocellulosic-containing media, suggesting that neither Asn-Phe nor HCLN media are suitable for MnP production by *P. cinnabarinus*. The absence of activity in agar-containing cultures suggests that the specific chemical nature of the lignocellulosic material rather than the solid, *per se*, is responsible for MnP production. There is also evidence that the production of ligninolytic enzymes by *P. chrysosporium* is affected by the nature of the substrate (lignocellulosic or synthetic) [183].

However, mineralization of side chain-labeled DHP suggests that MnP was produced in agar-containing HCLN media. Since no surfactant was used in the present experiments, it is possible that MnP remained bound to the mycelium in synthetic media and, thus, was not detected in the culture supernatant [184]. The results presented in Figure 3.5b indicate that *D. squalens* MnP activity was quite unstable in the HCLN liquid medium. It is possible that, in liquid cultures, *P. cinnabarinus* MnP was lost before it was detected. The enzyme may have been subject to autolysis, bleaching of the heme, or proteolysis. Proteases are commonly detected in ligninolytic cultures of white-rot basdiomycetes [185] and have been correlated with the decline of LiP activity in cultures of *P. chrysosporium* [186,187]. Acidic proteases have been identified in cultures of *P. coccineus* [188] and *P. sanguineus* [189], two white-rot basidiomycetes closely related to *P. cinnabarinus*. The presence of a similar protease in *P. cinnabarinus* cultures remains to be demonstrated.

As shown in Figures 3.2a and 3.2b, *P. cinnabarinus* can efficiently demethylate lignin. The results suggest that laccase is responsible for the demethylation of lignin since the highest rate of $^{14}CO_2$ release is obtained in nitrogen-rich media between days 3 and 12, coincident with the highest level of laccase production. The higher rate of $^{14}CO_2$ release observed in agar-containing cultures may be due to the higher enzyme concentration on the surface as compared with liquid media, and to better oxygen uptake by cultures grown in thin layers on the agar surface [40,190]. Immobilization of the enzyme in the gel may contribute to its stabilization [191,192]. Alternatively, it is possible that *P. cinnabarinus* produces MnP when grown on a solid medium which, in turn, contributes to the demethylation of lignin. The latter interpretation is supported by the mineralization of ^{14}C-side chain-labeled DHP observed in HCLN-containing agar (presented in Figure 3.3) indicating that *P. cinnabarinus* can only degrade side chain substructures in nitrogen-limited solid media. Similar results have been reported for the degradation of non-phenolic dimers by *P. coccineus* and *Flammulina velupites* [193]. This suggests that the physical properties of the solid support are required for production of an enzyme capable of cleaving the side chains of polymeric lignin. The absence of degradation in the Asn-Phe medium indicates that such an enzyme is only produced under nitrogen-limited conditions, the same conditions required for the production of MnP and LiP by *P. chrysosporium* [38].

The time course for side chain-labeled DHP mineralization presented in Figure 3.4 indicates that the lignocellulosic medium is more suitable for side-chain substructure degradation. The highest rate of CO_2 formation was observed between days 10 and 15. This period corresponds to the appearance of MnP in the medium as shown in Figure 3.1b. The degradation of side-chain substructures may, therefore, be associated with the onset of MnP production as has been described for *D. squalens* [151]. The highest rate of CO_2 formation also corresponds to a high level of laccase activity in the medium. Laccase alone cannot degrade side-chain lignin substructures. However, cooperation between laccase and MnP in the solubilization of natural lignin *in vitro* has been reported [84].

The cooperation between MnP and laccase was investigated and the results are reported in Figure 3.5a. Reactions were carried out in HCLN liquid media because of the low level of laccase produced and the apparent absence of MnP. Both factors limit the interaction between exogenously added enzymes and the enzymes produced endogenously. In addition, the results obtained in agar cultures suggest that nitrogen-rich media are not suitable for the mineralization of lignin *in vivo*. HCLN conditions were chosen to insure that the mineralization could be completed after the MnP had oxidized the ^{14}C-side chain-labeled DHP. Synergy between laccase and MnP could not be demonstrated in these experiments. The highest rate of mineralization was obtained in cultures containing MnP alone. The addition of laccase to the culture resulted in a 30% reduction in the rate of mineralization, suggesting that, under these conditions, laccase may counteract the action of MnP, possibly through an oxidative repolymerization of lignin. Laccase alone, or in conjunction with glucose oxidase, did not degrade more than 3% of the lignin. The slight increase in mineralization observed with the laccase-containing cultures supplemented with glucose oxidase is in agreement with the proposition that glucose oxidase partially prevents the repolymerization of lignin after oxidation by laccase [182]. However, in the present experiments, the difference in mineralization rates was too small to conclude that laccase and glucose oxidase act cooperatively.

The experiments carried out *in vitro* confirm that laccase is unable to depolymerize guaiacyl DHP under conditions suitable for depolymerization by MnP [164]. No change in M_r was observed after side chain-labeled DHP was incubated for 60 min with laccase whereas, under the same conditions,

significant depolymerization of the same substrate has been reported with
P. chrysosporium MnP [164]. However, as described in Figure 3.6b,
demethylation occurred. The maximum rate of demethylation was observed
during the first hour of reaction. Laccase produced by *P. cinnabarinus* is,
therefore, capable of oxidizing lignin, but the oxidation is limited to
demethylation of the methoxy group. No intermediate oligomers were detected
by gel permeation. Demethylation was confirmed by the detection of methanol
produced during the oxidation of unlabeled guaiacyl and syringyl DHP.
Laccase oxidized both lignins, producing methanol. Apparently syringyl lignin
is a better substrate for laccase than guaiacyl lignin since more than twice the
amount of methanol was produced from syringyl lignin. This may be interpreted
as a slower repolymerization rate of syringyl-type DHP due to the presence of
the second methoxyl group ortho to the phenol, blocking the repolymerization.

The selective degradation of the side-chain substructure under conditions
favoring the production of MnP by *P. cinnabarinus* suggests that this enzyme is
directly involved in the depolymerization of lignin. The absence of lignin
mineralization by the fungus in nitrogen-limited liquid media and the observation
that the onset of CO_2 formation is associated with the addition of exogenous
MnP provide evidence that MnP is responsible for the depolymerization of
lignin and initiates the mineralization of lignin *in vivo*. The role of laccase is
apparently limited to demethylation.

CHAPTER 4
PURIFICATION AND CHARACTERIZATION OF TWO MANGANESE PEROXIDASE ISOZYMES FROM THE WHITE-ROT BASIDIOMYCETE *DICHOMITUS SQUALENS*

4.1   Introduction

Two peroxidases, lignin peroxidase (LiP) and manganese peroxidase (MnP), appear to play a central role in lignin degradation by the best studied white-rot fungus, *Phanerochaete chrysosporium* [62,70,71,74,75]. Another white-rot fungus, *Dichomitus squalens* (*Polyporus anceps*), efficiently degrades both natural [35,49,50,194] and synthetic lignins (DHP) [151]. By comparing $^{14}CO_2$ evolution from ^{14}C-side chain-labeled and ^{14}C-ring-labeled DHP incubated in cultures of *D. squalens* in the presence and absence of Mn^{II}, we demonstrated the importance of Mn for lignin degradation by *D. squalens* and correlated the latter with the presence of extracellular manganese peroxidase (MnP) in the culture medium [151].

P. chrysosporium MnP is a heme glycoprotein of M_r 46,000 [62]. The primary role of this enzyme is to oxidize Mn^{II} to Mn^{III} [64] which, in turn, oxidizes phenolic compounds including lignin [63,64]. In the presence of H_2O_2, ferric MnP is oxidized by two electrons to form MnP compound I. The catalytic cycle of MnP is completed by sequential oxidation of two molecules of Mn^{II}, reducing compound I to the native enzyme with compound II as an intermediate [100]. Although phenolic compounds can be oxidized by compound I, they are not oxidized by compound II; therefore, the catalytic cycle is not completed in the absence of Mn^{II} [100]. The *in vitro* depolymerization of synthetic lignin by homogeneous MnP has been demonstrated recently [164]. MnP production by *P. chrysosporium* is regulated by Mn [165,166] at the level of gene transcription [166,195]. In this report, we describe the purification and partial characterization of two MnP isozymes from the white-rot basidiomycete *D. squalens*.

46

4.2 Material and Methods

4.2.1 Organism

A culture of *D. squalens* (Karst) Reid (*Polyporus anceps* Peck)
(CBS 432.34) was obtained from F. Zadrazil and maintained on potato dextrose-
yeast extract agar slants.

4.2.2 Culture conditions

2-L flasks containing 1 L of medium (35 mM nitrogen from asparagine and
phenylalanine and 2% glucose) [151] were inoculated with a mycelium
suspension and grown for 25 days at 28°C with agitation (150 rpm, 2.5 cm
radius). The flasks were flushed with pure oxygen daily after the sixth day of
growth.

4.2.3 Enzyme assays

MnP activity was determined by following the formation of a Mn^{III}-
malonate complex at 270 nm as described previously [112]. MnP activity was
also determined by following the oxidation of 2,6-dimethoxyphenol (2,6-DMP)
(0.2 mM) at 469 nm in the presence of 0.2 mM $MnSO_4$, and 0.1 mM H_2O_2 in
10 mM malonate, pH 4.5 [151].

4.2.4 Physical and chemical properties

Enzyme molecular weights were estimated by SDS PAGE in 7.5% and
10% acrylamide gel, at 200 V and 150 mA, and by gel filtration on a Superose 12
(Pharmacia), standardized with alcohol dehydrogenase, bovine serum albumin,
and egg albumin. Isoelectric focusing was carried out on vertical slab gels. The
gels were prepared according to O'Farrell [196], with Pharmalyte 2.5–5 from
Pharmacia. Focusing was carried out for 3 h at 200 V [197].

Protein concentrations were determined by BCA assay [197,198] using
bovine serum albumin as a standard. Enzyme carbohydrate content was
determined by the phenol-sulfuric acid method [163,199]. Iron content was
determined by atomic absorption on a Varian Techtron spectrometer at 248.3 nm.

4.2.5 Spectroscopic procedures

Absorption spectra were recorded and enzyme activities measured on a
Shimadzu UV-260 spectrophotometer at room temperature, using a 1-nm spectral

bandwidth and cuvettes of 1-cm light path. When not specified, spectra were taken on a 10-μM protein solution in 10 mM succinate buffer, pH 5.0. Protein heme content was determined by the pyridine hemochrome method [200,201] using a molar extinction coefficient of 3.32×10^4 at 557 nm and was carried out as described previously [62]. The ferrous enzyme was prepared under anaerobic conditions by purging the native enzyme with argon, then adding a sodium dithionite solution [62]. The reduced CO spectrum was obtained by purging the reduced enzyme with CO. Cyanide was added to the native enzyme solution to a final concentration of 10 mM [62]. A final azide concentration of 200 mM was used in an attempt to prepare the azide adduct. Compound I was obtained by adding 1 equiv hydrogen peroxide to the native enzyme. Compound I was also obtained by adding 1 equiv mCPBA or pNPBA organic peroxides [100]. Organic peroxides were dissolved in 2-methyl-2-propanol and diluted 100-fold in water as described [100]. Compound II was obtained by adding 1 equiv ferrocyanide followed by 1 equiv peroxide to the native enzyme. Compound III was obtained by adding 50 equiv H_2O_2 to compound II or by purging the reduced enzyme solution with O_2 [100]. The EPR spectra were taken at 100°K with a Varian E109 EPR spectrometer at 9.122 GHz with a scan range of 4,000 g [89].

4.2.6 Substrate specificity

The ability of MnP to oxidize various aromatic compounds was followed in 10 mM malonate buffer, pH 4.5, and 10 mM malonate, pH 3.0, in the presence of 0.2 mM $MnSO_4$ and 0.1 mM H_2O_2. The oxidation of 2,6-DMP, guaiacol and o-phenylenediamine was followed at 469, 450, and 440 nm, respectively. Veratryl alcohol oxidation was determined by HPLC on an Ultrasphere Octyl 5 μm column, 4.6 mm × 25 cm, equilibrated with 30% methanol in water.

4.2.7 Effect of chelators

The effect of chelators on MnP activity was determined by following 2,6-DMP oxidation at 469 nm in the presence of organic acids. Reaction mixtures contained MnP (1 μg/ml), $MnSO_4$ (0.2 mM), H_2O_2 (0.1 mM), and 2,6-DMP (0.5 mM) in chelator solutions adjusted to pH 4.5 with NaOH. Solutions (50 mM) of acetate, oxalate, glycolate, lactate, malonate, pyruvate, phenyllactate, fumarate, maleate, malate, tartrate, succinate, citrate gluconate, 2,2-dimethylsuccinate (DMS), and phosphate were used.

4.2.8 Mn^{II} binding

For determination of the binding constant, both the reference and sample cuvettes contained MnP ($10~\mu M$ MnP1 and $5~\mu M$ MnP2) in either succinate or malonate buffer. $MnSO_4$ was added only to the sample cuvette, and difference spectra were recorded between 450 and 350 nm. The apparent dissociation constants (K_D) were calculated from the expression $1/\Delta A = 1/(K_D \cdot \Delta A) \cdot 1/Mn + 1/\Delta A$ [202,203]. ΔA was measured as $A_{404}-A_{425}$, representing the difference between maximum and minimum absorption. ΔA is the absorbance difference obtained by addition of an infinite concentration of manganese. The number of binding sites was determined from the Hill equation: $\log[\Delta A/(\Delta A_\infty - \Delta A)] = h \cdot \log(Mn) + \log K_D$, where h is the number of binding sites near the heme [202].

4.2.9 Immunoblotting experiments

Following SDS PAGE, the proteins were electrophoretically transferred to a PVDF membrane (Immobilon-P, Millipore) using a Biorad apparatus as described previously [166,204,205]. Immunodetection was performed as previously described [166,206] using antibodies raised against *P. chrysosporium* MnP isozyme 1 and LiP isozyme 2 [207].

4.3   Results

4.3.1   Purification of extracellular MnP isozymes

Table 4.1   *D. squalens* MnP isozyme purification

	Volume	MnP act.[a]	Protein[b]	Spec act[c]	Recovery	Purification
Crude medium	4000 mL	15.0	1.095	13.70		
Freeze/thaw	3950 mL	14.0	1.050	13.33	92.17	0.97
Polyethyleneimine	3950 mL	13.33	1.029	12.95	87.75	0.94
DEAE Sepharose	250 mL	200	0.690	289.86	83.33	21.16
MonoQ Frac 190[d]	50 mL	385.32	0.190	2028.03	36.47[e]	148.03
MonoQ Frac 280	60 mL	193.30	0.09	2151.44	15.00[e]	157.04

[a] MnP activity is in $\Delta A/min/mL$, measured at 270 nm. [b] Protein concentrations are expressed in mg/mL. [c] Specific activity is in $\Delta A/min/mL$. [d] MnP1 characteristics are reported after the second chromatography on Mono Q. [e] Each value corresponds to the percentage of total initial activity.

Table 4.1 shows the results of purification of the two MnP isozymes. The cells were separated from the extracellular medium by filtration and the filtrate was frozen for at least 50 h at −20°C. After thawing, a colloidal precipitate was removed by filtering through cheesecloth. Polyethyleneimine (0.002% final concentration) was added to the medium and the mixture was allowed to stand for 2 h at 4°C [82]. A second precipitate was separated by filtration through diatomaceous earth. The MnPs were purified from this medium by ion exchange chromatography. The filtrate was adjusted to pH 5.5 and loaded on a DEAE Sepharose column previously equilibrated with 10 mM succinate, pH 5.5, containing 0.01% Tween 80 to prevent unwanted interaction with the gel. The active MnP fraction containing both isozymes was eluted from the column batchwise with a 200-mM solution of sodium chloride in the equilibrating buffer. The MnP fraction was concentrated by ultrafiltration on an Amicon Diaflo PM10 membrane (10,000 cutoff) and dialyzed against 10 mM bis-Tris, pH 7.0, containing 0.01% Tween 80. The fraction was loaded on a Mono Q HR5/5 column equilibrated with the same buffer, and the column was eluted with a stepwise sodium chloride gradient (Figure 4.1).

The use of Tween 80 during the chromatography maximized the recovery of MnP activity. When the chromatography was run in the absence of Tween 80, the protein appeared to lose its heme prosthetic group. The fraction eluting at 190 mM NaCl containing one of the MnP isozymes (MnP1) and laccase was dialyzed against 10 mM succinate buffer, pH 4.5. The proteins were loaded on the Mono Q column equilibrated with 10 mM succinate, pH 4.5. MnP1 was separated from laccase by using a linear gradient of NaCl from 0 to 150 mM.

4.3.2 Homogeneity and molecular mass of peroxidases

The purified proteins each exhibited a single band when subjected to SDS PAGE (Figure 4.2). The retention time for both MnPs by gel filtration was identical to the egg albumin retention time indicating that the native proteins have an approximate M_r of 45,000. The migration pattern on SDS PAGE suggested that the denatured MnP1 has a M_r of 46,000 and MnP2 has a M_r of 48,500. Phenol sulfuric acid assay indicated that both isozymes are glycoproteins and that carbohydrates contribute 8.5% and 10.3% to the masses of MnP1 and MnP2, respectively. Isoelectrofocusing indicated that MnP1 has a pI of 4.1 and, under these conditions, MnP2 is separated into two bands corresponding to estimated pIs of 3.9 and 3.85.
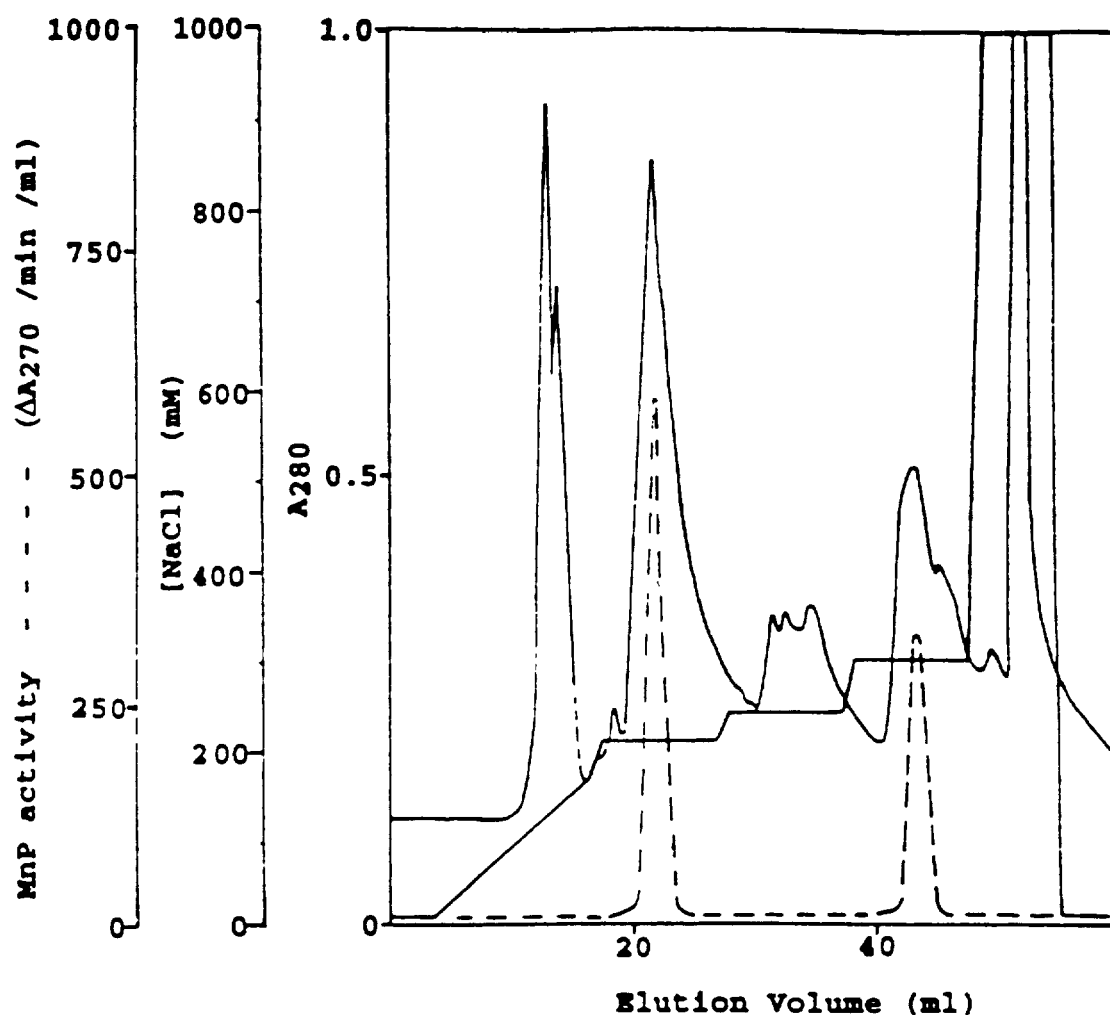
Figure 4.1 Separation of MnP isozymes by chromatography on FPLC.
DEAE Sepharose active fraction (200 mM) resolved on FPLC. Equilibrating
buffer: 10 mM bis-Tris, pH 7.0, 0.01% Tween 80. Eluting buffer: 10 mM bis-
Tris, pH 7.0, 0.01% Tween 80, 1 M NaCl. MnP1 elutes at 190 mM of NaCl
(second peak), laccase elutes at 220 mM (third peak), and MnP2 elutes at 280
mM (fourth peak). The first and fifth peaks have no oxidase or peroxidase
activity.

mole MnP2 were calculated. Atomic absorption measurements showed that each molecule of enzyme contained one iron, confirming the pyridine hemochrome results. The electronic absorption maxima of MnPs from *D. squalens* and *P. chrysosporium* are shown in Table 4.2.

