

Purification and Characterization of an Extracellular  
Manganese Oxidizing Peroxidase from the White Rot Fungus  
Phanerochaete chrysosporium

Jeffrey K. Glenn  
B.A., Northwestern University, 1974  
M.S., University of Wisconsin - Madison, 1978  
M.A., University of Wisconsin - Madison, 1979

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The dissertation "Purification and Characterization of an Extracellular Manganese Oxidizing Peroxidase from the White Rot Fungus Phanerochaete chrysosporium" by Jeffrey K. Glenn has been examined and approved by the following Examination Committee:

\_\_\_\_\_  
Michael H. Gold, Thesis Advisor  
Professor

\_\_\_\_\_  
Thomas Loehr  
Professor

\_\_\_\_\_  
William Pengelly  
Associate Professor

\_\_\_\_\_  
David Pribnow  
Assistant Professor

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## ABSTRACT

Purification and Characterization of an  
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from the White Rot Fungus Phanerochaete chrysosporium

Jeffrey K. Glenn, Ph.D.  
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Supervising Professor: Michael H. Gold

A correlation was found between lignin degradation and the decolorization of three polymeric dyes (Poly B, Poly R, and Poly Y) by Phanerochaete chrysosporium. Like ligninolytic activity, dye decolorization occurred only during secondary metabolism and was strongly dependent on oxygen concentration. A variety of inhibitors of lignin degradation also inhibited dye decolorization, and a mutant of P. chrysosporium lacking phenol oxidase also was not able to decolorize dyes.

In the presence of H<sub>2</sub>O<sub>2</sub>, medium from ligninolytic cultures of P. chrysosporium was able to decolorize Poly R and oxidize a variety of lignin model compounds including a diarylpropane and a  $\beta$ -ether dimer. Media from primary growth cultures or a non-ligninolytic mutant had no activity.

Chromatography on a blue agarose column revealed that the extracellular media contained two separable peroxidases. One of the enzymes catalyzed the  $\alpha,\beta$  cleavage of diarylpropane. The second enzyme required manganese(II) for activity, and was stimulated by lactate and protein.

This manganese-dependent peroxidase was purified to homogeneity by DEAE-sepharose ion-exchange chromatography, blue agarose chromatography, and gel filtration. The peroxidase has an  $M_r$  of 46,000 and contains one molecule of iron protoporphyrin IX. The spectra of the native enzyme and  $CN^-$ ,  $N_3^-$  and  $H_2O_2$  complexes resembled those of horseradish peroxidase. In the presence of Mn(II), NADH and a wide variety of dyes, including Poly B and Poly R were oxidized by the peroxidase. The enzyme rapidly and efficiently oxidises Mn(II) to Mn(III). Manganese(III) was detected and quantified by the characteristic spectra of Mn(III)-pyrophosphate and Mn(III)-lactate complexes.  $K_m$  values for manganese and  $H_2O_2$  are 80  $\mu$ M and 140  $\mu$ M respectively.  $MnO_2$  was also a product of the enzymatic oxidation of Mn(II). Manganese(III)-lactate was capable of oxidizing NADH and a variety of dyes including Poly B and Poly R. Herein, we propose that the principal function of the Mn-dependent peroxidase is manganese oxidation. The Mn(III) produced by the enzyme in turn oxidizes all the other substrates.

The Mn-peroxidase is not present in cultures of *P. chrysosporium* grown without manganese, but the enzyme rapidly accumulates if Mn(II) is added to the medium. The Mn-peroxidase appears to be the enzyme responsible for the oxidation of Poly R during the first few days of secondary metabolism, but other enzymes probably play a role in dye decolorization in older cultures.

## CHAPTER 1

### Introduction

#### Historical Perspective

Cellulose was first isolated from wood in about 1838 by the French chemist Anselme Payen<sup>1</sup>. This was accomplished by removing an "incrusting material" by alternately treating wood with nitric acid, alkali, alcohol, and ether. By analysis he concluded that his incrusting material, later named "lignin," had a considerably higher carbon content than cellulose.

Since Payen's time, a considerable amount of work has gone into the elucidation of the structure of lignin. Numerous detailed reviews of the chemistry of lignin are available;<sup>2-5</sup> a brief historical account follows.

In the manufacture of paper pulp, it is necessary to remove some of the lignin from wood. Two important processes for achieving this are the sulfite pulping process, invented by the Tilghman brothers around 1866<sup>6</sup> and the alkaline sulfide pulping (kraft) processes, pioneered by Watt and Burgess around 1854<sup>7</sup> and further developed by Dahl in 1884.<sup>8</sup> The invention of these processes led to the creation of a new industry and to the expansion of research into the nature of lignin and its chemical reactivity.

Initial progress in this field was slow. It was not until 1890 that Benedikt and Bamberger found that lignified materials but not cellulose contained methoxyl groups.<sup>9</sup> Beginning in 1893 Peter Klason published papers on the composition of waste liquors from the Kraft

and sulfite processes. His technique for the measurement of lignin in wood is still used today and bears his name.<sup>10</sup> In 1897 he advanced the idea, first suggested in 1875 by Tiemann and Mendelsohn,<sup>11</sup> that lignin was built up from coniferyl alcohol.<sup>12</sup>

By the 1930's Freudenberg had introduced successful procedures for the oxidative degradation of lignin, which produced large amounts of aromatic carboxylic acids and aromatic aldehydes.<sup>13</sup> This provided further evidence for the aromatic nature of lignin.

Although the detailed structure of lignin was still not known, Hibbert's work on ethanolysis,<sup>14</sup> and pressure hydrogenation of wood supported the theory that lignin was composed of building blocks of guaiacyl-propane and syringyl-propane (See next section for molecular structures). The analysis of isolated lignin preparations by Adkins and Harris<sup>15</sup> further supported this theory.

A major step in understanding the structure of lignin came in the early 1930's when Holger Erdtman found that the dehydrogenation product of isoeugenol had the structure of a phenylcoumaran. Similar compounds had been proposed by Freudenberg for lignin substructures. Erdtman proposed in 1933 that lignin might be formed in nature by dehydrogenation of phenylpropane compounds carrying an oxygenated side chain.<sup>16</sup> In 1937 Freudenberg found that the dehydrogenation of coniferyl alcohol with ferric chloride produced products similar to those reported for isoeugenol. In 1943 he began his studies on the enzymatic dehydrogenation for coniferyl alcohol,<sup>17</sup> a process still used today to produce synthetic lignin.

A detailed understanding of the structure of lignin was not

possible until a pure, relatively undegraded lignin could be obtained from wood. This occurred in 1954, when Bjorkman prepared milled wood lignin.<sup>18</sup> A thorough understanding of lignin's structure was achieved in the 1960s, when Adler completed analytical studies of milled wood lignin and lignin model compounds.<sup>19</sup>

### Lignin Structure and Biosynthesis

Lignin is found in all vascular plants and in certain primitive plant groups such as ferns and club mosses, but not in Bryophyta (true mosses) and algae.<sup>20</sup> Lignin occurs in the cell walls of vascular plants where secondary walls are present.<sup>21</sup> Lignification begins in the intercellular layers and spreads inward, so more of the lignin is found in the intercellular layer and primary walls than in secondary walls.<sup>21</sup> In addition to encrusting the cellulose microfibrils like a sheath,<sup>22</sup> it is physically and chemically bound to plant polysaccharides.<sup>23,24</sup> Lignin's most important role appears to be the rigidity it contributes to the cell wall. Thus cotton fibers, which contain no lignin, are much more flexible than jute or wood, which contain considerable amounts of lignin. Because of its resistance to degradation, lignin probably protects plants against pathogenic organisms.<sup>25</sup> Also, it minimizes water permeation across the cell walls of xylem tissue.<sup>21</sup>

As a result of both tracer experiments and enzymatic studies it is now possible to draw a fairly detailed picture of the biosynthetic pathways leading to lignin (Figure 1-1). The phenylpropanoid amino acids phenylalanine and tyrosine are synthesized via the shikimic acid

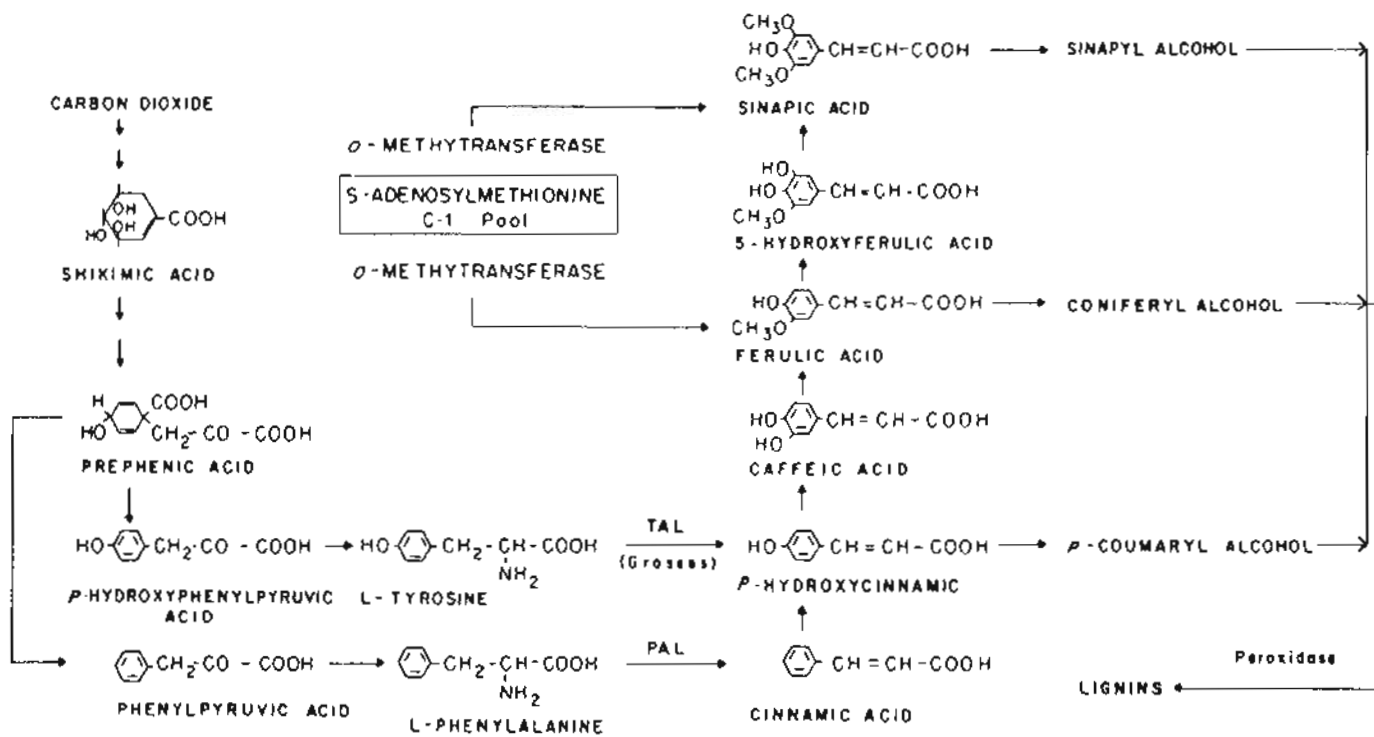


Fig. 1-1 Biosynthetic pathway from  $\text{CO}_2$  to lignin (From Ref. 23).



pathway from carbohydrate.<sup>26</sup> All of the steps to this point are aspects of general metabolism. The biosyntheses of lignin and other secondary plant products begins with the deamination of phenylalanine or tyrosine to cinnamate or p-hydroxycinnamate by the ammonia lyases (PAL and TAL).<sup>27</sup> While PAL exists in all plants which produce lignin, tyrosine ammonia lyase occurs only in grasses.<sup>28</sup>

After the production of cinnamic acid, the following steps are involved in its conversion to the immediate precursors of lignin: cinnamic acid is hydroxylated successively to p-coumaric and caffeic acids by specific hydroxylases. Caffeic acid is converted to ferulic acid by an O-methyltransferase; ferulic acid may be further hydroxylated to 5-hydroxyferulic acid, which is again methylated to produce sinapic acid. The reduction of the substituted cinnamic acids to the corresponding cinnamyl alcohols requires three enzymes. Hydroxycinnamate:CoA ligase and hydroxycinnamyl-CoA reductase reduce the acids to aldehydes, which are further reduced to the alcohols by hydroxycinnamyl alcohol oxidoreductase.<sup>26,27</sup>

These substituted cinnamyl alcohols are polymerized by a free radical mechanism initiated by peroxidases.<sup>29</sup> The first step is the enzymatic removal of an electron from a phenoxyl anion, producing a cinnamyl free radical which exists in a number of resonance forms. These can dimerize, producing compounds such as lignans,<sup>30</sup> or further polymerize, resulting in the formation of lignin, a highly branched molecule containing a variety of covalent bonds<sup>31</sup> (Figure 1-2). It is not surprising that a detailed understanding of the structure of lignin took such a long time. The mole-% composition of covalent bonds

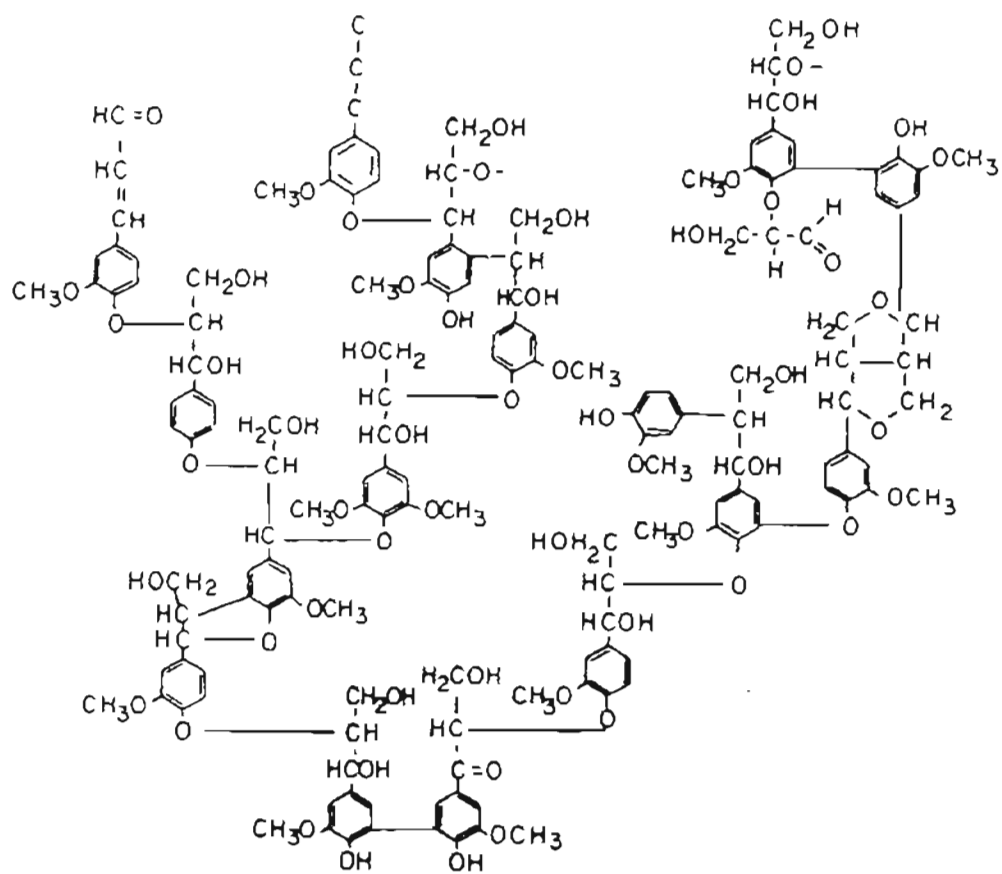


Fig. 1-2. Prominent structural features of conifer lignin (From Ref. 5).

in spruce lignin is shown in Table 1-1<sup>5</sup>. A majority are arylglycerol- $\beta$ -aryl ether linkages. Other important structures are biphenyl and phenylcoumaran groups. Extracted lignin has a molecular mass of under 20,000 daltons, but it is likely that in vivo natural lignin has a molecular mass average of at least 100,000.<sup>33</sup> The true size (or size range) of natural lignin remains undetermined.

Lignin normally contains all three of the substituted cinnamyl alcohols shown in Figure 1-1, but the proportions are dependent on the type of plant examined.<sup>26</sup> Guaiacyl lignin, found in most conifers, lycopods, ferns, and horsetails, is composed principally of coniferyl alcohol. The ratio of coniferyl to coumaryl to sinapyl alcohol in spruce lignin has been estimated to be 80:14:6.<sup>32</sup> Guaiacyl-syringyl lignin, found in dicotyledonous angiosperms and a few gymnosperms, contains large amounts of coniferyl alcohol and sinapyl alcohol with only small amounts of coumaryl alcohol. Beechwood lignin, for example has been found to contain a ratio of 49:5:46 for the three alcohols.<sup>34</sup> Finally, a guaiacyl-syringyl-p-hydroxyphenyl lignin, containing approximately equal amounts of all three cinnamyl alcohols is thought to be produced by the highly evolved grasses and in the compression wood of conifers,<sup>26</sup> produced on the underside of limbs. However, because large amounts of p-coumaric acid are bound to grass lignins as esters,<sup>35</sup> it is not considered to be incorporated into the lignin polymer.<sup>35</sup> Thus there may only be two major classes of lignin.

TABLE 1-1  
Proportions of some major types of bonds in spruce lignin<sup>a</sup>

Bond Type	Structure	Proportion(%)
Arylglycerol-B-aryl ethers		48
Noncyclic benzyl ethers		6 - 8
Biphenyl		9.5 - 11
1,2-Diarylpropane structures		7
Phenylcoumaran structures		9 - 12
Diphenyl ethers		3.5 - 4

<sup>a</sup>From Ref. 5.

### Ecological and Industrial Importance of Lignin

After cellulose, lignin is the most abundant organic polymer on earth, representing perhaps 15-20% of all photosynthetically fixed carbon and 20-30% of the dry weight of wood.<sup>20,21</sup> When plants die, the cellulose and other components are quickly converted back into carbon dioxide. However, higher plants act as unique reservoirs of fixed carbon because the lignins and tannins they produce are some of the most persistent naturally occurring carbon-oxygen compounds. Unlike polysaccharides and proteins, these compounds have no easily hydrolyzed ester or amide bonds. The complete recycling of lignin may take several thousand years,<sup>20</sup> and is accomplished almost entirely by microorganisms. Under anaerobic conditions, such as lake sediments, rumen fluid, etc., lignin may be modified but does not appear to be appreciably broken down.<sup>36</sup>

Because many invertebrates such as termites, millipedes, slugs, snails, and earthworms utilize lignocelluloses as an important component of their diets, some research has been conducted into the fate of cellulose and lignin passing through the guts of these organisms. It now appears that less than 20% of the carbon in leaf litter is metabolized by soil fauna.<sup>37</sup> However, these organisms contribute to the metabolism of the remaining plant material by providing mechanical disintegration. This enhances microbial attack by disrupting the protective sheath of lignin surrounding polysaccharides, and improving the aeration and water-holding capacities of the soil.<sup>37</sup> With few exceptions, most research suggests that during passage through an invertebrate's gut, cellulose

is degraded but lignin is not significantly mineralized (converted to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ ), although it may be chemically modified.<sup>38,39</sup> This is consistent with the current assumption that a termite's gut is an anaerobic environment.<sup>40</sup>

Man specifically exploits lignocellulose in the lumber and papermaking industries and as feed for livestock. These activities, along with agricultural practices, produce tremendous quantities of waste lignocellulose as shown in Table 1-2. Although estimates of the total amount of cellulosic wastes vary considerably, over 1 billion tons per year are probably produced in the United States alone.<sup>41,42</sup> At the present time, these wastes go largely unused. In the paper industry, for example, the waste liquor, containing large amounts of chemically modified lignins and some cellulosic material is normally burned or simply left in holding ponds. Considerable effort is required in order to rescue these potentially valuable materials.

Attempts have been made to develop industrial processes for the conversion of waste lignocellulose to useful products, but most have been largely unsuccessful. Biotransformation using microorganisms has been particularly unsuccessful in the past because the high lignin content of these wastes and the intimate association of lignin with polysaccharide presents a significant barrier to the utilization of more readily digested polysaccharides.<sup>33</sup> Microorganisms which are unable to attack the lignin do not have access to these carbohydrates unless they are exposed by chemical or physical treatment of the lignified materials.<sup>43</sup> This is a costly process, and does not result in the utilization of the lignin itself. Bioconversion technologies

TABLE 1-2  
Annual production of lignocellulosic  
wastes in the United States

Source	10 <sup>6</sup> Tons/Year	
	Estimate #1 <sup>a</sup>	Estimate #2 <sup>b</sup>
Agriculture	400	2347
Manure	200	218
Forestry	60	140
Urban	150	256
Industrial	45	110
Miscellaneous		70
Total	<u>925</u>	<u>3141</u>

<sup>a</sup>From Ref. 42.

<sup>b</sup>From Ref. 41.

which make use of both lignin and carbohydrate are both economical and desirable. As much as 40% of the caloric content of wood consists of lignin.<sup>44</sup>

There is great potential in developing methods of delignifying lignocellulosic materials using microbial techniques. It has long been known that the digestibility of forages by ruminants and other domestic animals increases as the lignin content decreases.<sup>45</sup> Treatments that expose cellulose in straws and feed lot wastes significantly improved their digestibility.<sup>46</sup> Reduction in the cost of paper making by reducing energy requirements is possible if microorganisms are used to partially remove lignin and thus aid in the pulping process.<sup>47</sup> Waste liquors and other lignin derived wastes could be used as carbon sources for lignin degrading organisms. If edible microorganisms are used, single cell protein can be obtained from them.<sup>41</sup> Thus, a valuable nutritional resource may be obtained while environmental pollution is reduced.<sup>48</sup> In addition, waste materials are a potential source of glucose,<sup>49</sup> organic acids,<sup>50</sup> and even lignin derived, low-molecular-mass phenolic compounds.<sup>51</sup>

#### Measurement of Lignin Degradation

In order to determine which organisms were active at degrading lignin, it was necessary to develop measures of lignin biodegradation or "ligninolytic activity." This required the preparation of purified lignin. Early studies used such preparations as "phenol lignin," "HCl lignin," and "Klason lignin." These lignin preparations are highly modified and are of questionable value in biodegradation studies.



Klason lignin, for example, is produced by treating lignified tissue with cold 72% sulfuric acid then refluxing with dilute acid.<sup>4,10</sup> The lignin produced is highly condensed and resinous.<sup>52</sup> Although it is of no value as a substrate for microorganisms, the technique is valuable for the determination of lignin content in plant tissues.<sup>53</sup>

A low-molecular-mass lignin is obtained by ethanol extraction of ground plant tissues.<sup>54</sup> This "Brauns' native lignin" is obtained in very low yield, and may contain appreciable quantities of non-lignin contaminants.<sup>55</sup>

Milled wood lignin is produced by extracting powdered plant tissues with neutral solvents, such as 9:1 dioxane-water, at room temperature.<sup>18,56</sup> Up to 50% of the lignin in wood can be extracted with this technique. The extracted lignin is usually purified by a series of solvent precipitations and also by gel filtration. Milled wood lignins represent an excellent preparation for microbiological studies because they are believed to be representative of bulk lignin in plants,<sup>5</sup> and have an average molecular mass of 15,000 daltons (about 83 condensed coniferyl alcohols).<sup>57</sup> A method for obtaining essentially unmodified lignin in quantitative yield is the treatment of powdered wood with cellulase and hemicellulase.<sup>58</sup> This lignin preparation still retains considerable carbohydrate, and is therefore unsuitable for some experiments.

Artificial lignin can be biochemically prepared by a procedure which models the biosynthetic pathway. This synthetic lignin, generally referred to as DHP (for dehydrogenation polymerizate), is produced by polymerizing chemically prepared substituted cinnamyl

alcohols using horseradish peroxidase and hydrogen peroxide.<sup>59</sup> DHPs, which are insoluble in water and have a size greater than 1500 daltons, contain molecular linkages similar to that found in natural lignin.<sup>59</sup> Another synthetic substitute for lignin has been prepared by polymerizing guaiacol (2-methoxyphenol) with peroxidase and  $H_2O_2$ .<sup>60</sup> This polyguaiacol is missing some of the bonds present in natural lignin, but is a useful model for some of the bonds in lignin. DHP and polyguaiacol are usually prepared using  $^{14}C$ -precursors. By using appropriately labeled hydroxycinnamyl alcohols,  $^{14}C$  can be placed specifically in the aromatic rings, propanoid side chains, or methoxyl groups.<sup>59</sup> This makes it possible to determine if microorganisms have the ability to attack particular portions of the lignin macromolecule.  $^{14}C$ -DHP is the best model presently available for biodegradation research. However, there are several disadvantages associated with its use.<sup>20</sup> Production of labeled DHPs requires ability in organic chemistry and experience in its preparation. Considerable expense is involved, both in labor costs and the expense of labeled precursors. Finally, unlike natural lignin, DHP is not associated with intact plant cell walls, and therefore, even if polysaccharides are also present, the mixture does not mimic exactly natural plant materials.

Considerable amounts of industrially produced lignins are byproducts of the kraft and sulfite pulping processes. During pulping, lignin is solubilized by degradation and/or derivatization in order to separate it from the cellulose fibers. Kraft and sulfite lignins are highly modified and biodegradation research using these materials may

not be completely relevant to the study of natural lignin.<sup>61</sup> However, the environmental significance of these materials makes the study of how microorganisms degrade them important in its own right. Kraft lignin preparations, such as indulin, are frequently studied without purification, and usually contain a variety of non-lignin products.<sup>62</sup>

Finally, "enzymatically liberated lignin"<sup>63</sup> is produced by allowing microorganisms which do not degrade lignin to grow on wood. Brown rot fungi are usually used for this process, but the lignin which is produced is not identical to sound lignin.<sup>64</sup>

#### Lignin Biodegradation

A wide variety of techniques have been devised to measure lignin biodegradation. The earliest methods, which are still used, involve physical measurements such as weight loss or change in optical density. In the soil block procedure,<sup>65</sup> wood wafers are placed on the surface of soil or vermiculite. After inoculating with a microorganism and incubating under defined conditions, samples are removed for analysis. After scraping free any surface growth, samples are typically milled and analyzed for Klason lignin,<sup>53</sup> total reducing sugars,<sup>66</sup> or polysaccharides.<sup>65</sup> While this is a good procedure, it requires a great deal of time, often months. In addition, it suffers from the problem that degradation is not a uniform process throughout a solid material, but occurs in zones.<sup>67</sup> Finally, the Klason procedure often gives only a rough estimate of the true lignin content of some tissues.<sup>68</sup>

Microscopic techniques are also used to measure microbial degradation of plant tissues. Scanning electron microscopy has been used to show, for example, that woods from various trees are degraded differently by the same fungus.<sup>69</sup> Using the electron microscope, it is possible to distinguish lignin from other polymeric materials. Examining the amount of lignin remaining in wood after microbial degradation may be a useful screening procedure for finding microorganisms which remove a large amount of lignin but consume only small amounts of other cell wall components.