Figures 4.3 and 4.4 show the electronic absorption spectra of native *D. squalens* MnP and various derivatives. The addition of 1 equiv H_2O_2 generated a new spectrum corresponding to compound I (Figures 4.3b and 4.4b). For both isozymes, the Soret bands were reduced to 40% of the native in compound I. The MnP2 Soret band was blue shifted to 400 nm (Table 4.1). The 650-nm peak characteristic of compound I was detected at 648 nm for MnP1 and 650 nm for MnP2. The addition of 1 equiv ferrocyanide generated compound II (Figures 4.3c and 4.4c). A species with a spectrum corresponding to compound III was generated by adding excess hydrogen peroxide (50 equiv) to compound II or by purging the reduced enzyme with O_2 (Figures 4.3d and 4.4d). The values reported in Table 4.2 were obtained for the ferrous-oxy form. The addition of peroxide to compound II resulted in an immediate bleaching of the proteins, decreasing the values of the maxima. The addition of cyanide to the native enzymes resulted in a red shift of the Soret band. A peak at 350 nm was also observed for this derivative (Table 4.2). The addition of azide (200 mM) did not significantly affect the spectral features of the native enzymes. In contrast to the results for *P. chrysosporium* MnP [62], a 20-fold excess of azide did not result in a red shift of the Soret band of the *D. squalens* MnPs. Reduction of the native form with sodium dithionite caused a shift of the Soret band to 434 nm and the formation of visible bands at 554 nm for MnP1 and 557 nm for MnP2. The binding of carbon monoxide to the reduced enzyme generated maxima at 422 nm, 540 nm, and 570 nm.

4.3.4 Organic peroxide

Both isozymes were oxidized to compound I upon the addition of 1 equiv mCPBA or pNPBA. A second equivalent of the oxidant resulted in the formation of compound II. The spectra of these compounds were not significantly different from those obtained with hydrogen peroxide (data not shown).
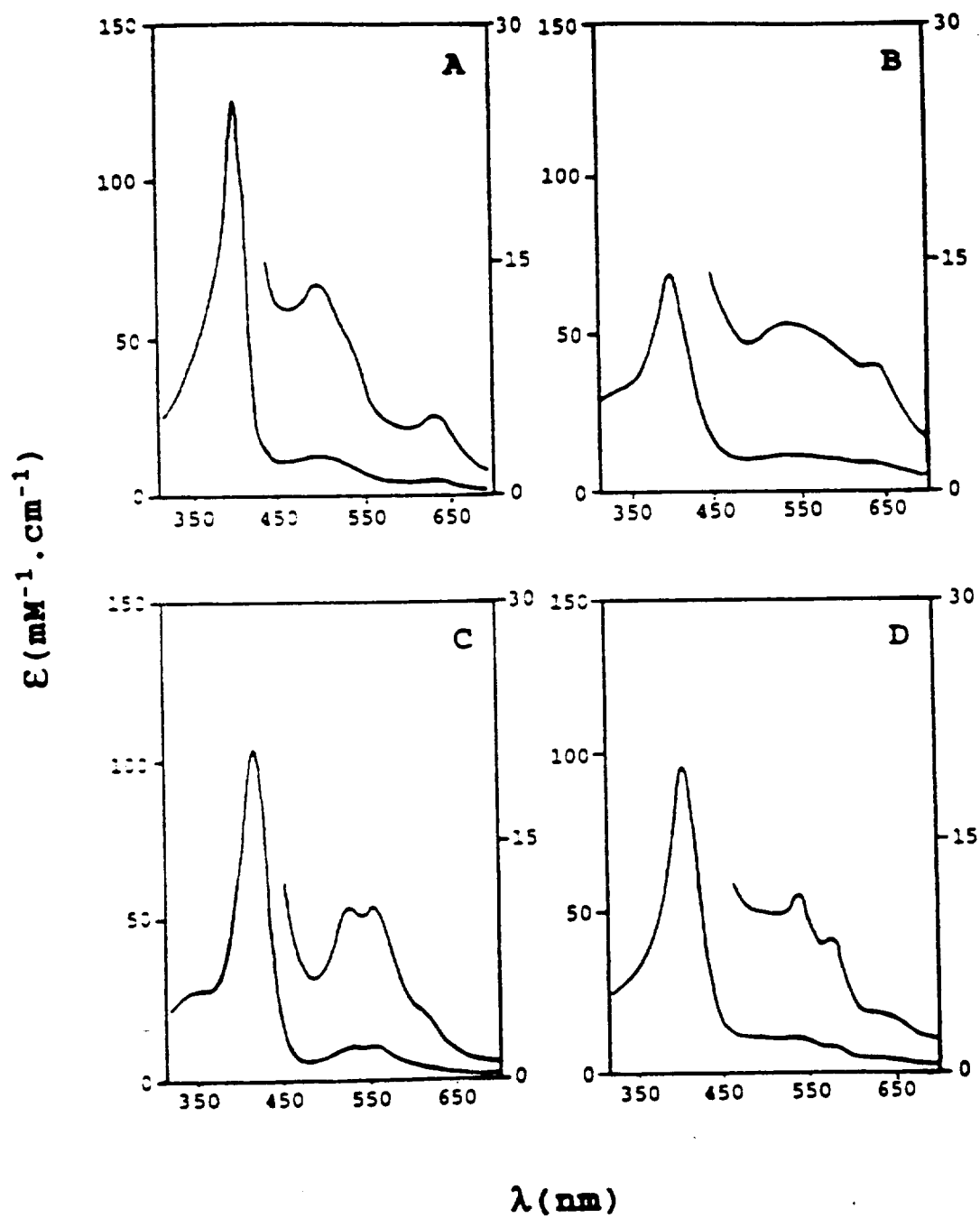
Figure 4.3 Electronic absorption spectra of MnP1.
A) Native form, (B) compound I, (C) compound II, (D) compound III.
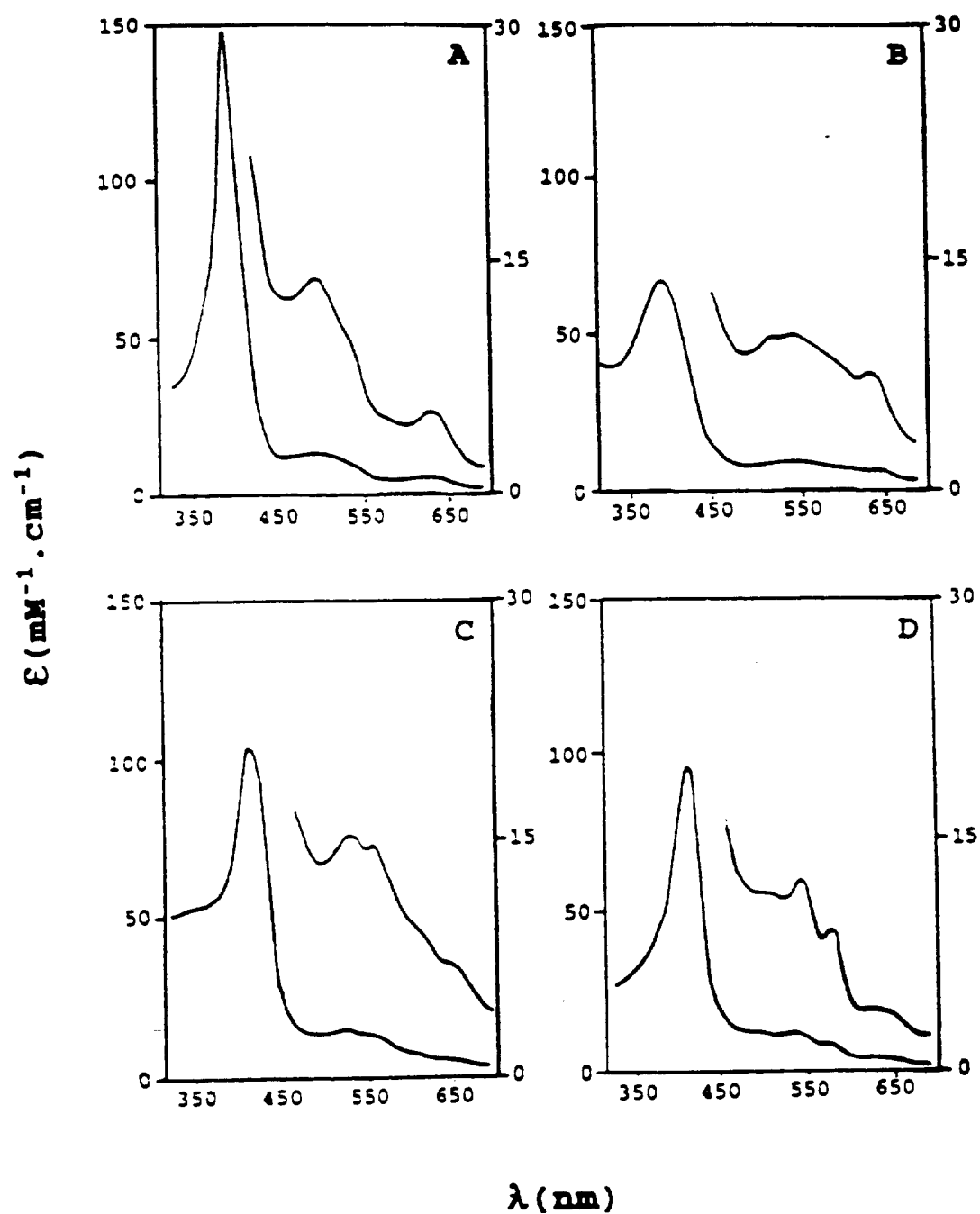
Figure 4.4 Electronic absorption spectra of MnP2.
(A) Native form, (B) compound I, (C) compound II, (D) compound III.

4.3.5 pH dependence

Both isozymes were assayed in 50 mM malonate with pH varying from 2.0 to 7.3. As shown in Figure 4.5, MnP1 had a pH optimum of 4.5 and MnP2 had a pH optimum of 5.0.

Table 4.3 Effect of chelators: Initial rate of 2,6-DMP oxidation by MnP1 and MnP2

	MnP1 (% of max)	MnP2 (% of max)
Acetate	2.0	3.3
Oxalate	87.5	79.0
Glycolate	99.1	73.2
Lactate	100.0	85.8
Malonate	77.2	68.5
Pyruvate	21.5	24.6
Phenyllactate	99.1	84.4
Fumarate	3.2	5.5
Maleate	2.6	5.5
Malate	73.2	80.3
Tartrate	68.7	81.7
Succinate	15.2	16.1
Citrate	36.3	55.4
Gluconate	79.8	100.0
2,2 DMS	2.4	4.8
Phosphate	4.5	8.0

The pH of each solution (50 mM) was adjusted to 4.5 with NaOH.

4.3.6 Substrate specificity

In the presence of Mn^{II}, both MnPs were able to oxidize phenolic compounds such as 2,6-DMP and guaiacol, or aromatic amines such as o-phenylenediamine. In the presence of H_2O_2 and Mn^{II}, neither MnP isozyme was able to oxidize the non-phenolic model compound veratryl alcohol.
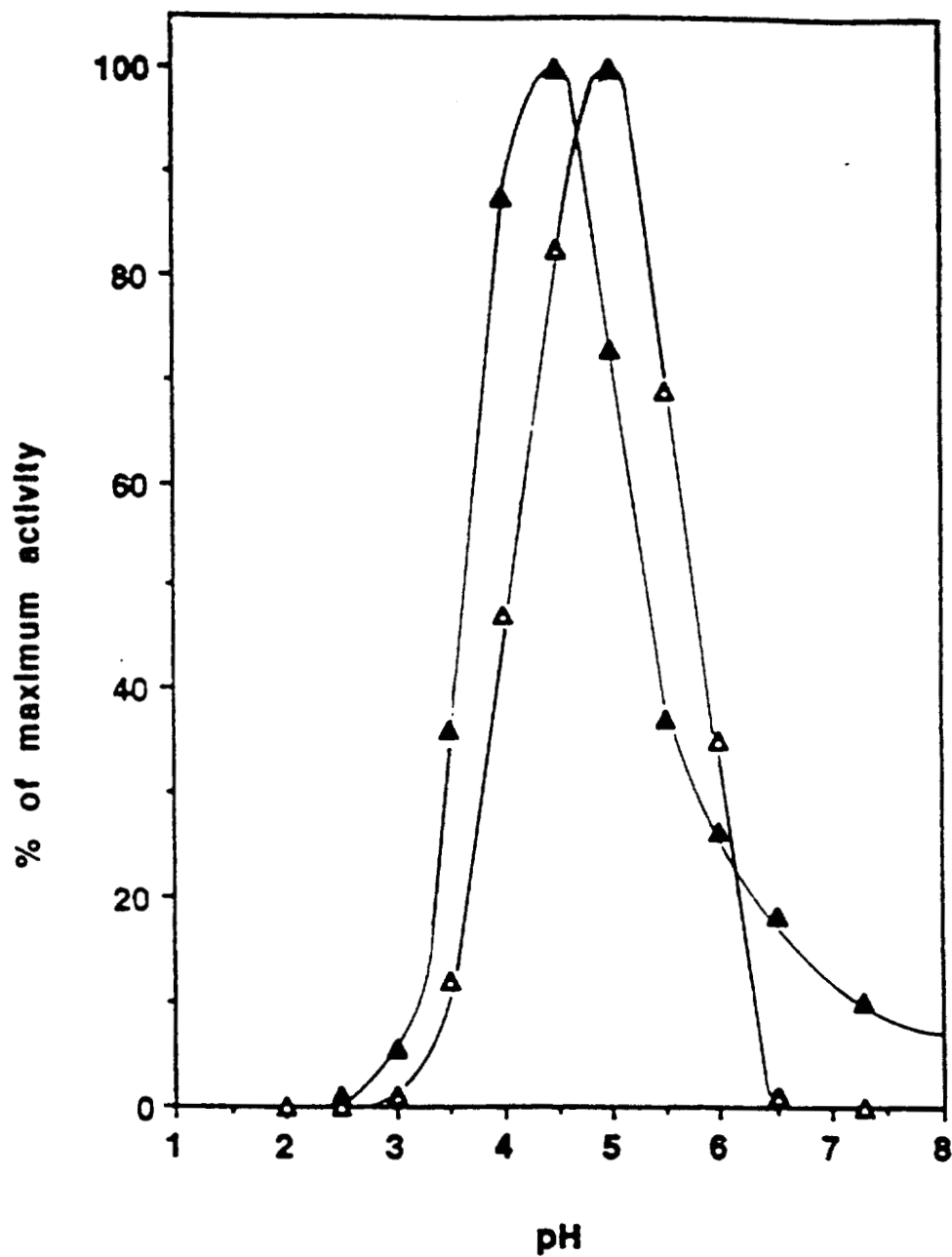
Figure 4.5 pH activity profile of MnPs. Activities were measured in malonate-buffered solutions, 50 mM, 0.2 mM $MnSO_4$, 0.1 mM H_2O_2. MnP1 (▲), MnP2 (Δ).

4.3.7 Effect of chelators

The relative activities of the enzymes in the presence of various chelators are shown in Table 4.3. The highest activities were obtained with dicarboxylic and α-hydroxy acids. The maximum MnP1 activity was obtained in lactate, whereas the maximum MnP2 activity was obtained in gluconate. Approximately 11% of the maximum activity measured was detected in succinate for both isozymes. In the presence of phosphate buffer, 5 and 8% of MnP1 and MnP2 maximal activity was observed. No activity was detected in water with MnP1.

4.3.8 Other metals as substrates for MnPs

MnP has been shown to have a high specificity for Mn^{II} [62,64]. Mn^{II} was replaced by Fe^{II}, Co^{II}, Ni^{II}, Cu^{II}, and Zn^{II} in the MnP reaction mixture and the oxidation of 2,6-DMP was followed as the final substrate of the MnP reaction. Surprisingly, MnP was able to oxidize 2,6-DMP in the presence of Co, although to a much lesser extent—7.35% with MnP1 and 2.2% with MnP2— than in the presence of Mn^{II}. Other metal ions such as Cu or Ni gave less than 2% of the activity obtained with Mn^{II}. Buffers other than malonate did not increase the activity observed with these metals. No detectable oxidation of 2,6-DMP was observed in the absence of metal ions.

4.3.9 Affinity for Mn

The K_m for Mn^{II} was measured in malonate buffer, pH 4.5, for MnP1 and in malonate buffer, pH 5.0, for MnP2 in the presence of 0.1 mM hydrogen peroxide. MnP1 and MnP2 had K_ms for Mn^{II} of 39 μM and 25 μM, respectively. The K_D for Mn^{II} was measured in malonate and succinate buffers at the pH optimum for each isozyme. Using the double reciprocal plot $1/\Delta A$ versus $1/[Mn]$, with $\Delta A = A_{404} - A_{425}$, the MnP1 K_D for Mn^{II} was 12.2 μM and the MnP2 K_D for Mn^{II} was 3.8 μM in succinate buffer. In malonate, the K_D for MnP1 and MnP2 was 21 μM (MnP1) and 11 μM (MnP2), respectively (Figures 4.6a and 4.6b). The Hill equation

$$\log[\Delta A/(\Delta A_\infty - \Delta A)] = h \cdot \log([Mn]) + \log K_D)$$

showed a linear relation between the two log expressions with a slope of 1, suggesting that there is a single binding site for Mn^{II} in the vicinity of the heme.
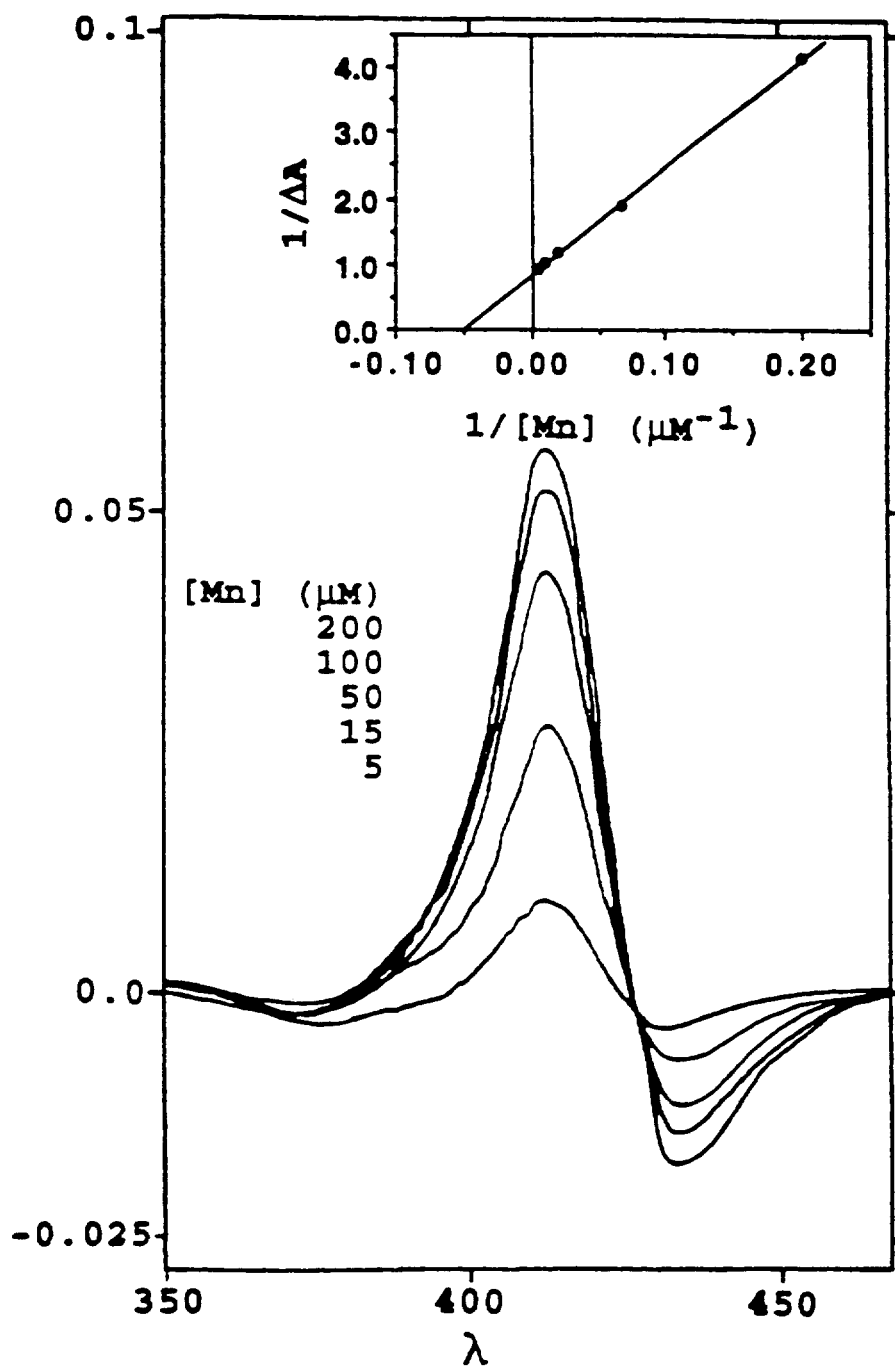
Figure 4.6 MnP1 affinity for Mn. Difference spectrum of MnP1 (10 µM) in 50 mM Na-malonate, pH 4.5, with several concentrations of $MnSO_4$ in the sample cuvette, 5 µM, 10 µM, 15 µM, 50 µM, 100 µM, and 200 µM. Double reciprocal plots of the difference of aborbance between 406 nm and 424 nm versus $[Mn^{II}]$.
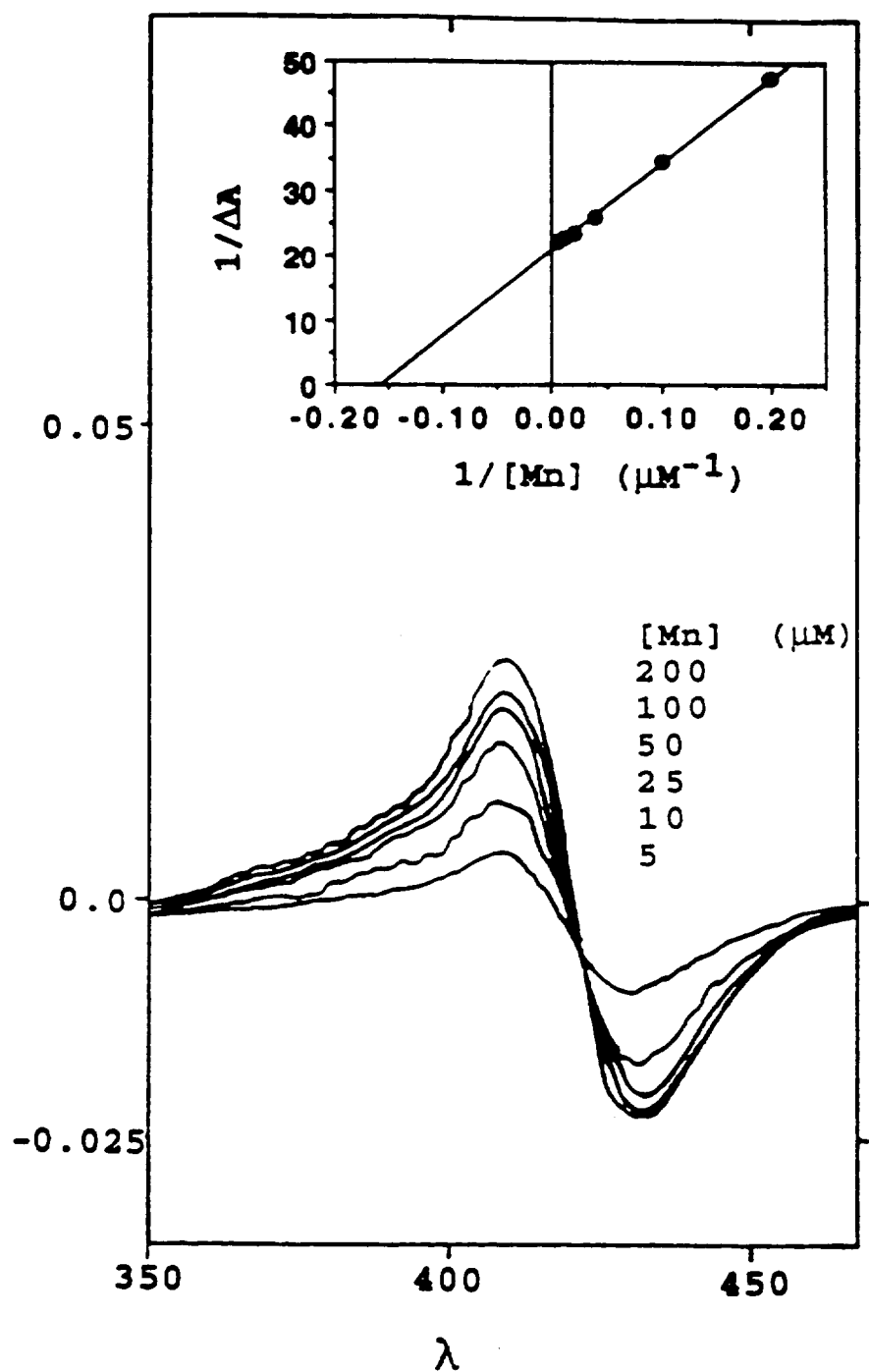
Figure 4.7 MnP2 affinity for Mn. Difference spectrum of MnP2 (5 μM) in 50 mM malonate, pH 5.0, with several concentrations of manganese in the sample cuvette, 5 μM, 10 μM, 25 μM, 50 μM, 100 μM, and 200 μM. Double reciprocal plots of the difference of absorbance between 406 nm and 424 nm versus [Mn].

4.3.10 Immunologic relatedness with *P. chrysosporium* MnP

Figure 4.8 shows the results of a western blot for MnP1 and MnP2 (lanes 1 and 2). Both isozymes reacted positively with polyclonal antibodies raised against *P. chrysosporium* MnP1. Culture media obtained after 25 days of growth in the presence and absence of Mn^{II} were also tested. MnP protein was detected from cultures grown in the presence of Mn^{II}, but was not detectable from cultures grown in the absence of Mn^{II}. The identical blots reprobed with polyclonal antibodies against LiP showed no reaction.

1   2   3   4   5
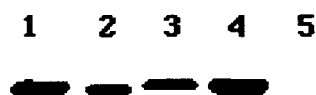
Figure 4.8 Immunodetection of MnP with MnP polyclonal antibodies raised against *P. chrysosporium* MnP. Lane 1: DEAE Sepharose active fraction (eluted at 200 mM); lane 2: MnP2 after FPLC; lane 3: MnP1 after FPLC; lane 4: culture medium containing 100 μM MnSO$_4$; lane 5: culture medium containing no manganese.

4.4   Discussion

Previous results [151] indicated that the appearance of MnP in the extracellular medium and the capacity to mineralize lignin were dependent on the presence of Mn in the *D. squalens* culture medium. The only other ligninolytic enzyme detected from *D. squalens* cultures was laccase. In particular, no LiP activity was present either in the culture medium or bound to the cells. Since *P. chrysosporium* MnP reactions via the oxidation of Mn^{II} to Mn^{III} appear to be limited to the attack of free phenol on the aromatic ring of lignin [14,18], it appeared of interest to determine whether these phenoloxidases were capable of degrading lignin. In particular, we wanted to determine whether *D. squalens* MnP had different properties from *P. chrysosporium* MnP or whether other factors were affecting the ability of *D. squalens* to degrade lignin.

MnP is a heme protein first discovered in the extracellular medium of *P. chrysosporium* cultures [71]. The enzyme requires H_2O_2 as a cosubstrate and oxidizes Mn^{II} to Mn^{III} [64], which acts as an obligatory redox couple, in turn oxidizing various organic substrates [14,62–64]. The activity is greatly enhanced by the presence of organic acid chelators such as dicarboxylic or α-hydroxy

acids. The prosthetic group of the protein is a protoporphyrin IX [64]. Studies of this enzyme using electronic absorption [62], EPR, and resonance Raman spectroscopy [90] indicate that iron in the native protein is in the high-spin, ferric, pentacoordinate state with histidine coordinated as the fifth ligand. MnP has a typical peroxidase catalytic cycle. The ferric enzyme is oxidized by two electrons to form compound I ($Fe^{IV}=O[P]^{+\bullet}$). Compound I is subsequently reduced via two single electron steps to compound II ($Fe^{IV}=O[P]$) and to the native enzyme [100]. In the presence of excess peroxide, the enzyme is oxidized to compound III ($Fe^{III}O_2^{-\bullet}$, $Fe^{II}O_2$), which is not part of the catalytic cycle [100]. *P. chrysosporium* expresses MnP only when Mn^{II} is present in the nitrogen-limited culture medium. Mn has been shown to regulate MnP at the level of the gene transcription [166,195]. MnP production by *D. squalens* is also regulated by Mn^{II} concentration, but does not depend upon nitrogen concentration. The purification of MnP from the crude medium of *D. squalens* yielded two isozymes with small differences in molecular mass, carbohydrate content, and chromatographic behavior.

4.4.1 Physical and chemical properties

The M_r of MnP1 and MnP2 are 46,000 and 48,500, respectively, within the range of values reported for *P. chrysosporium* MnP (46,000) [62], *Trametes versicolor* MnP (49,000) [83], *Lentines edodes* (44,600) [82], or *Rigidoporus lignosus* MnP (42,000) [84]. The pI values of 4.15 (MnP1) and 3.9 and 3.85 (MnP2) also fall within the range observed for the other MnPs: 4.2–4.9 for *P. chrysosporium* MnPs [73], 3.5–3.7 for *R. lignosus* MnPs [84], and 3.2 for *L. edodes* MnP [82]. The two pI values of 3.85 and 3.9 corresponding to MnP2 suggested that they present a very small difference in charge that could be explained by a difference in either the carbohydrate content or the amino acid sequence. The pH optima for these isozymes also corresponded to the optimum found for other MnPs [62,82–84].

The immunoblot experiments indicated that the structures of both isozymes may be closely related to those of *P. chrysosporium* MnPs. The absence of reaction with LiP antibodies confirmed the absence of LiP in *D. squalens* cultures and suggested that *D. squalens* MnPs had a higher degree of similarity with *P. chrysosporium* MnP than with *P. chrysosporium* LiP. Previous results [151] showing that MnP production depends upon the presence of Mn^{II} were confirmed by these experiments.

4.4.2 Electronic absorption spectroscopy

The optical absorption maxima of the native, liganded, oxidized, and reduced MnPs suggested that the heme environment was very similar to that of other well-characterized peroxidases [62,70,99]. The Soret maxima at 408 nm and 406 nm, and visible maxima at 500–501 nm and 634 nm of the native proteins, are characteristic of high-spin ferric heme proteins with histidine as the fifth ligand [62,64,90]. The Soret bands fell within 2 nm of the Soret band of horseradish peroxidase [85], *P. chrysosporium* MnP (406nm) [90], and *P. chrysosporium* LiP (407 nm) [89]. Likewise, the high-spin marker at 632–633 nm was within 2 nm of those of the above peroxidases. These data suggest that *D. squalens* MnPs were also pentacoordinated.

The modification of the spectra induced by cyanide binding suggested that the iron in this derivative became low-spin and hexacoordinated [62,64,89,90]. The enzyme reduction by sodium dithionite to form ferrous protein and the binding of CO to the reduced (ferrous) enzyme confirmed the similarity with *P. chrysosporium* MnP and LiP, and with HRP. These observations strongly suggest that the fifth ligand to the heme iron was likely to be a histidine or histidinate.

The different enzyme oxidation states characterized by Wariishi et al. [100] for *P. chrysosporium* MnP were also detected. Compound I, corresponding to the two-electron oxidized form of the enzyme, had a reduced Soret band intensity. This has been interpreted for HRP [92,93,208,209] and for *P. chrysosporium* MnP [100] as an indication of *p*-cation radical formation on the porphyrin ring of compound I. Compound II is obtained by a one-electron reduction of compound I with the addition of 1 equiv ferrocyanide or Mn^{II}. It took only 1 equiv organic peroxide to reduce compound I to compound II in the case of *D. squalens* MnP, where it takes 25 equiv to reduce *P. chrysosporium* MnP compound I [100]. This suggests that the active site of *D. squalens* compound I was either more accessible to bulky organic peroxides or was easier to reduce than *P. chrysosporium* compound I. Compound III corresponds to the ferrous oxy/ferric superoxide form of the enzyme [100]. Excess peroxide added to compound II generated a mixture of species containing compounds II and III. The enzyme started to bleach, probably owing to the oxidation of the heme, before a pure compound III could be obtained. 50 Equiv peroxide were sufficient to initiate bleaching. Hence, the exact quantity of peroxide needed to form compound III had not been determined. However, 250 equiv are required

to form compound III with *P. chrysosporium* MnP [100]. In this respect, *D. squalens* MnP was more similar to *P. chrysosporium* LiP which requires only 25 equiv H_2O_2 for compound III formation [99].

4.4.3 Catalytic activity

The substrate specificities of these MnPs appeared to be the same as *P. chrysosporium* MnP [62,63]. *D. squalens* MnPs preferentially oxidized phenolic substrates. In particular, veratryl alcohol, the non-phenolic substrate used in lignin peroxidase assay [75], was not oxidized by these MnPs. As described for *P. chrysosporium* MnP, *D. squalens* MnPs had a very high specificity for Mn^{II} [62,64]. Most other metal ions could not act as obligatory redox couples for the oxidation of phenol. Surprisingly, Co^{II} oxidizes 2,6-DMP with a relative rate of 7.3% (MnP1) and 3.3% (MnP2) compared to Mn^{II}. The other transition metals tested, Cu^{II} and Ni^{II}, had relative rates below 2%.

α-Hydroxy acids or certain dicarboxylic acids greatly enhance MnP activity via stabilization of the Mn^{III} high redox potential [62,63,100] and facilitation of Mn^{III} dissociation from the enzyme-Mn^{III} complex, [62,64,109]. Studies with *P. chrysosporium* MnP show that malonate is the most efficient chelator for the oxidation of Mn^{II} to Mn^{III}. Data obtained with the *D. squalens* MnPs confirmed the stimulation of MnP activity by small dicarboxylic and α-hydroxy acids, although some activity was detectable with acids such as succcinate or even acetate that do not enhance *P. chrysosporium* MnP activity [62]. MnP activity was also stimulated by bulky organic acids such as phenyllactate, indicating that the size of the chelator was not limiting MnP activity. The absence of MnP1 activity in water indicated that Mn^{III} was not spontaneously released from the enzyme. The activity found in succinate suggested that, in contrast with *P. chrysoporium* MnP, succinate interacts with the Mn^{III}-MnP complex to cause oxidation of 2,6-DMP. In contrast, it appeared that *D. squalens* native MnPs bind Mn^{II} more strongly than *P. chrysosporium* MnP in malonate. A comparison of K_D values suggests that *D. squalens* MnPs bind Mn^{II} 2–4 times more strongly in malonate buffer and almost 10 times more in succinate buffer. With a value of 1, the Hill coefficient indicated that MnP had a single binding site for Mn^{II} in the vicinity of the heme.

The two MnPs purified from the liquid culture medium of *D. squalens* were similar in many ways to those described for *P. chrysosporium* MnP. Immunologic cross-reactivity and spectral characterization strongly suggest that

the similarity of the enzymes covers both the general structure of the protein and the environment of the prosthetic group. However, some mechanistic differences seem to exist between *D. squalens* and *P. chrysosporium* MnPs, e.g., their abilities to bind Mn^{II} and Mn^{III}. In a previous report [151], it was suggested that *D. squalens,* unlike *P. chrysosporium,* degrades DHP in the presence of MnP and laccase, but in the absence of LiP. The present chapter further indicates that *D. squalens* MnPs are similar in mechanism to *P. chrysosporium* MnP. Additional comparison of MnPs from *D. squalens* and *P. chrysoporium* should help to elucidate the mechanism of this unique enzyme.

CHAPTER 5
PURIFICATION AND CHARACTERIZATION OF LACCASES FROM
PYCNOPORUS CINNABARINUS AND *DICHOMITUS SQUALENS*

5.1 Introduction

The study of lignin degradation by white-rot basidiomycetes has been
mainly focused on *Phanerochaete chrysosporium* [17,18], *Coriolus versicolor*
[34,210,211] and *Phlebia radiata* [43] because of their high rate of lignin
degradation [167]. Along with other peroxidases and oxidases, these fungi
produce high titers of lignin peroxidase, and some results suggest that this
enzyme is directly responsible for lignin mineralization *in vivo* [14,17,18,212].
Yet other white-rot fungi degrade lignin [39,168–170] without necessarily
producing LiP under ligninolytic conditions [44,67,151]. *P. cinnabarinus*
[39,40,43,171,172] and *D. squalens* [35,49,50,151,194] degrade lignin under
selected conditions without apparently producing any LiP. It was suggested
that the mineralization of lignin by *D. squalens* was dependent on the presence
of MnP and possibly laccase [151]. It is possible that *P. cinnabarinus* relies on
the same enzymes—MnP and laccase—to degrade lignin. The role of laccase in
the degradation of lignin remains undetermined [138,139], but results suggest
that laccase may be actively involved alone [141,148], in synergy with other
enzymes [84,182], or via the oxidation of intermediates that are subsequently
capable of oxidizing non-phenolic substrates [149,181]. The purification and
characterization of several fungal laccases have been reported for other
basidiomycetes [152,213–215]. These enzymes are glycoproteins containing four
copper atoms per molecule of protein and primarily oxidize phenolic substrates.
Each copper is spectroscopically distinct and can be characterized as type 1,
type 2, or type 3 copper [120]. Laccase from *Agaricus bisporus* and *P. radiata*
apparently contain only two copper atoms per molecule of protein. In this
chapter, the purification and partial characterization of *P. cinnabarinus* and
D. squalens laccases are described.

66

5.2 Material and Methods

5.2.1 Organisms

A culture of *D. squalens* (Karst) Reid (*Polyporus anceps* Peck) (CBS 432.34) was obtained from F. Zadrazil and maintained on potato dextrose-yeast extract agar slants. A culture of *P. cinnabarinus* (Jacq. ex Fr) Karst (IFO 6139) was obtained from H. Tanaka (Kinki University, Nara, Japan) and maintained on malt extract and yeast extract supplemented with Vogel salts [173,174].

5.2.2 Culture conditions

2-L flasks containing 1 L of medium were inoculated with a suspension of mycelium and grown at 28°C for 10 days with agitation (150 rpm, 2.5 cm radius). After 1 day of growth in air for *P. cinnabarinus* and after 6 days of growth in air for *D. squalens*, the flasks were purged daily with 100% oxygen. Both organisms were grown in a medium containing the following components (per liter): 20 g glucose, 2.5 g L-asparagine, 0.15 g L-phenylalanine, 2.5 mg cysteine, 30 mg adenine, 0.1 mg thiamine, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 10 mg $FeSO_4$, 12.5 mg $CuSO_4.5H_2O$, 1 mg $ZnSO_4$, as well as 0.01% Tween 80. The pH of the medium was adjusted to 5.3 with Na_2PO_4 (0.1 g/L approximately).

5.2.3 Enzyme isolation

Protein separations were carried out on a DEAE Sepharose column (15 cm × 3.5 cm) equilibrated with 10 mM succinate, pH 6.0, for *P. cinnabarinus* laccase (P-laccase), and with 10 mM succinate, pH 6.0, containing 0.01% Tween 80 for *D. squalens* laccase (D-laccase). Further anion exchange chromatography was carried out by FPLC (Pharmacia) using a Mono Q HR 5/5 column equilibrated with 10 mM bis-Tris buffer, pH 6.0, for *P*-laccase purification, and with 10 mM bis-Tris, pH 7.0, and 0.01% Tween 80 for *D*-laccase purification. *D*-laccase isozymes were separated in 10 mM succinate, pH 4.5. Concentration, desalting, and buffer exchange were carried out by ultrafiltration on an Amicon Diaflo PM 10 membrane (10,000 cutoff).

5.2.4 Physical and chemical properties

The molecular weights of the enzymes were estimated by SDS PAGE in 7.5 and 10% acrylamide, at 200 V 150 mA, and by gel filtration on a Superose 12 column (Pharmacia) calibrated with alcohol dehydrogenase (150,000), bovine

serum albumin (66,000), and egg albumin (43,000). Isolelectric focusing was carried out on vertical slab gels prepared according to O'Farrell [196] with Pharmalyte 2.5–5 (Pharmacia). Focusing was carried out for 3 h at 200 V.

Protein concentrations were determined by BCA assay [197,198], using bovine serum albumin as a standard. Carbohydrate content of the enzymes was determined by the phenol-sulfuric acid method [163,199]. Copper content was determined by atomic absorption on a Varian Techtron spectrometer at 324.65 nm.

5.2.5 Enzyme assay

Laccase activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol at 469 nm. The reaction mixture contained 2,6-DMP (0.2 mM) and the enzyme in 20 mM Na-succinate, pH 3.5 for P-laccase, and pH 3.0 for D-laccase.

5.2.6 pH dependence

The pH optimums of the enzymes were determined in 20 mM Na-succinate from pH 3.0 to 6.0, in 20 mM glycine-HCl from pH 2.5 to 3.5, in 20 mM HCl-KCl for pH 2.0, and in 20 mM Na-phosphate for pH 5.5 to 7.5.

5.2.7 pH and temperature stability

The effect of temperature on laccase stability was estimated by incubating a solution of enzyme in 10 mM Na-phosphate, pH 6.0, for 15 min at several temperatures. The enzyme solution was cooled on ice immediately and the activity was measured spectroscopically. The effect of pH on laccase stability was estimated by incubating the laccase at pH 2.5, 3.0, 5.0, 7.0, and 8.5, for 20 h at 28 °C, in the solutions described above. Laccase activity was assayed in 10 mM Na-succinate, pH 3.5.