Analysis of the degradation of lignin preparations such as milled wood lignin and DHPs, is frequently measured by observing decreases in absorbance at 280 nm. After incubation with microorganisms, residual lignin must be reisolated from the culture media by centrifuging or filtering off insoluble materials and dissolving lignin from this residue using 1:1 dioxane-water,<sup>70</sup> acetyl bromide-acetic acid,<sup>71</sup> or dilute sodium hydroxide.<sup>71</sup> Strong adsorption of lignin to cell walls may prevent an accurate measurement. Except in the case of acetyl bromide, which dissolves the entire residue, it is difficult to establish that all the lignin has been extracted. Another difficulty encountered with this technique is the possibility that spectral changes of lignin are produced by microbial modifications of its chemical structure, which may decrease the absorbance at 280 nm but not decrease the amount of lignin present.<sup>71</sup>

Chlorine consumption by plant tissue appears to be a linear function of lignin content.<sup>71</sup> The chlorine number has therefore been used as a chemical measure of lignin degradation. However, chlorine

number differs for different lignin preparations,<sup>72</sup> and may also be changed by microorganisms which modify but do not completely degrade lignin. This assay is sensitive to the presence of phosphate, which is common in growth media.

Due to the molecular complexity of lignin, it has been extremely difficult to study the biochemical mechanisms used by microorganisms to degrade lignin. But spectroscopic and chemical procedures on the degraded lignin, and chromatographic analysis of products produced from lignin have been used to establish what processes are occurring. For example, these techniques were used to demonstrate that the degradative process is mainly oxidative, with the oxidative cleavage of side chains, and the formation of substantial amounts of carboxylic acids in the lignin polymer.<sup>73-75</sup>

#### Lignin Models

In order to understand specific reactions, studies have been carried out on the microbial degradation of lignin model compounds.<sup>76,77</sup> Model compounds are small organic molecules containing chemical structures which are thought to exist in lignin. They are used as a substitute for lignin in degradation studies to obtain a better understanding of the individual steps in lignin degradation. It is then assumed that processes used in the degradation of the lignin model compounds are also involved in the degradation of natural lignin. Representative examples of these compounds are shown in Figure 1-3. Some monomeric compounds such as veratric and syringic acids are, however, degraded by a number of microorganisms which cannot

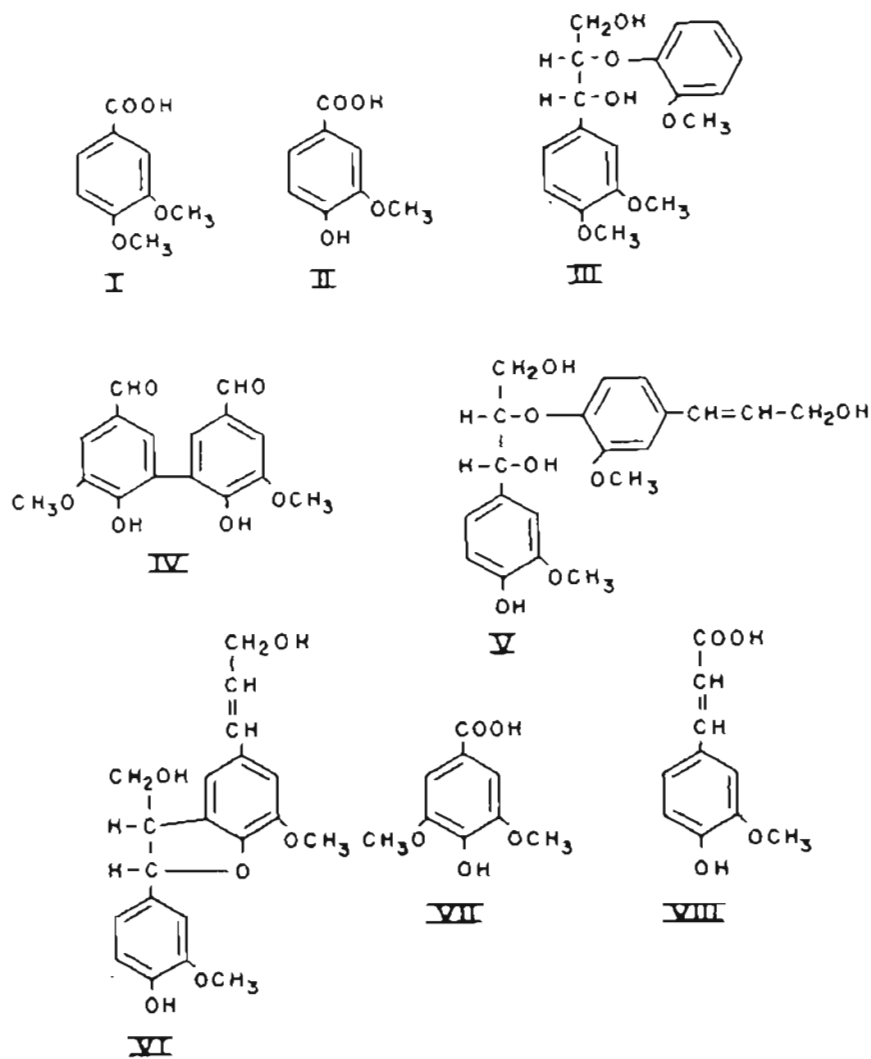


Fig. 1-3. Some representative lignin model compounds:  
 (I) veratric acid; (II) vanillic acid; (III) veratrylglycerol- $\beta$ -(o-methoxyphenyl)ether; (IV) dehydrodivanillin; (V) guaiacylglycerol- $\beta$ -coniferyl alcohol ether; (VI) dehydrodiconiferyl alcohol; (VII) syringic acid; (VIII) ferulic acid.

degrade lignin.<sup>78</sup> Specific pathways, which have been thoroughly studied, are used by a variety of microorganisms to oxidize simple aromatic molecules.<sup>79</sup> It has not been established that any of the enzymes involved in these pathways are used by lignin degrading organisms in the degradation of the lignin polymer, but they are likely to be involved in the degradation of products released from the polymer.

The greatest advances in the understanding of lignin biodegradation have come as a result of the use of radiolabeled lignin and model compounds.<sup>80</sup> Using  $^{14}\text{C}$  as a tag, it is possible to follow the fate of carbon atoms during the degradation process. This has allowed the unequivocal and highly sensitive determination of lignin degradation. By incubating a known quantity of  $^{14}\text{C}$  labeled lignin or model compound with microorganisms in a closed container, the amount of total degradation can be measured by collecting  $^{14}\text{CO}_2$ .<sup>59</sup> Using gas sampling ports, intermittent or continuous aeration of cultures allows the trapping of  $\text{CO}_2$  in aqueous  $\text{NaOH}$  or in an organic base. Quantitation of  $^{14}\text{CO}_2$  is accomplished with liquid scintillation counting. In one common method,  $\text{CO}_2$  is trapped directly in a toluene-based scintillation fluid containing ethanolamine.<sup>59</sup> Other radioactive gases such as  $^{14}\text{CH}_4$  can be trapped and counted separately,<sup>81</sup> or gas chromatography/gas-flow proportional counting techniques<sup>36</sup> can be used to quantitate all of the gases. Water soluble lignin breakdown products can be extracted and separated by chromatographic techniques before liquid scintillation counting.<sup>82</sup> Using high specific activity lignin, it is possible to measure lignin

degradation in liquid cultures within a few hours.

The pathway of lignin biosynthesis was largely determined by following the dilution of radioactivity during incorporation of labeled compounds into the lignin of growing plants.<sup>34</sup> Using the same techniques, lignin can be labeled for the purpose of studying its degradation. Precursors such as  $^{14}\text{C}$ -phenylalanine<sup>80</sup> or  $^{14}\text{C}$ -cinnamic acids<sup>83</sup> are used for preferential incorporation into lignin. Radiolabeled lignocellulose produced from these precursors must be used with caution because label may wind up in components other than lignin,<sup>84</sup> even after careful purification. A typical procedure for the preparation of  $^{14}\text{C}$ -lignin is the cut stem method,<sup>80</sup> in which a small limb or stalk is cut from a plant of interest and immersed in dilute buffer containing  $^{14}\text{C}$ -precursor. Normally 10-50  $\mu\text{Ci}$  of high specific activity precursor is used. When the buffer has been almost completely taken up by the limb, more buffer is added and the plant is allowed to continue metabolizing for another week. The sapwood is then removed (or the whole stalk in the case of grasses), dried, ground, and extracted to obtain a lignin preparation.

#### Lignin Degrading Microorganisms

Progress in the understanding of lignin biodegradation has been slow partly because the structure of lignin was not elucidated until the 1960s. Earlier it was not possible to completely rely on the assumption that model compounds represent substructures in lignin, or to relate the products of biodegradation to structures in the polymer. In addition, lignin is radically different from the other natural



polymers that had been previously studied. New techniques for isolation and analysis of products were required. The complexity of lignin not only hampered structural determination, it also made it difficult for those who did not have organic chemical expertise to synthesize model compounds or analyze degradation products.

The first review devoted to lignin biodegradation, written in 1936,<sup>85</sup> contains much valuable information. The polymer was recognized to be the most resistant component in plant materials, but studies showed that it was attacked fairly readily by basidiomycetes and decomposed by a group of basidiomycetes described as white rot fungi. Isolated lignin was not degraded by bacteria, but it was recognized that the harsh treatment used for isolation of the lignin might have altered its structure. Studies using pure fungal cultures were just beginning.

Reviews in 1944<sup>86</sup> and 1951<sup>87</sup> also stated that higher fungi degrade lignin in nature and further conclude that research was hampered by the lack of understanding of lignin structure. A good correlation was found between formation of colored products from tannic acid and other phenols, and lignin degradation among higher fungi. While no lignin-degrading enzymes had been found, the color reaction with phenols was an indication of phenol oxidase activity. The relationship between phenol-oxidizing enzymes and lignin biodegradation received considerable attention in an extensive review in 1954.<sup>88</sup>

Important developments in lignin biodegradation research occurred in the 1950s and 1960s.<sup>52</sup> The soft rot type of wood decay was described, and evidence was presented that bacteria may degrade

lignin. Differences between the effects of white rot and brown rot fungi on wood were described, as were some of the chemical changes in lignin produced by these fungi. Degradation was seen as primarily oxidative, and the role of phenol oxidases in lignin biodegradation was investigated. Model compounds were first used in biodegradation research during this period.

During the 1970s,<sup>89</sup> biodegradation assays based on <sup>14</sup>C-lignins were developed. These provided evidence that actinomycetes, soft rot and brown rot fungi produced partial decomposition of lignin to CO<sub>2</sub>. Culture conditions were defined and optimized for lignin degradation by white rot fungi, and the white rot fungus Phanerochaete chrysosporium was selected as a suitable experimental organism for detailed study.

By the early 1980s,<sup>90</sup> the physiology of lignin metabolism and preliminary genetics of P. chrysosporium were described. Lignin degradation negative mutants had been isolated. Pathways for model compound metabolism by this organism had been described in detail.<sup>76,77,90</sup> Although no direct evidence for the mechanism of lignin degradation was available, active oxygen species (such as hydroxyl radical) were postulated as the cause of degradation.

It is now clear that a wide variety of microorganisms are capable of transforming lignin, although white rot fungi produce the most complete degradation. In addition to fungi, some transformation of lignin may also be produced by bacteria.

Brown rot fungi are usually defined as wood rotting fungi that decompose and remove carbohydrate but leave a residue of modified lignin.<sup>47</sup> This lignin is typically dark brown and is almost equal in

weight to the lignin in the original wood. Analysis by numerous investigators has shown that brown rotted lignin has a decreased methoxyl content.<sup>64,68</sup> In addition to extensive demethylation, brown rot fungi introduce other chemical modifications into the lignin polymer. More carboxyl and conjugated carbonyl groups are present, and additional phenolic hydroxyl groups are introduced.<sup>68</sup> Brown rot fungi have been examined for their abilities to convert  $^{14}\text{C}$ -DHPs to  $^{14}\text{CO}_2$ . Gloeophyllum trabeum and Poria cocos have been shown to degrade  $^{14}\text{C}$ -methoxy-DHP faster than  $^{14}\text{C}$ -side chain-DHP which in turn degraded more rapidly than  $^{14}\text{C}$ -ring-DHP.<sup>59</sup> However, in one experiment involving G. trabeum and other brown rot fungi, no  $^{14}\text{C}$ -DHPs were converted to  $^{14}\text{CO}_2$ .<sup>92</sup> These results generally support the view that brown rot decay of lignin is largely oxidative and that demethylation is the major degradative reaction.<sup>20</sup>

Soft rot fungi attack moist wood, producing a characteristic softening of the surface of the wood<sup>47</sup> and extensive lignin degradation. Chaetomium globosum was found to remove up to 45% of the lignin from beechwood.<sup>93</sup> Studies using  $^{14}\text{C}$ -DHPs show that soft rot fungi are able to convert all structural elements of DHP to  $^{14}\text{CO}_2$ .<sup>94</sup> Although it seems likely that soft rot fungi have an important role as lignin degraders in nature, laboratory culture of these organisms is still at a primitive stage, and essentially nothing is known of the enzymes they use to degrade lignin.

Several soil fungi that are not readily classifiable into specific decay groups are also thought to degrade lignin. Fusarium species, for example, are capable of degrading a variety of lignin model

compounds<sup>95</sup> and chemically modifying DHPs.<sup>96</sup> Ligninolytic activity has also been reported in Aspergillus,<sup>97</sup> Penicillium,<sup>98</sup> and Alternaria<sup>99</sup> species. However, these studies need to be done more rigorously. They can only be considered preliminary results. Other research involving Aspergillus and other soil fungi failed to produce any  $^{14}\text{CO}_2$  from  $^{14}\text{C}$ -DHP over a period of two months.<sup>92</sup> Before a detailed understanding of the role of soil fungi in lignin degradation is realized, more research using specifically labeled  $^{14}\text{C}$ -DHPs will be required.

Numerous bacteria have also been reported to decompose lignin, but weaknesses in experimental methods have produced questionable results.<sup>20</sup> Many studies have used nonrepresentative or modified lignins, and measurement of residual lignin frequently used only absorbance at 280 nm. Results with  $^{14}\text{C}$ -substrates has proven more conclusive. Nocardia species could release  $^{14}\text{CO}_2$  from DHPs containing  $^{14}\text{C}$ -labeled methoxyl groups, side chains, or rings,<sup>78,100</sup> but these rates were very slow. Numerous Streptomyces species are capable of releasing  $^{14}\text{CO}_2$  from  $^{14}\text{C}$ -lignocelluloses.<sup>83</sup> Several of the strains which were examined released  $^{14}\text{CO}_2$  much better from ring labeled lignin than from side chain labeled lignin. A most interesting discovery is the finding that a Xanthomonas strain decomposed dioxane-lignin as a sole carbon and energy source.<sup>101</sup> After 15 days of growth, 77% of the lignin was degraded. However it is not clear whether the organism was growing on lignin or attached carbohydrate. At the present time the evidence for the degradation of pure high-molecular-mass lignin by eubacteria is not

sufficient to convince all researchers of its occurrence.<sup>102</sup>

Actinomycetes (filamentous bacteria) such as Streptomyces and Nocardia produce limited degradation of intact wood,<sup>103</sup> and appear to produce a slow partial degradation of isolated lignocellulose,<sup>104</sup> but not DHP.

White rot fungi are a group of basidiomycetes known for their ability to extensively decompose all the important structural components of wood, including cellulose and lignin.<sup>87</sup> Under proper environmental conditions, lignin is converted completely to CO<sub>2</sub> and H<sub>2</sub>O. The name "white rot" comes from the observation that wood being decayed by these organisms turns white as the lignin is removed. These organisms are the most active lignin degrading microorganisms. The physiology and biochemistry of several white rot fungi, especially Phanerochaete chrysosporium, is becoming well understood, and this organism has become the model for most current studies of lignin degradation.

While all white rot fungi degrade both lignin and wood polysaccharides, the rate at which the various wood components are degraded is dependent on the species of fungus. For example, Pycnoporus cinnabarinus can degrade as much as 12.5% of the lignin in pinewood blocks without the loss of cellulose or mannan if the wood is supplemented with malt extract.<sup>65</sup> Other white rot fungi, such as Ganoderma applanatum, degrade the carbohydrates in wood more rapidly than lignin.<sup>105</sup>

Elemental and chemical analyses of white rotted lignins provide evidence that white rot fungi degrade lignin by oxidative processes.

White-rotted lignin contains less carbon and hydrogen, and more oxygen than sound lignin.<sup>73</sup> It also shows a decrease in methoxyl groups but an increase in carbonyl and carboxyl groups.<sup>73,74</sup> White rotted lignin is still polymeric, and has a molecular mass similar to sound lignin.<sup>106</sup> The substitution of oxygen for carbon and hydrogen within the structure produces a unit molecular formula with approximately the same molecular mass as the original lignin.<sup>73,74</sup>

Quantitative chemical analysis of degraded lignins<sup>73,74</sup>, and structural analysis of lignin fragments released from decayed wood<sup>75</sup> indicate that aromatic ring oxidation and cleavage may occur within the polymer. This conclusion has been substantiated by research with specifically labeled <sup>14</sup>C-DHPs. Some white rot fungi are capable of converting <sup>14</sup>C-ring-DHP to <sup>14</sup>CO<sub>2</sub> faster than <sup>14</sup>C-side chain-DHP.<sup>107</sup> This further supports the idea that cleavage of aromatic rings occurs while they are still bound in the polymer. However, other research with Coriolus versicolor and Phanerochaete chrysosporium using <sup>14</sup>C-DHPs found the following order for the rate of <sup>14</sup>CO<sub>2</sub> production: <sup>14</sup>C-methoxyl > <sup>14</sup>C-side chain > <sup>14</sup>C-ring.<sup>59</sup>

#### Lignin Model Compound Degradation

The study of lignin model compound degradation offers the most straightforward procedure for understanding the steps in lignin degradation. However, model compounds must be used with caution, since many examples are known in which specific microorganisms can completely degrade model compounds but are incapable of degrading lignin. A rule of thumb for this area of research is: lignin-degrading organisms are

able to degrade lignin models, but model degraders are not necessarily degraders of lignin.<sup>20</sup> In the case of P. chrysosporium, vanillic acid<sup>108</sup> and other simple aromatic compounds are not the most suitable model compounds because they are also degraded at times when ligninolytic activity is suppressed. However, they do have value in studying how small fragments of lignin may be ultimately converted to CO<sub>2</sub>. The dimeric compounds 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -guaiacyl ether<sup>76</sup> and 1-(3',4'-diethoxyphenyl)-1,3-dihydroxy-2-(4"-methoxyphenyl)-propane<sup>77</sup> are excellent model compounds. They resemble structures present in lignin and are degraded only under conditions in which lignin is also degraded.<sup>76,77</sup> Analysis of degradation products (Figure 1-4) indicates that  $\beta$ -ether cleavage and cleavage between the alpha- and beta-carbons of the alkyl sidechain occur. The benzaldehydes which are produced by these reactions are rapidly reduced by the fungus to benzyl alcohols.

Lignin, containing many aromatic groups, is highly reduced and releases large amounts of energy when completely oxidized. Therefore, the oxidative degradation of lignin by white rot fungi should provide sufficient energy for growth and further lignin degradation. However, this does not appear to be the case. Lignin decomposition by white rot fungi is strongly affected by the presence or absence of carbohydrate in the growth medium. Early research using Brauns' native lignin suggested that certain white rot fungi could use lignin as the sole source of carbon and energy.<sup>109,110</sup> But it is difficult to interpret these studies because Brauns' lignin is probably not representative of the bulk lignin in wood, and may contain carbohydrate contaminants.

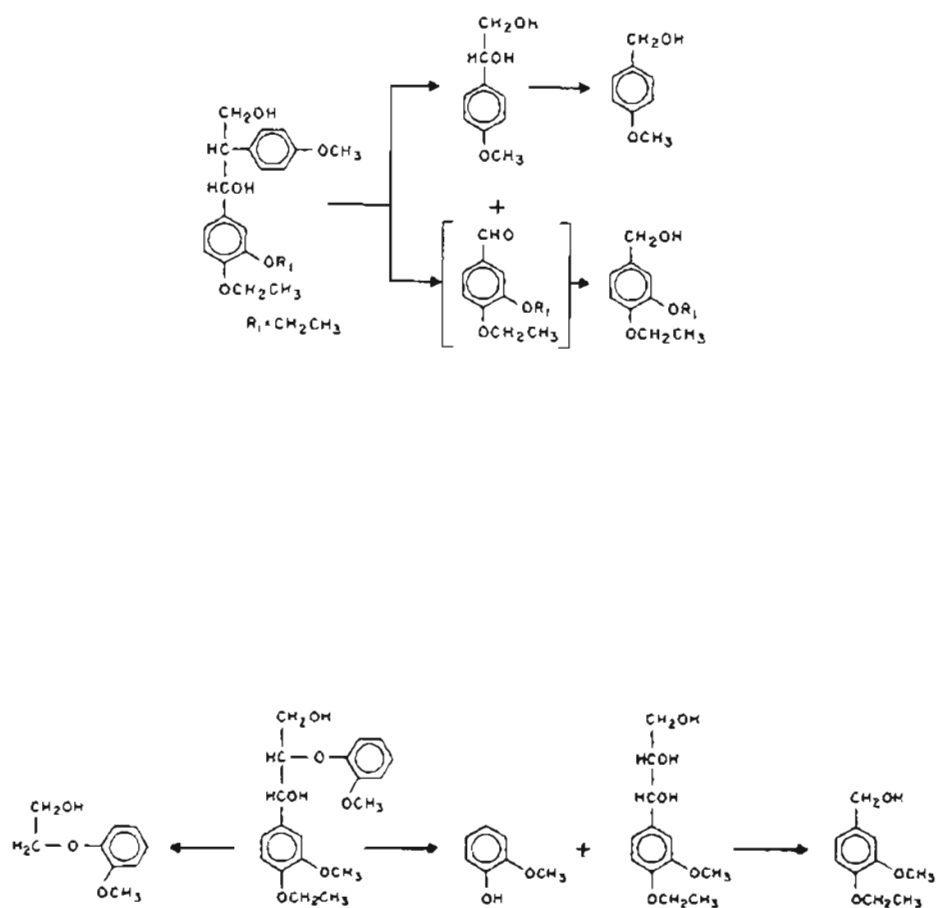


Fig. 1-4. Metabolic schemes for the fungal degradation of lignin model compounds. Top scheme: diarylpropane, 1-(3',4'-diethoxyphenyl)-1,3-dihydroxy-2-(4''-methoxyphenyl)-propane (From Ref. 77). Bottom scheme:  $\beta$ -ether dimer, 4-ethoxy-3-methoxyphenylglycerol- $\beta$ -guaiacyl ether (From Ref. 76).



Phanerochaete chrysosporium and other well-studied white rot fungi do not degrade  $^{14}\text{C}$ -DHP to  $\text{CO}_2$  in the absence of a readily utilizable growth substrate such as cellulose, glucose, or glycerol.<sup>111</sup>

In addition to the requirement for an energy source, other environmental conditions must be met before P. chrysosporium will degrade lignin. At the present time, the physiological controls of lignin degradation have been thoroughly studied in this organism alone. P. chrysosporium only degrades lignin during secondary metabolism, that is, when primary growth ceases because of the depletion of certain nutrients. Limiting nitrogen, carbon, or sulfur leads to secondary metabolism in P. chrysosporium.<sup>112</sup> Most research on lignin degradation is performed with cultures limited in nitrogen. Lignin degradation stops when primary growth is restored by adding excess carbon or nitrogen to cultures limited for that nutrient.<sup>112</sup> Perhaps because nitrogen is limiting in wood,<sup>113</sup> cultures depleted in nitrogen are capable of sustained degradation of lignin. However, cultures limited for nutrient carbon degrade lignin only transiently, until they deplete their own reserves.<sup>112</sup>

The concentrations of intracellular glutamate and cyclic adenosine monophosphate (cAMP) have been studied with respect to the transition between primary and secondary metabolism. The addition of ammonium or glutamate to nitrogen starved cultures raises the concentration of intracellular glutamate,<sup>114</sup> sharply decreases the concentration of cAMP,<sup>115</sup> restores primary growth,<sup>114</sup> and stops lignin degradation.<sup>114</sup>

During secondary metabolism, P. chrysosporium synthesizes the

secondary metabolite veratryl alcohol (3,4-dimethoxybenzyl alcohol).<sup>116</sup> The entire pathway of veratryl alcohol biosynthesis, starting from phenylalanine, has been described.<sup>117</sup> The ligninolytic system appears to be required for the final step in veratryl alcohol biosynthesis.<sup>117</sup>

Molecular oxygen exerts a strong influence on lignin degradation by P. chrysosporium.<sup>118</sup> At a 5% or lower concentration of O<sub>2</sub> in N<sub>2</sub>, there is no conversion of <sup>14</sup>C-lignin to <sup>14</sup>CO<sub>2</sub>. Degradation is better at ambient concentrations of oxygen (21%), and increases two- to threefold under 100% O<sub>2</sub>. Increasing oxygen not only increases the rate at which the ligninolytic system operates, it also increases the overall capacity of the system.<sup>119</sup> Elevating the oxygen concentration also increases the rate of degradation of lignin model compounds,<sup>120</sup> and increases the level of veratryl alcohol in cultures.<sup>117</sup>

Lignin degradation by P. chrysosporium is very pH dependent. The optimum for conversion of <sup>14</sup>C-DHP or <sup>14</sup>C-lignin to <sup>14</sup>CO<sub>2</sub> is between pH 4.0 and 4.5, while optimum pH for growth is somewhat higher.<sup>118</sup>

The ligninolytic system of P. chrysosporium is synthesized in the absence of lignin as part of secondary metabolism. Thus lignin degradation cannot be induced by the addition of lignin to primary cultures.<sup>121</sup> The addition of small amounts of DHP (50 mg/l) to secondary cultures does not increase ligninolytic activity,<sup>121</sup> but the addition of large amounts of lignin (1000-2000 mg/l) produces an increase in activity over a 24 hour period.<sup>122</sup> The time requirement and the large amount of lignin required suggests that a lignin

degradation product is responsible for the increase.

White rot fungi characteristically produce extracellular phenol oxidases.<sup>123</sup> Nonligninolytic fungi usually do not have these enzymes.<sup>52</sup> Traditionally, three distinct types of enzymes have been considered phenol oxidases.<sup>47</sup> Tyrosinases use oxygen to monohydroxylate phenols, yielding ortho-diphenols or ortho-quinones. They can also oxidize catechols to o-quinones. Laccase catalyses the oxidation of a large variety of phenolic compounds by abstraction of an electron and a hydrogen ion from a hydroxyl group forming an aryloxy free radical. The radicals which are produced undergo disproportionation or polymerization. Laccase uses oxygen as the co-substrate, which is ultimately reduced to water by the electrons and hydrogen ions removed from the phenols. Peroxidase performs the same reaction as laccase, but uses hydrogen peroxide rather than oxygen as the oxidizing co-substrate.

The correlation between phenol oxidase and ligninolytic activity is supported by genetic studies.<sup>124,125</sup> Mutants of P. chrysosporium which have lost phenol oxidase activity have also lost the ability to degrade lignin. These mutants were also unable to degrade lignin model compounds and did not produce veratryl alcohol. Revertants which have regained phenol oxidase activity have also regained ligninolytic activity and other aspects of secondary metabolism.