5.2.8 Substrate specificity

K_m measurements were carried out with an oxygen electrode (Rank Brothers Ltd.) at 25°C, with 2.5 μg/mL of enzyme in 20 mM Na-succinate, pH 3.5, for P-laccase 1, and in 20 mM Na-succinate, pH 3.0, for D-laccase 1. Oxidation of o-phenylene diamine, veratryl alcohol, and ABTS was monitored with the oxygen electrode or spectroscopically at 440, 310, and 415 nm, respectively.

5.2.9 Spectroscopic procedures

Absorption spectra were recorded and enzyme activities were measured on a Shimadzu UV-260 spectrophotometer at room temperature using a 1-nm spectral bandwidth and cuvettes of 1-cm light path. EPR spectra were recorded on a Varian E109 EPR spectrometer at approximately 9 GHz, 40°K. Spectra of the enzymes were recorded in 20 mM Na-succinate, pH 3.0, and in 20 mM Na-phosphate, pH 7.0. Integration of the EPR signal was estimated by comparison with a standard solution of Cu-EDTA. The pH dependence of extinction coefficients for P-laccase 1 was followed by recording the UV vis spectra of the enzyme in 20 mM Na-succinate, pH 3.0 and 4.5, and in 20 mM Na-phosphate, pH 6.0, 7.1, and 8.0.

5.3 Results

5.3.1 Purification of laccases

Tables 5.1a and 5.1b show the results of purification of P- and D-laccases. The cells were separated from 4 L of extracellular culture fluid by filtration, and the medium was frozen for at least 50 h at −20 °C. After thawing, a colloidal precipitate was removed by filtration. The laccase activity remained in the filtrate.

5.3.1.1 Purification of P-laccases

Table 5.1a Purification of P-laccases

	V. (mL)	Activity (U/mL) a	Prot conc mg/mL	Spec act (U/mg)	Rec.	Purif.
Crude medium	4000	12	1.51	7.95		
Freeze/thaw	3980	12	1.45	8.28	0.995	1.04
Act. charcoal	3980	12	1.40	8.57	0.995	1.08
DEAE Sepharose	70	743	4.06	183.0	1.08	23.0
Mono Q pH 6 L1[b]	10	2004	0.75	2668	0.42	3377
Mono Q pH 6 L2[b]	10	990	0.43	2302	0.21	2914

[a]1 U= DA$_{469}$/min [b]L1: P-laccase 1; L2: P-laccase 2.

P. cinnabarinus produces abundant red/brown pigments eliminated from the culture fluid by adsorption on activated charcoal. 2% PEG 3000-3500 was added prior to the activated charcoal treatment to maximize laccase recovery. Activated charcoal (2-5%, 400 mesh) was progressively added to the solution until the pigments were completely removed. The activated charcoal was removed from the enzyme solution by filtering the solution through diatomaceous earth. The filtrate, adjusted to pH 6.0, was loaded on a DEAE Sepharose column equilibrated with 10 mM Na-phosphate, pH 6.0. The active fraction was eluted with a NaCl gradient from 0 to 500 mM (Figure 5.1a). The active fraction was concentrated and equilibrated with 10 mM bis-Tris, pH 6.0, and loaded on a Mono Q column equilibrated with the same buffer. The column was eluted with a stepwise gradient of NaCl from 0 to 1 M (Figure 5.2a). Two active fractions were collected, corresponding to *P*-laccase 1 and *P*-laccase 2, respectively.

5.3.1.2 Purification of *D. squalens* laccases

Table 5.1b Purification of *D*-laccases

	Vol. (mL)	Activity (U/mL) [a]	Prot.conc (mg/mL)	Spec act (U/mg) [a]	Rec.	Purif
Crude medium	4000	9.5	0.93	10.2		
Freeze/thaw	3900	9.6	0.93	10.3	0.98	1
Polyethyleneimine	3900	9.4	0.92	10.2	0.97	0.99
DEAE Sepharose	180	253.3	0.21	1183	1.2	116
Mono Q pH 7	35	827	0.65	1272	0.76	125
MonoQ pH 4.5 L1[b]	3.5	5707	2.5	2283	0.52	224
MonoQ pH 4.5 L2[b]	6	450	0.2	2251	0.07	220.7

[a] 1 U= ΔA_{469}/min [b] L1: *D*-laccase 1; L2: *D*-laccase 2.

After filtering out the colloidal precipitate, the filtrate was treated with 0.001% polyethylene imine and allowed to stand for 2 h at 4°C. A second precipitate was separated by filtering the solution through diatomaceous earth. The filtrate, adjusted to pH 6.0, was loaded on a DEAE Sepharose column equilibrated with 10 mM Na-succinate, pH 6.0, containing 0.01% Tween 80. A single active fraction was recovered by eluting the column batchwise with a

a.

b.
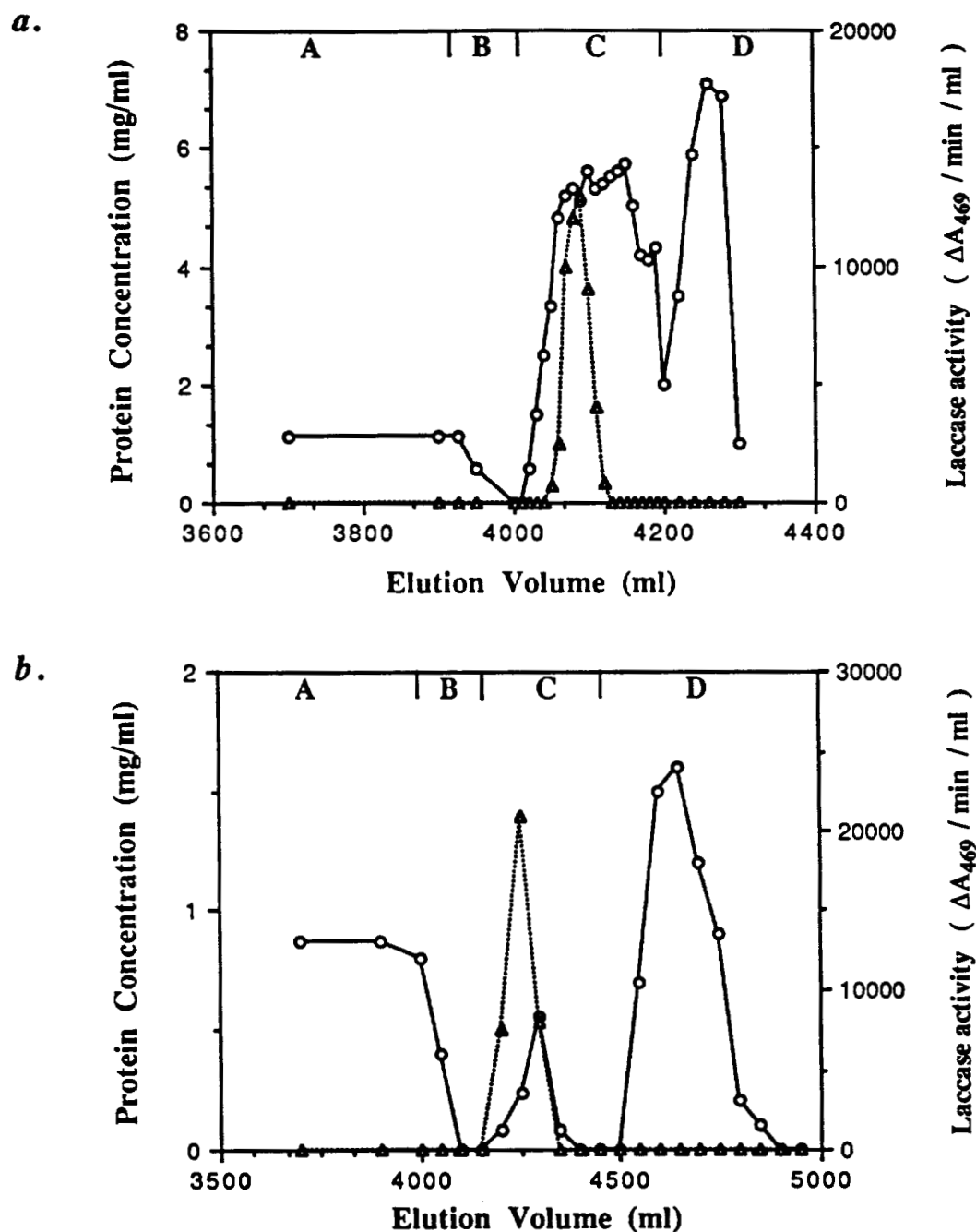
Figure 5.1 Elution profile of laccases on a DEAE Sepharose column.
(a) Elution of *P*-laccase: (A) filtrate flow-through; (B) 10 mM phosphate, pH 6.0; (C) linear NaCl gradient from 0 to 500 mM; (D) 1 M NaCl in 10 mM·phosphate, pH 6.0.
(b) Elution of *D*-laccase: (A) filtrate loaded on the column; (B) 10 mM phosphate, pH 6.0; (C) 200 mM NaCl in 10 mM phosphate, pH 6.0; (D) 1 M NaCl in 10 mM phosphate, pH 6.0.
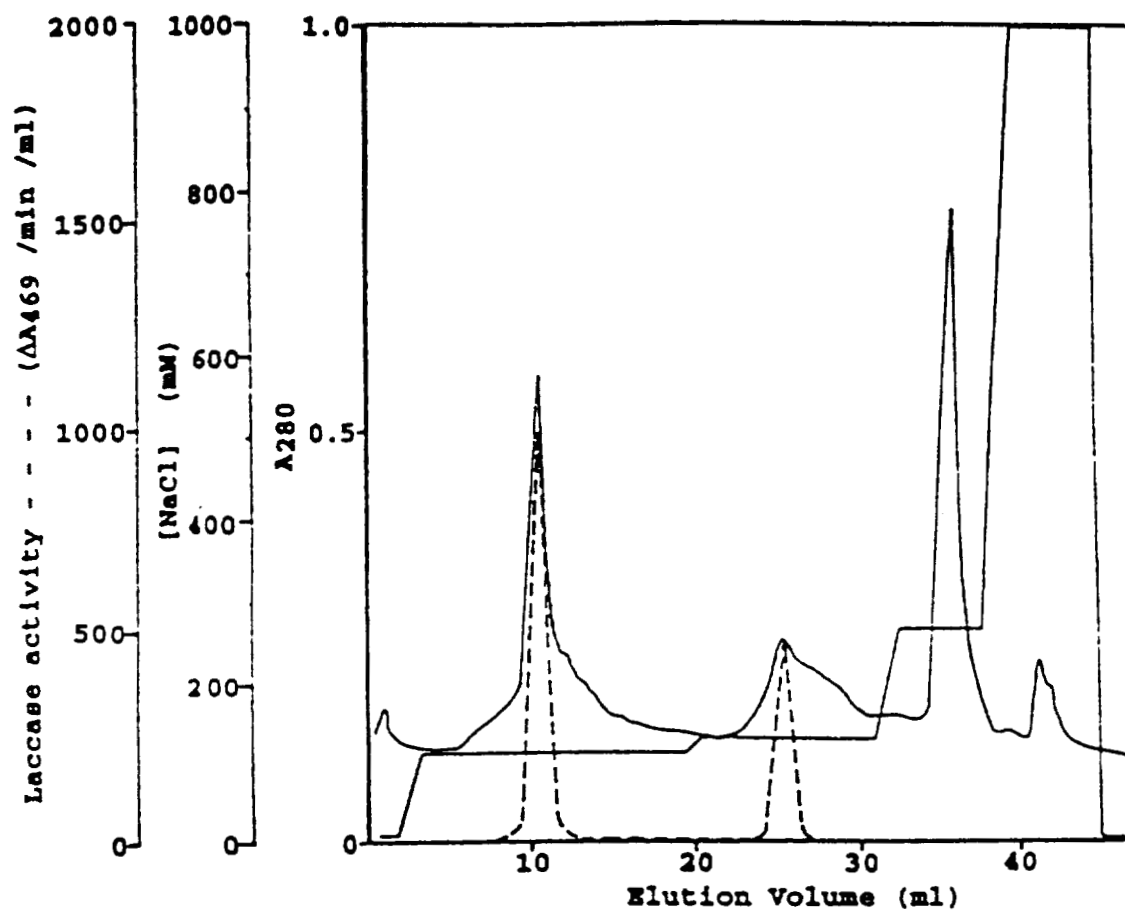
Figure 5.2 Elution profile of *P*-laccase on a Mono Q column. Elution profile of *P*-laccases on a Mono Q HR 5/5 column equilibrated with 10 mM bis-Tris, pH 6.0. NaCl gradient from 0 to 1 M.
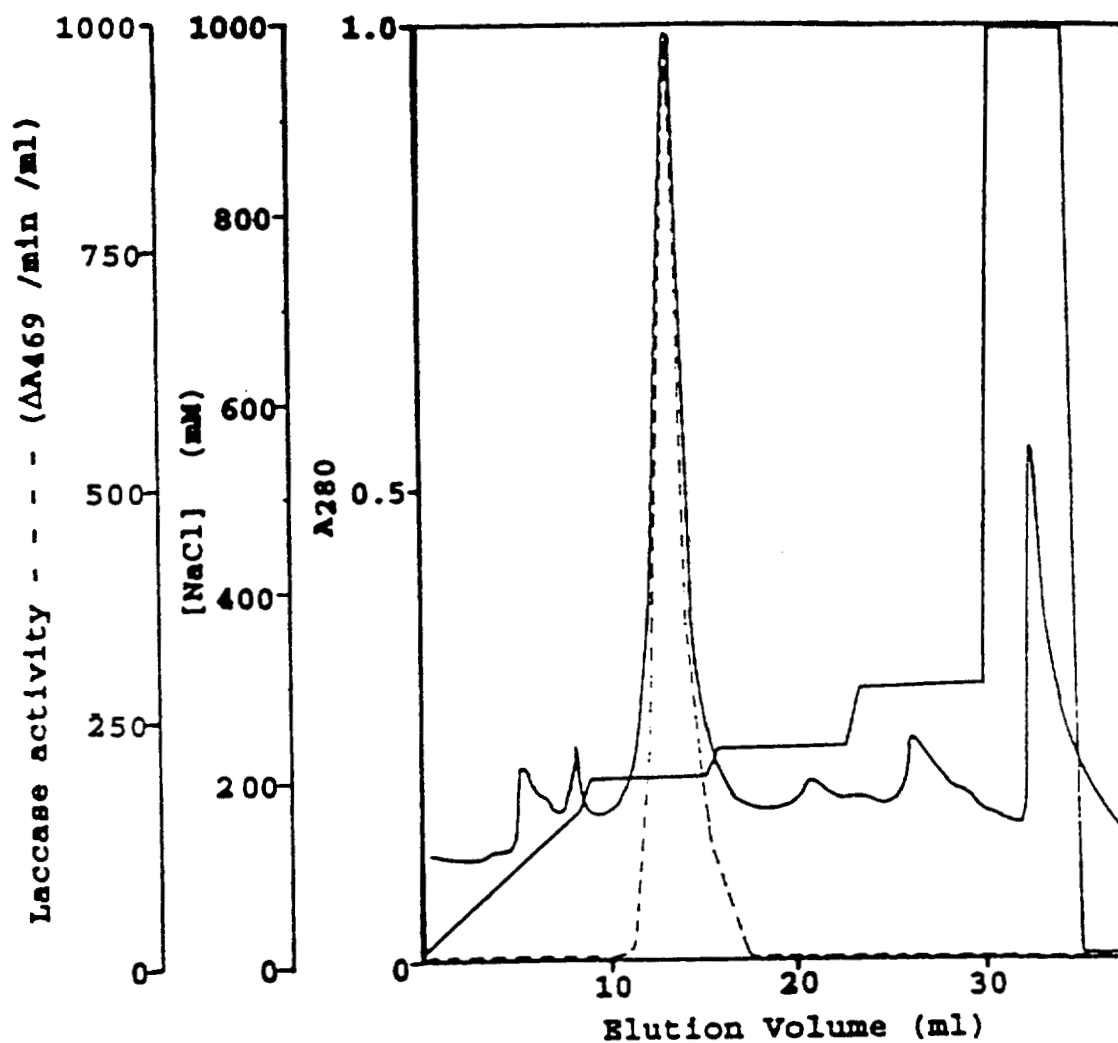
Figure 5.3 Elution profile of *D*-laccase on a Mono Q column, pH 7.0.
Elution profile of *D*-laccases on a Mono Q HR 5/5 column equilibrated with 10 mM bis-Tris, pH 7.0, and 0.01% Tween 80. NaCl gradient from 0 to 1 M.
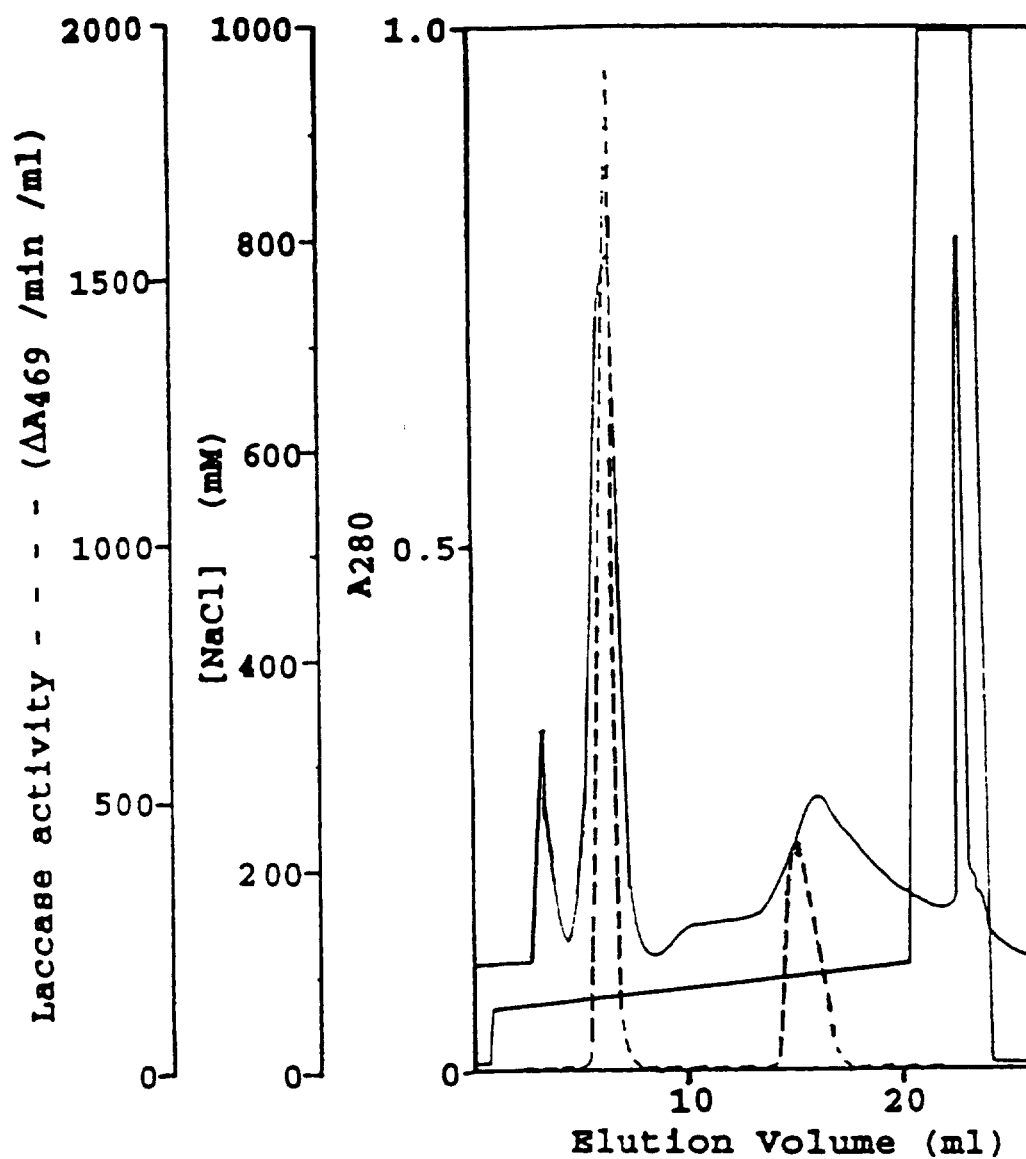
Figure 5.4 Elution profile of *D*-laccase on a Mono Q column, pH 4.5.
Elution profile of *D*-laccases on a Mono Q HR 5/5 column equilibrated with 10 mM succinate, pH 4.5. Linear NaCl gradient was from 50 to 100 mM.

200 mM solution of NaCl in the equilibrating buffer (Figure 5.1b). The active fraction was concentrated and equilibrated in 10 mM bis-Tris, pH 7.0, containing 0.01% Tween 80 and loaded on a Mono Q column equilibrated with the same buffer. A single active fraction was eluted using a stepwise NaCl gradient from 0 to 1 M (Figure 5.3). The active fraction was dialyzed and concentrated in 10 mM Na-succinate, pH 4.5 ,and was loaded on a Mono Q column equilibrated with the same buffer. Two isozymes, D-laccase 1 (Major) and D-laccase 2 (Minor), were separated by a linear NaCl gradient from 50 to 100 mM (Figure 5.4).

5.3.2 Homogeneity and molecular mass of laccases

Table 5.2 Physical and chemical characterisitics of P- and D-laccases

	P-laccase 1	P-laccase 2	D-laccase 1	D-laccase 2
M_r	62 000	60 300	65 800	63 900
Carbohydrate content	14%	13.5%	10.5%	8%
Copper content	3.9	3.8	3.9	4
pI	3.5	3.4, 3.65, 3.8	3.5	3.6

Table 5.2 summarizes the physical properties of the laccases. The M_r of D. *squalens* laccases were approximately 66,000 by gel filtration. The purified proteins each exhibited a single band when subjected to SDS PAGE (Figure 5.5).

The band at 36,000 in lane 3 probably corresponds to the autolytic degradation of the protein during freeze drying and was not observed in fresh preparations of the enzyme. The migration pattern suggested M_rs of approximately 60,000 and 62,000 for P-laccase 1 and 2 and approximately 64,000 and 66,000 for D-laccase 1 and 2, respectively. Phenol sulfuric acid assays indicated that the laccases are glycoproteins and that the carbohydrates contribute 14% and 13.5% to the mass of P-laccase 1 and 2, and 10.5% and 8% to the mass of D-laccase 1 and 2, respectively. P-laccases were separated into four isozymes with pIs of 3.4, 3.5, 3.65, and 3.8 by isoelectrofocusing (Figure 5.5b, lane 2). P-laccase 1 corresponded to the isozyme with a pI of 3.5, whereas D-laccase 1 exhibited a single band at pI 3.5 and D-laccase 2 exhibited a single band at pI 3.6 (Figure 5.5b, lane 3).

a)

b)
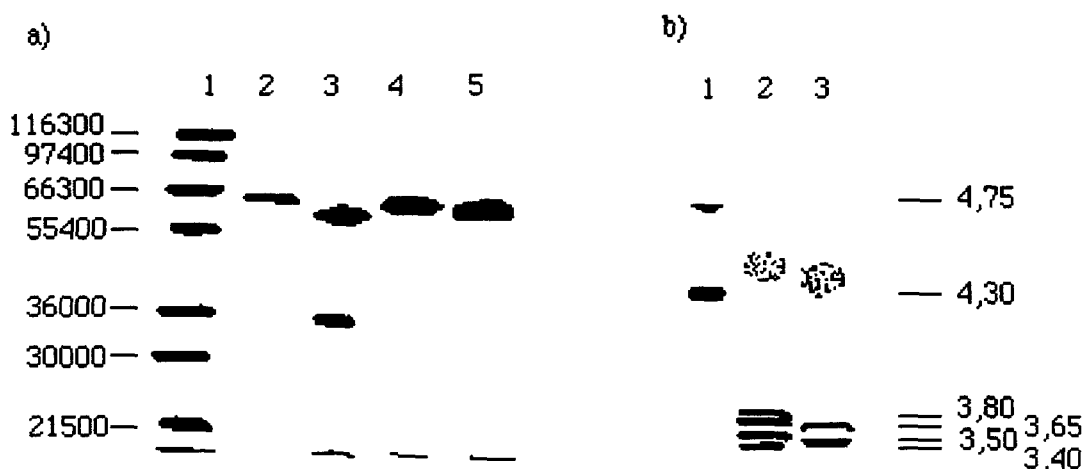
Figure 5.5 Gel electrophoresis of D- and P-laccases. SDS PAGE (a) and IEF (b) of the laccases after FPLC at pH 6.0 for P-laccases and pH 7.0 for D-laccases. β-galactosidase, phosphorylase B, bovine serum albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase were used for SDS PAGE M_r determination, and IEF mix 3.6–6 (Sigma) was used for IEF calibration. The pIs were determined by measuring the pH gradient of the gel.

5.3.3 pH optimum, pH stability, and temperature stability of P-laccase 1

The rate of oxidation of 2,6-DMP was measured over the pH range 2.0–7.5. Maximum activity was at pH 3.5 for P-laccase and 3.0 for D-laccase. Less than 50% of the maximum activity was measured above pH 4.5. No activity could be detected at pH 6 and higher, or below pH 2.5.

Laccase slowly lost activity below pH 3.0 with 75% loss at pH 2.0 after 5 h. In contrast, the enzyme was stable for several days at pH 6 and above. After 15 min at 60°C, P-laccase 1 retained 100% of its activity. The enzyme lost 50% activity after 15 min at 77°C and was completely inactivated at 90°C.

5.3.4 Substrate specificity

Table 5.3 shows the K_ms of P-laccase 1 and D-laccase 1 for o- and p-hydroxy- and o- and m-methoxy-phenol derivatives, as well as for di- and tri-methoxy phenols. Both enzymes have a high affinity for either methoxy- or hydroxy-substituted phenols provided that the meta position is not occupied. The two enzymes had approximately the same affinities for the various substrates. ABTS and o-phenylenediamine were also readily oxidized by both enzymes (data not shown). In contrast, no oxidation products were observed spectroscopically and no oxygen consumption was detected with either tyrosine or the non-phenolic substrate veratryl alcohol.

Table 5.3 Substrate specificity of P-laccase 1 and D-laccase 1

	P. cinnabarinus L1	*D. squalens* L1
p-hydroquinone	0.290	0.305
catechol	0.330	0.320
Guaiacol	0.728	1.1
3-methoxyphenol	24.91	N.D.
2,6 dimethoxyphenol	0.152	0.167
3,4-dimethoxyphenol	0.417	0.415
2,3-dimethoxyphenol	18.05	17.01
3,4,5 trimethoxyphenol	5.39	N.D.

K_m (mM) was determined at 25°C with an oxygen electrode.

5.3.5 Spectral characteristics

Figure 5.6 shows the UV and visible spectra of P- and D-laccases at pH 3.0 and 7.0. The laccases had an absorption maximum at about 610 nm, characteristic of type 1 copper. A secondary maximum at 425 nm associated with type 1 copper also was detected at pH 3.0. A shoulder at 330 nm corresponds to the signal associated with type 3 copper. A small shoulder at 720 nm, also associated with type 3 copper, was also observed (spectra not shown).

Table 5.4 shows the extinction coefficients of P-laccase 1 and D-laccase 1 at pH 3.0 and 7.0. The absorbances at 610 nm of both enzymes were lower at pH 7.0 and the absorbances at 330 nm were lower at pH 3.0. P-laccase

Table 5.4 UV visible maxima of P-laccase 1 and D-laccase 1

	P. cinnabarinus laccase1	*D. squalens* laccase1
ε_{610} at pH 3.0	4385	5200
ε_{610} at pH 7.0	3045	4930
ε_{330} at pH 3.0	4255	4700
ε_{330} at pH 7.0	5500	5800
rz $\varepsilon_{280}/\varepsilon_{610}$	14.7	18
EPR		
$g\perp$	2.04	2.04
$g//$	2.20	2.20
$A//$	92G	90G

Extinction coefficients were measured against the anaerobically reduced enzyme with ascorbic acid in the reference cuvette. EPR values were obtained at 9.123 GHz at 40°K.
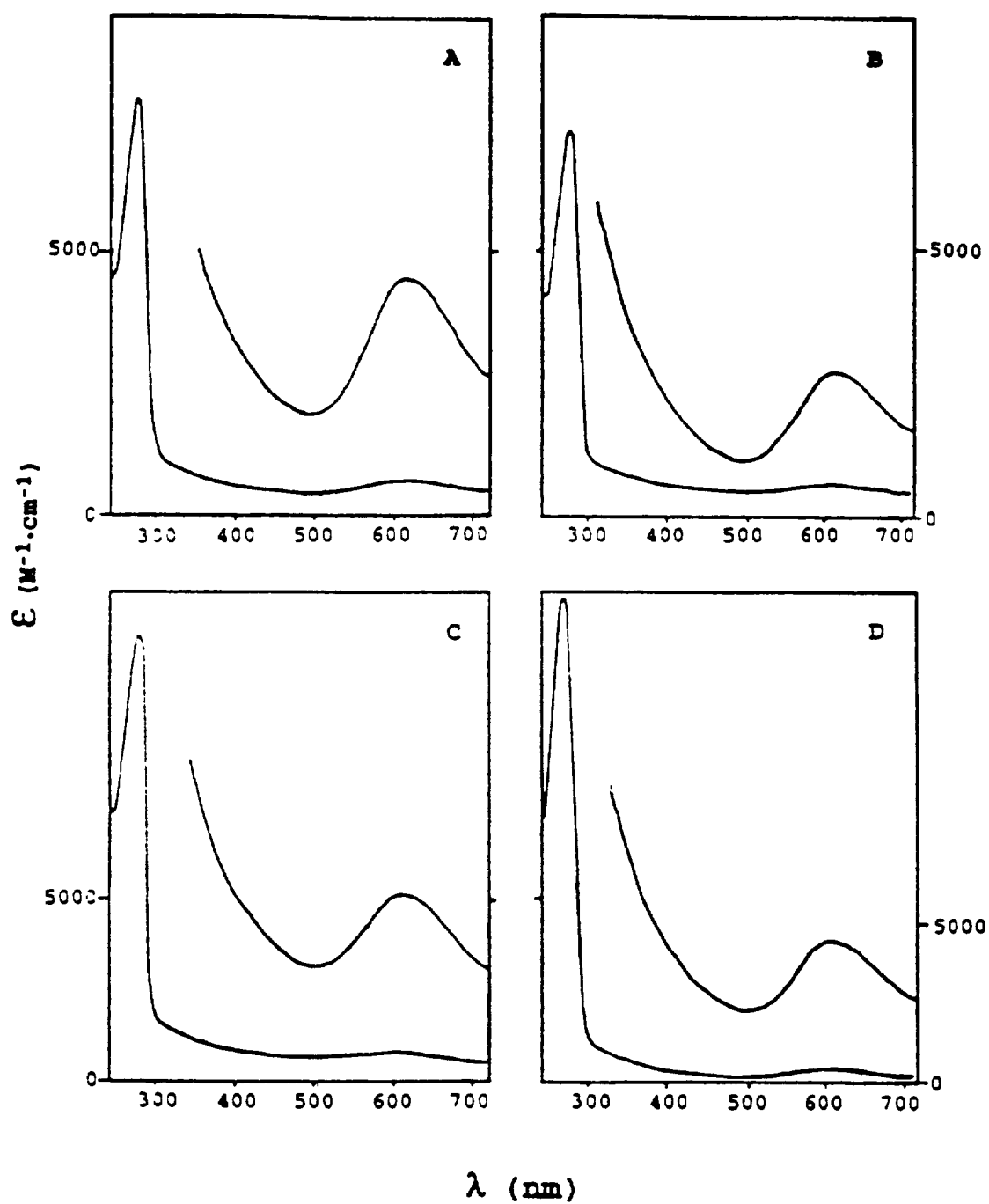
Figure 5.6 **UV visible spectra of** *P*-**laccase 1 and** *D*-**laccase 1**. *P*-laccase 1:
(A) at pH 3.0, and (B) at pH 7.0. *D*-laccase 1: (C) at pH 3.0, and (D) at pH 7.0.
Visible region is expanded 10×.

appeared to be more sensitive to pH changes than D-laccase. Figure 5.7 shows the dependence of extinction coefficients of P-laccase 1 at 610 and 330 nm on pH. Increasing the pH above 4.5 resulted in a sharp decrease in the extinction coefficient at 610 nm, whereas the extinction coefficient at 330 nm increased sharply between pH 3.0 and 4.5 and remained relatively stable above this pH. The peak at 330 nm formed a more defined shoulder as the pH increased (spectra not shown). The decrease in intensity at 610 nm was reversible and an extinction coefficient of approximately 4500 $M^{-1} cm^{-1}$ was measured after the pH was lowered from 8.0 to 3.0. EPR spectroscopy detected type 1 and type 2 copper signals with similar values to those reported for other fungal laccases (Figure 5.8 and Table 5.4). The integration of the signals of P-laccase 1 at pH 3.0 and 7.0 (Figure 5.9) indicated that, at both pHs, two coppers were detectable. The type 2 signal, as observed at the low flux density end and in the perpendicular region of the spectrum, appeared to be stronger at pH 7.0 than at 3.0.

5.4   Discussion

Because laccases have been found in cultures of wood rotting fungi, they have been implicated in the biodegradation of lignin [5,178,179]. Laccases have been detected in most wood rotting fungi although not in *P. chrysosporium*. These enzymes are also found in other fungal species (e.g., *Neurospora* [115] and *Aspergillus* [114]). Specific roles for laccase in the degradation of lignin have not been clearly determined. Evidence does suggest that laccase could be involved in lignin degradation alone [141,148], or in cooperation with other enzymes [84, 182], or via the mediation of oxidizable substrates [149,181]. As part of our attempt to characterize the lignin degradation systems of *P. cinnabarinus* and *D. squalens*, the laccases produced by these two white-rot fungi were characterized.

5.4.1   Purification

The presence of large amounts of pigment in the culture of *P. cinnabarinus* required the use of activated charcoal prior to laccase isolation. These pigments have been described as phenoxazinone derivatives [216] and are responsible for the dark red color of the mycelium. To prevent the passive adsorption of laccase to the activated charcoal and to maximize the elimination
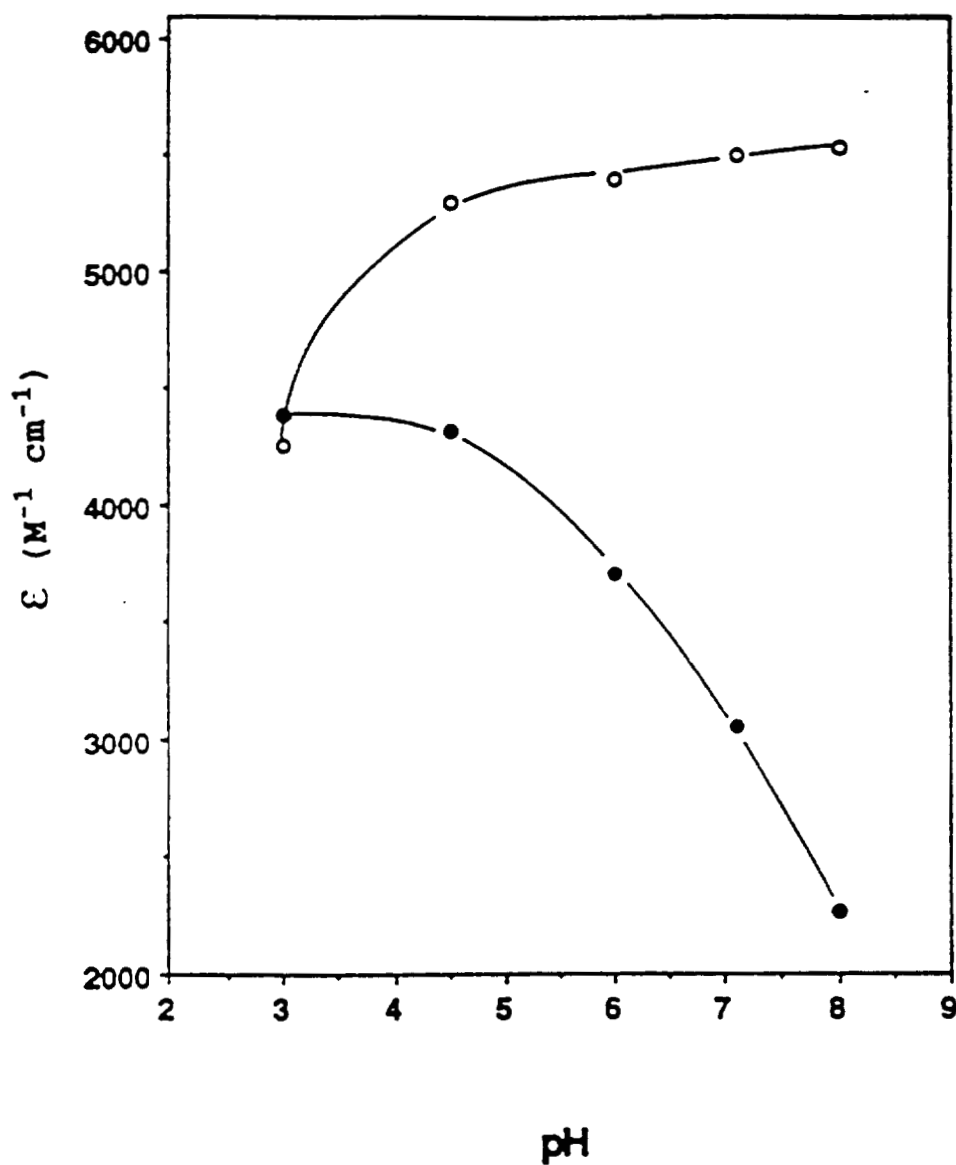
Figure 5.7 pH effect on *P*-laccase 1 ε_{330} and ε_{610}. Extinction coefficients were measured in 10 mM Na-succinate, pH 3.0 and 4.5, and in 10 mM phosphate, pH 6.0, 7.1, and 8.0.