Numerous attempts have been made to degrade lignin using purified laccase and peroxidase. Some modification of lignin may be produced by laccase,<sup>52</sup> but the primary effect is further polymerization.<sup>126,127</sup> Horseradish peroxidase/H<sub>2</sub>O<sub>2</sub> has been found to release a volatile

$^{14}\text{C}$ -labeled product (probably methanol) from  $^{14}\text{C}$ -methoxyl-polyguaiacol,<sup>128</sup> but this demethoxylation is very limited; under 6%. There is no sound experimental evidence that any phenol oxidase alone can mediate extensive lignin degradation. Other roles for phenol oxidases produced by white rot fungi have been suggested. 1) Phenol oxidases may act to detoxify low-molecular-mass phenols released during lignin degradation.<sup>126</sup> 2) Phenol oxidases may produce a specific chemical transformation of lignin required for further degradation by other enzymes.<sup>129</sup> 3) Phenol oxidases may be involved in regulating the production of lignin degrading enzymes<sup>124</sup> or other required substances, such as  $\text{H}_2\text{O}_2$ .<sup>130</sup>

#### Summary of Research

When we began the research with Phanerochaete chrysosporium described in this thesis, the physiology of lignin degradation in P. chrysosporium had been well studied. As described above, most of this physiological research was based on the conversion of  $^{14}\text{C}$ -lignin (or DHP) to  $^{14}\text{CO}_2$ . While this is the only entirely accurate measure of lignin degradation, it is an expensive and somewhat tedious assay. Because  $^{14}\text{CO}_2$  is produced, all experiments must be performed in closed containers, which may lead to uncharacterized changes in the culture atmosphere as the fungus respire. To obtain a measurable quantity of  $^{14}\text{CO}_2$ , in six hours, very small quantities (e.g. 50  $\mu\text{g}$ ) of high specific activity DHP (e.g.  $10^6$  cpm/mg) are required. In many experiments, measurements are only taken every day or two. Thus rapid changes in ligninolytic activity are hard to follow. This

measure of ligninolytic activity is useful for studying mutants of P. chrysosporium and for investigating the ligninolytic activity of other organisms. However, this assay is not suitable for selecting mutants or new lignin degrading organisms from vast numbers of colonies.

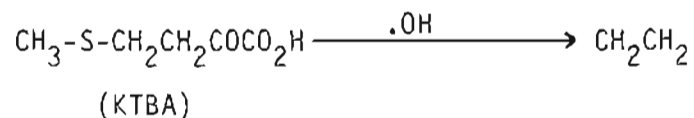
In an effort to find a fast, simple, and quantitative substitution for the traditional ligninolytic assay, we examined three polymeric dyes (Poly B-411, Poly R-481, and Poly Y-606) as alternative substrates. This research is presented in Chapter 2 (and Ref. 131). The Phenol oxidase indicator o-anisidine had been used previously in agar plate assays to detect phenol oxidase mutants of P. chrysosporium, but this is not a quantitative assay as used. Three properties of the polymeric dyes allows their decolorization to be quantified: a) Changes in spectra could be used to distinguish adsorption to fungal mycelium from enzymatic decolorization. b) The absorbance changes produced by oxidation were not reversible. c) These dyes were not easily reduced. High concentrations of dithionite were required to decolorize the dyes, and subsequent reoxidation of the dyes by  $O_2$  occurred rapidly. The colored products produced from the oxidation of o-anisidine or guaiacol are unstable, and are rapidly decolorized by mild reducing agents. Unlike o-anisidine and guaiacol, the large size of the polymeric dyes (40,000 d) insures that they will not be oxidized by intracellular phenol oxidases. Because the initial steps in lignin degradation occur outside the cell, it seemed possible that the same enzymes might be responsible for oxidizing both lignin and polymeric dyes.

Like lignin degradation, the decolorization of these dyes by P.

chryso sporium occurred during secondary metabolism, was suppressed by nutrient nitrogen, and was strongly dependent on oxygen concentration. A variety of inhibitors of lignin degradation, including thiourea, azide, and 4'-O-methylisoeugenol, also inhibited dye decolorization. A pleiotropic mutant of P. chryso sporium, 104-2, lacking phenol oxidase and ligninolytic activity was also not able to decolorize the polymeric dyes, whereas a phenotypic revertant strain, 424-2, regained this capacity. All of these results suggested that the ligninolytic degradation activity of the fungus was responsible for the decolorization of these dyes. The readily visible decolorization of Poly B and Poly R in plates allows them to be used to select ligninolytic mutants of P. chryso sporium and find other microorganisms that may degrade lignin.

By this point in our research, several laboratories were proposing that lignin degradation by white rot fungi was directly caused by active oxygen species such as singlet oxygen,<sup>132</sup> superoxide radical,<sup>133,134</sup> or hydroxyl radical<sup>135,136</sup> reported to be produced by these organisms. Singlet oxygen was quickly eliminated as the cause of lignin degradation,<sup>137,138</sup> and the evidence pointing to the involvement of superoxide in this process was slight.<sup>102</sup> Hydroxyl radical (.OH), however, was considered a likely effector of lignin degradation. Chemically generated .OH could degrade lignin,<sup>139</sup> and ligninolytic cultures of P. chryso sporium were capable of generating ethylene from 2-keto-4-thiomethylbutyric acid (KTBA)<sup>135,136</sup> or methional.<sup>135</sup> This reaction had been considered strong evidence that hydroxyl radical is being produced,<sup>140</sup> although other organic

radicals are also capable of the same reaction.<sup>141</sup>



Hydroxyl radical could be generated in vitro by exposing water to gamma radiation<sup>142</sup> or by exposing hydrogen peroxide solutions to ultraviolet radiation.<sup>143</sup> We observed that oxidation of the polymeric dyes by hydroxyl radicals produced complete bleaching rather than the selective changes in the spectra produced by ligninolytic cultures of P. chrysosporium. Also, as described in Chapter 2, dye decolorization by the fungus was not inhibited by high concentrations of mannitol, a well-known scavenger of hydroxyl radical.<sup>144</sup> On the other hand, horseradish peroxidase/H<sub>2</sub>O<sub>2</sub> produced the same spectral changes in solutions of Poly B as produced by the fungus. Researchers in our lab also discovered that hydroxyl radical and P. chrysosporium produced different reaction products with several lignin model compounds. We hypothesized that an enzyme(s) produced by P. chrysosporium might be capable of oxidizing all three polymeric dyes and directly oxidizing KTBA to ethylene. It was well known that P. chrysosporium had phenol oxidase activity, but no one had found an extracellular phenol oxidase from this fungus. Because it was also known that white rot fungi produce hydrogen peroxide, we decided to test the extracellular medium from P. chrysosporium for peroxidase activity.

At this point I would like to acknowledge that M. Morgan and M. B. Mayfield contributed significantly to the research described in Chapter

3. Unlike a previous attempt at detecting peroxidase activity from this fungus,<sup>134</sup> we used glucose/glucose oxidase to generate low continuous levels of  $H_2O_2$ . The results we obtained are described in Chapter 3 (and Ref. 145). The medium was capable of generating ethylene from KTBA and oxidizing a variety of lignin model compounds including the diarylpropane 1-(4'-ethoxy-3'-methoxyphenyl)-1,3-dihydroxy-2-(4''-methoxyphenyl)propane, a  $\beta$ -ether dimer 1-(4'-ethoxy-3'-methoxyphenyl)-glycerol- $\beta$ -guaiacyl ether, and an olefin 1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propene. The products found were equivalent to the metabolic products previously isolated from intact ligninolytic cultures. The medium was also capable of partially degrading DHP. Media from primary growth cultures or a nonligninolytic mutant had no activity. Inactivation of all activity was produced by heating, incubation with pepsin, a proteolytic enzyme, and by the addition of inhibitors. This was strong evidence that one or more enzymes were involved.

In order to determine how many peroxidases were present in the medium of ligninolytic cultures of *P. chrysosporium*, a partial purification was performed with blue agarose column chromatography. This work is presented in Chapter 4 (and Ref. 146). The research described in Chapter 4 was very much a collaborative effort. Chromatography, gel filtration,<sup>180</sup> incorporation experiments, and the protocol for acetone precipitation were carried out by Dr. M. Kuwahara and M. Morgan. Two peroxidases were found. The first fraction eluting from the column generated ethylene from KTBA in the presence of veratryl alcohol, and catalyzed the  $\alpha,\beta$  cleavage of the diarylpropane model



compound. The second enzyme, which bound to blue agarose, required manganese(II) for activity, and was stimulated by lactate and protein. This enzyme catalyzed the oxidation of a variety of dyes, including Poly R, and also decarboxylated vanillic acid.

Chapter 5 (and Ref. 130) describes the purification and characterization of the Mn(II)-dependent peroxidase. The extracellular medium was separated from the fungus by filtration and protein was precipitated with acetone at  $-10^{\circ}\text{C}$ . After dissolving the soluble proteins in buffer, the Mn(II)-dependent peroxidase was purified by DEAE-Sepharose ion-exchange chromatography (as first performed in Ref. 147), Blue Agarose chromatography, and gel filtration on Sephadex G-100. The peroxidase has an Mr of 46,000 and contained one molecule of heme as iron protoporphyrin IX. The spectra of the native enzyme and  $\text{CN}^-$ ,  $\text{N}_3^-$ , and  $\text{H}_2\text{O}_2$  complexes resembled those of horseradish peroxidase. This peroxidase activity was dependent on Mn(II), with maximal activity attained above 100  $\mu\text{M}$ . The enzyme was also stimulated to varying degrees by  $\alpha$ -hydroxy acids (e.g. malic, lactic) and protein (e.g. gelatin, albumin). The peroxidase was capable of oxidizing a wide variety of dyes, including Poly B and Poly R. In the absence of  $\text{H}_2\text{O}_2$  it was also capable of oxidizing NADH. Several of the substrates were oxidized by this enzyme at considerably faster rates than those catalyzed by horseradish peroxidase. The enzyme rapidly oxidized Mn(II) to Mn(III); the latter was detected by the characteristic absorption spectrum of its pyrophosphate complex. Inhibition of the oxidation of the substrate diammonium 2,2-azino-bis(3-ethyl-6-benzothiazolinesulfonate) (ABTS) by Na

pyrophosphate suggested that Mn(III) plays a role in the enzyme mechanism.

The degree of stimulation of enzyme activity by lactate and protein varied widely with the substrate being oxidized. This effect would not be easily explained by assuming that these cofactors were directly activating the enzyme, which would probably increase the oxidation of all substrates equally. In addition, the high levels of Mn(II), lactate, and protein required to produce optimal activity (e.g. 60 mM lactate), and the shapes of the activity vs concentration curves also suggested that these cofactors were not behaving as allosteric effectors. Because Mn(III) is a powerful oxidant,<sup>148</sup> it seemed possible that the chief function of the fungal peroxidase might be to oxidize Mn(II) to Mn(III), which in turn oxidised the other substrates present. The lactate and protein might then stimulate oxidation by interacting with the Mn(III) or the other reactant rather than the enzyme. Evidence supporting this hypothesis is presented in Chapter 6 wherein we further examine the properties of the fungal peroxidase and investigate the oxidation of dyes and NADH by Mn(III).

The oxidation of Mn(II) by the fungal peroxidase was an efficient process. At least 90% of the oxidizing capacity of the hydrogen peroxide was available to oxidize Mn(II); i.e. 72  $\mu\text{M}$  Mn(III)-pyrophosphate was produced when Mn(II) was oxidized in the presence of 40  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . This oxidation was not inhibited by superoxide dismutase. This suggests that  $\text{O}_2^-$  is not involved in the oxidation mechanism. Among the compounds tested [Mn(II), Co(II), Ni(II), NADH, and guaiacol], only Mn(II) was found to reduce the

oxidized form of the enzyme at equimolar concentrations (0.85  $\mu\text{M}$ ). At 850  $\mu\text{M}$ , Co(II) was oxidized at a rate 50-fold slower than Mn(II), and Ni(II) was not oxidized. The  $K_m$  values for manganese and  $\text{H}_2\text{O}_2$  are 80  $\mu\text{M}$  and 140  $\mu\text{M}$  respectively. Enzyme pH profiles based on the formation of Mn(III)-lactate or -pyrophosphate complexes produce an optimum between pH 4 and 5. Manganese(III)-lactate was capable of oxidizing NADH and a variety of dyes, including Poly B and Poly R. The relative rates of oxidation of NADH and the dyes phenol red, pinacyanol chloride and ABTS by Mn(III)-lactate were about the same as observed for enzymatic oxidation. With most of the other dyes, there was under a ten-fold difference between the relative rates of oxidation by the enzyme and Mn(III)-lactate. As with enzymatic oxidations, lactate and protein were capable of stimulating substrate oxidation by Mn(III)-lactate under some conditions. This provides further evidence that enzymatic oxidations are mediated by higher oxidation states of manganese. The enzyme could generate insoluble manganese oxides, and was capable of oxidizing Poly B across an ultrafiltration membrane. As discussed in Chapter 6, these activities may allow the fungus to oxidize components within the wood matrix.

It had previously been reported that cultures of *P. chrysosporium* grown without manganese were capable of degrading lignin.<sup>112</sup> Because it is likely that the fungal peroxidase requires Mn(II) for any significant activity, we wanted to confirm that Mn(II) was not required for lignin degradation. If this were true, it would suggest that the manganese peroxidase was not required for ligninolytic activity, at least in liquid culture. We also wished to determine if peroxidase

levels and rate of dye decolorization were affected by manganese concentration. This research is presented in Chapter 7.

The production of Mn-peroxidase is manganese-dependent. No peroxidase is produced by cultures grown in the absence of Mn. In cultures grown without manganese, the addition of Mn to the medium leads to the rapid production of the enzyme; with 100  $\mu$ M Mn producing 4-fold higher levels of activity than 10  $\mu$ M Mn. Both of the protein synthesis inhibitors cycloheximide and 5-fluorouracil inhibit enzyme production when added simultaneously with Mn. The rapid initial rise of peroxidase activity in cultures grown with Mn corresponds to a rapid increase in the rate of decolorization of the dye Poly R. In cultures grown without Mn, dye decolorization activity develops much more slowly. This suggests that initial decolorization activity is caused by the Mn-peroxidase, but other enzymes are produced which can also decolorize Poly R. Using small amounts of high specific activity lignin we find that ligninolytic activity is the same in liquid cultures of *P. chrysosporium* grown with and without Mn. Therefore, the Mn-peroxidase is not required for lignin degradation under these conditions. However, during the fungal degradation of wood this enzyme may be responsible for the detoxification of soluble phenolic compounds and may also play a role in lignin degradation.

In summary, my research started with the development of a useful dye decolorization assay and was completed with the discovery, purification, and characterization of a novel manganese oxidizing peroxidase.

## CHAPTER 2

## Decolorization of Several Polymeric Dyes by the Lignin-Degrading

Basidiomycete Phanerochaete chrysosporium

Jeffrey K. Glenn and Michael H. Gold

## Introduction

A variety of  $^{14}\text{C}$ -radiolabeled and unlabeled substrates have been used to measure ligninolytic activity in Phanerochaete chrysosporium and other white rot fungi. Radiolabeled substrates such as synthetic  $^{14}\text{C}$ -dehydropolymerizates,<sup>118</sup>  $^{14}\text{C}$ -natural plant lignins,<sup>149</sup> C-polyguaiacol,<sup>60</sup> and  $^{14}\text{C}$ -dimeric<sup>120,125</sup> and -monomeric<sup>117,125</sup> model compounds have been utilized in studies of the physiology and genetics of lignin degradation. Assays with these substrates, however, are relatively slow and measure, at least in the case of the polymers, the result of a relatively large number of reactions. In addition, these substrates are not commercially available, and their synthesis requires considerable labor and expertise. Unlabeled dimeric compounds have also been used to elucidate the individual reactions involved in lignin degradation. However, assays with these substrates, although chemically elegant, are cumbersome. They minimally require gas chromatographic analysis and are not suitable for routine assays. Lignin degradation can be conveniently divided into depolymerization reactions, which are extracellular, and the further metabolism of fragments released from the polymer, which is likely to be intracellular. Since the localization of the metabolism of dimeric compounds has not been rigorously examined in previous studies, these

compounds may not in all cases be suitable models for lignin depolymerization. The production of ethylene from  $\alpha$ -keto- $\gamma$ -methylthiobutyric acid, a measure of fungal  $\cdot\text{OH}$  radical production,<sup>135,136</sup> has been proposed as a measure of ligninolytic activity.<sup>135</sup> However, only indirect evidence<sup>136</sup> exists suggesting that fungal ligninolytic activity actually catalyzes this reaction. Finally, none of the assays described above readily lends itself to simple selection processes for the isolation of ligninolytic mutants.

In this report, we describe the decolorization of three polymeric dyes by *P. chrysosporium*. These substrates are readily soluble and stable in the absence of the fungus, are inexpensive, are obtained commercially in high purity, and possess high extinction coefficients and low toxicity toward *P. chrysosporium*. The evidence presented here indicates that these dyes serve as substrates of the fungal lignin degradation system (LDS) and also that they have value for the determination of the onset of secondary metabolism in this organism. Further, these dyes can be used in simple, rapid, and quantitative spectrophotometric assays, which can easily be modified for the selection of lignin degradation mutants of this organism.

#### Materials and Methods

Culture methods. Cultures of *P. chrysosporium* wild-type (ME-446) and mutant strains were maintained on slants as previously described.<sup>150</sup> Erlenmeyer flasks (250 ml) containing 25 ml of medium were inoculated with  $\sim 5 \times 10^7$  conidia and incubated at 37°C in high

humidity to reduce evaporation. The medium, containing 2% glucose and 1.2 mM  $(\text{NH}_4)_2$  tartrate and buffered with 20 mM sodium 2,2-dimethylsuccinate, pH 4.5, was as previously described.<sup>118</sup> Cultures, which were 6 days old and described as  $\text{O}_2$  grown, were allowed to grow for 3 days in air and subsequently were fitted with ports and periodically purged with  $\text{O}_2$  as described previously.<sup>59,120</sup> For the time dependence experiments, flasks were purged with  $\text{O}_2$  after inoculation and at 2-day intervals thereafter.

For assays on petri dishes, the same medium was used except 1.5% agar and either 1% glucose for the minimal plates or 4% sorbose for the induction of colonies<sup>150</sup> were added. The plates were incubated at 28°C.

Decolorization assay. Dye was added to the liquid medium as an aqueous solution to a final concentration of 0.02% with gentle swirling. Directly after its addition and at the indicated intervals, 0.1 ml of the extracellular culture medium was removed and diluted 10-fold with water. The wavelengths in nanometers used for the absorbance ratios of Poly B-411 (Poly B), Poly R-481 (Poly R), and Poly Y-606 (Poly Y) were  $A_{593}/A_{483}$ ,  $A_{513}/A_{362}$ , and  $A_{430}/A_{392}$  respectively. Visible absorption spectra and routine absorbance measurements of the dyes were determined on a Cary 15 spectrophotometer.

Chemicals. Poly B, Poly R, and Poly Y, synthesized as previously described,<sup>151</sup> and thiourea were obtained from Sigma Chemical Co. 4'-O-Methylisoeugenol was synthesized as reported earlier.<sup>76</sup> All other chemicals were reagent grade.

## Results

The chemical structure and visible spectrum of each of the three polymeric dyes used in this study are shown in Fig. 2-1. Because fungal adsorption, as well as fungal transformation, reduces the intensity of the dyes in solution, it was necessary to measure soluble dye absorbance at two wavelengths. The determination of dye (Poly R) adsorption to the mycelium is shown in Fig. 2-2. In this experiment, cultures were inactivated with sodium azide before the addition of Poly R. The absorbance at both wavelengths decreased significantly, whereas the absorbance ratio ( $A_{513}/A_{362}$ ) remained almost constant (Fig. 2-2). This was predictable because adsorption leads to a proportional decrease in absorbance at all wavelengths, resulting in a minimal change in absorbance ratios. Similar results were obtained with Poly B and Poly Y. The two wavelengths indicated were chosen to produce the greatest change in the absorbance ratio as the dyes were degraded. As the change in the absorbance ratio during dye degradation indicates, decolorization did not occur equally at all wavelengths. This led to a change in color from blue to light brown for Poly B, from red to orange for Poly R, and from yellow to light green-yellow for Poly Y.

Since the LDS in the fungus is expressed as a secondary metabolic process,<sup>118,121,125</sup> the exhaustion of certain nutrients from the medium leads to a cessation of growth and derepression of the LDS.<sup>118,121</sup> The effect of nutrient nitrogen concentration on the fungal decolorization of the polymeric dyes is shown in Fig. 2-3. Cells grown in high-N [12mM  $(\text{NH}_4)_2$  tartrate] medium were less than 5% as effective as cells grown in low-N [1.2 mM  $(\text{NH}_4)_2$  tartrate] medium



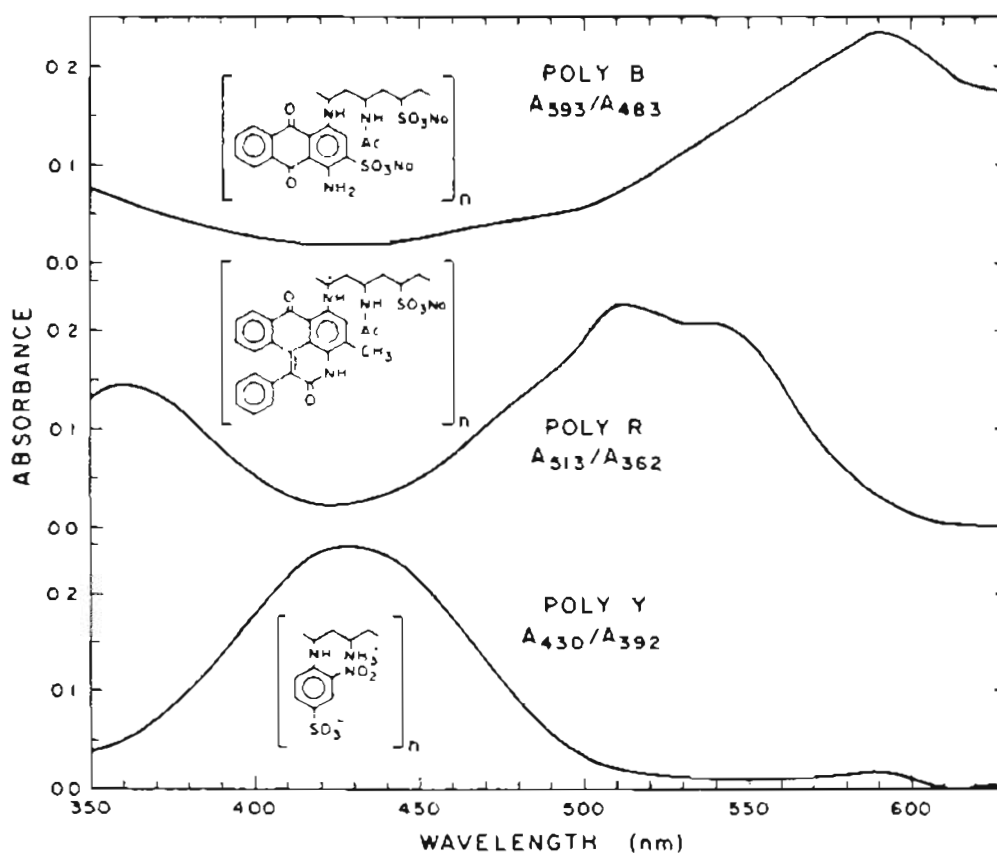


Fig. 2-1. Chemical structures and visible spectra of the polymeric dyes Poly B, Poly R, and Poly Y. The spectra of the dyes (0.002%) in sodium 2,2-dimethylsuccinate, pH 4.5, were taken with a Cary 15 spectrophotometer.

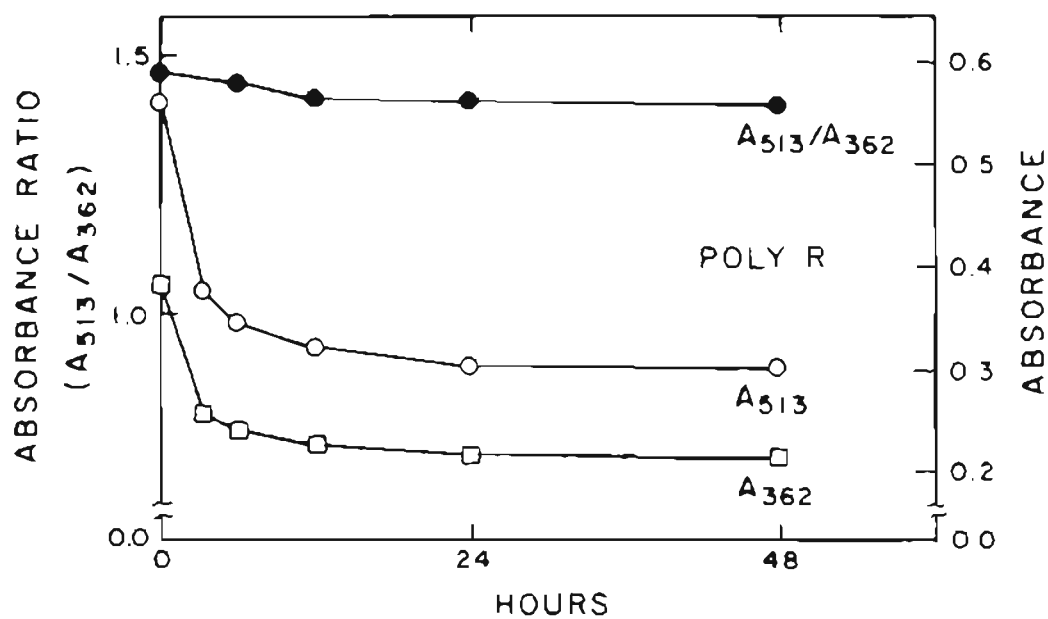


Fig. 2-2.  $A_{513}$  (○),  $A_{362}$  (□), and  $A_{513}/A_{362}$  ratio (●) of Poly R in culture filtrates. Cultures were 100%  $O_2$  grown, after which they were inactivated with  $N_3^-$ , and the dye was added as described in the text. Aliquots (0.1 ml) of medium were removed at the times indicated and diluted 10-fold with water.