0.22   0.24   0.26   0.28   0.30   0.32   0.34   0.36

Magnetic Flux Density (T)
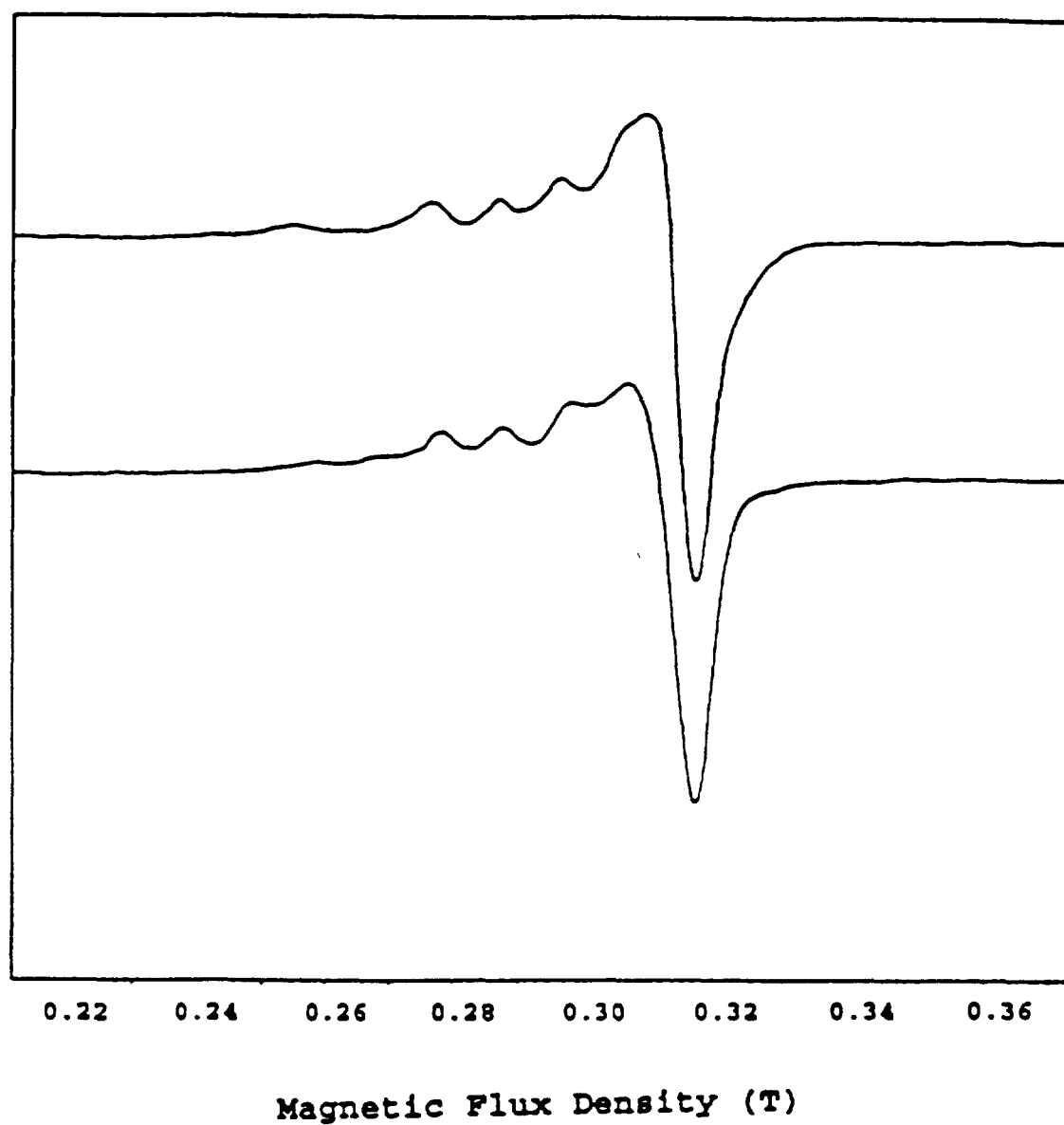
Figure 5.8 EPR spectra of *P*-laccase 1 and *D*-laccase 1 at pH 7.0. Top line corresponds to the *P*-laccase spectrum; bottom line corresponds to the *D*-laccase spectrum. Spectra were recorded at 9.123 GHz and 40°K, with a scan range of 1600 *g*.
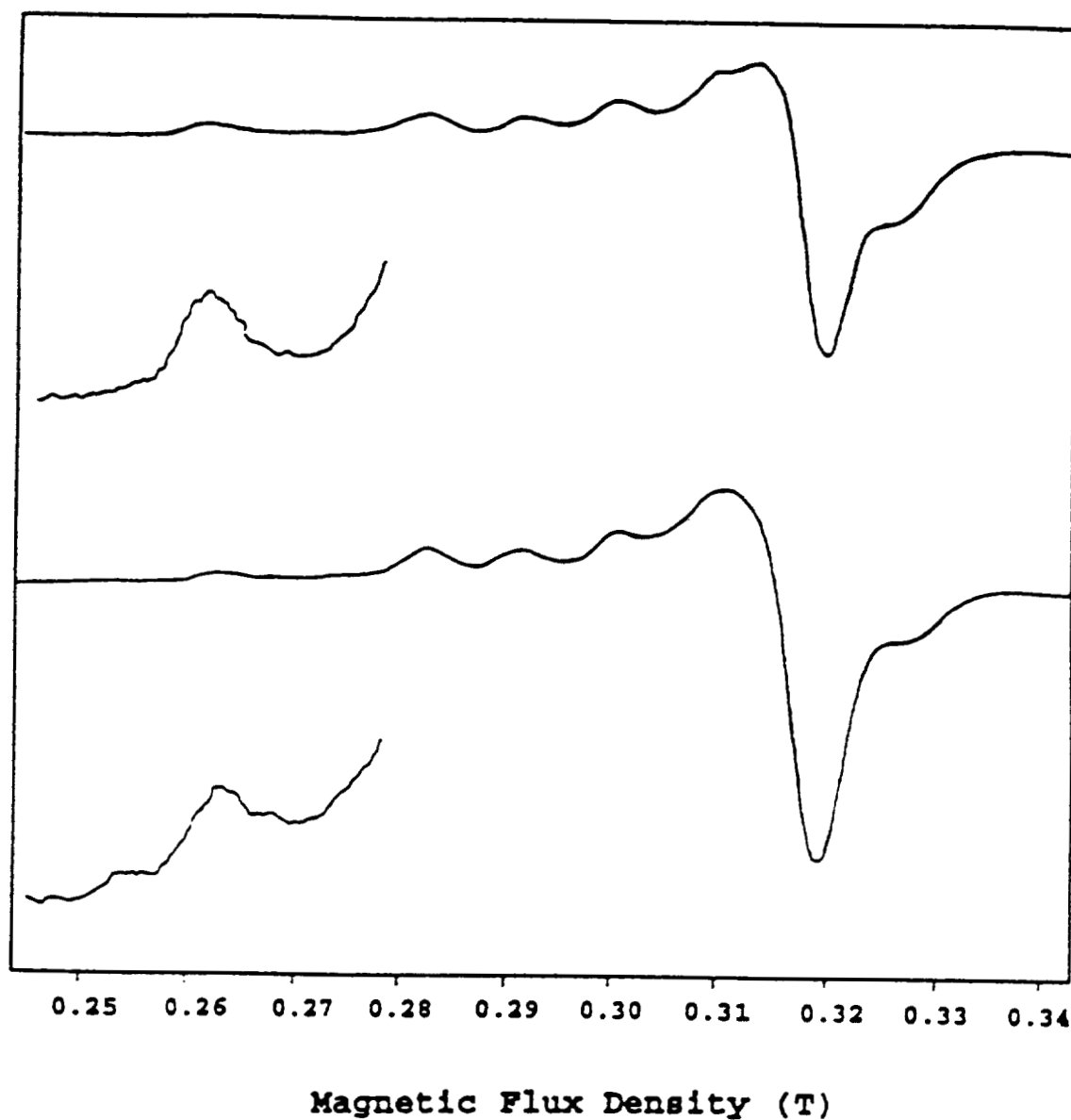
Figure 5.9 Effect of pH on EPR spectra of *P*-laccase. At pH 7.0 (top line) and pH 3.0 (bottom line). The low flux ends of the spectra were expanded 5×. The spectra were recorded at 9.125 GHz, 40°K, with a scan range of 1000 *g*.

of pigments, PEG 3000-3500 was added to the culture medium. This step appeared to be a very efficient alternative to the commonly-used chromatography on hydroxyapatite, [213,215]. *D. squalens* did not produce large amounts of pigments, but the presence of phenolic compounds produced during the growth of the fungus required the use of polyethyleneimine [82] to maximize the resolution of the chromatography steps. Two steps of ion exchange chromatography enable purification of the laccases from both *D. squalens* and *P. cinnabarinus* to homogeneity as determined by SDS PAGE.

5.4.2 Characterization

The M_{rs} of these fungal laccases were in the range of 60,000 to 66,000, similar to those reported for laccases from *Rigidoporus lignosus* (52,000 - 55,000) [84,213], *Pleurotus ostreatus* (59,000) [118], *Coriolus versicolor* (64,650) [152], *Lentines edodes* (66,000) [214], and *Pycnoporus coccineus* (68,000–70,000) [215]. Comparison of the M_r determination of native enzymes with denatured enzymes indicates that the laccases are produced as a single polypetide chain in *P. cinnabarinus* or *D. squalens*. pIs of 3.5 and 3.6 for *D*-laccases and of 3.4 to 3.8 for *P*-laccases also fall within the range of pIs observed for other fungal laccases: 2.9 for *P. ostreatus* [118]; 3.0 for *L. edodes* [214]; 3.5 for *P. coccineus* [215]; and 3.32–3.8 for *R. lignosus* [84,213]. The pH optima of 3.5 for *P*-laccase and 3.0 for *D*-laccase are at the acidic end of the range of pH optima exhibited by fungal laccases: 4.5 for *C. versicolor* [152], *L. edodes* [214], and *P. coccineus* [215], and 6.0 for *R. lignosus* [213]. Comparative studies of model compound oxidation with these various laccases would be of great interest since the nature of the product of the oxidation by laccase may be dependent upon the pH [217]. With the exception of those reported for *A. bisporus* [218] and *P. radiata* laccases [219], fungal laccases, including *P. cinnabarinus* and *D. squalens* laccases, contain 4 moles of copper per mole of enzyme. The absorbance maximum at 610 nm, characteristic of type 1 copper, was detected.

5.4.3 Spectroscopic characteristics

Both *P*- and *D*-laccases exhibit extinction coefficients at pH 3.0 in the range of values commonly found in laccases [120]. The extinction coefficients were considerably reduced at pH 7.0, suggesting that the Cu-S environment of type 1 copper is sensitive to pH, and that the deprotonation of one or several

groups, possibly a histidinate close to type 1 copper, affects the oxidation state of copper, causing its partial reduction. Similar results have been observed with *Rhus* laccase as well as ascorbate oxidase [220]. This is in agreement with the decrease in the laccase redox potential of approximately 15 mV per pH unit observed between pH 3.4 and 9.5 [221]. Modification of the type 3 signal may be explained in the same way by a partial reduction of type 3 copper at low pH, leading to decoupling of the pair of type 3 copper ions and resulting in the loss of absorbance at 330 nm [130,222]. The increase in the EPR signal detected at pH 7.0 suggested that type 2 copper was not fully detected and possibly was partially reduced at pH 3.0. Modification of the EPR signal in the perpendicular region of the spectrum has been reported to be due to deprotonation of a water molecule or one of the imidazole ligands coordinated to type 2 copper [220]. Other explanations, such as the ferromagnetic coupling of type 2 copper with type 3 copper resulting in the partial silencing of type 2 copper [222], cannot be ruled out. However, no evidence was found to suggest that such a coupling occurs for these particular laccases. All these results indicated that copper was not fully oxidized in the resting enzyme. This is in agreement with the detection of Cu^I in resting laccase [130,223]. This also suggests that the integration of the signal using Cu-EDTA as a standard apparently overestimated the amount of copper.

5.4.4 Substrate specificity

The substrate specificity of various fungal laccases has been investigated by several groups [224,225]. It is generally accepted that laccase-catalyzed oxidation of lignin is limited to phenolic subunits [15]. The absence of oxidation of veratryl alcohol confirms the inability of laccases to oxidize a non-phenolic compound. The effect of the number and position of hydroxyl and methoxyl groups on the ring on the activity of *P*- and *D*-laccases was analyzed. At least one phenol or amine appears to be necessary for oxidation to take place. *P*- and *D*-laccase have similar affinities for hydroquinone and catechol. When one hydroxy is replaced by a methoxy (catechol to guaiacol), the affinity of the enzyme for the substrate was noticeably decreased. When the *o*-methoxy was replaced with the *m*-methoxy derivative, the enzyme affinity was decreased by a factor of 34, confirming that the meta position is not favorable for oxidation by laccase. 2,6-DMP appeared to be the preferred substrate for both enzymes. It is noteworthy that, although *P*-laccase 1 apparently reduced itself more easily than

D-laccase 1, both laccases appeared to have the same substrate specificity and approximately the same affinity for a given substrate. The pH sensitivity of the redox potential of *P*-laccase 1 did not affect its activity against organic substrates, suggesting that the state of oxidation of type 1copper was the critical factor in the oxidation of phenols by this laccase.

Those results indicate that both *P*- and *D*-laccases possess the characteristics commonly associated with other laccases. Previous results indicated that *P. cinnabarinus* (Chapter 3) and *D. squalens* [151] degrade lignin even though they do not produce detectable LiP. The differences in ability to degrade lignin observed in *in vivo* experiments, based on culture medium composition, were confirmed by the present *in vitro* activities analysis of these laccases.

CHAPTER 6
FINAL COMMENTS

6.1   D. squalens

Several types of synthesized lignin (DHP) and natural lignin were extensively degraded by *D. squalens* under conditions allowing for the selective expression of MnP and laccase and under conditions related to a natural environment. ^{14}C-labeled DHPs were degraded to [14]CO_2 demonstrating the ability of this fungus to completely mineralize synthetic lignin. These experiments suggested that the presence of manganese was necessary for the mineralization of DHP and natural lignin. In the absence of manganese, depolymerization was not observed, and *D. squalens* could not grow on wood. Only the methoxyl-labeled lignin was degraded to [14]CO_2 when the fungus was cultured in the absence of manganese, suggesting that laccase alone was not able to cause lignin mineralization *in vivo*. The concentration of nitrogen did not significantly affect the expression of MnP, and the fungus was able to express MnP and to degrade lignin in the presence of excess nitrogen in the culture.

D. squalens did not express any LiP, therefore, the enzymes involved in the oxidation of lignin were presumably a series of MnP isozymes and laccases. The MnPs shared the spectral and mechanistic properties of *P. chrysosporium* MnP isozymes. The laccases were partially characterized and shared the spectral and mechanistic features of other fungal laccases. They belong to the group of acidic laccases with an optimum pH close to 3.

6.2   P. cinnabarinus

Because it produced a high concentration of laccase under selected conditions, *P. cinnabarinus* was used to determine the role of laccase in the *in vivo* degradation of several DHPs. *P. cinnabarinus* was able to generate [14]CO_2 from methoxyl-labeled lignin, but little or no mineralization was observed with

side chain- and ring-labeled lignin. Under most conditions, the only oxidizing enzyme produced by this fungus was a laccase. This enzyme shared the spectral and mechanistic characteristics of fungal laccases. With an optimum pH of 3, like *D. squalens* laccase, it belongs to the group of acidic fungal laccases. Traces of MnP activity could be detected when the fungus was grown on natural lignin, but MnP isolation was not feasible under these conditions and the characterization of this MnP was not undertaken.

6.3   The Role of MnP and Laccase in the Biodegradation of Lignin

Based on the results of lignin degradation by *P. chrysosporium*, *P. radiata* and *C. versicolor*, as well as mechanistic studies, two enzymes—LiP and MnP—have been considered the core of the ligninolytic system of white-rot basidiomycetes. Its unusually high redox potential and its ability to oxidize non-phenolic compounds suggested that LiP was the major ligninolytic enzyme. In this scheme, MnP played a secondary role in lignin biodegradation, limited to the oxidation of phenolic derivatives. Along with *in vitro* experiments presenting evidence that MnP can depolymerize several types of lignin, and that under selected conditions MnP can oxidize non-phenolic compounds, the apparent absence of LiP in *D. squalens* cultures suggested that MnP played a key role in *in vivo* lignin biodegradation. This role may possibly be extended to basidiomycetes capable of producing LiP. Lignin degradation by *D. squalens* was as extensive as the degradation observed with other well-studied white-rot basidiomycetes producing LiP, such as *P. chrysosporium*, *C. versicolor*, or *P. radiata*. The absence of MnP in the cultures of *P. cinnabarinus* and *D. squalens* cultured in the absence of manganese suggested that the presence of MnP and manganese were necessary to the degradation of lignin and that laccase alone could not mineralize lignin.

The differences observed in the expression of *D. squalens* MnP and *P. chrysosporium* MnP suggest that they may contribute to lignin degradation under different conditions. A comparison of the two MnP genes, including sequencing of the *D. squalens* MnP gene promoter region, should allow a determination of the promoter region of the *D. squalens* MnP gene critical to manganese-regulated expression.

Because no obvious role for laccase alone has been determined, the activity of laccase with other enzymes, quinone reductases, hydroxylases,

oxygenases, etc., should be studied to describe the role of laccase, not as the sole enzyme involved in lignin degradation, but working in synergy with other enzymes. The products of oxidation by laccase should be studied as potential substrates for these enzymes. In particular, the role of demethoxylation in lignin depolymerization should be examined. One possible hypothesis is that laccase generates sites bearing two hydroxyl groups in ortho position suitable for attack by an oxygenase responsible for the opening of the ring. Other pathways may be described and may lead to new schemes for lignin depolymerization involving the active contribution of laccase.

One example of synergistic mechanisms has been described with *R. lignosus*, and the proposed results suggested that MnP and laccase together cause a higher level of lignin solubilization than each enzyme taken alone. Such synergy was not observed during the mineralization experiments carried out with *D. squalens*, suggesting that at least one of the *D. squalens* enzymes—MnP or laccase—presents different substrate specificity or mechanistic behavior as compared to that of *R. lignosus*. A detailed mechanistic study of the two enzymes should reveal the exact role of each in lignin degradation.

In vitro experiments of DHP depolymerization with combinations of enzyme mixtures should indicate the role of each enzyme in the degradation of lignin. A cross-mixing of *R. lignosus* and *D. squalens* MnP and laccase should help to determine if one of these enzymes has a particular activity. *In vivo* experiments of lignin biodegradation of DHPs by *D. squalens* and *R. lignosus* in cultures complemented with laccase purified from cultures of *R. lignosus* and *D. squalens*, respectively, may provide some pieces of evidence regarding the contribution of each laccase. If any of these effects are observed, a generalization of these results should be demonstrated with other laccases belonging to the acidic and neutral groups of laccases.

The oxidation of non-phenolic compounds, observed during mineralization of lignin, attributed at least partially to MnP or laccase, remains to be explained. The oxidation of intermediates, such as thiol and other radical precursors, has been suggested as part of an oxidative pathway for non-phenolic compounds. Such intermediates need to be identified in cultures of white-rot fungi lacking LiP to validate this hypothesis.

LITERATURE CITED

1. Lewis, N. G. and Paice, M. G. (1989) *Plant Cell Wall Polymers* (M. J. Comstock, Ed.) ACS Symposium Series 399, American Chemical Society, Washington, DC.

2. Adler, E. (1977) Lignin chemistry—Past, present and future. *Wood Sci. Technol.* **11**, 169-218.

3. Adler, E. (1961) Present status of lignin research. *Papier* **15**, 604-609.

4. Higuchi, T. (1980) Lignin structure and morphological distribution in plant cell walls. In *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications* (T. K. Kirk, T. Higuchi, and H.-M. Chang, Eds.) CRC Press, Boca Raton, FL, pp. 557-578.

5. Higuchi, T. (1971) Formation and biological degradation of lignins. *Adv. Enzymol.* **34**, 207-283.

6. Higuchi, T. (1985) Biosynthesis of lignin. In *Biosynthesis and Biodegradation of Wood Components* (T. Higuchi, Ed.) Academic Press, Orlando, FL, pp. 141-160.

7. Higuchi, T. (1990) Lignin biochemistry: Biosynthesis and biodegradation. *Wood Sci. Technol.* **24**, 23-63.

8. Freudenberg, K. (1963) Recent contributions to the formation and constitution of lignin. *Brennst. Chem.* **44**, 328-331.

9. Freudenberg, K. (1963) Formation of lignin in the tissue and *in vitro*. In *Formation of Wood Forest Trees*, 2nd Symposium, Petersham, MA.

10. Freudenberg, K. (1965) Lignin: Its constitution and formation from p-hydroxy-cinnamyl alcohol. *Science* **148**, 595-600.

11. Freudenberg, K. (1965) Biosynthesis of lignin. *Bertr. Biochem. Physiol. Naturstoffen, Festchr.* 167-175.

12. Freudenberg, K. (1968) The constitution and biosynthesis of lignin. In *Constitution and Biosynthesis of Lignin* (K. Freudenberg and A. C. Neish, Eds.) Springer, Berlin, pp. 47-122.

13. Sarkanen, K. V. (1971) Precursors and their polymerization. In *Lignins: Occurrence, Formation, Structure and Reactions* (K. V. Sarkanen and C. H. Ludwig, Eds.) John Wiley & Sons, New York, pp. 93-163.

14. Gold, M. H., Wariishi, H., and Valli, K. (1989) Extracellular peroxidases involved in lignin degradation by the white-rot basidiomycete *Phanerochaete chrysosporium. Am. Chem. Soc. Symp. Ser.* **389**, 127-140.

15. Higuchi, T. (1989) Mechanisms of lignin degradation by lignin peroxidase and laccase of white-rot fungi. In *Plant Cell Wall Polymers. Biogenesis and Biodegradation* (N. G. Lewis and M. G. Paice, Eds.) American Chemical Society, Washington, DC, pp. 482-502.

16. Higuchi, T. (1982) Biodegradation of lignin: biodegradation and potential applications. *Experientia* **38**, 159-166.

17. Buswell, J. A. and Odier, E. (1987) Lignin biodegradation. *Crit. Rev. Biotechnol.* **6**, 1-60.

18. Kirk, T. K. and Farrell, R. L. (1987) Enzymatic "combustion:" The microbial degradation of lignin. *Annu. Rev. Microbiol.* **41**, 465-505.

19. Fengel, D. and Wegener, G. (1989) *Wood: Chemistry, Ultrastructure, and Reactions* (W. D. Gruiter, Ed.) Berlin.

20. Saka, S. and Goring, D. A. I. (1985) Biosynthesis of lignin. In *Biosynthesis and Biodegradation of Wood Components* (T. Higuchi, Ed.) Academic Press, Orlando, FL.

21. Fry, S. C. and Miller, J. G. (1989) Toward a working model of the growing plant cell wall: Phenolic cross-linking reactions in the primary cell walls of dicotyledon. In *Plant Cell Wall Polymers. Biogenesis and Biodegradation* (N. G. Lewis and M. G. Paice, Eds.) American Chemical Society, Washington, DC, pp. 33-46.

22. Yamamoto, E., Bokelman, G. H., and Lewis, N. G. (1989) Phenylpropanoid metabolism in cell walls. In *Plant Cell Wall Polymers. Biogenesis and Biodegradation* (N. G. Lewis and M. G. Paice, Eds.) American Chemical Society, Washington, DC, pp. 68-88.

23. Bjorkman, A. (1954) Isolation of lignin from finely divided wood with neutral solvent. *Nature* **174,** 1057-1058.

24. Freudenberg, K. (1964) A schematic constitutional formulation for spruce lignin. *Holzforschung* **18,** 3-9.

25. Nimz, H. (1974) Beech lignin. Draft of a constitution scheme. *Angew. Chem.* **86,** 336-344.

26. Pometto, A. L., III, Lee, B., and Johnson, K. E., (1992) Production of an extracellular polyethylene-degrading enzyme(s) by *Streptomyces* species. *Appl. Environ. Microbiol.* **58,** 731-733.

27. Eslyn, W. E., Kirk, T. K., and Effland, M. J. (1975) Changes in the chemical composition of wood caused by six soft-rot fungi. *Phytopathology* **65,** 473-476.

28. Ander, P., Eriksson, K.-E., and Yu, H.-S. (1984) Metabolism of lignin-derived aromatic acids by wood-rotting fungi. *J. Gen. Microbiol.* **130,** 63-68.

29. Ander, P., Stoytschev, I., and Eriksson, K. E. L. (1988) Cleavage and metabolism of methoxyl groups from vanillic and ferulic acids by brown-rot and soft-rot fungi. *Cellul. Chem. Technol.* **22,** 255-266.

30. Rayner, A. D. M. and Body, L. (1988) *Fungal Decomposition of Wood. Its Biology and Ecology,* John Wiley & Sons, Chichester.

31. Higuchi, T. (1953) Biochemical study of wood rotting fungi. I. Studies on the enzymes which cause Bavendamm's reaction. *J. Jap. For. Soc.* **35,** 77-86.

32. Kirk, T. K. (1975) Effect of a brown fungus, *Lenzites trabea,* on lignin in spruce wood. *Holzforschung* **29,** 99-107.