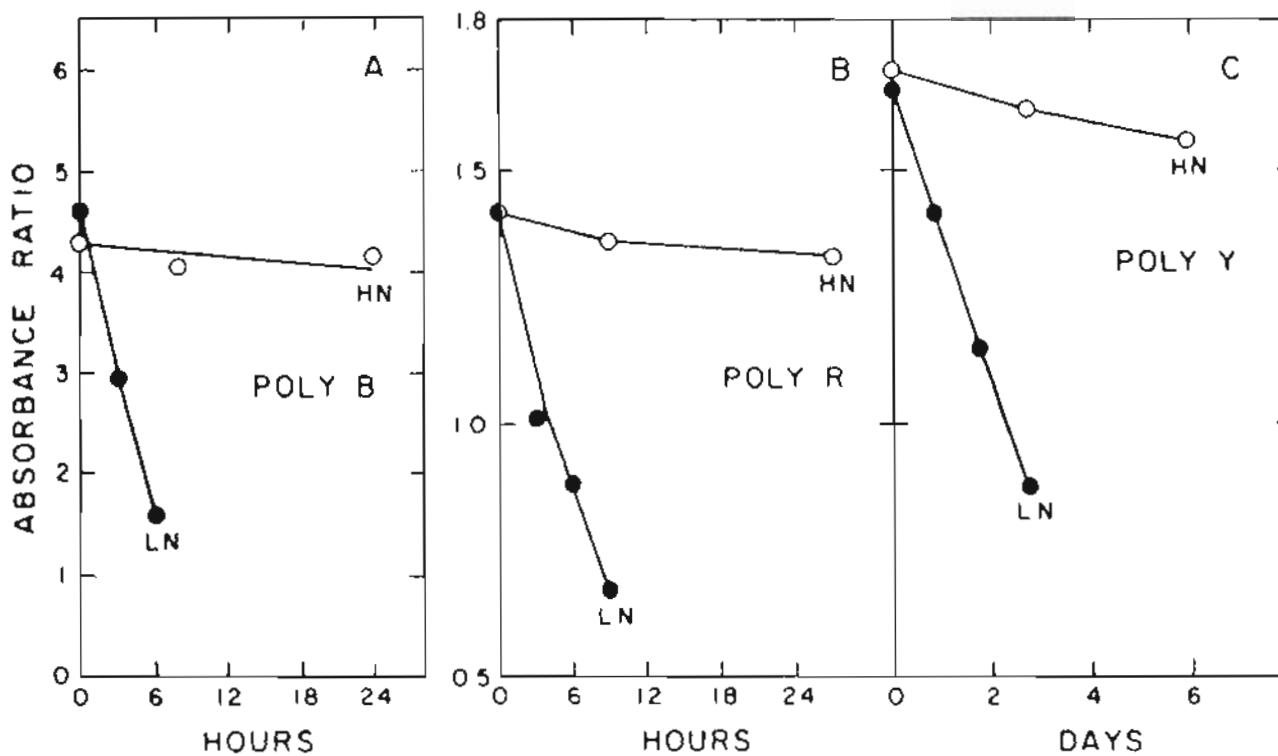


Fig. 2-3. Effect of Nutrient nitrogen on dye decolorization. The dyes were added to duplicate 6-day-old cultures previously grown in the presence of 1.2 mM  $(\text{NH}_4)_2$  tartrate (low N, LN) (●) or 12 mM  $(\text{NH}_4)_2$  tartrate (high N, HN) (○). As indicated, samples were removed and diluted, and the absorbance ratios were measured as described in the text.

at decolorizing all three dyes. The repressive effect of high nitrogen on the decolorization of these dyes was similar to that described for the effect of nitrogen on the metabolism of  $^{14}\text{C}$ -lignin to  $^{14}\text{CO}_2$ .<sup>118</sup>

The temporal dependence of the decolorization of Poly B and Poly R by cultures of *P. chrysosporium* ME-466 is shown in Fig. 2-4. Decolorization started after an initial lag of approximately 3 days and continued through the 14-day course of the experiment. Growth of the organism in stationary cultures as measured by dry weight (data not shown) was essentially complete after 2 days, confirming earlier experimentation.<sup>118,120</sup> Poly B decolorization reached a peak at about day 8 and then declined. Ligninolytic activity in this organism peaks at approximately day 6.<sup>121</sup> Of note, Poly R decolorization, like the fungal decolorization of Kraft  $E_1$  effluent,<sup>152</sup> remained optimal over a longer period of time. The temporal dependence of Poly Y decolorization was not examined because of the significantly slower rate of decolorization for this dye.

Several studies have described the dependence of lignin<sup>118,119</sup> and model compound metabolism<sup>117,120</sup> on the oxygen concentration of the fungal cultures. The effect of the oxygen concentration of the cultures on Poly B and Poly Y decolorization is shown in Fig. 2-5. With the dye Poly B, the fastest rate of decolorization ( $\Delta$  absorbance ratio per hour = 0.62) occurred in cultures purged with 100%  $\text{O}_2$ , whereas the slowest rate of decolorization ( $\Delta$  absorbance ratio per hour = 0.16) occurred in cultures purged with air. Intermediate rates were observed in cultures grown under oxygen and purged with air after the

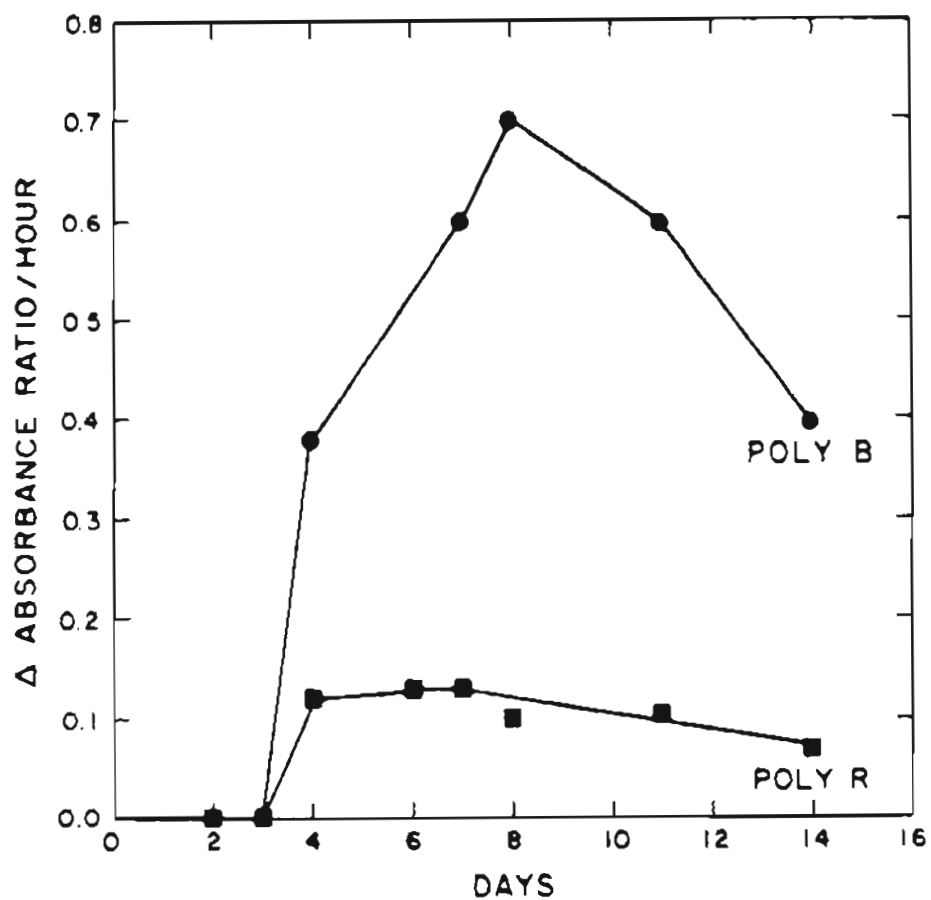


Fig. 2-4. Dependence of dye decolorization on culture age. To measure dye decolorization, Poly B (●) or Poly R (■) was added to individual flasks of  $O_2$ -grown cultures on days 2, 3, 4, 6, etc., as indicated, and the initial rate of decolorization was determined as described in the text.

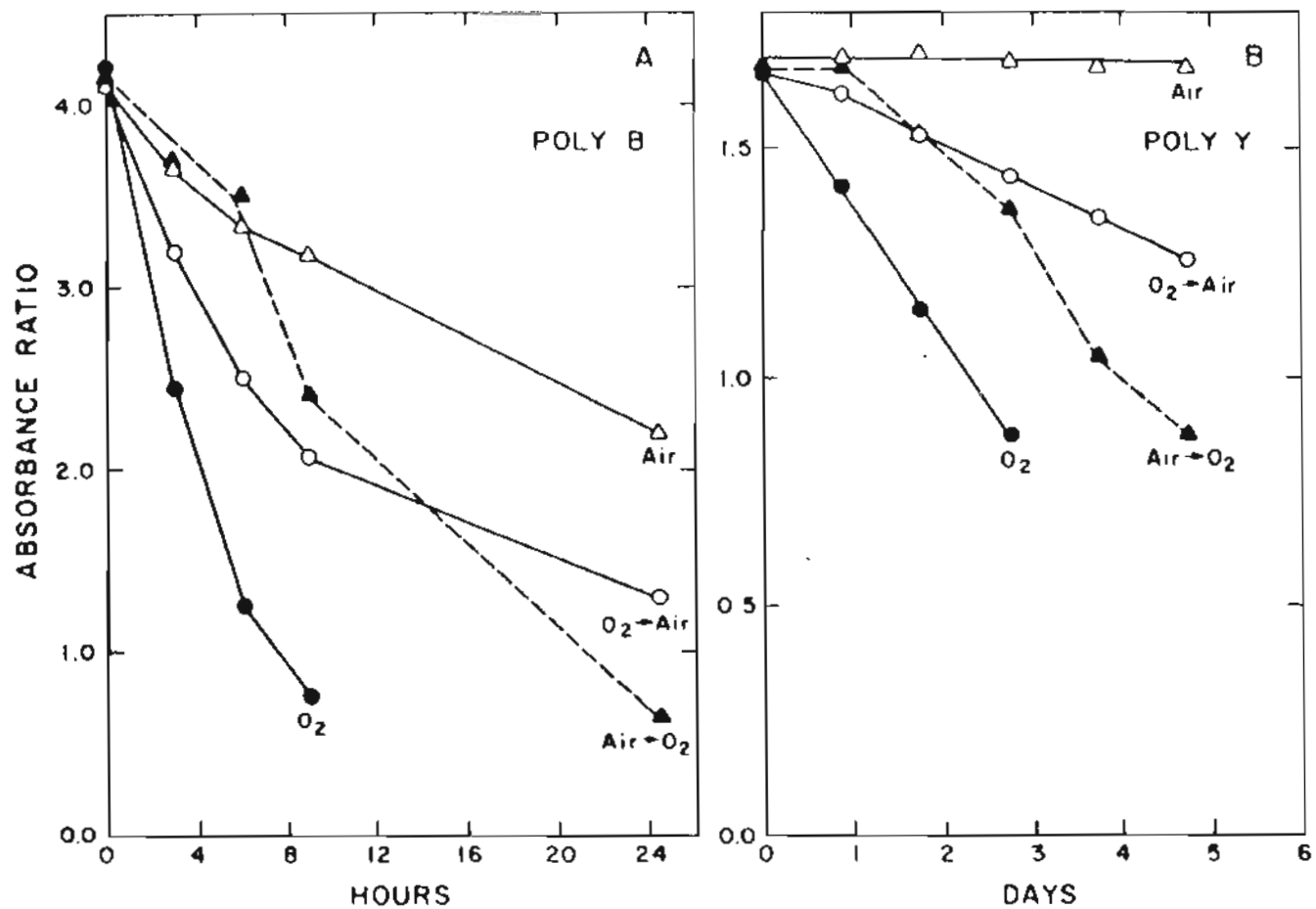


Fig. 2-5. Effect of oxygen concentration on dye decolorization. Six-day-old O<sub>2</sub>-grown or air-grown cultures were utilized as indicated. Decolorization of Poly B (A) and Poly Y (B) was examined. The following regimes were utilized: ●, O<sub>2</sub>-grown cultures, O<sub>2</sub> after the addition of dye; ○, O<sub>2</sub>-grown cultures, transferred to air after the addition of dye; △, air-grown cultures, air after the addition of dye; ▲, air-grown cultures, transferred to O<sub>2</sub> after the addition of dye.

dye addition and in cultures grown in air and purged with oxygen after the dye addition. Essentially identical results were obtained with Poly R (data not shown). Similar, but more pronounced, results were observed when Poly Y was used. The latter was not appreciably decolorized in cultures under an air atmosphere. Cultures grown in  $O_2$  and purged with air after the addition of the dye showed an intermediate rate of decolorization. The activation by 100%  $O_2$  ( $O_2 \rightarrow O_2$  versus  $O_2 \rightarrow \text{air}$ ) of the dye-degrading system(s) was approximately two fold with Poly B and three fold with Poly Y. In addition,  $O_2$ -grown cultures continued to degrade Poly Y long after they were transferred to an air atmosphere, suggesting that the induced condition is permanent.

The effect of various lignin biodegradation inhibitors<sup>135,136</sup> on the fungal decolorization of Poly B, Poly R, and Poly Y is shown in Table 2-1. Sodium azide (1 mM) and thiourea (5 mM), potent scavengers of hydroxyl radicals,<sup>144</sup> effectively inhibited dye decolorization. This was consistent with previous results obtained for lignin biodegradation.<sup>135,136</sup> Urea (5 mM), which served as a control for thiourea, was not an inhibitor. Sufficient preincubation with urea, however, resulted in repression related to the repression of secondary metabolism. Cyanide acted as a temporary inhibitor, producing 80 to 95% inhibition for ~6 h with Poly B and Poly R, and then rapidly lost effectiveness. Chemically synthesized lignin (synthetic  $^{14}C$ -dehydropolymerizate)<sup>125</sup> and 4'-O-methylisoeugenol probably acted as competitive inhibitors. These two agents have been previously shown to inhibit lignin biodegradation and the production of ethylene

TABLE 2-1

Effect of various inhibitors on the fungal decolorization of Poly B, Poly R, and Poly Y<sup>a</sup>

Inhibitor	% Inhibition		
	Poly B	Poly R	Poly Y
Standard condition	0	0	0
NaN <sub>3</sub> (1 mM)	100	100	100
Thiourea (5 mM)	100	100	100
Urea (5 mM)	0	0	0
Cyanide (1 mM)	95	80	65
Lignin (10 mg per flask)	45	40	ND <sup>b</sup>
4'-O'Methylisoeugenol (1 mM)	30	35	ND
Mannitol (0.1 M)	0	0	0

<sup>a</sup>Cultures were incubated under 100% O<sub>2</sub> for 6 days, after which inhibitors and dyes were simultaneously added. Dye decolorization was measured at 3-, 6-, and 9-h intervals for Poly B and Poly R and at 24-h intervals for Poly Y as described in the text.

<sup>b</sup>ND, Not determined.



form methional by this organism.<sup>136</sup> In contrast, mannitol, another (although less effective) hydroxyl radical scavenger,<sup>144</sup> had no effect on dye decolorization at a concentration of 0.1 M.

The decolorization of Poly B, Poly R, and Poly Y by the wild-type and two mutant strains of P. chrysosporium is shown in Fig. 2-6. The lignin degradation mutant 104-2<sup>125</sup> was unable to decolorize any of the dyes, whereas a phenotypic revertant strain, 424-2, which partially regained the capacity to degrade lignin<sup>125</sup> also partially regained the capacity to degrade the dyes. Similar results were obtained with the wild-type and mutant strains on solid medium. With solid medium containing colony-inducing agents and Poly B or Poly R, the colonies produced a clear halo in the colored background, allowing the possible visual selection of new mutants of lignin biodegradation.

### Discussion

In this paper we have examined the degradation of three polymeric dyes by P. chrysosporium. Throughout, we have attempted to correlate dye decolorization and the ligninolytic activity by P. chrysosporium by comparing the effect of various physiological parameters, mutations, and inhibitors on both processes. Dye decolorization, like ligninolytic activity, appears to be a secondary metabolic process. It was repressed by nutrient nitrogen and only occurred after the nitrogen in the cultures had been consumed. Dye decolorization paralleled lignin degradation temporally; the rate of dye decolorization reached an optimum in ~8-day-old cultures. Further, the amount of dye decolorization in a secondary metabolic mutant<sup>125</sup> and a phenotypic

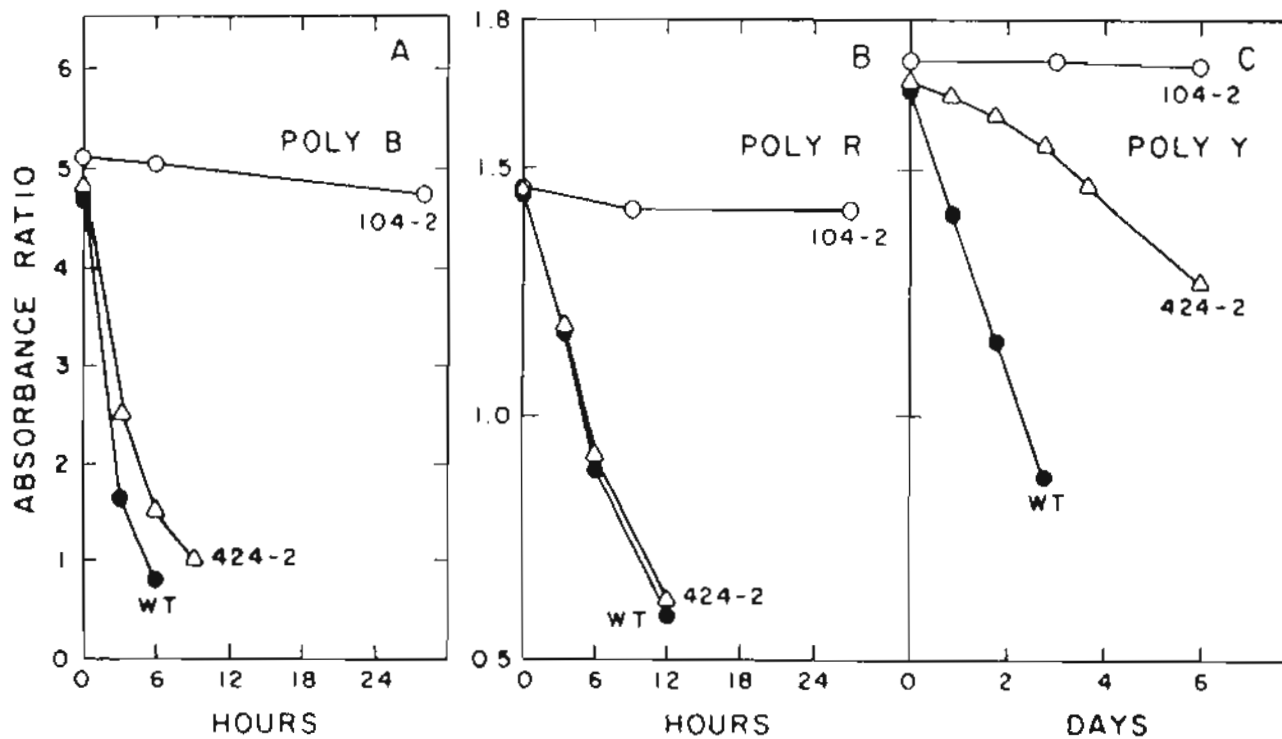


Fig. 2-6. Dye decolorization by several mutant strains of *P. chrysosporium*. Poly B (A), Poly R (B), or Poly Y (C) was added to 6-day-old  $O_2$ -grown duplicate cultures of the wild-type (●), 104-2 mutant (○), and 424-2 revertant (Δ) strains. Samples were removed at the times indicated and diluted, and the absorbance ratios were measured as described in the text.

revertant strain paralleled the capacity of these strains to degrade lignin. All of these results indicate that dye decolorization is a secondary metabolic process and suggest that the LDS or a part of it is responsible for decolorization.

As with the LDS,<sup>119</sup> oxygen appears both to induce fungal dye decolorization and to activate the catalytic system when it is already present. An example of this is in the case of Poly B or Poly R. The transfer of cultures from a 100% O<sub>2</sub> atmosphere to an air atmosphere after the addition of the dye reduced the rate of decolorization by ~50%. In addition, the transfer of cultures from an air atmosphere to a 100% O<sub>2</sub> atmosphere increased the rate of decolorization by three- to fourfold. Similar results are obtained when <sup>14</sup>C-lignins<sup>118,119</sup> and <sup>14</sup>C-lignin model compounds<sup>117,120</sup> are used as substrates. This also suggests that the LDS is responsible for dye decolorization. The effect of various lignin degradation inhibitors on dye decolorization was examined to test this hypothesis. Azide and thiourea, potent •OH scavengers<sup>144</sup> and inhibitors of lignin degradation, were very effective at inhibiting dye decolorization, although mannitol, a weaker inhibitor of lignin degradation, appeared to have no effect on dye decolorization. The strong inhibitory effect of azide on respiration may also contribute to its inhibition of dye degradation. 4'-O-Methylisoeugenol and lignin were also effective as inhibitors of dye decolorization and may have been acting as competitive inhibitors. KCN (1mM) was also an effective inhibitor, but its effect only lasted ~6 h. It is not clear whether this transitory effect was caused by a loss through purging with oxygen, cyanide-insensitive

respiration, or fungal transformation of the cyanide, although the latter appears most likely.

We have examined the use of these dyes as possible substrates for the LDS for several reasons. The dyes allow a simple, quantitative spectrophotometric assay. Due to the rapid spectral changes incurred, this assay can be performed more rapidly than previous assays based on radiolabeled substrates. Second, these attributes may allow the use of the dyes for the isolation of possible enzymes or other catalysts involved in lignin degradation. Finally, the dyes provide an inexpensive and effective method for the selection of fungal mutants with alterations in lignin metabolism in addition to providing a method for the possible screening of additional organisms capable of degrading lignin. The particular dyes described in this paper were chosen because they are polymeric, inexpensive, and commercially available in high purity. Their polymeric nature assures that at least the initial steps in their degradation are extracellular. We have found, recently, that many dyes, including blue dextran, Coomassie brilliant blue, methylene blue, and methyl red are also degraded by ligninolytic cultures of *P. chrysosporium*. This suggests the possible use of this organism for wastewater treatment in dye-related industries.<sup>153</sup>

The specific catalysts and reactions involved in dye degradation by the fungus are unknown. Observed differences between the fungal degradation of lignin and the dyes may be more related to physical, rather than chemical, properties. Lignin is a three-dimensional, heterogeneous, hydrophobic polymer at physiological pH which appears to be degraded only after strong specific binding to the fungus.<sup>154</sup>

These polymeric dyes are linear, charged, and hydrophilic. Binding of the dyes appears to be nonspecific and related to the net charge on the molecule. Despite these differences, the results described here suggest that there is probably a single catalytic system responsible for the degradation of both lignin and the dyes but that specific reactions are likely to be dependent on the chemical and physical properties of the substrates. All of the dyes are rapidly bleached by the hydroxyl radical ( $\cdot\text{OH}$ ) produced via Fenton reagent or via UV light- $\text{H}_2\text{O}_2$  (unpublished observation). This is consistent with the possible involvement of  $\text{OH}$  in the fungal degradation of lignin as described earlier.<sup>135,136</sup> Further research on the use of these dyes for the isolation and characterization of the catalytic system responsible for their degradation is planned.

## CHAPTER 3

An Extracellular  $H_2O_2$ -Requiring Enzyme Preparation Involved In  
Lignin Biodegradation By The White Rot Basidiomycete  
Phanerochaete chrysosporium

Jeffrey K. Glenn, Meredith A. Morgan, Mary E. Mayfield,  
Masaaki Kuwahara and Michael H. Gold

## Introduction

The specific catalysts involved in lignin degradation by white rot fungi have previously not been determined. A number of studies,<sup>60,61,76,77,155</sup> however, indicate that the catalysts may be nonspecific. Additional reports<sup>119,120,156-159</sup> indicate that the process is oxidative. It has been postulated recently that a form of free activated oxygen may be involved in lignin biodegradation.<sup>160</sup> In this study, we reinvestigated the involvement of extracellular enzymes in lignin degradation. We report on the characterization of an enzyme(s) preparation found in the cell free medium of competent P. chrysosporium cultures. This preparation is able to oxidatively cleave a variety of lignin model compounds, to generate ethylene from KTBA,<sup>161</sup> and to depolymerize lignin. Additional characterization indicates this enzyme(s) is part of the ligninolytic system of this organism.

## Materials and Methods

Chemical and culture preparation. KTBA, glucose oxidase Type V, and porcine pepsin were obtained from Sigma. [ $^{14}C$ ]-labeled lignin and lignin model compounds were synthesized as previously

described.<sup>76,77,125,137,162</sup> P. chrysosporium ME 446 and mutant strains were maintained on slants as described previously.<sup>150</sup> Stationary cultures in 250 ml flasks were prepared using 25 ml of a medium containing 2% glucose, 1.2 mM (NH<sub>4</sub>)<sub>2</sub> tartrate, 20 mM Na 2,2-dimethylsuccinate buffer and salts as previously described.<sup>118</sup> High N cultures contained 12 mM (NH<sub>4</sub>)<sub>2</sub> tartrate.<sup>121</sup> Cultures were inoculated with ~5 x 10<sup>7</sup> conidia and incubated at 38°C under air for three days after which the flasks were stoppered and purged with 100% O<sub>2</sub>.

Enzyme preparation. Medium from 6-day-old cultures was centrifuged at 18,000xg for 30 min to remove mycelial and spore contaminants. The supernatant was stored at 4°C for up to 24 hours before use. Reaction mixtures consisted of medium enzyme (1-5 ml), and H<sub>2</sub>O<sub>2</sub> generating system consisting of 0.02 units/ml of glucose oxidase and glucose (3 mM), and substrate. Reactions were purged with 100% O<sub>2</sub> and incubated at 37°C for the indicated time period.

Assay of ethylene production and lignin model compound oxidation. Reaction mixtures (2 ml) contained enzymes, glucose and KTBA (1 mM). After 1 hr at 37°C, 1-2.5 ml of gas was removed from the headspace and ethylene was measured by GC as previously described.<sup>136</sup> The following model compounds in N,N-dimethyl formamide were added to a final concentration of 0.02% (w/v):

1-(3',4'-diethoxyphenyl)-1,3-dihydroxy(4"-methoxyphenyl)propane (I);  
 1-(3',4'-diethoxyphenyl)-1-oxo-3-hydroxy-2-(4"-methoxyphenyl)propane (II);  
 1-(3',4'-diethoxyphenyl)-1-hydroxy-2-(4"-methoxyphenyl)ethane (III);  
 1-(4'-ethoxy-3'-methoxyphenyl)glycerol-β-guaiacyl ether (IV);

1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propane diol (V);  
1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propene (VI);  
3,4-diethoxybenzaldehyde (VII); 4-ethoxy-3-methoxybenzaldehyde  
(VIII); 1-(4'-methoxyphenyl)-1,2-dihydroxyethane (IX);  
1-(4'-methoxyphenyl)-1-oxo-2-hydroxyethane (X);  
4-ethoxy-3-methoxyphenylglycerol (XI). Reactions were carried out in a  
total volume of 5.0 ml for 16 hr as described above. Substrates and  
products were extracted, identified and quantitated by GC and GC/MS  
76,77,137,162 as previously described, except that products were  
silylated with bis-(N,O-trimethylsilyl)trifluoroacetamide:γ-collidine  
(3:1 v/v). <sup>14</sup>C-ring labeled lignin (50,000 cpm) ( $3 \times 10^5$  cpm/mg)  
was added to the reaction mixture as described above. After incubation  
(18 hr), the entire reaction mixture was gel filtered using a Sephadex  
LH-20 column in dioxane:H<sub>2</sub>O (9:1) as previously described.<sup>163</sup>

## Results

The results in Table 3-1 indicate that an extracellular enzyme preparation from 6-day-old competent cultures in combination with an H<sub>2</sub>O<sub>2</sub> generating system was capable of generating 0.2 μmol of ethylene from KTBA in 1 hr. In the absence of the H<sub>2</sub>O<sub>2</sub> generating system, or if a boiled preparation was used, no ethylene was generated.

Preincubation of the enzyme preparation with pepsin (300 units, 25°C, 30 min) inactivated greater than 80% of the subsequent ethylene generation activity. Thiourea and NaN<sub>3</sub> (1 mM) completely inhibited the enzyme. KCN and EDTA (1 mM) inhibited the enzymes 96% and 92% respectively. In contrast, mannitol (100 mM), a known hydroxyl radical



TABLE 3-1  
 Generation of ethylene from KTBA by a Phanerochaete  
chrysosporium extracellular enzyme preparation.

Conditions <sup>a</sup>	Ethylene generated (1 hr)	% of complete system
Complete system: extracellular enzyme preparation, glucose oxidase, glucose + KTBA	0.2 $\mu$ Moles	100
Extracellular enzyme preparation + KTBA		0.0
Complete system: heat killed extracellular enzyme preparation		0.0
Complete system: extracellular enzyme preparation from high N cultures		<0.01
Complete system: extracellular enzyme preparation from mutant strain 104-2		<0.01

<sup>a</sup>Enzyme was prepared from ligninolytic or high N cultures of the wild type or mutant strains. Reaction mixtures were prepared and ethylene was measured as described in the text.

scavenger, had no effect on the reaction.

The results in Figure 3-1 indicate that this enzyme system was able to oxidize a variety of lignin model compounds. The diarylpropane (I) was cleaved between the  $\alpha$  and  $\beta$  carbons to yield the diethoxybenzaldehyde (VII) and the phenylglycol (IX) as major products, in addition to a small amount of the phenylketol (X). When this experiment was performed under argon with the slow introduction of 0.05 mM  $H_2O_2$  the same products were formed. The diarylethane (III) was also cleaved at its  $\alpha,\beta$  bond to yield the diethoxybenzaldehyde (VII), anisylalcohol (XII), and anisylaldehyde (XIII). The  $\beta$ -ether dimer (IV) was also slowly oxidized by this enzyme system to yield the benzaldehyde (VIII) and a small amount of the phenylglycerol (XI). Finally, the olefin (VI) was rapidly hydroxylated and cleaved to yield the diol (V) and the benzaldehyde (VIII).

None of the above reactions, including ethylene generation, occurred when the enzyme was prepared from high N cultures, when a boiled enzyme preparation was used, or when cultures of the ligninolytic mutant 104-2<sup>125</sup> were used as a source of the enzyme preparation. Finally, EDTA, thiourea, KCN and azide also inhibited the oxidation of the lignin model compounds. Incubation of <sup>14</sup>C-ring labeled lignin with the enzyme preparation led to ~10% degradation as determined by gel filtration. Comparison of the filtration profile with that of the control indicated that the radioactivity in the high molecular weight material decreased with a corresponding increase in radioactivity in a low molecular weight fraction.

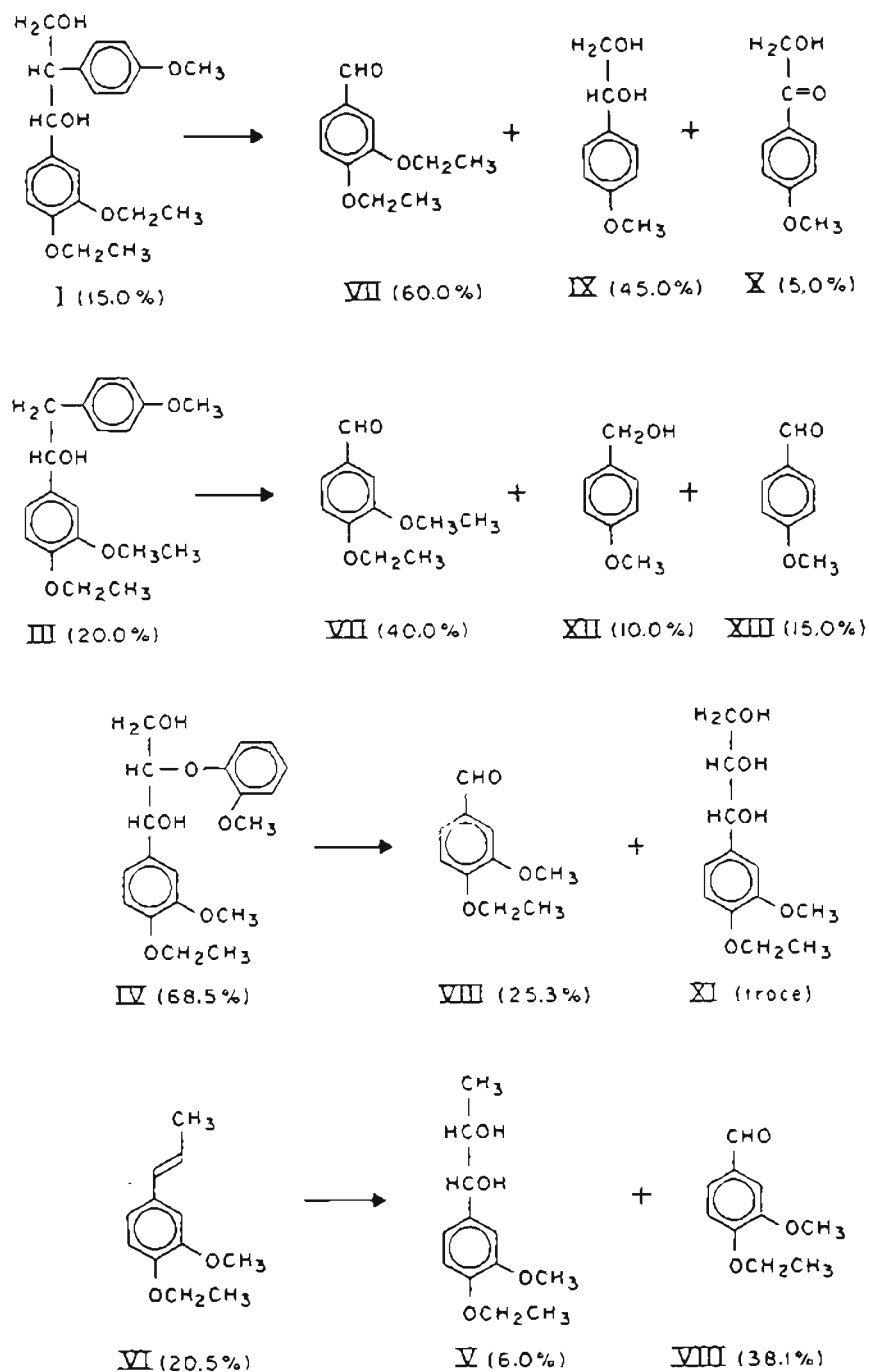


Fig. 3-1. Products identified and proposed pathway for the oxidation of several lignin model compounds by an extracellular enzyme preparation from *P. chrysosporium*. Enzyme was prepared from ligninolytic cultures, reactions were carried out, and products were extracted and identified as described in the text. Numbers in parenthesis refer to amount of remaining substrate or products formed, with respect to the amount of initial substrate. % = mole of product formed/mole of initial substrate x 100.

## Discussion

Ligninolytic activity<sup>118,121</sup>, model compound degradation<sup>120</sup> and ethylene production from KTBA<sup>135,136</sup> are expressed as secondary metabolic events in *P. chrysosporium*. The above activities are optimal in 5- to 7-day-old stationary, low N cultures which are grown under 100% oxygen.<sup>119,120,159</sup> Extracellular medium from 6-day-old cultures grown under the above conditions was, therefore, used as a source of enzyme. A mixture of glucose oxidase, glucose and O<sub>2</sub> was routinely used to produce a continuous low concentration of H<sub>2</sub>O<sub>2</sub> during the course of the reactions; however, the slow addition of H<sub>2</sub>O<sub>2</sub> to the enzyme under an argon atmosphere also resulted in similar activity. Inactivation of the preparation by pepsin treatment and by boiling both indicate that an enzyme(s) is, in fact, responsible for the catalytic activities.

The results in Table 3-1 indicate that this enzyme preparation, rather than free hydroxyl radical as reported earlier<sup>135,136</sup>, is responsible for the generation of ethylene from KTBA in intact cultures of *P. chrysosporium*. The effect of thiourea on the process and the nature of the reactivity of KTBA suggest, however, that radicals or equivalent species<sup>137,163</sup> may play a role in the mechanism of this enzyme(s). Suppression of this enzyme activity in high nitrogen cultures and its absence in cultures of the ligninolytic mutant 104-2<sup>125</sup> strongly suggest that it is a component of the ligninolytic system.

The pathways for the enzymic degradation of the lignin model compounds used in this study are shown in Figure 3-1. All of the

compounds have been shown to be degraded by P. chrysosporium cultures grown under ligninolytic conditions. Both the diarylpropane (I) and the diarylethane (III) are rapidly cleaved at their respective  $\alpha, \beta$  bonds, indicating that this crude enzyme preparation, like the fungus and photosensitizing riboflavin<sup>60,76,77,137,155</sup>, is relatively nonspecific. The diarylpropane derivative (II) with an  $\alpha$ -carbonyl at its  $\alpha$ -carbon was not cleaved directly, confirming the importance of the hydroxyl group on the  $\alpha$ -carbon.<sup>77,164</sup> The olefin (VI) was both hydroxylated to form the diol (V) and cleaved to form the benzaldehyde (VIII) concurrently. Finally, the  $\beta$ -ether dimer was slowly cleaved to yield the benzaldehyde (VIII) and a small amount of the phenylglycerol (XI). All of these reactions are equivalent to those previously observed in intact cultures<sup>76,77,125,164</sup> except that in intact cultures, benzaldehydes are rapidly reduced to benzyl alcohols<sup>165,166</sup>. These results indicate that this enzyme preparation is at least partially responsible for the oxidative cleavage reactions carried out by intact cultures. The results with enzymic degradation of <sup>14</sup>C-ring labeled lignin also indicate that this extracellular enzyme preparation is an integral part of the lignin degradative system. Through characterization of the enzyme(s) involved in these reactions awaits possible separation and purification.

## CHAPTER 4

Separation and Characterization of Two Extracellular  $H_2O_2$ -Dependent  
Oxidases from Ligninolytic Cultures of  
Phanerochaete chrysosporium

Masaaki Kuwahara, Jeffrey K. Glenn, Meredith A. Morgan  
and Michael H. Gold

## Introduction

Recently, we reported on an  $H_2O_2$ -requiring enzyme preparation found in the extracellular medium of ligninolytic cultures of Phanerochaete chrysosporium.<sup>91,145</sup> This activity was not found in nonligninolytic cultures nor in cultures of a nonligninolytic mutant<sup>125</sup> of this organism. This extracellular preparation is able to generate ethylene from KTBA,<sup>145,161</sup> to oxidize a variety of lignin model compounds,<sup>145,167</sup> to depolymerize lignin,<sup>145,167</sup> and to decolorize the polymeric dye Poly R.<sup>91,131</sup> In this report we describe the separation of this crude preparation into two  $H_2O_2$ -requiring enzyme fractions by blue agarose affinity chromatography. The first fraction, containing an apparent  $H_2O_2$ -requiring oxygenase, is responsible for the cleavage of KTBA and the oxidation of a variety of lignin model compounds, including the diarylpropane (I). The second enzyme is a Mn (II)-dependent, lactate-activated peroxidase.

## Materials and Methods

KTBA, phenol red and Poly R-481 were obtained from Sigma.  
<sup>14</sup>COOH-labeled vanillic acid (<sup>14</sup>COOH-4-hydroxy-3-methoxybenzoic

acid) was obtained from Research Products International. Lignin model compounds were synthesized as described previously.<sup>76,77</sup> P. chrysosporium ME 446 was maintained on slants as previously described.<sup>150</sup> Stationary cultures in 2 liter flasks containing 150 ml of medium were inoculated, incubated at 28°C and purged with 100% O<sub>2</sub> as previously described.<sup>145</sup> Crude enzyme fractions were prepared as previously described.<sup>145</sup> Acetone (-10°C) was added to the crude enzyme fraction to 66% v/v. The precipitated protein was centrifuged at 12,000 x g at -10°C for 15 min and resuspended in 20 mM Na succinate, pH 4.5, one-fiftieth of the original volume. Insoluble material was removed by recentrifugation and the supernatant was dialyzed against 20 mM Na succinate, pH 4.5.

The acetone concentrate (6 ml) was adsorbed to a column of reactive blue 2-cross linked agarose (Sigma Chem. Co.) (1.0 x 17 cm) equilibrated with 20 mM Na succinate, 100 mM NaCl, pH 4.5. The column was eluted with 25 ml of the equilibration buffer after which it was eluted with 20mM Na succinate, 250 mM NaCl, pH 4.5. Fractions (2 ml) were collected. Enzyme peaks were pooled and 1 ml of each fraction was applied to a Sephadex G-100 column (1.7 x 50 cm) previously equilibrated with 20 mM Na succinate, 100 mM NaCl, pH 4.5. Fractions were pooled and assayed for activity.

Ethylene generation from KTBA was measured as previously described<sup>145</sup> except that veratryl alcohol (3,4-dimethoxybenzyl alcohol) (1 mM) was added to all reactions. Peroxidase activity was measured by a modification of a previously described procedure.<sup>168</sup> Reaction mixtures (1 ml) consisted of phenol red (0.01%), lactate (25

mM),  $\text{MnSO}_4$  (100  $\mu\text{M}$ ), egg albumin (0.1%), and  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ) in 1.0 ml of 20 mM Na succinate buffer, pH 4.5. Reactions were carried out at 30°C for 5 min and terminated with the addition of 2 N NaOH (40  $\mu\text{l}$ ). Absorbance was read at 610 nm.

Model compound oxidations were carried out in 1 ml of Na-tartrate, 20 mM, pH 3.0 containing 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  or glucose/glucose oxidase as previously described<sup>145</sup> and enzyme. 1-(3',4'-Diethoxyphenyl)-1,3-dihydroxy(4"-methoxyphenyl)propane (I) or veratryl alcohol were added to reaction mixtures to a final concentration of 0.02% (w/v). Substrates and products were extracted, and identified by GC and GC/MS.<sup>76,77,137</sup>  $^{14}\text{COOH}$ -vanillic acid decarboxylation was measured as previously described.<sup>125</sup>

Diarylpropane (I) and veratryl alcohol oxidation by purified fractions were routinely monitored spectrophotometrically at 310 nm. Reaction mixtures (1 ml) consisted of substrate (3 mM),  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$ ) and enzyme in Na tartrate buffer (pH 3.0).

In experiments to measure the incorporation of  $^{18}\text{O}$ , reaction vessels contained two compartments, one containing the enzyme and  $\text{H}_2^{16}\text{O}_2$ , the other the diarylpropane (I). Reaction vessels were evacuated, flushed with scrubbed argon, re-evacuated and finally equilibrated with  $^{18}\text{O}_2$ . Reactions were started by mixing the contents of the vessel and incubating at 37°C for 2 h. Extraction and mass analysis were as previously described.<sup>76,77</sup>

## Results and Discussion

Approximately 60% of the KTBA and phenol red oxidase activities



were recovered in the acetone concentrate. The results in Fig. 4-1 indicate that the two enzyme fractions were separated by affinity chromatography on blue agarose. KTBA cleavage activity eluted with 0.1 M NaCl while the phenol red peroxidase eluted only after the salt concentration of the eluant was increased to 0.25 M. Absorbance of the fractions at 410 nm parallels the activity peaks, suggesting that both enzymes are heme proteins. Preliminary spectral evidence also indicates that both oxidases are heme proteins.

Fractions from each of the two major activity peaks were pooled and used for enzyme characterization and gel filtration experiments. Fig. 4-2 shows a Sephadex G-100 gel filtration profile for each enzyme activity. Each enzyme elutes as a single symmetrical peak. The molecular weight of each enzyme as determined by the method of Andrews<sup>169</sup> was ~41,000. SDS polyacrylamide gel electrophoresis indicates, however, that the dye peroxidase has a Mw of ~46,000.

The dye peroxidase displayed maximal activity at pH 4.5 and showed an absolute dependency for Mn(II). Maximal stimulation occurs at 100  $\mu$ M Mn(II). Peroxidase activity was also stimulated by both lactate and a variety of proteins with optimal activity occurring at approximately 25 mM lactate and 0.1% egg albumin. Optimal activity also occurred at approximately 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>. The peroxidase was inhibited completely by NaN<sub>3</sub>, KCN and EDTA, each at 1 mM. The enzyme oxidized a variety of dyes including phenol red, *o*-dianisidine and Poly R<sup>131</sup> and was also capable of decarboxylating <sup>14</sup>COOH-vanillic acid to yield <sup>14</sup>CO<sub>2</sub>. However, the latter activity did not appear to be Mn(II) dependent. The exact role of the enzyme in lignin degradation is being investigated.

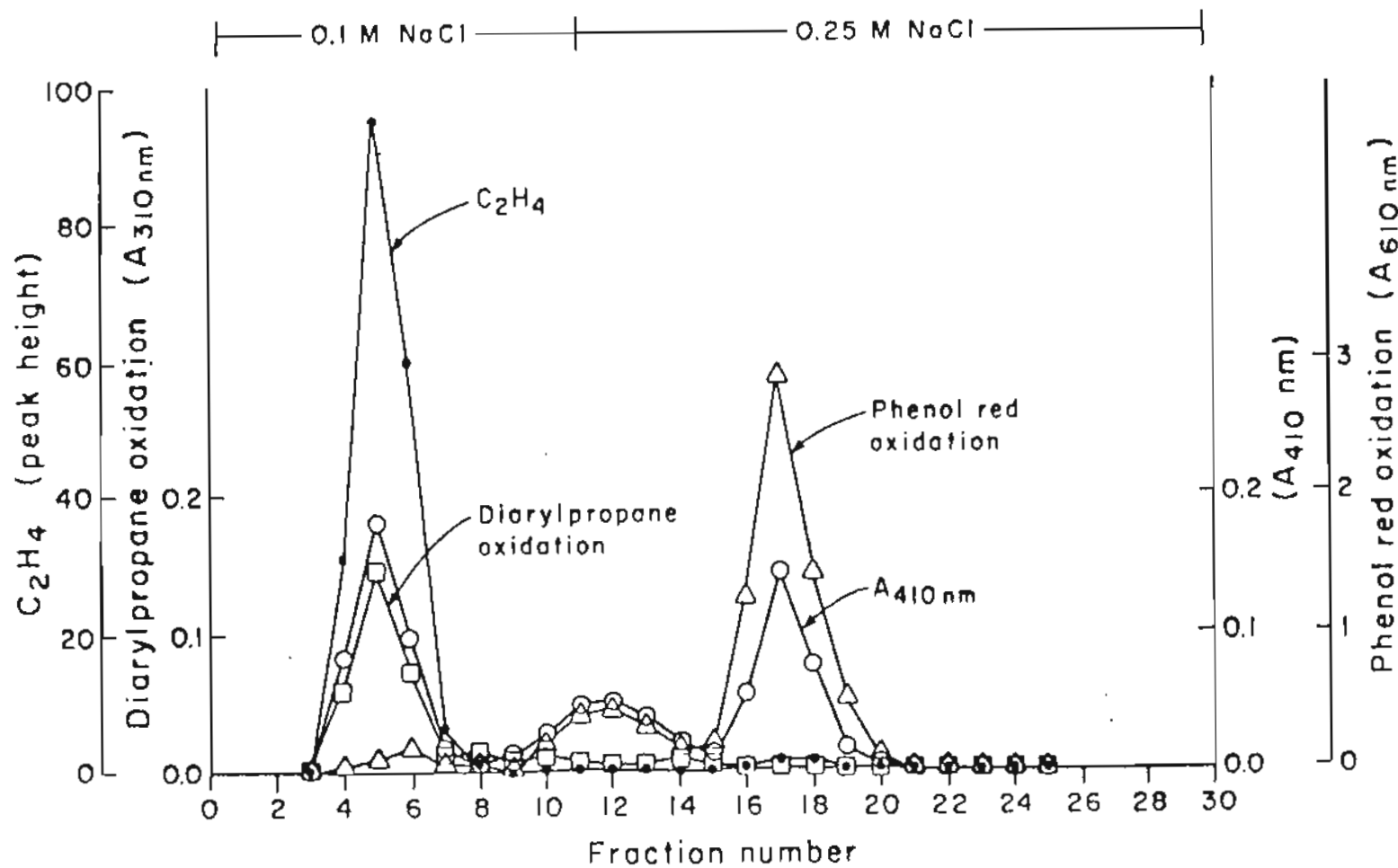


Fig. 4-1. Separation of two extracellular oxidases by blue agarose affinity chromatography. (□) Diarylpropane oxidation measured at 310 nm; (●) ethylene generation from KTBA, (Δ) phenol red oxidation; and (○) absorbance of the fractions at 410 nm. Experimental procedures and assays are described in the text.

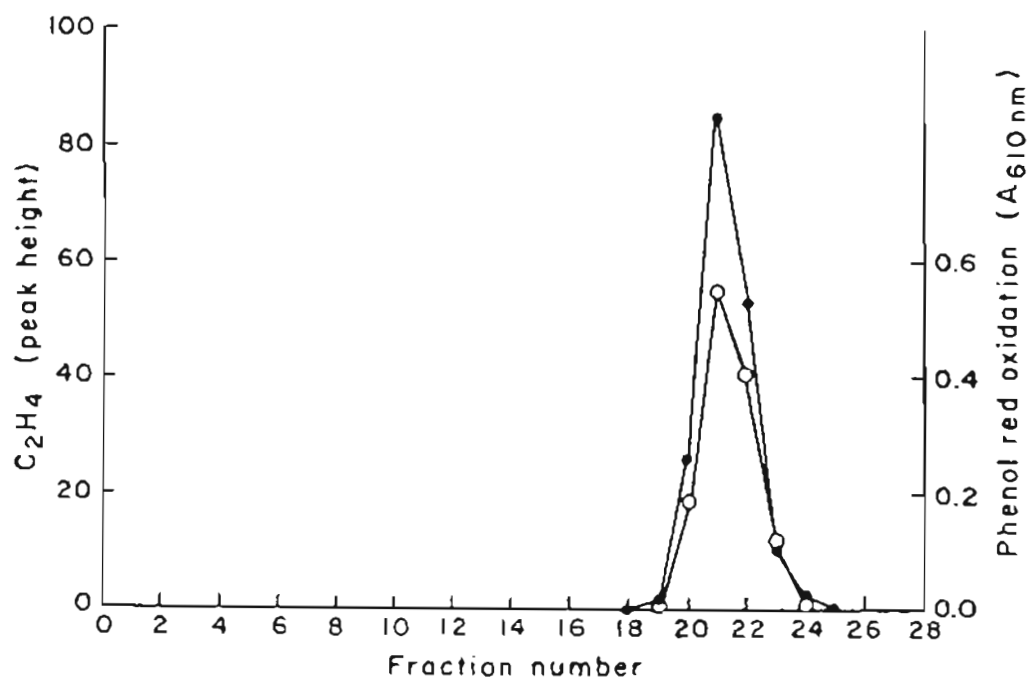


Fig. 4-2. Gel filtration profiles of two oxidases on Sephadex G-100. (O) Phenol red oxidation; (●) ethylene generation. Experimental procedures and details are described in the text.

The second enzyme separated by blue agarose chromatography (Fig.4-1) cleaved KTBA in the presence of the secondary metabolite veratryl alcohol (1 mM).<sup>116</sup> This enzyme also catalyzed the cleavage of the diarylpropane (I) at the  $\alpha,\beta$  bond to yield 3',4'-diethoxybenzaldehyde (II) and 1-(4'methoxyphenyl)-1,2-dihydroxyethane (III) (Fig. 4-3). Finally, veratryl alcohol was also oxidized to veratrylaldehyde as measured at 310 nm and by thin layer chromatography. Unlike KTBA cleavage, diarylpropane cleavage by this enzyme was not dependent on veratryl alcohol. The diarylpropane oxidase displayed maximal activity at pH 3.0 and approximately 50-100  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . In the absence of  $\text{H}_2\text{O}_2$ , no products were formed. This enzyme was also completely inhibited by  $\text{NaN}_3$ , KCN, EDTA and thiourea, each a 1 mM.

As shown in Fig. 4-3, when the diarylpropane cleavage was performed in the presence of  $\text{H}_2\text{O}_2 + ^{18}\text{O}_2$ ,  $^{18}\text{O}$  was incorporated into the diol (III). The mass spectrum of the TMSi derivative of the diol product included the following ion peaks (m/e,rel. int.): 314, 0.02% $\text{M}^+$ ; 299, 0.71% (M- $\text{CH}_3$ ); 211, 100% (M- $\text{CH}_3$ -OTMS). Intensity of the ion peaks at 297 and 209 was less than 5% of those at 299 and 211. When the same experiment was conducted under  $^{16}\text{O}_2$ , the major fragment ions were found at m/e 297 and 209, the same as in the chemically synthesized standard. This indicates that one  $^{18}\text{O}$  atom was incorporated specifically into the  $\alpha$ -position of the diol (III) ( $\beta$ -position of the diarylpropane) and that this oxygen atom is donated by  $\text{O}_2$  rather than by  $\text{H}_2\text{O}_2$  as previously implied.<sup>91</sup> The results are the same as those obtained with  $^{18}\text{O}$  incorporation into the

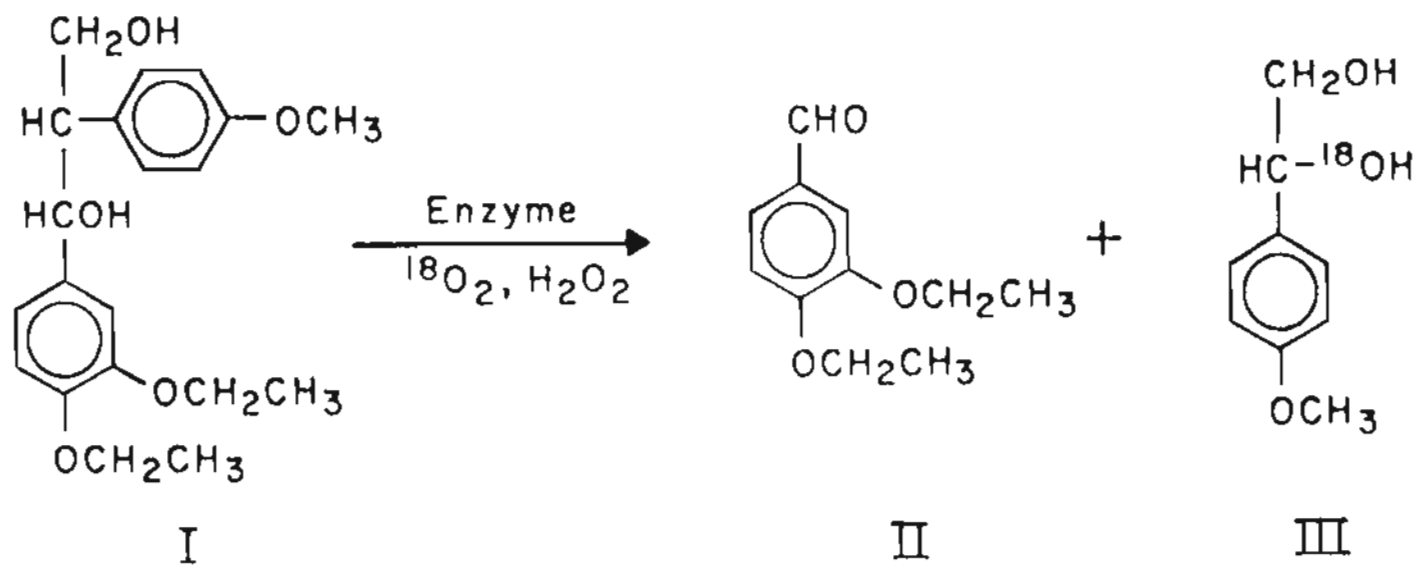


Fig. 4-3. Incorporation of  $^{18}\text{O}$  from  $^{18}\text{O}_2$  into the diol product (III) during the  $\alpha, \beta$  cleavage of the diarylpropane (I) by an oxygenase found in the extracellular medium of *P. chrysosporium*. Experimental details are described in the text.

diarylpropane (I) in intact cultures.<sup>163,170</sup> These results indicate that this enzyme is a novel H<sub>2</sub>O<sub>2</sub>-dependent oxygenase. Further characterization of these enzymes is in progress.