33. Kirk, T. K. and Adler, E. (1970) Methoxyl-deficient structural elements in lignin of sweetgum decayed by a brown-rot fungus. *Acta Chem. Scand.* **24**, 3379-3390.

34. Crawford, R. L. (1981) *Lignin Biodegradation and transformation*. John Wiley & Sons, New York.

35. Blanchette, R. A., Otjen, L., and Carlson, M. C. (1987) Lignin distribution in cells walls of birch wood decayed by white-rot basidiomycetes. *Phytopathology* **77**, 684-690.

36. Ruel, K. and Joseleau, J. P. (1991) Involvement of an extracellular glucan sheath during degradation of populus wood by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **57**, 374-384.

37. Keyser, P., Kirk, T. K., and Zeikus, J. G. (1978) Ligninolytic enzyme system of *Phanerochaete chrysosporium* synthesized in the absence of lignin in response to nitrogen starvation. *J. Bacteriol.* **135**, 790-797.

38. Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J. G. (1978) Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. *Arch. Microbiol.* **117**, 227-285.

39. Hatakka, A. I. and Uusi-Rauva, A. K. (1983) Degradation of ^{14}C-labeled poplar wood lignin by selected white-rot fungi. *Eur. J. Appl. Microbiol. Biotechnol.* **17**, 235-242.

40. Reid, I. D. and Seifert, K. A. (1982) Effect of an atmosphere of oxygen on growth, respiration, and lignin degradation by white-rot fungi. *Can. J. Bot.* **60**, 252-260.

41. Yu, H.-S. and Eriksson, K. E. (1985) Influence of oxygen on the degradation of wood and straw by white-rot fungi. *Svensk Papperstidn.* **88**, 57-60.

42. Faison, B. D. and Kirk, T. K. (1985) Factors involved in the regulation of ligninase activity in *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **49**, 299-304.

43. Ander, P. and Eriksson, K.-E. (1977) Selective degradation of wood components by white-rot fungi. *Physiol. Plant.* **41**, 239-248.

44. Nerud, F., Zouchová, Z., and Misurcová, Z. (1991) Ligninolytic properties of different white-rot fungi. *Biotech. Lett.* **13**, 657-660.

45. Ohtakara, A., Mitsutomi, M., and Uchida, Y. (1984) Purification and enzymatic properties of α-galactosidase from *Pycnoporus cinnabarinus*. *Agric. Biol. Chem.* **48**, 1319-1327.

46. Mitsutomi, M. and Ohtakara, A. (1984) A simplified procedure for purification and crystallization of thermostable α-galactosidase from *Pycnoporus cinnabarinus*. *Agric. Biol. Chem.* **48**, 3153-3155.

47. Peck, C. H. (1895) New species of fungi. *Bull. Torrey Bot. Club* **22**, 198.

48. Babcock, E. C. (1944) Methods for obtaining fructification of wood-rotting fungi in culture. *Trans. Brit. Mycol. Soc.* **26**, 127-132.

49. Myers, G. C., Leatham, G. F., Wegner, T. H., and Blanchette, R. A. (1988) Fungal pretreatment of aspen chips improves strength of refiner mechanical pulps. *Tappi.* **71**, 105-108.

50. Zadrazil, F. and Brunnert, H. (1982) Solid state fermentation of lignocellulose containing plant residues with *Sporotrichum pulverulentum* Nov. and *Dichomitus squalens* (Karst) Reid. *Eur. J. Appl. Microbiol. Biotechnol.* **16**, 45-51.

51. Matteau, P. and Bone, D. H. (1980) Solid-state fermentation of maple wood by *Polyporus anceps*. *Biotechnol. Lett.* **2**, 127-132.

52. Agosin, E., Rouau, X., and Brillouet, J. M. (1987) Fermentation of wheat straw xylan by the white-rot fungus *Dichomitus squalens*. *Can. J. Microbiol.* **33**, 1050-1054.

53. Petroski, R. J., Peczynska-Czoch, W., and Rosazza, J. P. (1980) Analysis, production and isolation of an extracellular laccase from *Polyporus anceps*. *Appl. Environ. Microbiol.* **40**, 1003-1006.

54. Pham, T. T. T., Slezec, A. M., and Odier, E. (1991) Fruiting in *Dichomitus squalens* (Kast) Reid. *Cryptogamie Mycol.* **12**, 1-11.

55. Rouau, X. and Odier, E. (1986) Production of extracellular enzyme by the white-rot fungus *Dichomitus squalens* in cellulose containing liquid culture. *Enzyme Microbiol. Technol.* **8**, 22-26.

56. Rouau, X. and Foglietti, M. (1985) Purification and partial characterization of three endo-glucanases from *Dichomitus squalens.* *Carbohydr. Res.* **142**, 299-314.

57. Rouau, X. and Odier, E. (1986) Purification and properties of two enzymes from *Dichomitus squalens* which exhibit both cellobiohydrolase and xylanase activity. *Carbohydr. Res.* **14**, 279-292.

58. Brillouet, J. M., Moulin, J. C., and Agosin, E. (1985) Production, purification, and properties of an α-L-arabinofuranosidase from *Dichomitus squalens. Carbohydr. Res.* **144**, 113,126.

59. Bavendamm, W. (1928) Uber des vorkommen und den nachweis von oxydasen bei holzzerstorenden pilzen. *Z. Pflanzenkr.* **38**, 257-276.

60. Kirk, T. K. and Kelman, A. (1965) Lignin degradation as related to the phenol oxidases of selected wood-decaying basidiomycetes. *Phytopathology* **55**, 739-745.

61. Kersten, J. and Kirk, T. K. (1987) Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium. J. Bacteriol.* **169**, 2195-2202.

62. Glenn, J. K. and Gold, M. H. (1985) Purification and characterization of an extracellular Mn(II)-dependent peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **242**, 329-341.

63. Paszczyñski, A., Huynh, V. B., and Crawford, R. (1986) Comparison of ligninase-I and peroxidase M2 from the white-rot fungus *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **244**, 750-765.

64. Glenn, J. K., Akileswaran, L., and Gold, M. H. (1986) Mn(II) oxidation is the principal function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **251**, 688-696.

65. Kuwahara, M., Ishida, Y., Miyagama, Y., and Kawakami, C. (1984) Production of extracellular NAD and NADP by a lignin-degrading fungus *Phanerochaete chrysosporium. J. Ferment. Technol.* **62**, 237-242.

66. Bourbonnais, R. and Paice, M. G. (1988) Veratryl alcohol oxidases from the lignin-degrading basidiomycete *Pleurotus sajor-caju. Biochem. J.* **255**, 445-450.

67. Waldner, R., Leisola, M. S. A., and Fiechter, A. (1988) Comparison of ligninolytic activities of selected white-rot fungi. *Appl. Microbiol. Biotechnol.* **29**, 400-407.

68. Tien, M. and Kirk, T. K. (1983) Lignin degrading enzyme from the hymenomycete *Phanerochaete chrysosporium. Science* **221**, 661-663.

69. Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M., and Gold, M. H. (1983) An extracellular H_2O_2 requiring enzyme preparation involved in lignin degradation by the white-rot fungus *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* **114**, 1077-1083.

70. Gold, M. H., Kuwahara, M., Chiu, A. A., and Glenn, J. K. (1984) Purification and characterization of an extracellular H_2O_2 requiring diarylpropane oxygenase from the white-rot basidiomycete *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **234**, 353-362.

71. Kuwahara, M., Glenn, J. K., Morgan, M. A., and Gold, M. H. (1984) Separation and characterization of two extracellular H_2O_2 dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium. FEBS Lett.* **169**, 247-250.

72. Kirk, T. K., Croan, S., Tien, M., Murtagh, K. E., and Farrell, R. L. (1986) Production of multiple ligninases by *Phanerochaete chrysosporium*: Effect of selected conditions and use of a mutant strain. *Enzyme Microbiol. Technol.* **8**, 27-32.

73. Leisola, M. S., Kozulic, B., Meussdoerffer, F., and Fiechter, A. (1987) Homology among multiple extracellular peroxidases from *Phanerochaete chrysosporium. J. Biol. Chem.* **262**, 419-424.

74. Renganathan, V., Miki, K., and Gold, M. H. (1985) Multiple molecular forms of diarylpropane oxygenase an H_2O_2 requiring, lignin degrading enzyme from *Phanerochaete chrysosporium*. *Arch. Biochem. Biophys.* **241**, 304-314.

75. Tien, M. and Kirk, T. K. (1984) Lignin degrading enzyme from *Phanerochaete chrysosporium*: Purification characterization and catalytic properties of an unique H_2O_2 requiring oxygenase. *Proc. Natl. Acad. Sci. USA* **81**, 2280-2284.

76. Dodson, J., Evans, C. S., Harvey, J., and Palmer, J. M. (1987) Production and properties of an extracellular peroxidase from *Coriolus versicolor* which catalyses $C\alpha$-$C\beta$ cleavage in a lignin model compound. *FEMS Microbiol. Lett.* **42**, 17-22.

77. Niku-Paavola, M. L., Karhunen, E., Salola, and Raunio, V. (1988) Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochem. J.* **254**, 877-884.

78. Hatakka, A. (1988) Production of ligninases by *Phlebia radiata*. *VTT Symp.* **88**, 85-95.

79. Niku-Paavola, M. L. (1988) Ligninases of the white-rot fungus *Phlebia radiata*. *VTT Symp.* **88**, 74-84.

80. Leontevskii, A. A., Mishurtsova, Z., and Golovleva, L. A. (1988) Ligninolytic activity of the fungus *Panus tigrinus* 8/18 during submerged and solid-state cultivation. *VTT Symp.* **88**, 96-102.

81. Forrester, I. T., Grabski, A. C., Mishra, C., Kelley, B. D., Strickland, W. N., Leatham, G. F., and Burgess, R. R. (1990) Purification and characterization of a manganese-dependent peroxidase from *Lentinula edodes*. In *Application of Biotechnology in Pulp and Paper Manufacture* (T. K. Kirk and H. M. Chang, Eds.) Butterworths, Stoneham.

82. Forrester, I. T., Grabski, A. C., Mishra, C., Kelley, B. D., Strickland, W. N., Leatham, G. F., and Burgess, R. R. (1990) Characteristics and N-terminal amino acid sequence of a manganese peroxidase purified from *Lentinula edodes* cultures grown on commercial wood substrate. *Appl. Microbiol. Biotechnol.* **33**, 359-365.

83. Johansson, T. and Nyman, O. (1987) A manganese (II)-dependent extracellular peroxidase from the white-rot fungus *Trametes versicolor. Acta Chem. Scand.* **B41**, 762-765.

84. Galliano, H., Gas, G., Séris, J. L., and Boudet, A. M. (1991) Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn-peroxidase and laccase. *Enzyme Microb. Technol.* **13**, 478-482.

85. Dunford, H. B. and Stillman, J. S. (1976) On the function and mechanism of action of peroxidases. *Coord. Chem. Rev.* **19**, 187-251.

86. La Mar, G. N. and de Ropp, J. S. (1979) Assignment of exchangeable proximal histidine resonances in high-spin ferric hemoproteins: Substrate binding in horseradish peroxidase. *Biochem. Biophys. Res. Commun.* **90**, 36-41.

87. Kobayashi, K., Tamura, M., Hayashi, K., Hori, H., and Moritomo, H. (1980) Electron paramagnetic resonance and optical absorption spectrum of the pentacoordinated ferrihemoproteins. *J. Biol. Chem.* **225**, 2239-2242.

88. Sono, M. and Dawson, J. H. (1982) Formation of low spin complexes of ferric cytochrome P-450-CAM with anionic ligands. *J. Biol. Chem.* **257**, 5496-5502.

89. Andersson, L. A., Renganathan, V., Chiu, A. A., Loehr, T. M., and Gold, M. H. (1985) Spectral characterization of diarylpropane oxygenase, a novel peroxide-dependent, lignin-degrading heme enzyme. *J. Biol. Chem.* **260**, 6080-6087.

90. Mino, Y., Wariishi, H., Blackburn, N. J., Loehr, T. M., and Gold, M. H. (1988) Spectral characterization of manganese peroxidase, an extracellular heme enzyme from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium. J. Biol. Chem.* **263**, 7029-7036.

91. Kirk, T. K., Tien, M., Kerstein, J., and Kalyanamaran, B. (1986) Ligninase of *Phanerochaete chrysosporium.* Mechanism of its degradation of non-phenolic arylglycerol β-aryl ether substructure of lignin. *Biochem. J.* **236**, 279-292.

92. Dunford, H. B. (1982) Peroxidases. In *Advances in Inorganic Chemistry* (G. L. Eichhorn and L. G. Marzilli, Eds.) Elsevier, New York, pp. 41-68.

93. Dolphin, D., Forman, A., Borg, D. C., and Felton, R. H. (1971) Compounds I of catalase and horseradish peroxidase: π-cation radicals. *Proc. Natl. Acad. Sci. USA* **68**, 614-618.

94. Yonetani, T. and Asakura, T. (1968) Cytochrome c peroxidase. XI. A crystalline enzyme reconstituted from apoenzyme and manganese protoporphyrin IX. *J. Biol. Chem.* **243**, 3996-3998.

95. Yonetani, T. and Asakura, T. (1968) Cytochrome c peroxidase. XII. Crystalline synthetic enzymes containing unnatural heme prosthetic groups. *J. Biol. Chem.* **243**, 4715-4721.

96. Keilin, D. and Mann, T. (1937) Hematin compound of peroxidase. *Proc. Roy. Soc. London* **122**, 119-133.

97. Blumberg, W. E., Peisach, J., Wittenberg, B. A., and Wittenberg, J. B. (1968) The electronic structure of protoheme proteins. I. An electron paramagnetic resonance and optical study of horseradish peroxidase and its derivatives. *J. Biol. Chem.* **243**, 1854-1868.

98. Wittenberg, J. B., Noble, R. W., Wittenberg, B. A., Antonini, E., Brunori, M., and Wyman, J. (1967) Studies on the equilibria and kinetics of the reactions of peroxidase with ligands. II. The reaction of ferroperoxidase with oxygen. *J. Biol. Chem.* **242**, 626-634.

99. Renganathan, V. and Gold, M. H. (1986) Spectral characterization of the oxidized states of lignin peroxidase, an extracellular heme enzyme from the white-rot basidiomycete *Phanerochaete chrysosporium. Biochemistry* **25**, 1626-1631.

100. Wariishi, H., Akileswaran, L., and Gold, M. H. (1988) Manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*: Spectral characterization of the oxidized states and the catalytic cycle. *Biochemistry* **27**, 5365-5370.

101. Hammel, K. E., Tien, M., Kalyanaraman, B., and Kirk, T. K. (1985) Mechanism of C_α-C_β cleavage of a lignin model dimer by *Phanerochaete chrysosporium* ligninase. Stoichiometry and involvement of free radical. *J. Biol. Chem.* **260**, 8348-8353.

102. Hammel, K. E., Kalyanamaran, B., and Kirk, T. K. (1986) Substrates free radicals are intermediates in ligninase catalysis. *Proc. Natl. Acad. Sci. USA* **83**, 3708-3712.

103. Kersten, J., Tien, M., Kalyanamaran, B., and Kirk, T. K. (1985) The ligninase of *Phanerochaete chrysosporium* generates cation radicals from methoxybenzenes. *J. Biol. Chem.* **260**, 2609-2612.

104. Renganathan, V., Miki, K., and Gold, M. H. (1986) Role of molecular oxygen in lignin peroxidase reactions. *Arch. Biochem. Biophys.* **246**, 155-161.

105. Schoemaker, H. E., Harvey, J., Bowen, R. M., and Palmer, J. M. (1985) On the mechanism of enzymic lignin breakdown. *FEBS Lett.* **183**, 7-12.

106. Schoemaker, H. E. (1990) On the chemistry of lignin degradation. *Recl. Trav. Pays Bas* **109**, 255-272.

107. Haemmerli, S. D., Leisola, M. S. A., and Fiechter, A. (1986) Polymerisation of lignins by ligninases from *Phanerochaete chrysosporium*. *FEMS Microbiol. Lett.* **35**, 33-36.

108. Hammel, K. E. and Moen, M. A. (1991) Depolymerization of a synthetic lignin *in vitro* by lignin peroxidase. *Enzyme Microb. Technol.* **13**, 15-18.

109. Wariishi, H., Dunford, H. B., MacDonald, I. D., and Gold, M. H. (1989) Manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Transient state kinetics and reaction mechanism. *J. Biol. Chem.* **264**, 3335-3340.

110. Forrester, I. T., Grabski, A. C., Burgess, R. R., and Leatham, G. F. (1988) Manganese, Mn-dependent peroxidases and the biodegradation of lignin. *Biochem. Biophys. Res. Commun.* **157**, 992-999.

111. Wariishi, H., Valli, K., and Gold, M. H. (1989) Oxidative cleavage of a phenolic diarylpropane lignin model dimer by manganese peroxidase from *Phanerochaete chrysosporium. Biochemistry* **28**, 6017-6023.

112. Wariishi, H., Valli, K., Renganathan, V., and Gold, M. H. (1989) Thiol-mediated oxidation of nonphenolic lignin model compounds by manganese peroxidase of *Phanerochaete chrysosporium. J. Biol. Chem.* **264**, 14185-14191.

113. Yoshida, H. (1883) Zur chemie des Urushi-firniss. *J. Chem. Soc.* **63**, 472-486.

114. Kurtz, M. B. and Champe, S. P. (1982) Purification and characterization of the conidial laccase of *Aspergillus nidulans. J. Bacteriol.* **151**, 1338-1345.

115. Froehner, S. C. and Eriksson, K.-E. (1974) Purification and properties of *Neurospora crassa* laccase. *J. Bacteriol.* **120**, 458-465.

116. Durrens, P. (1981) The phenoloxidases of the ascomycete *Podospora anserina*: The three forms of the major laccase activity. *Arch. Microbiol.* **130**, 121-124.

117. Dubernet, M., Ribereau-Gayon, P., Lerner, H. R., Harel, E., and Mayer, M. (1977) Purification and properties of laccase from *Botrytis cinerea. Phytochemistry* **16**, 191-193.

118. Sannia, G., Giardina, P., Luna, M., Rossi, M., and Buonocore, V. (1986) Laccase from *Pleurotus ostreatus. Biotechnol. Lett.* **8**, 797-800.

119. Omura, T. (1961) Studies on laccases of lacquer trees. I. Comparison of laccase obtained from *Rhus vernicifera* and *Rhus succedanea. J. Biochem.* **50**, 264.

120. Reinhammar, B. (1984) Laccase. In *Copper Proteins and Copper Enzymes* (R. Lontie, Ed.) CRC Press, Boca Raton, FL, pp. 1-35.

121. Zouari, N., Romette, J. L., and Thomas, D. (1987) Purification and properties of two laccase isoenzymes produced by *Botrytis cinerea. Appl. Biochem. Biotechnol.* **15**, 213-25.

122. McMillin, D. R., Rosenberg, R.C., and Gray, H. B. (1974) Preparation and spectroscopic studies of cobalt(II) derivatives of blue copper proteins. *Proc. Natl. Acad. Sci. USA* **71**, 4760-4762.

123. McMillin, D. R., Holwerda, R. A., and Gray, H. B. (1974) Preparation and spectroscopic studies of cobalt (II)-stellacyanin. *Proc. Natl. Acad. Sci. USA* **71**, 1338-1341.

124. Solomon, E. I., Rawlings, J., McMillin, D. R., Stephens, J., and Gray, H. B. (1976) Infrared and visible circular dichroism and magnetic circular dichroism studies of cobalt (II)-substituted blue copper proteins. *J. Am. Chem. Soc.* **98**, 8046-8048.

125. Vänngard, T. (1972) Copper proteins. In *Biological Applications in Electron Spin Resonance* (H. Swartz, J. R. Bolton, and D. C. Borg, Eds.) Wiley-Interscience, New York, pp. 411-447.

126. Markley, J. L. (1975) Observation of histidine residues in proteins by nuclear magnetic resonance spectroscopy. *Acc. Chem. Res.* **8**, 70-80.

127. Cole, J. L., Clark, A., and Solomon, E. I. (1990) Spectroscopic and chemical studies of the laccase trinuclear cluster copper active site: Geometric and electronic structure. *J. Am. Chem. Soc.* **112**, 9534-9548.

128. Colman, N., Freeman, H. C., Guss, J. M., Murata, M., Norris, V. A., Ramshaw, J. A. M., and Venkatappa, M. P. (1978) X-Ray crystal structure analysis of plastocyanin at 2.7 Å resolution. *Nature* **272**, 319-324.

129. Adman, E. T., Stenkamp, R. E., Sicker, L. R., and Jensen, L. H. (1978) A crystallographic model for azurin at 3 Å resolution. *J. Mol. Biol.* **123**, 35-47.

130. Kau, L.-S., Spira-Solomon, D. J., Penner-Hahn, J. E., Hodgson, K. O., and Solomon, E. I. (1987) X-Ray absorption edge determination of the oxidation state and coordination number of copper: Application to the type 3 site in *Rhus vernicifera* laccase and its reaction with oxygen. *J. Am. Chem. Soc.* **109**, 6433-6442.