## CHAPTER 5

Purification and Characterization of an Extracellular  
Mn(II)-Dependent Peroxidase from the  
Lignin-Degrading Basidiomycete,  
Phanerochaete chrysosporium

Jeffrey K. Glenn and Michael H. Gold

## Introduction

Numerous investigations have shown that white rot fungi, those capable of degrading lignin, produce phenol oxidases.<sup>20,47,123</sup> Additionally, genetic studies<sup>124,125</sup> have demonstrated a relationship between lignin degradation and phenol oxidase production by the white rot basidiomycete, Phanerochaete chrysosporium. Phenol oxidases are capable of oxidizing a variety of phenolic lignin model compounds,<sup>20</sup> yet alone their major effect on lignin appears to be further polymerization.<sup>126</sup>

A correlation has also been demonstrated between lignin degradation and polymeric dye decolorization by P. chrysosporium.<sup>131</sup> This led to studies on the demonstration and partial purification of the enzyme activity involved in dye decolorization. A crude extracellular enzyme preparation from ligninolytic cultures of P. chrysosporium catalyzed the oxidation of a variety of lignin model compounds<sup>91,145,167</sup> and several polymeric dyes in the presence of  $H_2O_2$ .<sup>91</sup> The extracellular enzyme preparation has been separated into two  $H_2O_2$ -requiring fractions by Blue Agarose chromatography.<sup>146</sup> The first fraction containing an  $H_2O_2$ -requiring oxygenase was

responsible for the oxidative degradation of a variety of lignin model compounds.<sup>76,77,91,145,146,167</sup> This oxygenase has now been purified to homogeneity from both large, agitated cultures<sup>147</sup> of P. chrysosporium. The second enzyme fraction to elute during Blue Agarose chromatography contained a Mn-(II)-dependent peroxidase.<sup>146</sup>

In this report we describe the purification and properties of this novel P. chrysosporium peroxidase. The enzyme is a heme protein with a  $M_r$  of 46,000. It is dependent on  $H_2O_2$  and Mn(II) for activity, and is stimulated by  $\alpha$ -hydroxy acids and protein. The enzyme catalyzes the oxidation of Mn(II) and a variety of organic compounds, including NADH and the polymeric dyes, Poly R-481 and Poly B-411.

#### Methods and Materials

Cultural conditions. The strain of P. chrysosporium and culture conditions used for production of the enzyme were as previously described.<sup>147</sup> Two 2-liter Erlenmeyer flasks containing 1 liter of a medium consisting of 2% glucose, 1.2mM  $(NH_4)_2$  tartrate, 20 mM 2,2-dimethylsuccinate, and minimal salts<sup>118</sup> were inoculated with a suspension of mycelial fragments.<sup>147</sup> The cultures were incubated at 28°C on a rotary shaker for 6 days. The organism was grown under air for the first 3 days and then purged with 100%  $O_2$  at 24-h intervals. Cultures were filtered through glass wool and the resultant filtrate was stored at 4°C.

SDS electrophoresis. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out in standard 8% gels as described previously.<sup>172</sup> Protein samples were dissolved in 10 mM



Na phosphate, 2% SDS, 2% 2-mercaptoethanol, and 5 M urea. After electrophoresis, the gels were fixed overnight in H<sub>2</sub>O:methanol:acetic acid (5:5:1), and then stained with Coomassie blue R-250 and destained as previously described.<sup>172</sup>

Enzyme assays. The standard reaction mixture consisted of 50 mM Na succinate, 50 mM DL-lactate, 100  $\mu$ M MnSO<sub>4</sub>, pH 4.5, containing predissolved gelatin (3 mg/ml) and substrate. When pinacyanol was used as a substrate the reaction mixture also contained 10% DMSO (v/v). Unless otherwise indicated, reaction mixtures contained 0.1  $\mu$ g/ml of P. chrysosporium peroxidase or 0.085  $\mu$ g/ml of horseradish peroxidase (HRP), an equivalent amount of each enzyme, based on an M<sub>r</sub> of 46,000 for P. chrysosporium peroxidase and 40,000 for HRP.<sup>173</sup> Unless indicated otherwise, reactions were initiated by the addition of H<sub>2</sub>O<sub>2</sub> to a final concentration of 50  $\mu$ M. The stimulating effects of Mn(II), lactate, and protein were determined by comparing the complete reaction mixture with those lacking one of the components. Dye concentrations and the wavelengths used to follow the reactions are given in Table 5-2. ABTS, phenol red, and guaiacol have been used previously as peroxidase substrates.<sup>168,174,175</sup> To our knowledge, except for our previous publications<sup>91,46</sup> where Poly R was used, this is the first use of pinacyanol, variamine blue salt, Poly B, Poly R, and indigo trisulfonate as peroxidase substrates. The oxidation of phenol red was carried out in 1-ml reaction mixtures at 30°C for 5 min and was terminated by the addition of 5 N NaOH (20  $\mu$ l). Absorbance was read at 610 nm.<sup>146,168</sup> For all other dyes, the reactions were continuously measured spectrophotometrically. Poly R oxidation

required 1  $\mu\text{g/ml}$  P. chrysosporium peroxidase, or 85  $\mu\text{g}$  HRP. NADH oxidation was followed at 340 nm. Reaction mixtures (1 ml) contained 0.1 mg NADH and reactions were initiated by the addition of 0.1  $\mu\text{g}$  P. chrysosporium peroxidase.

Oxidation of Mn(II) to Mn(III) was determined spectrophotometrically at 258 nm by measuring the formation of the Mn(III)-pyrophosphate complex.<sup>176</sup> Oxidation was measured in a 1-ml reaction mixture containing 1  $\mu\text{g}$  peroxidase,  $\text{MnSO}_4$  (0.1 mM), and Na pyrophosphate in either 10mM Na phosphate, pH 6.0, or 20 mM Na acetate, pH 4.5. The reaction was initiated by the addition of 10 nmol  $\text{H}_2\text{O}_2$ .

Spectroscopic procedures. Absorption spectra were recorded on a Shimadzu UV-260 spectrophotometer at room temperature using a spectral bandwidth of 1.0 nm and cuvettes of 1-cm light path essentially as described previously.<sup>147</sup> Measurements were taken on the protein (0.15  $\mu\text{g/ml}$ ) dissolved in 20 mM Na succinate, pH 4.5. The ferrous enzyme was prepared under anaerobic conditions by purging the native enzyme with scrubbed argon in a septum-filled cuvette, after which a small crystal of  $\text{Na}_2\text{S}_2\text{O}_4$  was added. The reduced-CO complex was generated by purging the native protein with CO, adding a small crystal of  $\text{Na}_2\text{S}_2\text{O}_4$ , and subsequently purging again with CO. KCN and  $\text{NaN}_3$  were added to the enzyme to final concentrations of 5 and 10 mM, respectively. The  $\text{H}_2\text{O}_2$ -enzyme complex was generated at room temperature by the addition of 10 nmol  $\text{H}_2\text{O}_2$  to 1 ml of enzyme solution, after which the spectrum was recorded immediately. The pyridine hemochrome was determined essentially as described

previously.<sup>147,177</sup> Pyridine (300  $\mu$ l) was added to the protein solution (300  $\mu$ l). Subsequently, 375  $\mu$ l of NaOH (0.2N) was slowly added with gentle shaking. The cuvette was then evacuated, Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub> was added under N<sub>2</sub>, and the spectrum was determined after 15 min.

Protein determination. Protein concentration was determined by the method of Lowry et al.,<sup>178</sup> using bovine serum albumin as the standard.

Chemicals. Poly B-411, Poly R-481, variamine blue RT salt, phenol red, pinacyanol chloride, K-indigo trisulfonate, DEAE-Sephacrose, reactive Blue 2-crosslinked Agarose, hexadecyl trimethyl ammonium bromide (Cetrimide), HRP fractions II and VI, catalase, ovalbumin, glyceraldehyde-3-P-dehydrogenase, myoglobin, and glucose oxidase were obtained from the Sigma Chemical Company. ABTS<sup>174</sup> was obtained from the Boehringer Mannheim Company. Bactogelatin was purchased from Difco. All other chemicals were reagent grade.

## Results

Purification of the extracellular *P. chrysosporium* peroxidase. The initial steps in the purification of the peroxidase were identical to those described for the purification of the *P. chrysosporium* diarylpropane oxygenase.<sup>147</sup> In summary: (a) The protein was concentrated from the crude culture filtrate by precipitation with 66% acetone at -10°C. (b) The precipitated protein was resuspended in 20 mM Na succinate buffer, pH 4.5, containing Cetrimide (buffer A). (c) The dialyzed protein was applied to a DEAE-Sephacrose column

equilibrated with buffer A and the column was washed with buffer A. The peroxidase activity did not bind to the DEAE-Sepharose column under these conditions, and was eluted in the breakthrough fractions. The concentrate contained 45 ml (180  $\mu\text{g}$  protein/ml) with specific activity for ABTS oxidation of  $0.20 \Delta A \text{ min}^{-1} \mu\text{g}^{-1}$ . Further purification was as described below; all steps were performed at  $4^\circ\text{C}$ .

1. To remove the Cetrimide detergent, the breakthrough and wash fractions from the DEAE-Sepharose column were passed through an octyl-Sepharose column (1.7 X 20 cm) previously equilibrated with 50 mM Na phosphate, pH 7.0. The column was eluted with the same buffer and 5-ml fractions were collected. The fractions containing heme protein (absorbance at 410 nm) were pooled, washed, and concentrated by membrane ultrafiltration using 50 mM Na succinate, pH 4.5 (buffer B). The concentrate contained 2 ml (4.0 mg protein/ml).

2. All of the above protein was adsorbed to a column of reactive Blue 2-cross linked agarose (0.8 X 18 cm) equilibrated with buffer B. The column was washed with buffer B and the enzyme was eluted at pH 4.5 with a linear gradient consisting of buffer B in the mixing chamber and buffer B + 400 mM NaCl in the reservoir. The total volume of the gradient was 100 ml. Fractions (1.5 ml) were assayed for activity and one major peak of activity was obtained (Fig. 5-1). This peak was pooled and concentrated to 1 ml (2.0 mg protein/ml) with a specific activity of  $1.0 \Delta A \text{ min}^{-1} \mu\text{g}^{-1}$ .

3. This concentrated Blue Agarose eluate was applied to a Sephadex G-100 column (1.7 X 50 cm) previously equilibrated with buffer B + 100 mM NaCl. Fractions (1.5 ml) were collected and assayed for enzyme

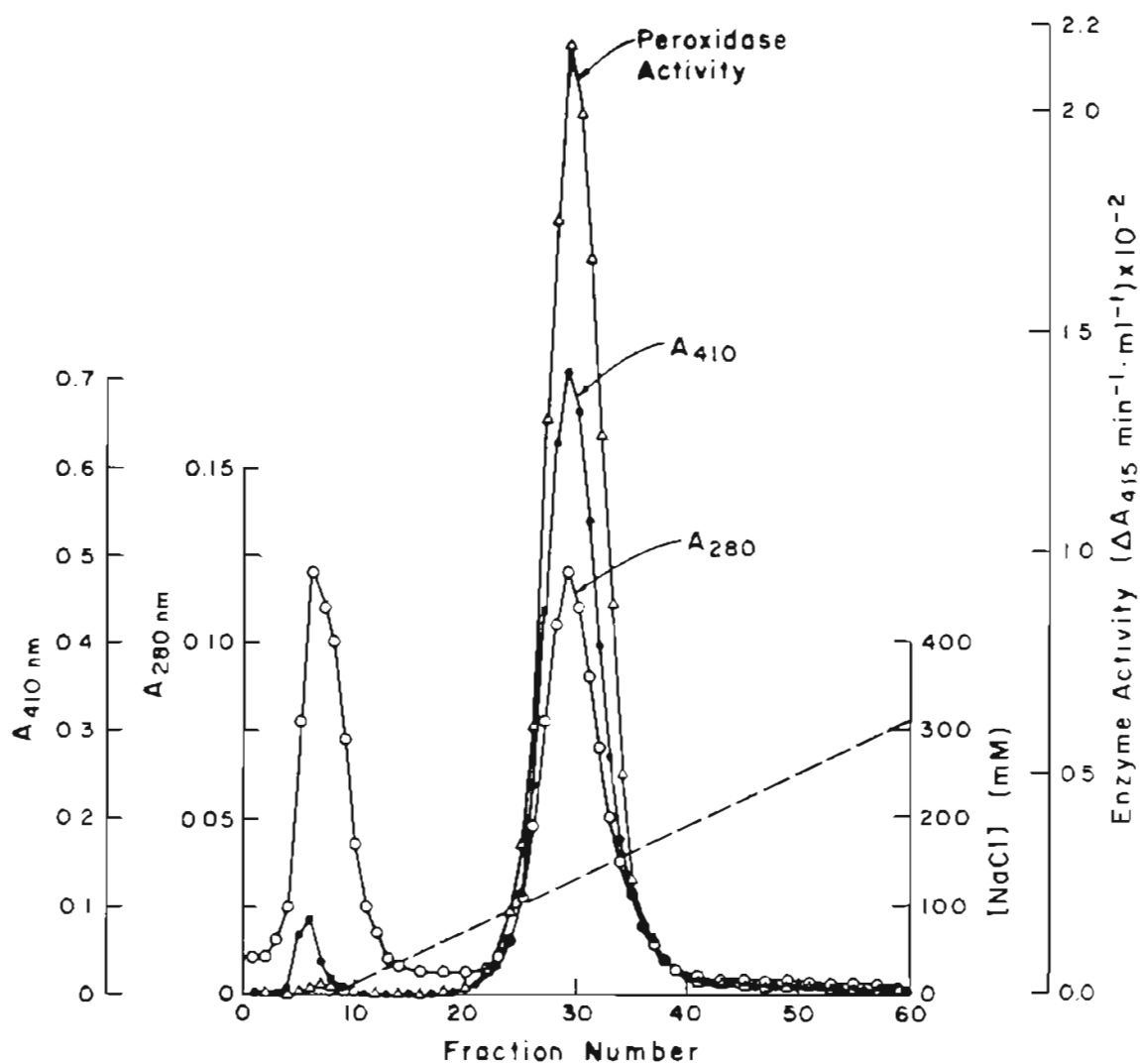


Fig. 5-1. Chromatography of the *Phanerochaete* peroxidase on Blue Agarose. After adsorption of the protein solution the column (0.8 x 18 cm) was washed with 10 ml of buffer B. The enzyme was eluted with a linear gradient consisting of 50 ml of buffer B in the mixing chamber and 50 ml of the same buffer containing 400 mM of NaCl in the reservoir. Fractions (1.5 ml) were collected. Absorbance at 410 nm (●); absorbance at 280 nm (O); peroxidase activity as measured by ABTS oxidation (Δ). Experimental procedures and assays are described in the text.

activity. The results in Fig. 5-2 show the Sephadex G-100 elution profile for the peroxidase. The enzyme eluted as a single symmetrical peak. The  $M_r$  of the enzyme as determined by the method of Andrews<sup>169</sup> was  $\sim 41,000$ . The concentrate contained 1 ml (1.6 mg protein/ml) with a specific activity of  $1.2 \Delta A \text{ min}^{-1} \mu\text{g}^{-1}$ . The yield of the purified enzyme was 25% with respect to the crude filtrate, with a 12-fold increase in specific activity. However, the amount of enzyme in the culture filtrate of individual batches varied considerably. The peroxidase retained full activity for at least three months when stored at  $-20^\circ\text{C}$  as a concentrated solution (1 mg/ml).

#### Homogeneity and molecular mass of the extracellular peroxidase.

When the purified enzyme was subjected to SDS-PAGE only one band of protein was detected (Fig. 5-2 inset). A plot of the mobilities of standard proteins versus log of molecular mass gave a  $M_r$  for P. chrysosporium peroxidase of  $\sim 46,000$ .

Spectral properties of the peroxidase. The absorption spectrum of the native enzyme, shown in Fig. 5-3A, has a maximum of 406 nm with smaller peaks at 502 and 632 nm. This spectrum did not change with the addition of 10 mM Na lactate or 0.1 mM  $\text{MnSO}_4$ . The millimolar extinction coefficient at 406 nm of 129.3 was determined by measuring the protein concentration of a solution of known absorbance and using an  $M_r$  of 46,000. The  $A_{406}/A_{280}$  is equal to 6.15.

The absorption spectra of the reduced and reduced-CO forms of the enzyme are shown in Fig. 5-3B. The maxima of the  $\text{Na}_2\text{S}_2\text{O}_4$ -reduced enzyme are at 433 and 554 nm with a shoulder at 585 nm. The reduced-CO complex had maxima at 423, 541, and 570 nm. The Soret maximum shifts

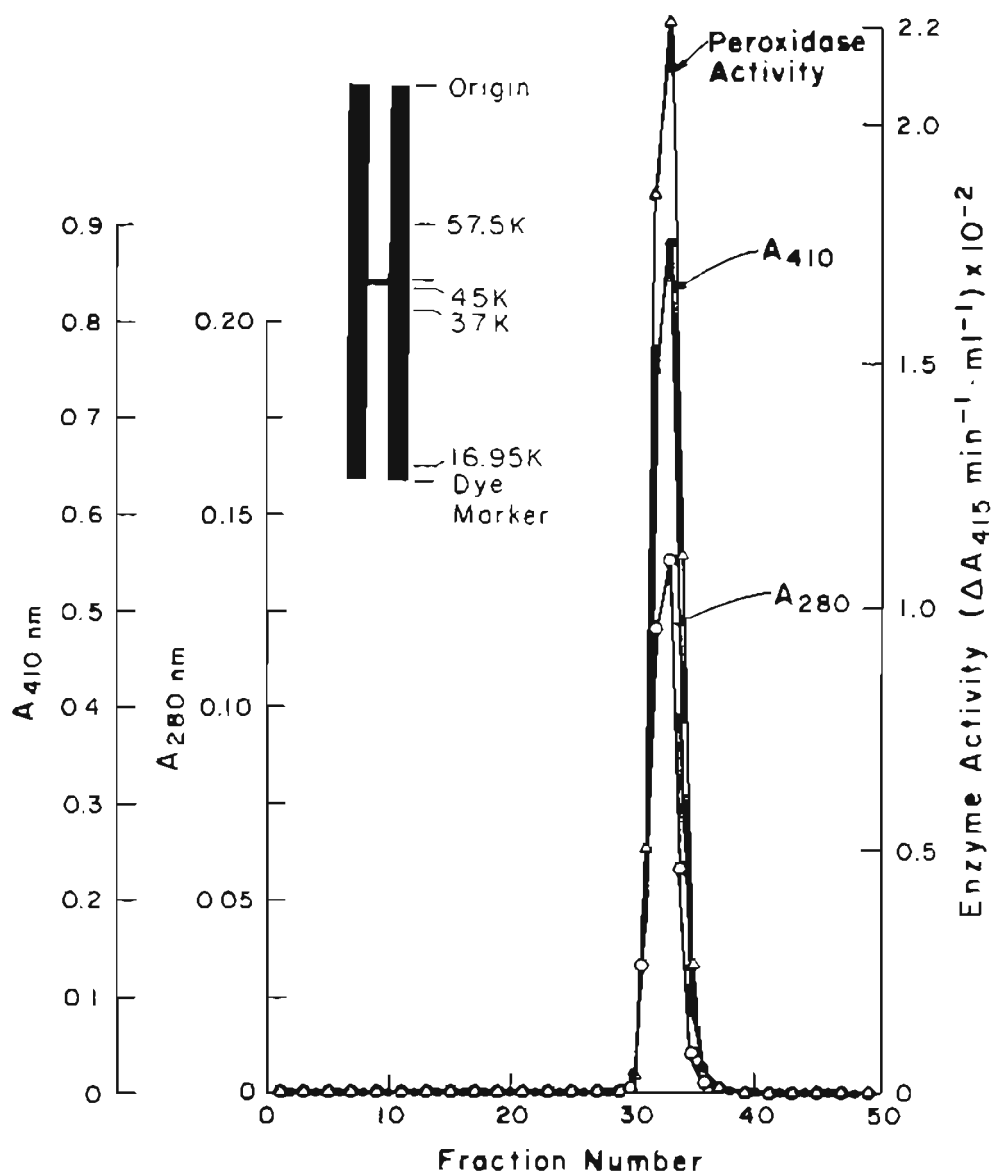


Fig. 5-2. Gel filtration of the *Phanerochaete* peroxidase on Sephadex G-100 (50 x 1.5 cm). The column was eluted with buffer B + 100 mM NaCl, and fractions (1.5 ml) were collected and assayed. Absorbance at 410 nm (●); absorbance at 280 nm (○); peroxidase activity (Δ). Inset: SDS-PAGE of the purified enzyme. The gel was stained with Coomassie blue R-250. The following marker proteins were electrophoresed on a separate gel: catalase, 57.5K; ovalbumin, 45K; glyceraldehyde-3-phosphate dehydrogenase, 37K; and myoglobin, 16.95K. Other procedures used are described in the text.

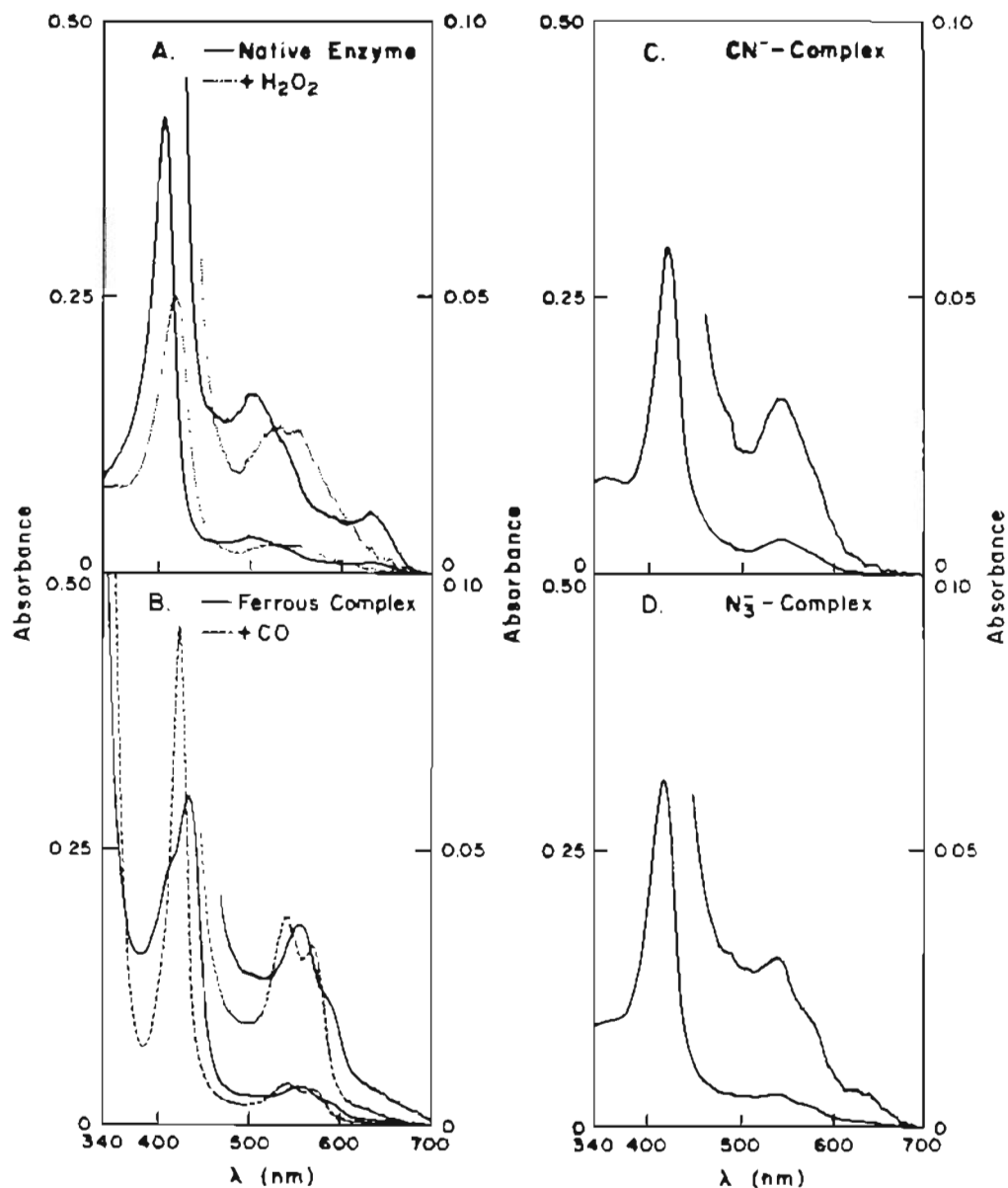


Fig. 5-3. Absorbance spectra of the enzyme and enzyme complex: (A) Spectra of the native enzyme (—) and  $\text{H}_2\text{O}_2$  adduct (···). (B) Spectra of the ferrous enzyme (—) and ferrous-CO complex (---). (C) Spectrum of the cyanide complex. (D) Spectrum of the azide complex. Spectra of the enzyme and enzyme complexes in 20 mM Na succinate buffer, pH 4.5, were recorded with the same buffer in the reference cuvette. Procedures for preparation of the complexes were as described in the text.



to 421 nm in the ferric cyanide complex (Fig. 5-3C) and to 417 nm in the ferric azide complex (Fig. 5-3D). An additional peak appeared at 352 nm in the cyanide complex.

The addition of 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  to the native enzyme at room temperature produced a spectrum with a maximum of 420 nm (Fig. 5-3A). This spectrum was stable for at least 5 min and did not change upon the addition of 10 mM KCN. With the addition of 0.1 mM Mn(II) to the  $\text{H}_2\text{O}_2$  complex the spectrum returned rapidly to that of the native enzyme. The pyridine hemochrome spectrum of the peroxidase gave absorbance maxima at 419, 475, 524, and 557 nm. The pyridine hemochrome spectrum of myoglobin determined under the same conditions appears identical. Using an  $\epsilon$  of  $33.2 \times 10^3$  at 557 nm,<sup>177</sup> a heme content of 0.94 mol heme/mol enzyme was calculated.

Optimal conditions for enzyme activity. Using either ABTS or phenol red as a substrate, the pH profile was essentially symmetrical, with an optimum at pH 4.5. Activity was negligible below pH 2.0 and above pH 6.5. Activity of the purified enzyme was dependent on  $\text{H}_2\text{O}_2$  (Fig. 5-4A), with maximal activity occurring at approximately 75  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . Higher concentrations of  $\text{H}_2\text{O}_2$  decreased the enzyme activity for the oxidation of ABTS. The enzyme has an absolute requirement for Mn(II) (Fig. 5-4B). Activity (90%) occurred at approximately 100  $\mu\text{M}$  Mn(II); further addition of Mn(II) up to 1 mM had only a slight stimulating effect. Although manganese sulfate was used routinely, identical results were produced when the sulfate counterion was replaced by chloride or acetate. No activity was observed when Mn(II) was replaced by Fe(III), Cu(II), Co(II), Ni(II), Zn(II), Mg(II),

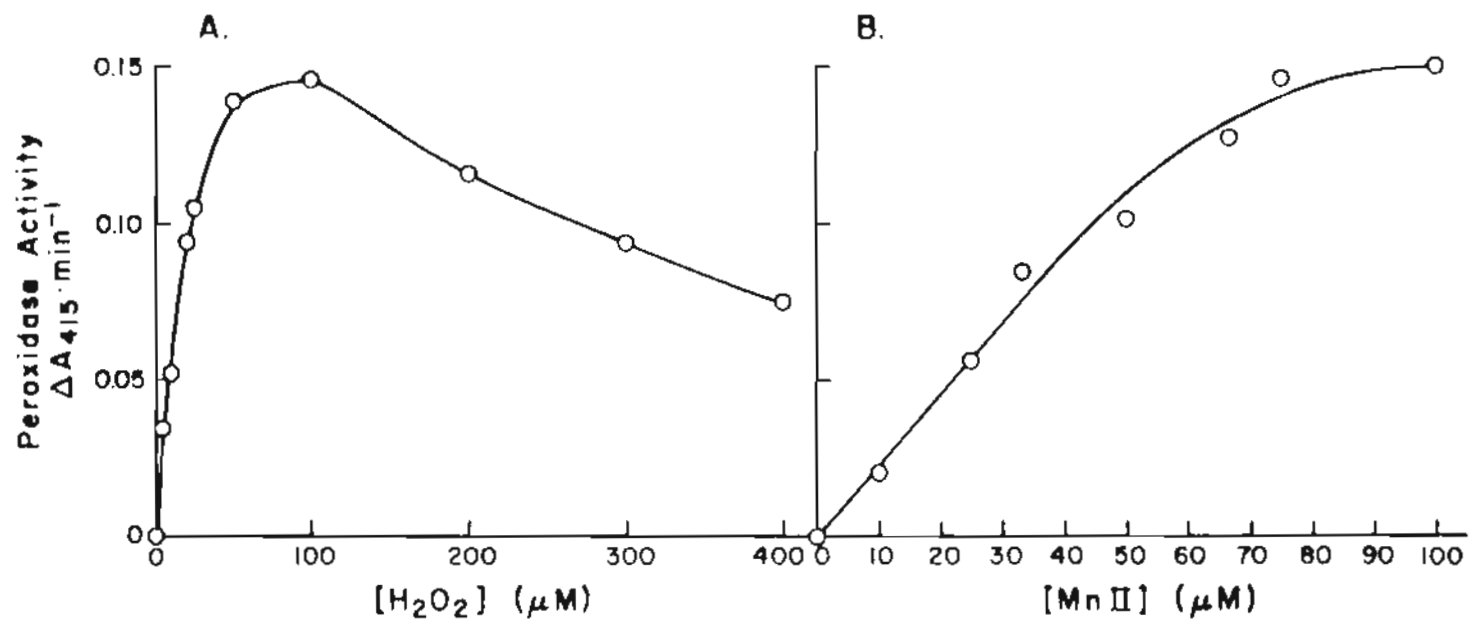


Fig. 5-4. Dependence of *Phanerochaete* peroxidase on  $\text{H}_2\text{O}_2$  and  $\text{Mn(II)}$ . (A) Reaction mixture (1 ml) consisted of enzyme (70 ng) in buffer B containing 50 mM Na lactate, 0.1 mM  $\text{MnSO}_4$ , 3 mg/ml gelatin, and 40  $\mu\text{g/ml}$  ABTS. Reactions were initiated with  $\text{H}_2\text{O}_2$ , and the initial rate of oxidation was determined by following the increase in absorbance at 415 nm. (B) Same as above except that 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  was used and the  $\text{MnSO}_4$  concentration was varied as indicated.

or Al(III), each at 0.1 mM. All peroxidase activity was lost after dialyzing against 20 mM Na succinate, indicating that the Mn(II) is loosely bound. Activity was rapidly restored upon addition of Mn(II) to an otherwise complete reaction mixture.

ABTS oxidation by the peroxidase was dependent on  $\alpha$ -hydroxy acids (Fig. 5-5A, Table 5-1). The dependence of peroxidase activity (ABTS oxidation) on malate and lactate is shown in Fig. 5-5A. The optimal concentrations of malate and lactate were 10 and 50 mM, respectively. A comparison of the stimulatory activity of several  $\alpha$ -hydroxy acids and related compounds is shown in Table 5-1. At a concentration of 10 mM, L-malate was most effective. In the presence of 2-hydroxybutyrate, enzyme activity was approximately 43% of the maximum.

Bulk protein also stimulated peroxidase activity to varying degrees depending on the substrate used. The effect of gelatin on the oxidation of ABTS and phenol red is shown in Fig. 5-5B. Other proteins, such as bovine serum albumin, egg albumin and ribonuclease A, were as effective as gelatin.

Substrate specificity. The rates of oxidation of a variety of compounds by the P. chrysosporium peroxidase and HRP are compared in Table 5-2, ABTS, phenol red, and guaiacol, standard peroxidase substrates,<sup>168,174,175</sup> were tested along with a variety of other oxidizable compounds to determine requirements and rates of oxidation.

Numerous dyes were oxidized by the P. chrysosporium peroxidase (Table 5-2). These dyes differed in their chemical structures and ease of oxidation. Poly B and Poly R are degraded by ligninolytic cultures of P. chrysosporium<sup>131</sup> and by the culture medium from these

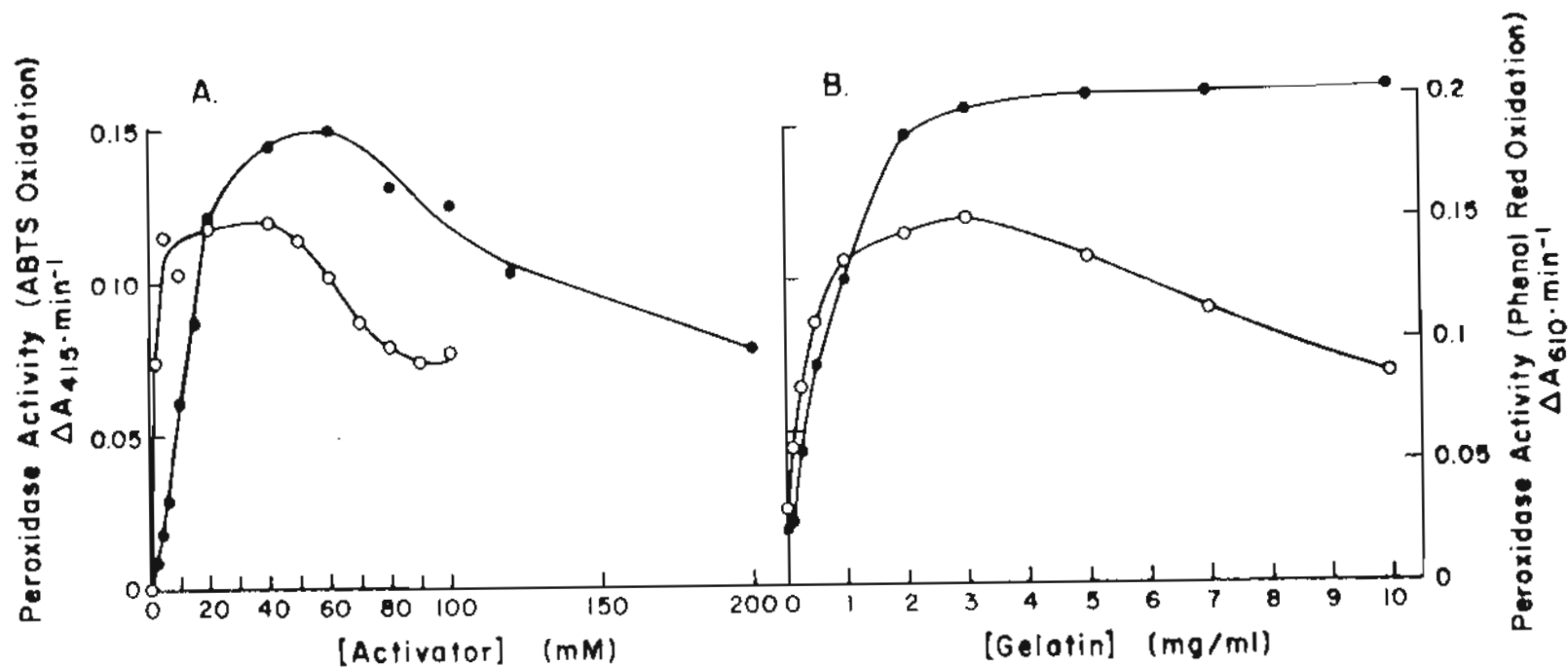


Fig. 5-5. Stimulation of *Phanerochaete* peroxidase activity by  $\alpha$ -OH acids and protein. (A) The reaction mixture (1 ml) consisted of enzyme (70 ng) in buffer B containing 0.1 mM  $\text{MnSO}_4$ , 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 40  $\mu\text{g/ml}$  ABTS. The concentrations of L-malate (o) and L-lactate (●) were varied as indicated. (B) The reaction mixture (1 ml) consisted of enzyme in buffer B containing 0.1 mM  $\text{MnSO}_4$ , 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , 50 mM L-lactate, and either 40  $\mu\text{g/ml}$  ABTS (●) or 100  $\mu\text{g/ml}$  phenol red (o).

TABLE 5-1  
 Relative rates of stimulation of the  
Phanerochaete peroxidase by  $\alpha$ -hydroxy  
 acids and related compounds<sup>a</sup>

Compound (10 mM)	Percentage of malate
L-Malate	100
D-Tartrate	91
Citrate	68
L-Lactate	68
DL-Lactate	66
Phenyl-DL-Lactate	61
Glycolate	59
2-Hydroxybutyrate	43
3-Hydroxybutyrate	0
Glycine	0
Alanine	0
Acetate	0
Pyruvate	0
2-Chloropropionate	0
None	0

<sup>a</sup>Rate of oxidation of ABTS (40  $\mu$ g/ml) by 0.1  $\mu$ g/ml of peroxidase. Assays were carried out in 1 ml of 20 mM Na succinate, pH 4.5, containing 0.1 mM  $MnSO_4$ , 50  $\mu$ M  $H_2O_2$ , and 3 mg/ml gelatin as described in the text.

TABLE 5-2  
Oxidation of various substrates by Phanerochaete peroxidase<sup>a</sup>

Substrate	(nm)	Abs min <sup>-1</sup> enzyme <sup>-1</sup> μg	Stimulation <sup>b</sup>			Phanerochaete <sup>c</sup> peroxidase/ HRP
			Mn(II)	Lactate	Protein	
ABTS	415	2.29	A <sup>d</sup>	A	8.7	0.37
Guaiacol	450	0.14	A	1.3	14.0	0.74
Phenol Red	610	1.42	A	8.4	4.8	0.78
Pinacyanol <sup>e</sup>	603	25.1	100	19.3	15.7	1.3
Poly B-411	597	0.49	A	23.2	1.5	44.4
Indigo trisulfonate	603	0.93	A	22.1	5.8	186 <sub>f</sub>
Mn(II)	256	0.02	-	-	-	250 <sub>f</sub>
NADH	340	3.51	A	3.0	2.5	300 <sub>f</sub>
Poly R-481	517	0.002	A	3.4	0.3	558 <sub>f,9</sub>
Variamine blue RT salt	376	1.30	A	3.0	1.8	>1000 <sub>f</sub>

<sup>a</sup>Rates of oxidation were followed spectrophotometrically at room temperature at the wavelengths indicated in the table. The complete reaction mixture (1 ml) contained 0.1 μg of Phanerochaete peroxidase or 0.086 μg of HRP and H<sub>2</sub>O<sub>2</sub> (50 μM) in 50 mM Na succinate, pH 4.5, and where indicated, 50 mM Na Lactate, 0.1 mM MnSO<sub>4</sub>, and 3 mg/ml gelatin. Concentrations of substrates were Poly B, Poly R, Phenol Red, guaiacol, and NADH, 100 μg/ml; ABTS, 40 μg/ml; indigo trisulfonate, 25 μg/ml; variamine blue RT salt, 10 μg/ml; pinacyanol chloride, 5 μg/ml. Oxidation of 0.1 mM Mn(II) was measured in 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 20 mM Na acetate adjusted to pH 4.5.

<sup>b</sup>Stimulation was calculated as the ratio of the rate of oxidation with one cofactor absent/rate of oxidation using the complete reaction mixture.

<sup>c</sup>Ratio of rate of oxidation of substrates by Phanerochaete peroxidase over rate of oxidation by HRP under the conditions used.

<sup>d</sup>A, no oxidation was observed in the absence of this compound.

<sup>e</sup>Assay mixture also contained 10% v/v dimethyl sulfoxide.

<sup>f</sup>In order to observe the reaction, a 100-fold excess of HRP was required. The ratio calculation was based on equivalent amounts of enzyme.

<sup>9</sup>Phanerochaete peroxidase (1 μg/ml) was used. The reactions were carried out in the absence of gelatin.

cultures.<sup>91,146</sup> The rates of oxidation of the various dyes used by HRP and the P. chrysosporium peroxidase differed significantly. The ratio of the rate of P. chrysosporium peroxidase over the rate of HRP varied from >1000 with variamine blue salt to 0.37 with ABTS. The three compounds which were degraded faster by HRP were ABTS, phenol red, and guaiacol. These compounds are easily oxidized by both peroxidases. The oxidation of all of the dyes by the P. chrysosporium peroxidase was strongly dependent on Mn(II), and was further stimulated to varying degrees by lactate and bulk protein. For example, the oxidation of Poly B-411, pinacyanol, and indigo trisulfonate were stimulated 20-fold by lactate, while the oxidations of Poly R-481 and variamine blue salt were stimulated only 3-fold. Likewise, protein stimulated pinacyanol oxidation 16-fold but it inhibited Poly R-481 oxidation. Increasing the concentration of the Poly dyes 2-fold in the presence of gelatin led to their precipitation. This may explain the anomalous protein effect with Poly R.

The oxidation of NADH by the fungal peroxidase is also shown in Table 5-2. Mn(II) is required for this reaction; however, H<sub>2</sub>O<sub>2</sub> is not. Lactate increased the rate of NADH oxidation but only after a 2-min lag period which was not observed in the absence of lactate. The P. chrysosporium peroxidase catalyzed the oxidation of NADH 300-fold faster than HRP. The enzymatic oxidation of NADH was completely inhibited by the introduction of 1000 units of catalase.

In the presence of H<sub>2</sub>O<sub>2</sub>, this peroxidase readily oxidized Mn(II), as measured by the formation of Mn(III)-pyrophosphate (Fig. 5-6). The spectrum of the Mn(III)-pyrophosphate (max at 258 nm) formed

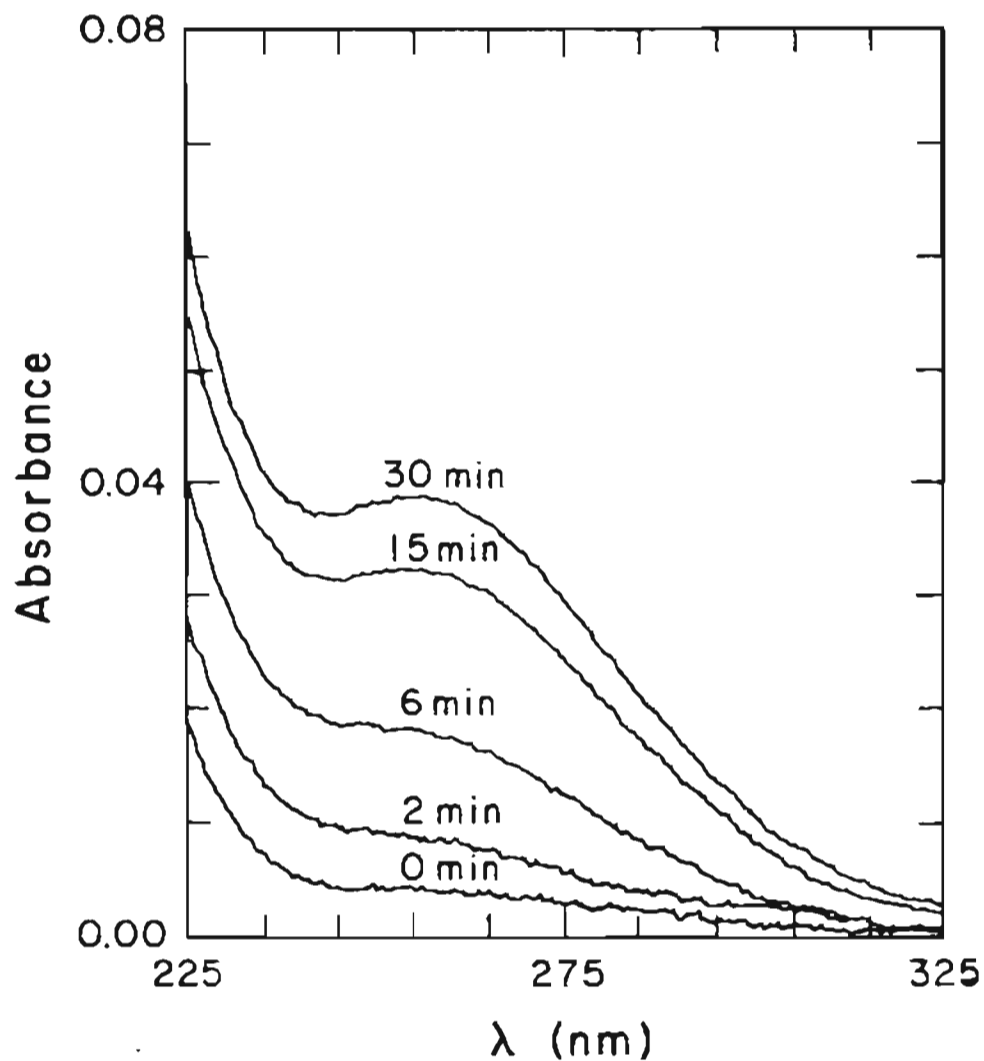


Fig. 5-6. Production of Mn(III)-pyrophosphate from the oxidation of Mn(II) by *Phanerochaete* peroxidase. The reaction mixture (1 ml) contained 1  $\mu$ g enzyme, 0.1 mM  $\text{MnSO}_4$ , and 0.5 mM Na pyrophosphate in 10 mM Na phosphate, pH 6.0. The reaction was started with the addition of  $\text{H}_2\text{O}_2$  to a final concentration of 10  $\mu$ M. Spectra were recorded at the indicated intervals.



closely resembles that produced previously upon the oxidation of Mn(II) to Mn(III) by superoxide anion.<sup>176</sup> The rate of formation of Mn(III) is significantly faster when the reaction is carried out at pH 4.5 in 20 mM Na acetate. In that case the Mn(III)-pyrophosphate complex spectrum had a maximum at 256 nm. At pH 4.5 the P. chrysosporium peroxidase oxidized Mn(II) approximately 250-fold faster than an equivalent amount of HRP (Table 5-2).

Inhibitors. Cyanide and azide ions strongly inhibited the P. chrysosporium peroxidase. Fifty percent inhibition was produced by 6  $\mu$ M cyanide and by 8  $\mu$ M azide. EDTA inhibited the peroxidase by sequestering Mn(II). The inhibition by as much as 1 mM EDTA could be reversed by the addition of excess Mn(II). In the presence of 1 mM Na pyrophosphate led to 26, 43, and 52% inhibition, respectively, of the ABTS oxidation by the peroxidase. The presence of superoxide dismutase (1000 units) in the reaction mixture only led to 12% inhibition of activity.

#### Discussion

Previous reports indicated that the fungal biodegradation of lignin is an oxidative process. P. chrysosporium degrades lignin approximately threefold faster when cultured under 100% O<sub>2</sub> rather than air,<sup>119,120,159</sup> and chemical and spectroscopic studies on heavily degraded lignin<sup>156-158,179</sup> have indicated that biodegradation is oxidative. The nature and structure of lignin—optically inactive, heterogeneous, and random<sup>180</sup>—also suggests that nonspecific enzymes are probably involved in its degradation. The first candidates

considered for this process were phenol oxidases. The relationship between ligninolytic activity and phenol oxidase activity has been pondered for 50 years,<sup>123,181</sup> and all white rot basidiomycetes examined produce phenol oxidases.<sup>20,121,181,182</sup> Both ligninolytic activity and phenol oxidases are expressed during secondary metabolism by P. chrysosporium.<sup>118,125</sup> Additionally, genetic studies<sup>124,125</sup> have demonstrated a relationship between ligninolytic activity and phenol oxidase production by this organism. Most previous work on the role of phenol oxidases in lignin degradation has been performed with Coriolus versicolor laccase.<sup>20,126,183</sup> However, the role of phenol oxidase in the degradation of lignin by P. chrysosporium can only be understood using the enzyme(s) derived from this organism.

The initial steps in the purification of the P. chrysosporium peroxidase were the same as those used to purify the P. chrysosporium diarylpropane oxygenase.<sup>147</sup> After an acetone precipitation the peroxidase was separated from the diarylpropane oxygenase and other proteins by passage through DEAE-Sepharose in the presence of Cetrimide. The detergent was removed from the peroxidase preparation by passage through octyl-Sepharose. Blue Agarose chromatography then separated one major peroxidase activity peak (Fig. 5-1). Subsequent gel filtration yielded a homogeneous protein which had an  $M_r$  of 46,000 by SDS-PAGE.

Although a previous study found no endogenous peroxidase in P. chrysosporium,<sup>134</sup> we found that the amount of peroxidase represents a significant fraction of the total protein in the culture filtrate. As shown in Fig. 5-3 and Table 5-3, the absorption spectrum of the native

TABLE 5-3  
Spectroscopic characteristics of Phanerochaete  
and horseradish peroxidases<sup>a</sup>

	Phanerochaete peroxidase		
	Wave-length (nm)	$\epsilon$ (mM cm <sup>-1</sup> )	HRP <sup>b</sup> (nm)
Native enzyme	406	129.3	403
	502	9.9	498
	632	3.4	640
Reduced enzyme	433	109	440
	-	-	510 sh
	554	13	557
	585	sh	580
Reduced-CO complex	423	140.2	423
	541	11.6	542
	570	9.9	572
N <sub>3</sub> <sup>-</sup> complex	417	99.6	416
	-	-	495 sh
	542	9.4	534
	580	6.2	565
	640	sh	635
CN <sup>-</sup> Complex	352	27.4	-
	421	91.9	422
	487	sh	439
	546	9.9	580
Native Enzyme +H <sub>2</sub> O <sub>2</sub>	420	77.8	420 <sup>c</sup>
	530	7.7	527
	554	7.7	554
Pyridine hemo-chrome	419	188.4	-
	475	12.4	-
	524	18.4	-
	557	30.8	-

<sup>a</sup>Procedures for preparation of the Phanerochaete peroxidase and enzyme complexes were as described in the text. Spectra were recorded at room temperature in a cell with a 1-cm light path vs. a blank containing only 20 mM Na succinate, pH 4.5.

<sup>b</sup>From Ref. 173

<sup>c</sup>HRP compound II.

enzyme strongly suggests the presence of a heme (iron porphyrin) prosthetic group. The Soret absorption maximum shifts to 433 nm in the reduced enzyme and to 423 nm in the reduced-CO complex. These absorption maxima are very similar to those reported for HRP.<sup>173</sup> The pyridine hemochrome<sup>147,177</sup> yields a spectrum (Table 5-3) which is indistinguishable from that of myoglobin, thus indicating that the prosthetic group of the peroxidase is iron protoporphyrin IX. Based upon the previously reported extinction for the pyridine hemochrome at 557 nm,<sup>177</sup> 0.94 molecule of heme/molecule of peroxidase was determined. Both  $\text{CN}^-$  and  $\text{N}_3^-$  readily bind to the native (ferric) enzyme, indicating an available coordination site or a loosely associated sixth ligand such as  $\text{H}_2\text{O}$ . The similarity of the absorption spectrum of the native enzyme to that of high spin HRP,<sup>173</sup> in addition to the evidence for  $\text{CN}^-$ ,  $\text{N}_3^-$ , and CO binding, suggests that the both native enzyme and the ferrous enzyme contain high-spin iron. The spectrum of the hydrogen peroxide complex of the P. chrysosporium peroxidase (Fig. 5-3A), with a Soret maximum at 420 nm, resembles that of HRP compound II.<sup>173</sup> However, the extinction of the Soret is considerably decreased compared to that of HRP(II).<sup>173</sup> The complex is stable in the presence of 10 mM  $\text{CN}^-$ ; similar results have been reported for HRP compound II.<sup>184</sup>

Hydrogen peroxide is required for the oxidation of all of the substrates examined except NADH. The optimal concentration of  $\text{H}_2\text{O}_2$  is  $\sim 75 \mu\text{M}$ . Loosely bound Mn(II) strongly stimulates the oxidation of a variety of substrates by the enzyme. With most compounds examined, the rate of oxidation in the absence of Mn(II) is negligible. To our

knowledge, only four manganese-requiring enzymes involved in oxidation-reduction have been studied in detail: manganese superoxide dismutase, isolated from a variety of sources;<sup>185-187</sup> 3,4-dihydroxyphenylacetate-2,3-dioxygenase from Bacillus brevis;<sup>188</sup> pseudocatalase from Lactobacillus plantarum;<sup>189</sup> and an Mn-containing protein involved in photosynthesis.<sup>190,191</sup> Although Mn(II) stimulates the oxidation of NADH<sup>192,193</sup> and 2,3-diketogulonate<sup>194</sup> by HRP, H<sub>2</sub>O<sub>2</sub> is not added exogenously as a cosubstrate in those reactions. Furthermore, Mn(II) appears to have no effect on most peroxidase-catalyzed reactions. Therefore, the P. chrysosporium enzyme can be considered a novel Mn(II)-dependent peroxidase. It has been suggested that Mn(II) stimulates the HRP oxidation of NADH by acting as a bridge between NADH and superoxide anion.<sup>192</sup> However, at pH 4.5 many of the substrates of the P. chrysosporium peroxidase are either neutral or positively charged (pinacyanol, variamine blue salt). Since the presence of Mn(II) stimulates the oxidation of these substrates, it is unlikely to function as a bridge in this system. The oxidation of 2,3-diketogulonate by HRP requires Mn(II)<sup>194</sup> which apparently is oxidized to Mn(III) during the course of the reaction. HRP can slowly oxidize Mn(II) directly<sup>195</sup> as measured by the formation of a Mn(III)-pyrophosphate complex. The inhibition produced by pyrophosphate, a known chelator of Mn(III),<sup>176,195</sup> in the Phanerochaete peroxidase system suggests that Mn(III) is involved in the enzyme mechanism. In addition, the P. chrysosporium peroxidase readily oxidizes Mn(II) to Mn(III) as measured by the formation of a Mn(III) pyrophosphate complex (Fig. 5-6). The reduction of the enzyme-peroxo adduct by Mn(II) also

suggests that Mn is, in turn, oxidized during this reaction. Finally, preliminary experiments (J. K. Glenn and M. H. Gold, in preparation) indicate that Mn(III)-acetate alone readily oxidizes the organic substrates (listed in Table 5-2) in aqueous solution. This strongly suggests that the enzyme produces Mn(III) which in turn oxidizes the substrates. The oxidation of a variety of organic substrates by Mn(III) has been reported.<sup>196-198</sup>

Lactic acid stimulates P. chrysosporium peroxidase oxidation of various substrates from 2- to 20-fold (Table 5-2). Neither the charge of the substrate nor its ease of oxidation appear to correlate with the extent of lactate stimulation. As shown in Table 5-1, other  $\alpha$ -hydroxy acids can replace lactate in the stimulation of ABTS oxidation. The results in Fig. 5-5A show the dependence of ABTS oxidation on L-lactate and L-malate. Malate produces optimal stimulation at a significantly lower concentration. Additional experiments are planned to determine whether  $\alpha$ -hydroxy acids stabilize the Mn(III) produced in this reaction.

Protein also stimulates the oxidation of most substrates by the P. chrysosporium peroxidase (Table 5-2). As with lactate, the degree of stimulation by protein is dependent on the substrate. Pinacyanol oxidation is stimulated 16-fold, but Poly B oxidation is stimulated only 2-fold. The results indicate that the bulk protein is probably not merely stabilizing the enzyme. Stimulation of other oxidases by protein has been reported.<sup>199</sup> The concentration of Poly R is near that at which precipitation with gelatin occurs. This may be a factor in the inhibition of Poly R oxidation by protein. The P. chrysosporium

peroxidase oxidized NADH in the absence of  $H_2O_2$  (Table 5-2). The inhibition of this reaction by catalase suggests that  $H_2O_2$  is necessary for the reaction, and is probably produced by the oxidation of NADH. This was confirmed by adding ABTS to the reaction and observing the oxidation of this dye in the presence of NADH, the enzyme, and Mn(II). Under the conditions used (Table 502) the P. chrysosporium peroxidase catalyzed the oxidation of NADH 300-fold faster than an equivalent amount of HRP.