131. Allendorf, M. D., Spira, D. J., and Solomon, E. I. (1985) Low temperature magnetic circular dichroism studies of native laccase: Spectroscopic evidence for exogenous ligand bridging at a trinuclear active site. *Proc. Natl. Acad. Sci. USA* **82**, 3063-3067.

132. Meyer, T. E., Marchesini, A., Cusanovich, M. A., and Tollin, G. (1991) Direct measurement of intramolecular electron transfer between type 1 and type 3 copper centers in the multi-copper enzyme ascorbate oxidase and its type 2 copper-depleted and cyanide-inhibited forms. *Biochemistry* **30**, 4619-4623.

133. Reinhammar, B. R. M. and Vänngard, T. I. (1971) The electron-accepting sites in *Rhus vernicifera* laccase as studied by anaerobic oxidation-reduction titration. *Eur. J. Biochem.* **18**, 463-468.

134. Aasa, R., Brändén, R., Deinum, J., Malmström, B. G., Reinhammar, B., and Vänngard, T. (1976) An oxygen 17 effect on the EPR spectrum of the intermediate in the dioxygen-laccase reaction. *Biochem. Biophys. Res. Commun.* **70**, 1204-1209.

135. Brändén, R. and Deinum, J. (1977) Type 2 copper (II) as a component of the dioxygen reducing site in laccase: Evidence from EPR experiments with ^{17}O. *FEBS Lett.* **73**, 144-146.

136. Latour, J. M. (1988) Les sites actifs binucléaires des protéines à cuivre. *Bull. Soc. Chim. Fr.* **3**, 508-523.

137. Mälmstrom, B. G. (1982) Enzymology of oxygen. *Annu. Rev. Biochem.* **51**, 21-29.

138. Ishihara, T., (1980) The role of laccase in lignin biodegradation. In *Lignin Biodegradation. Microbiology, Chemistry and Potential Applications* (T. K. Kirk, T. Higuchi, and H.-M. Chang, Eds.) CRC Press, Boca Raton, FL, pp. 17-31.

139. Evans, C. S. (1985) Laccase activity in lignin degradation by *Coriolus versicolor in vivo* and *in vitro* studies. *FEMS Microbiol. Lett.* **27**, 339-343.

140. Gierer, J. and Opara, A. E. (1973) Studies on the enzymatic degradation of lignin. The action of peroxidase and laccase on monomeric and dimeric model compounds. *Acta Chem. Scand.* **27**, 2909-2922.

141. Morohoshi, N., Fujita, K., Wariishi, H., and Haraguchi, T. (1987) Degradation of lignin by the extracellular enzymes of *Coriolus versicolor*. V. Degradation of isolated lignin fractions by laccases I, II, and III. *Mokuzai Gakkaishi* **4**, 310-315.

142. Wariishi, H., Morohoshi, N., and Haraguchi, T. (1987) Degradation of lignin by the extracellular enzymes of *Coriolus versicolor*. VII: Effective degradation of syringyl type β-aryl ether lignin model compound by laccase III. *Mokuzai Gakkaishi* **33**, 892-898.

143. Kawai, S., Umezawa, T., Shimada, M., Higuchi, T., Koide, K., Nishida, T., Morohoshi, N., and Haraguchi, T. (1987) Cα-Cβ cleavage of phenolic β-1 lignin substructure model compound by laccase of *Coriolus versicolor*. *Mokuzai Gakkaishi* **33**, 792-797.

144. Kawai, S., Umezawa, T., Shimada, M., and Higushi, T. (1988) Aromatic ring cleavage of 4,6-di(tert-butyl)guaiacol, a phenolic lignin model compound, by laccase of *Coriolus versicolor*. *FEBS Lett.* **236**, 309-311.

145. Ishihara, T. and Miyazaki, M. (1974) Demethylation of lignin and lignin models by fungal laccase. *Mokuzai Gakkaishi* **20**, 39-41.

146. Leonowicz, A. and Bollag, J.-M. (1984) Formation of 2-methoxy-1,4-benzoquinone from vanillic acid by fungal laccases at various pH values. *Experientia* **40**, 544-545.

147. Leonowicz, A., Edgehill, R. U., and Bollag, J.-M. (1984) The effects of pH on the transformation of syringic and vanillic acids by the laccases of *Rhizoctonia* and *Trametes versicolor*. *Arch. Microbiol.* **137**, 89-96.

148. Morohoshi, N., Nakamura, M., Katayama, Y., Haraguchi, T., Fujii, T., and Hiroi, T. (1989) Degradation of protolignin by laccase III. *Int. Biodeterior.* **25**, 7-14.

149. Bourbonnais, R. and Paice, M. G. (1990) Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. *FEBS Lett.* **267**, 99-102.

150. Archibald, F. and Roy, B. (1992) Production of manganic chelates by laccase from the lignin degrading fungus *Trametes versicolor*. *Appl. Environ. Microbiol.* **58**, 1496-1499.

151. Périé, F. H. and Gold, M. H. (1991) Manganese regulation of manganese peroxidase expression and lignin degradation by the white-rot fungus *Dichomitus squalens*. *Appl. Environ. Microbiol.* **57**, 2240-2245.

152. Fåhraeus, G. and Reinhammar, B. (1967) Large scale production and purification of laccase from cultures of the fungus *Polyporus versicolor* and some properties of laccase A. *Acta Chem. Scand.* **21**, 2367-2378.

153. Niku-Paavola, M. L., Karhunen, E., Kantelinen, A., Viikari, L., Lundell, T., and Hatakka, A. (1990) The effect of culture conditions on the production of lignin-modifying enzymes by the white-rot fungus *Phlebia radiata*. *J. Biotechnol.* **13**, 211-221.

154. Fukuzumi, T. (1987) Lignolytic enzymes of *Pleurotus sajor-caju*. In *Lignin Enzymic and Microbial Degradation* (E. Odier, Ed.) Institut National de la Recherche Agronomique, Paris, pp. 137-142.

155. Muheim, A., Waldner, R., Leisola, M. S. A., and Fiechter, A. (1990) An extracellular aryl alcohol oxidase from the white-rot fungus *Bjerkendera adusta*. *Enz. Microb. Technol.* **12**, 204-209.

156. Galliano, H., Gas, G., and Boudet, A. (1988) Biodegradation of *Hevea brasiliensis* lignocellulose by *Rigidoporus lignosus*: Influence of cultures, conditions and involvement of oxidizing enzymes. *Plant Physiol. Biochem.* **26**, 619-627.

157. Chang, H. M., Chen, C. L., and Kirk, T. K. (1980) The chemistry of lignin degradation by white-rot fungi (*Polyporys anceps, Coriolus versicolor*). In *Lignin Biodegradation: Microbiology, Chemistry and Applications* (T. K. Kirk, T. Higuchi, and H. M. Chang, Eds.) CRC Press, Boca Raton, FL, pp. 215-230.

158. Fincham, J. R. S., Day, R., and Radford, A. (1979) Fungal genetics. In *Botanical Monographs*, Vol. 4., Blackwell Scientific, Berkeley, CA.

159. Gold, M. H., Mayfield, M. B., Chang, K., Krisnangkura, M., Shimada, M., Enoki, A., and Glenn, J. K. (1982) A *Phanerochaete chrysosporium* mutant defective in lignin degradation as well as several other secondary metabolic functions. *Arch. Microbiol.* **132**, 115-122.

160. Hanson, R. S. and Phillips, J. A. (1981) Chemical composition. In *Manual of Methods for General Bacteriology* (R. G. E. M. Gerhardt, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips, Eds.) American Society for Microbiology, Washington, DC.

161. Yemm, E. W. and Cocking, E. C. (1954) The determination of amino acids with ninhydrin. *Analyst* **80**, 209-213.

162. Blankenship, D. T., Krivanek, M. A., Ackerman, B. L., and Cardin, A. D. (1989) High sensitivity amino acid analysis by derivatization with O-phthalaldehyde and 9-fluoreryl methyl chloroferumate using fluorescence detection, application in protein structure determination. *Anal. Biochem.* **178**, 227-232.

163. Ashwell, G. (1966) New colorimetric methods of sugar analysis. *Methods Enzymol.* **8**, 85-94.

164. Wariishi, H., Valli, K., and Gold, M. H. (1991) *In vitro* depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* **176**, 269-275.

165. Bonarme, P. and Jeffries, T. W. (1990) Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white-rot fungi. *Appl. Environ. Microbiol.* **56**, 210-217.

166. Brown, J. A., Glenn, J. K., and Gold, M. H. (1990) Manganese regulates expression of manganese peroxidase by *Phanerochaete chrysosporium. J. Bacteriol.* **172**, 3125-3130.

167. Yang, H. H., Effland, M., and Kirk, T. K. (1980) Factors influencing fungal decomposition of lignin in a representative, thermomechanical pulp. *Biotechnol. Bioeng.* **22**, 65-77.

168. Blanchette, R. A., Burnes, T. A., Leatham, G. F., and Effland, M. J. (1988) Selection of white-rot fungi for biopulping. *Biomass* **15**, 93-101.

169. Otjen, L. and Blanchette, R. (1987) Assessment of 30 white-rot basidiomycetes for selective lignin degradation. *Holzforschung* **41**, 343-349.

170. Reid, I. D. (1989) Solid-state fermentations for biological delignification. *Enzyme Microb. Technol.* **11**, 786-803.

171. Hatakka, A. I. (1985) Degradation of veratric acid and other lignin-related aromatic compounds by the white-rot fungus *Pycnoporus cinnabarinus. Arch. Microbiol.* **141**, 22-28.

172. Sutherland, J. B. (1984) Biodegradation of lignocellulose from cotton-gin trash by *Pycnoporus cinnabarinus. Mycologia* **76**, 369-372.

173. Smith, M. H. and Gold, M. H. (1979) *Phanerochaete chrysosporium* β-glucosidases. Induction, cellular localization and physical characterization. *Appl. Environ. Microbiol.* **37**, 938-942.

174. Vogel, H. J. (1964) Distribution of lysine pathways among fungi, evolutionary implications. *Am. Nat.* **98**, 435-446.

175. Dawes, E. A., McGill, D. J., and Midgley, M. (1971) Analysis of fermentation products. In *Methods in Microbiology* (J. R. Norris and D. W. Ribbon, Eds.) Academic Press, London, pp. 98-99.

176. Boos, R. N. (1948) Quantitative colorimetric microdetermination of methanol with chromotropic acid reagent. *Anal. Chem.* **20**, 964-965.

177. Gold, M. H., Wariishi, H., and Valli, K. (1989) Extracellular peroxidases involved in lignin degradation by the white-rot basidiomycete *Phanerochaete chrysosporium*. In *Biocatalysis in Agricultural Biotechnology*, American Chemical Society, Washington, DC.

178. Kirk, K. T., Connors, W. J., and Zeikus, J. G. (1977) Advances in understanding the microbiological degradation of lignin. In *Recent Advances in Phytochemistry* (F. A. Loewus and V. C. Runeckles, Eds.) Plenum Press, New York, pp. 369-394.

179. Kirk, T. K. (1971) Effects of microorganisms on lignin. *Annu. Rev. Phytopathol.* **9**, 185-210.

180. Lundquist, K. and Kristersson, P. (1985) Exhaustive laccase-catalysed oxidation of a lignin model compound (vanillyl glycol) produces methanol and polymeric quinoid products. *Biochem. J.* **229**, 277-279.

181. Bourbonnais, R. and Paice, M. G. (1992) Demethylation and delignification of kraft pulp by *Trametes versicolor* laccase in the presence of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonate). *Appl. Microbiol. Biotechnol.* **36**, 823-827.

182. Szklarz, G. and Leonowicz, A. (1986) Cooperation between fungal laccase and glucose oxidase in the degradation of lignin derivatives. *Physiochemistry* **25**, 2537-2539.

183. Datta, A., Bettermann, A., and Kirk, T. K. (1991) Identification of a specific manganese peroxidase among lignolytic enzymes secreted by *Phanerochaete chrysosporium* during wood decay. *Appl. Environ. Microbiol.* **57**, 1453-1460.

184. Asther, M., Corrieu, G., Drapron, R., and Odier, E. (1987) Effect of Tween 80 and oleic acid on ligninase production by *Phanerochaete chrysosporium* INA 12. *Enzyme Microb. Technol.* **9**, 245-249.

185. Eriksson, K.-E. and Pettersson, B. (1982) Purification and partial characterization of two acidic proteases from the white-rot fungus *Sporotrichum pulverulentum*. *Eur. J. Biochem.* **124**, 635-642.

186. Dosoretz, C. G., Chen, H.-C., and Grethlein, H. E. (1990) Effect of environmental conditions on extracellular protease activity in lignolytic cultures of *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **56**, 395-400.

187. Dosoretz, C. G., Dass, S. B., Reddy, C. A., and Grethlein, H. E. (1990) Protease-mediated degradation of lignin peroxidase in liquid cultures of *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* **56**, 3429-3434.

188. Kumagai, H., Matsue, M., Majima, E., Tomoda, K., and Ichishima, E. (1981) Carboxyl proteinase from the wood deteriorating basidiomycete *Pycnoporus coccineus*: Substrate specificity with oxidized insulin peptide B1-B16 and B15-B24, angiotensin and proangiotensin. *Agric. Biol. Chem.* **45**, 981-985.

189. Ichishima, E., Yoshimura, K., and Tomoda, K. (1983) Acid carboxypeptidase from a wood-deteriorating basidiomycete *Pycnoporus sanguineus*. *Phytochemistry* **22**, 825-829.

190. Leisola, M., Ulmer, D., and Fiechter, A. (1983) Problem of oxygen transfer during degradation of lignin by *Phanerochaete chrysosporium*. *Eur. J. Appl. Microbiol. Biotechnol.* **17**, 113-116.

191. Leonowicz, A., Sarkar, J. M., and Bollag, J. (1988) Improvement in stability of an immobilized fungal laccase. *Appl. Microbiol. Biotechnol.* **29**, 129-135.

192. Shuttleworth, K. L. and Bollag, J.-M. (1986) Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. *Enzyme Microb. Technol.* **8**, 171-177.

193. Enoki, A. and Takahashi, M. (1983) Metabolism of lignin-related compounds by various wood-decomposing fungi, In *Recent Advances in Lignin Biodegradation Research* (T. Higuchi, H.-M. Chang, and T. K. Kirk, Eds.) UNI Publishing Co., Tokyo, pp. 119-133.

194. Kirk, T. K. and Chang, H. M. (1974) Decomposition of lignin by white-rot fungi. I. Isolation of heavily degraded lignins from decayed spruce. *Holzforschung* **28**, 217-222.

195. Brown, J. A., Alic, M., and Gold, M. H. (1991) Manganese peroxidase gene transcription in *Phanerochaete chrysosporium*: Activation by manganese. *J. Bacteriol.* **173**, 4101-4106.

196. O'Farrell, H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021.

197. Bollag, D. M. and Edelstein, S. J. (1990) *Proteins Methods*, Wiley-Liss, Geneva.

198. Smith, K., Krohm, R. J., Hermanson, G. T., Malhia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.

199. Rao, P. and Pattabiraman, T. N. (1989) Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses. *Anal. Biochem.* **181**, 18-22.

200. Pajot, P. and Groudinsky, O. (1970) Molecular weight and quaternary structure of yeast L-lactate dehydrogenase (cytochrome *b*2). Revised heme extinction coefficients and minimal molecular weight. *Eur. J. Biochem.* **12**, 158-164.

201. Appleby, C. A. and Morton, R. K. (1959) Lactic dehydrogenase and cytochrome *b*2 of baker's yeast. Enzymic and chemical properties of the crystalline enzyme. *Biochem. J.* **73**, 539-550.

202. Casella, L., Gullotti, M., Poli, S., Bonfà, M., and Ferrari, R. P. (1991) Spectroscopic and binding studies on the stereoselective interaction of tyrosine with horseradish peroxidase and lactoperoxidase. *Biochem. J.* **279**, 245-250.

203. Renganathan, V., Miki, K., and Gold, M. H. (1987) Haloperoxidase reactions catalyzed by lignin peroxidase, an extracellular enzyme from the basidiomycete *Phanerochaete chrysosporium*. *Biochemistry* **26**, 5127-5132.

204. Hames, B. D. (1981) An introduction to polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins: A Practical Approach* (B. D. Hames and D. Ricwood, Eds.) IRL Press, Oxford, pp. 1-91.

205. Towbin, H., Staehelin, T., and Goedon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.

206. Leary, J. J., Brigati, D. J., and Ward, D. C. (1983) Rapid and sensitive colorimetric method for visualizing biotin labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose bio blots. *Proc. Natl. Acad. Sci. USA* **80**, 4045-4049.

207. Pribnow, D., Mayfield, M. B., Nipper, V. J., Brown, J. A., and Gold, M. H. (1989) Characterization of a cDNA encoding a manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. *J. Biol. Chem.* **264**, 5036-5040.

208. Schulz, C. E., Devaney, W., Winkler, H., Debrunner, G., Doan, N., Chiang, R., Rutter, R., and Hager, L. P. (1979) Horseradish peroxidase compound I: Evidence for spin coupling between the heme iron and a free radical. *FEBS Lett.* **103**, 102-105.

209. Rutter, R., Valentine, M., Hendrich, M. P., Hager, L. P., and Debrunner, G. (1983) Chemical nature of the porphyrin π cation radical in horseradish peroxidase compound I. *Biochemistry* **22**, 4769-4774.

210. Kamaya, Y. and Higuchi, T. (1984) Metabolism of 3,4-dimethoxycinnamyl alcohol and derivatives by *Coriolus versicolor*. *FEMS Microbiol. Lett.* **24**, 225-229.

211. Kawai, S., Umezawa, T., and Higuchi, T. (1985) Metabolism of a non-phenolic β-O-4 lignin substructure model compound by *Coriolus versicolor*. *Agric. Biol. Chem.* **49**, 2325-2330.

212. Perez, J. and Jeffries, T. W. (1990) Mineralization of ^{14}C-ring-labeled synthetic lignin correlates with the production of lignin peroxidase, not of manganese peroxidase or laccase. *Appl. Environ. Microbiol.* **56**, 1806-1812.

213. Geiger, J. P., Rio, B., Nandris, D., and Nicole, M. (1986) Laccases of *Rigidoporus lignosus* and *Phenillus noxius*. I. Purification and some physicochemical properties. *Appl. Biochem. Biotechnol.* **12**, 121-133.

214. Kofujita, H., Ohta, T., Asada, Y., and Kuwahara, M. (1991) Purification and characterization of laccase from *Lentines edodes*. *Mokuzai Gakkaishi* **37**, 562-569.

215. Oda, Y., Adachi, K., Aita, I., Ito, M., Aso, Y., and Igarashi, H. (1991) Purification and properties of laccase excreted by *Pycnoporus coccineus*. *Agric. Biol. Chem.* **55**, 1393-1395.

216. Sullivan, G. and Henry, E. D. (1971) Occurrence and distribution of phenoxazinone pigments in the genus *Pycnoporus*. *J. Pharm. Sci.* **60**, 1097-1098.

217. Ishihara, T. (1983) Effect of pH in the oxidation of syringic acid by fungal laccase. *Mokuzai Gakkaishi* **29**, 801-805.

218. Wood, D. A. (1980) Production, purification and properties of extracellular laccase of *Agaricus bisporus*. *J. Gen. Microbiol.* **117**, 327-338.

219. Karhunen, E., Niku-Paavola, M.-L., Viikari, L., Haltia, T., Van der Meer, R. A., and Duine, J. A. (1990) A novel combination of prosthetic groups in a fungal laccase: PQQ and two copper atoms. *FEBS Lett.* **267**, 6-8.

220. Sakurai, T., Suzuki, S., and Chikira, M. (1990) pH and microwave power effects on the electron spin resonance spectra of *Rhus vernicifera* laccase and *Cucumis sativus* ascorbate oxidase. *J. Biochem.* **107**, 37-42.

221. Nakamura, T. (1958) Purification and physico-chemical properties of laccase. *Biochim. Biophys. Acta* **30**, 44-52.

222. Calabrese, L., Carbonaro, M., and Musci, G. (1988) Chicken ceruloplasmin. Evidence in support of a trinuclear cluster involving type 2 and type 3 copper centers. *J. Biol. Chem.* **263**, 6480-6483.

223. Hanna, M., Tamilarasan, R., and McMillin, D. R. (1988) Cu(I) analysis of blue copper proteins. *Biochem. J.* **256**, 1001-1004.

224. Felici, M., Luna, M., Artemi, F., and Badiani, M. (1984) Phenols and alccase activity. *Agrochimica* **28**, 274-279.

225. Felici, M., Artemi, F., Luna, M., and Speranza, M. (1985) Determination of laccase activity with various aromatic substrates by high performance liquid chromatography. *J. Chromatog.* **320**, 435-439.

BIOGRAPHICAL SKETCH

Frédéric H. Périé was born in Paris, France, on June 8, 1962. He received his Bachelor of Science degree from ENSIA (Ecole Nationale Supérieure des Industries Agricoles et Alimentaires, Massy, France) in June 1986, and his Master of Science degree at Compiegne Technological University (UTC) in November 1986. In October 1987, he started work at the Oregon Graduate Institute of Science & Technology in the laboratory of Prof. M. H. Gold and, in September 1988, he began his studies for the Ph.D. degree.