A number of studies have recently indicated that P. chrysosporium generates  $H_2O_2$  during the time when it is competent to degrade lignin.<sup>135,136,200</sup> Thus, this extracellular peroxidase may be a source of  $H_2O_2$  by oxidizing the extracellular NADH and NADPH found in culture medium of ligninolytic cells.<sup>201</sup> This would be analogous to the production of  $H_2O_2$  in plant cells. In the latter case a wall-bound peroxidase apparently oxidizes NADH produced by a malate dehydrogenase.<sup>202</sup> It is noteworthy that the pH optimum of the P. chrysosporium peroxidase (4.5) is the same as that reported for the pH optimum of lignin degradation by this organism.<sup>118</sup>

The P. chrysosporium peroxidase oxidizes many substrates at rates considerably faster than HRP (Table 5-2). For example, the rate of oxidation of Poly R and variamine blue salt were 558 and >1000 times greater. The only compounds which are degraded faster by HRP are the peroxidase substrates ABTS, phenol red, and guaiacol. The apparent greater capacity of the P. chrysosporium peroxidase for the oxidation of more stable substrates may be related to its role in lignin biodegradation. Although P. chrysosporium peroxidase appears to

catalyze most typical peroxidase oxidations, its dependence on Mn(II) and its stimulation by  $\alpha$ -hydroxy acids and protein suggest that it may perform additional functions. Studies are in progress to determine the physiological role of this enzyme in  $H_2O_2$  production and lignin biodegradation by the fungus, and to better determine the roles of Mn(II), Mn(III), lactate, and protein in the catalytic mechanism of this enzyme.



## CHAPTER 6

Manganese Oxidation is the Principal Function of an  
Extracellular Mn-Dependent Peroxidase from the  
White Rot Fungus Phanerochaete chrysosporium

## Introduction

Lignin is a heterogeneous, random, phenolic polymer produced by higher plants.<sup>33</sup> Because white rot fungi are the most active lignin degraders, most research on lignin biodegradation has been conducted with these organisms. Owing to lignin's large size, the initial steps in its biodegradation are likely to be extracellular, but until recently, little was known of the mechanism used by white rot fungi to degrade lignin. Chemical analysis of degraded lignin and physiological studies have shown that lignin biodegradation is oxidative,<sup>110,156,158</sup> and that high levels of oxygen stimulate ligninolytic activity.<sup>119,120,159</sup> This was supported by observations that the production of hydrogen peroxide and phenol oxidase activity are both associated with white rot fungi capable of degrading lignin.<sup>123-125,134,136,203,204</sup> The close correlation between ligninolytic activity and dye degradation<sup>131</sup> also suggested a role for phenol oxidases in lignin degradation.

Progress in understanding the mechanism of lignin degradation came with the discovery that an extracellular enzyme preparation from ligninolytic cultures of the white rot fungus Phanerochaete chrysosporium was capable of partially degrading lignin<sup>145,167</sup> and oxidizing a variety of lignin model compounds<sup>145</sup> in the presence of

$H_2O_2$ . This preparation also had phenol oxidase activity.<sup>91</sup> Purification of this activity revealed two types of peroxidases.<sup>146</sup> One of the enzymes (ligninase, diarylpropane oxygenase, lignin peroxidase) is a heme protein<sup>147,171</sup> which exists in multiple forms.<sup>205</sup> This enzyme is capable of oxidative cleavage of carbon-carbon and carbon-oxygen (ether) bonds in a variety of lignin model compounds.<sup>205</sup> Much effort has been devoted to assessing the physical properties and mechanism of ligninase.<sup>147,171,205-210</sup>

The second peroxidase, which has received much less attention, had the dye oxidizing ability of a classical phenol oxidase, but only when manganese(II) was present.<sup>146</sup> In a previous paper we described the purification and physical properties of this manganese-dependent extracellular peroxidase from *P. chrysosporium*.<sup>130</sup> Like other peroxidases,<sup>211-213</sup> this *Phanerochaete* peroxidase is a heme protein, which can oxidize NADH and a wide variety of dyes. However, it oxidized some dyes 1000-fold faster than horseradish peroxidase.<sup>130</sup> Besides requiring Mn(II) for activity, the extracellular peroxidase was stimulated by alpha-hydroxy acids and protein. The amount of stimulation by lactate and protein was strongly dependent on the substrate. We also reported that the fungal peroxidase rapidly oxidized Mn(II) to Mn(III), as measured by the formation of Mn(III)-pyrophosphate complex. In addition, pyrophosphate inhibited enzymatic dye oxidation. All of these observations led us to suggest that this enzyme was not directly oxidizing dyes, but was producing Mn(III), which then oxidized other compounds.

The variable degree of stimulation of this peroxidase activity by

lactate and protein<sup>130</sup> could not be explained easily by assuming that these cofactors were directly activating the enzyme. In addition, the high levels of Mn(II), lactate, and protein required to produce optimal activity (e.g. 60 mM lactate),<sup>130</sup> and the shapes of the activity vs concentration curves also suggested that these cofactors were not behaving as allosteric effectors. If Mn(III) is the direct oxidant of the other substrates, then the lactate and protein might stimulate oxidation by interacting with the Mn(III) or the other reactant rather than with the enzyme.

In this paper we provide evidence that the principal function of this peroxidase is the oxidation of Mn(II). We also show that the product, Mn(III), is capable of oxidizing NADH and a variety of dyes in a manner similar to that of the enzyme system. This is the first report of a purified enzyme which functions to specifically oxidize manganese.

#### Methods and Materials

Enzyme. Purification of the Phanerochaete peroxidase (Mn-peroxidase) was described previously.<sup>130</sup> Concentration of the enzyme was based on an extinction coefficient of 129.3/mM/cm at 406 nm.

Preparation of Mn(II) complexes. Manganese(III) acetate was dissolved in 2M Na lactate, pH 4.5, or in 50 mM Na acetate, 10 mM Na pyrophosphate, pH 7.0, to a final concentration of 4 mM. Both solutions were kept at 0 - 4°C. The Mn(III)-lactate was always freshly prepared and used within one hour.

Spectroscopic procedures. All spectra and absorbance measurements

were performed on a Shimadzu UV-260 spectrophotometer with a 1 nm spectral band width, and cuvettes of 1 cm light path. Scanning speed was 400 nm/min. All reactions were performed at room temperature unless otherwise stated.

The enzymatic oxidation of Mn(II) was followed spectroscopically in 50 mM Na lactate, pH 4.5, containing 1 ug/ml purified Mn-peroxidase, 1 mM MnSO<sub>4</sub>, and 40 μM H<sub>2</sub>O<sub>2</sub>.

To determine the extinction coefficient of Mn(III)-lactate, the concentrated solution described above was diluted into 35 mM Na lactate buffer, pH 4.5, to give a final concentration of 30 μM Mn(III) in 50 mM lactate.

The spectra of the lactate and pyrophosphate complexes at different pH values were determined by diluting the concentrated complex solutions into the appropriate buffers (described below). At 256 nm the absorbance of Mn(III)-pyrophosphate varied about 25% over the pH range. Based on an extinction coefficient of 6200 at pH 6.5,<sup>176</sup>  $\epsilon$ 's were calculated for all pH values. For example, at pH 3.0  $\epsilon$ =5200, and at pH 9.0  $\epsilon$ =7000.

The reduction of the singly oxidized form of Mn-peroxidase (compound II) back to the native form was followed by scanning the Soret region of the enzyme and following the increase in absorbance at 405 nm. The native enzyme (0.85 μM) in 20 mM Na succinate, pH 4.5, was converted to compound II by the addition of an equimolar amount of H<sub>2</sub>O<sub>2</sub> (0.85 μM).

Enzyme pH profile. For Mn(III)-lactate formation, reactions were run between pH 4.5 and 8.5 in 50 mM Na lactate, 25 mM Na phosphate

containing 0.1 mM  $\text{MnSO}_4$  and 0.1  $\mu\text{g/ml}$  enzyme. The Mn(III)-pyrophosphate complex was produced from pH 3.0 to 9.0 in 25 mM Na acetate, 25 mM Na phosphate, 1 mM Na pyrophosphate, 0.1 mM  $\text{MnSO}_4$ , and 1  $\mu\text{g/ml}$  enzyme. Reactions were initiated by the addition of  $\text{H}_2\text{O}_2$  to a final concentration of 100  $\mu\text{M}$ , and the initial rates of formation of the lactate and pyrophosphate complexes were followed at 240 nm and 256 nm respectively. At 0.1  $\mu\text{g/ml}$  the enzyme activity was low enough to ensure that the enzymatic production of Mn(III) rather than formation of Mn(III)-lactate was rate limiting.

The pH profile of enzymatic ABTS oxidation was carried out in 50 mM Na succinate, 50 mM Na lactate, containing 0.1 mM  $\text{MnSO}_4$ , 3 mg/ml BSA, 0.1  $\mu\text{g/ml}$  enzyme, and 40  $\mu\text{g/ml}$  ABTS. The initial increase at 415 nm was used as the measure of ABTS oxidation. The pH profile for NADH oxidation was carried out in 25 mM Na acetate, 25 mM Na phosphate, containing 0.1 mM  $\text{MnSO}_4$ , 0.1  $\mu\text{g}$  enzyme, and 0.13 mM NADH. Enzymatic NADH oxidation was obtained by subtracting the non-enzymatic oxidation, measured in the absence of enzyme, from the total rate. NADH oxidation was followed at 340 nm, and an extinction coefficient of  $6620^{214}$  was used to calculate the true rate of oxidation. Both the ABTS and NADH reactions were initiated by the addition of  $\text{H}_2\text{O}_2$  to a final concentration of 100  $\mu\text{M}$ .

Other enzyme reactions. NADH oxidation was carried out in the absence of exogenous  $\text{H}_2\text{O}_2$  in 50 mM Na succinate, 50 mM Na lactate, pH 4.5, containing 0.1 mM  $\text{MnSO}_4$ , 3 mg/ml BSA, and 0.1  $\mu\text{g/ml}$  Mn-peroxidase. The reaction was initiated by the addition of NADH to a final concentration of 0.1 mg/ml, and oxidation was followed as the

decrease in absorbance at 340 nm. When ABTS was also present (40  $\mu\text{g/ml}$ ), the reaction was performed twice, first observing NADH oxidation at 340 nm, then following ABTS oxidation at 415 nm.  $\text{NAD}^+$  was reduced using glycerol dehydrogenase as previously described.<sup>215</sup> After the NADH oxidation was complete,  $\text{NAD}^+$  reduction was produced by the following method: To 1 ml reaction mixture, 0.1 ml of 0.5 M  $\text{Na}_2$  carbonate containing 0.1 M Na EDTA and 0.8 M ammonium hydroxide was added. The pH was then about 10.1. Glycerol dehydrogenase (0.25 units in 10  $\mu\text{l}$ ) was added, and the reaction initiated by the addition of glycerol solution (30  $\mu\text{l}$ ) to a final concentration of 0.15 M. The final volume was 1.14 ml.

Pinacyanol oxidation was carried out in 50 mM Na succinate, pH 4.5, containing 10% dimethylsulfoxide,<sup>130</sup> 5  $\mu\text{g/ml}$  pinacyanol chloride, and 0.1  $\mu\text{g/ml}$  fungal peroxidase or 0.085  $\mu\text{g/ml}$  horseradish peroxidase. The reaction was initiated by the addition of  $\text{H}_2\text{O}_2$  to a final concentration of 100  $\mu\text{M}$ , and the initial decrease in absorbance at 603 nm was followed. The rate of oxidation was based on an extinction coefficient for pinacyanol we have determined to be  $1.2 \times 10^5$ .

Rates of manganese oxidation used in Lineweaver-Burk plots were based on the oxidation of Mn(II) to Mn(III) complex. Manganese (III)-lactate (measured at 240 nm) was produced in 50 mM Na lactate, pH 4.5, containing 0.1  $\mu\text{g/ml}$  enzyme. Manganese(III)-pyrophosphate (measured at 256 nm) was produced in 50 mM Na acetate, pH 4.5, containing 1 mM pyrophosphate, and 1  $\mu\text{g/ml}$  enzyme. Reactions were initiated by the addition of  $\text{H}_2\text{O}_2$ . Concentration of  $\text{H}_2\text{O}_2$  was based on the extinction coefficient of 40.0/M/cm. at 240 nm.<sup>216</sup> When

Mn(II) concentration was varied to determine the  $K_m$  for manganese,  $H_2O_2$  was kept constant at 100  $\mu$ M. For determination of the  $K_m$  of  $H_2O_2$ , Mn(II) was kept constant at 0.1 mM in pyrophosphate or 1.0 mM in lactate.

As evidence that Mn(III), rather than the Mn-peroxidase, directly oxidized Poly B, the following experiment was performed. Two ml of Na lactate, pH 4.5, containing 1 mM  $MnSO_4$ , 1  $\mu$ g/ml Phanerochaete peroxidase (or 8.5  $\mu$ g/ml horseradish peroxidase - 10-fold greater concentration, based on  $M_r$ ), 10 mM glucose, and 0.01 U glucose oxidase, were placed on the top of a Centricon 10 microconcentrator (10K dalton cutoff). A 0.5% Poly B solution (20  $\mu$ l) was placed in the bottom of the microconcentrator. This was centrifuged at 2000xg at 25°C. After one hour the absorbance of the Poly B solution, diluted by the top solution, was read at 597 nm. Reaction mixtures lacking either enzyme or Mn(II) were run as controls.

Manganese dioxide (the +4 state of Mn) was produced by 1  $\mu$ g enzyme in 1 ml of 5 mM Na succinate, pH 5.0, containing 1 mM  $MnSO_4$ . This solution was placed on top of a 1 mM  $H_2O_2$  solution but separated from it by a membrane of dialysis tubing. This system was left standing for 48 hrs. The presence of  $MnO_2$  was confirmed as previously described<sup>217</sup> using 10%  $H_2O_2$ , 0.1% benzidine in 1% acetic acid, and 0.2% hydroquinone in  $H_2O$ .

Oxidations by Mn(III)-lactate. All reactions were initiated by the addition of the concentrated Mn(III)-lactate solution described above, and initial rates of absorbance change were followed at the wavelengths indicated in Table 6-2. Determination of the pH dependence of ABTS

oxidation by Mn(III)-lactate was carried out in 25 mM Na acetate, 25 mM Na phosphate containing 40 µg/ml ABTS. Other oxidations were carried out in 50 mM Na succinate, pH 4.5. The addition of concentrated Mn(III)-lactate to the reaction mixture produced a lactate concentration of 5 mM. The effect of lactate and protein concentration on ABTS oxidation by Mn(III) was determined by adding Na lactate or BSA as indicated. Oxidation of ABTS was also produced by the addition of solid Mn(III) acetate in an inert matrix of Na<sub>2</sub>SO<sub>4</sub>. Sodium sulfate was used as a carrier of Mn(III) to obtain uniform and reproducible quantities of oxidant. This procedure also prevented the formation of insoluble manganese oxides. Anhydrous Na<sub>2</sub>SO<sub>4</sub> (5 g) was thoroughly mixed with 10 mg Mn(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>3</sub>·3H<sub>2</sub>O. Aliquots of this mixture containing an average weight of 13.5 mg were added to 10 ml of Na succinate buffer, pH 4.5, containing 40 µg/ml ABTS. Immediately after addition of the mixture, the solution was vortexed for five sec. and the absorbance at 415 nm was taken after an additional 15 sec. The initial Mn(III) concentration was 10 µM.

Chemicals. Bovine serum albumin, horseradish peroxidase, glucose oxidase, glycerol dehydrogenase, NADH, Poly B-411 and Poly R-481 were obtained from Sigma Chemical Company. ABTS [2,2-azino-bis(3-ethyl-6-benzothiazolinesulfonate)] was obtained from Boehringer Mannheim Company. Manganese(III) acetate, guaiacol, phenol red, pinacyanol chloride, potassium indigotrisulfonate, and variamine blue RT salt were obtained from Aldrich Chemical Company. All other chemicals were reagent grade or better.



## Results

The spectra produced by the oxidation of Mn(II) in lactate buffer are shown in Fig. 6-1. After one minute a single absorption peak at 238 nm was produced. By the second minute the peak at 238 nm had shifted to 240 nm and a shoulder had appeared at 290 nm. The spectrum after 10 min. was essentially the same as that observed after two min. No spectrum was produced in the absence of Mn(II), enzyme, or  $H_2O_2$ . Also shown in Fig. 6-1 is the spectrum of Mn(III)-lactate produced by dissolving Mn(III) acetate in 2M Na lactate buffer (concentrated Mn(III)-lactate) and diluting this into 50 mM Na lactate, pH 4.5. From this we calculated an extinction coefficient at 240 nm of 7800/M/cm. The spectrum of the Mn(III)-lactate was indistinguishable from the stable spectrum produced enzymatically. Because one molecule of  $H_2O_2$  was two oxidizing equivalents, the Mn-peroxidase has the potential of producing 80  $\mu$ M Mn(III)-lactate or Mn(III)-pyrophosphate when the initial concentration of  $H_2O_2$  is 40  $\mu$ M. Under these conditions the absorbance increase of 0.46 at 240 nm (Fig. 6-1) allowed an estimation of 59  $\mu$ M Mn(III)-lactate, from which we calculated the efficiency of Mn(II) oxidation is 74%. About 72  $\mu$ M Mn(III)-pyrophosphate was produced at pH 4.5 and 7.5. From this result, an efficiency of 90% was determined. At pH 7.5, the rate of formation of Mn(III)-pyrophosphate was reduced from 6.6  $\mu$ moles/min to 6.3  $\mu$ moles/min in the presence of 250 U superoxide dismutase.

The concentrated Mn(III)-lactate solution had a half-life of about ten hours at 20°C, while a similarly prepared pyrophosphate solution was completely stable for over two days.

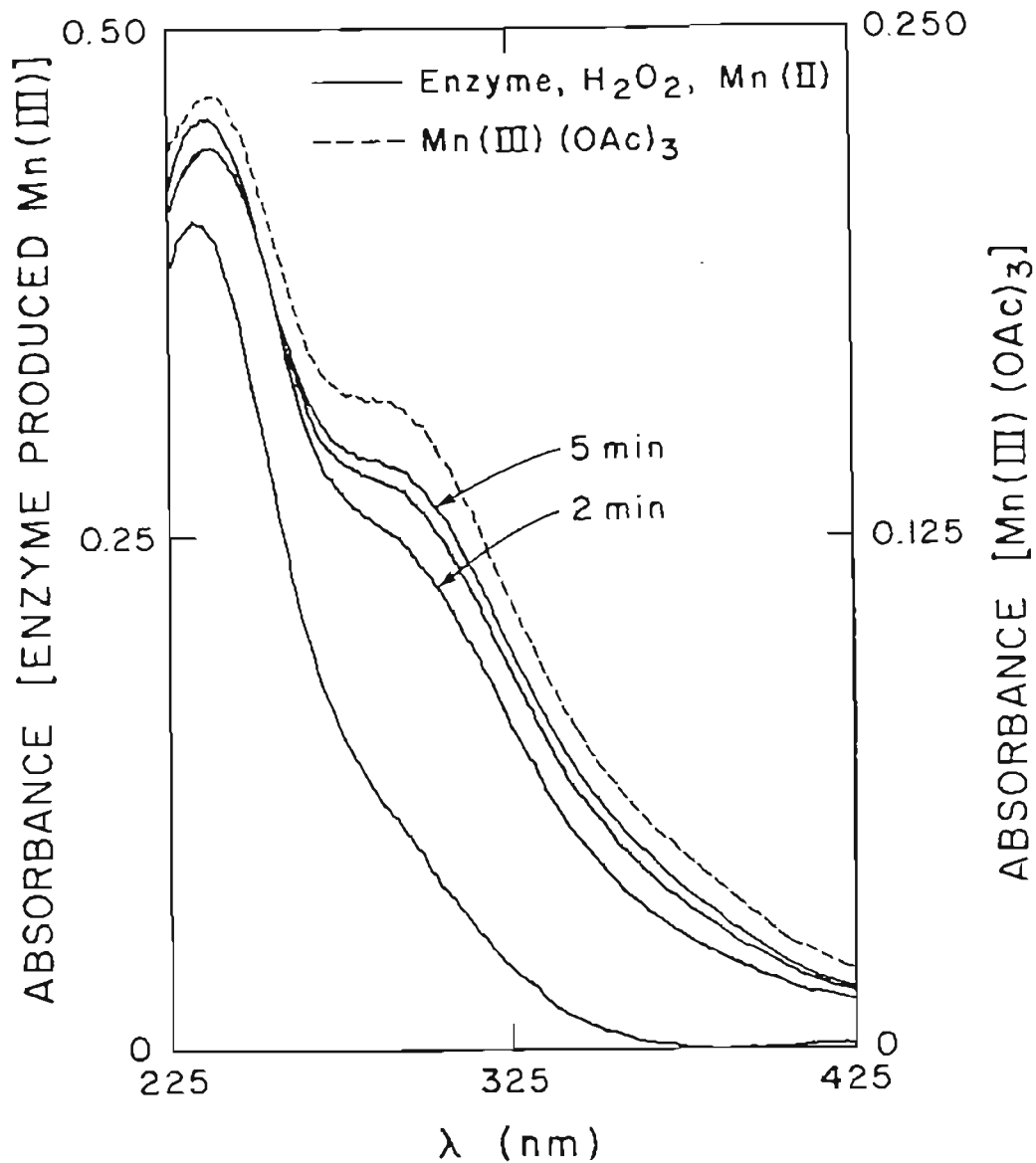


Fig. 6-1. Enzymatic oxidation of manganese(II) to Mn(III)-lactate by the *Phanerochaete* Mn-peroxidase. The reaction mixture (1 ml) contained 1  $\mu$ g enzyme and 1 mM MnSO<sub>4</sub> in 50 mM Na lactate, pH 4.5. The reaction was initiated by the addition of H<sub>2</sub>O<sub>2</sub> to a final concentration of 40  $\mu$ M. Reference cuvette contained the same as above, but without the H<sub>2</sub>O<sub>2</sub>. Spectra were recorded at the indicated intervals. The dashed line is a spectrum of 30  $\mu$ M Mn(III)-lactate in 50 mM Na lactate, pH 4.5, as described in Methods and Materials (scale on right).

Figure 6-2A shows the pH profile of the enzyme, as measured by the formation of Mn(III)-lactate or -pyrophosphate complexes. The spectrum and extinction coefficient at 240 nm for the lactate complex did not change significantly with pH above 4.0. The spectrum of the pyrophosphate complex did not change in appearance, but compensation was made for the small change in extinction coefficient with pH. The peak activity for the production of Mn(III)-lactate, at pH 5.0, was 180  $\mu\text{moles}/\text{min}/\text{mg}$  enzyme. This is considerably faster than the maximum rate of Mn(III)-pyrophosphate production. The formation of Mn(III)-pyrophosphate produced two optima, at pHs of 4.0 and 7.5 with rates of 6.8 and 6.6  $\mu\text{moles}/\text{min}/\text{mg}$  enzyme respectively.

Additional pH profiles were obtained by examining the oxidation of other compounds (Fig. 6-2B). Using optimum conditions for enzymatic oxidation of the dye ABTS (50 mM Na lactate, 50 mM Na succinate, 3 mg/ml BSA, and 0.1 mM Mn(II)),<sup>130</sup> peak activity occurred at pH 4.5. The enzymatic oxidation of NADH in phosphate acetate buffer produced a peak activity of 472  $\mu\text{moles}/\text{min}/\text{mg}$  enzyme at pH 4.0.

Figure 6-2B also shows the rate of oxidation of ABTS by Mn(III)-lactate complex. Between pH 5.5 and 6.5, ABTS is oxidized very slowly. Below this pH range, the rate of oxidation increased rapidly with decreasing pH.

The double reciprocal plot of rate of Mn(III)-lactate formation vs Mn(II) concentration was linear (Fig. 6-3A) and allowed a  $K_m$  of 80  $\mu\text{M}$  to be calculated for Mn(II). The double reciprocal plot of rate of Mn(III)-lactate formation vs  $\text{H}_2\text{O}_2$  concentration was not linear (data not shown). For low  $\text{H}_2\text{O}_2$  concentrations the Y-axis intercept

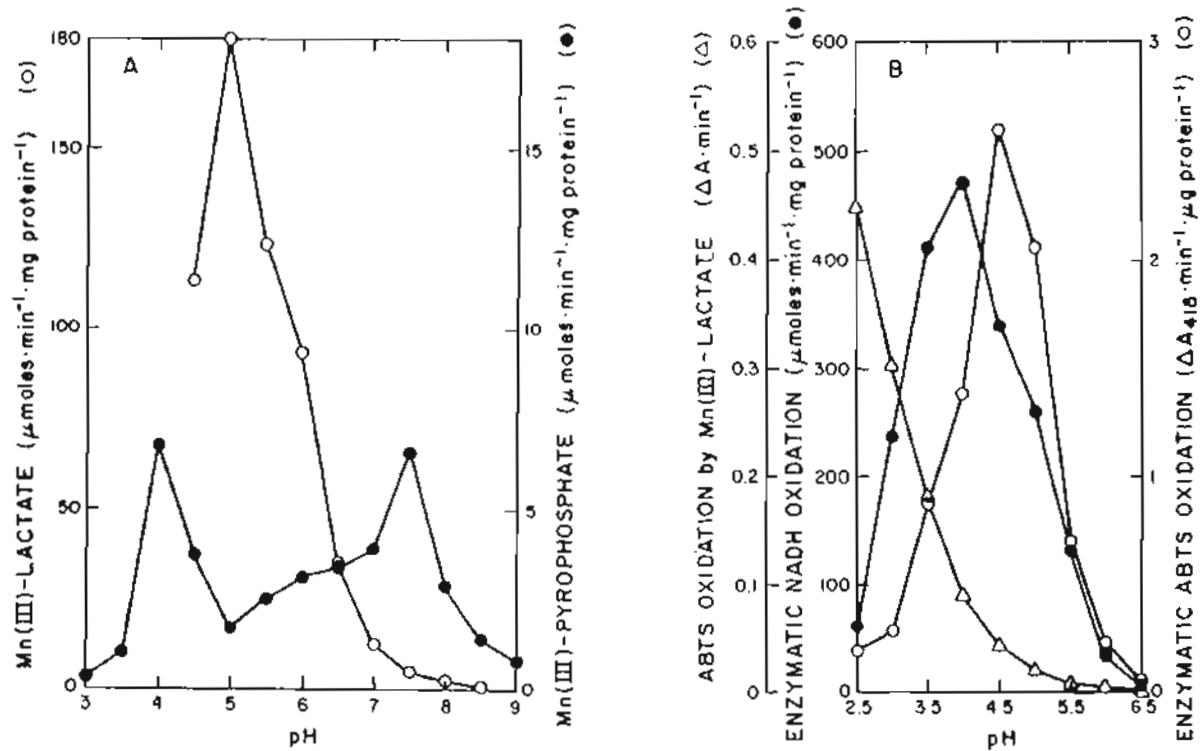


Fig. 6-2. Mn-oxidase and Mn(III)-lactate pH profiles. (A) Initial rate of Mn(III)-lactate (O) or Mn(III)-pyrophosphate (●) formation, based on absorbance increase at 240 and 256 nm respectively. Reaction mixtures (1 ml) contained 0.1 mM  $\text{MnSO}_4$  and enzyme in lactate-phosphate or pyrophosphate-acetate-phosphate buffer as described in Methods and Materials. The reaction was initiated by the addition of  $\text{H}_2\text{O}_2$  to 100  $\mu\text{M}$ . (B) Enzymatic oxidation of NADH (●): The reaction mixture (1 ml) contained 0.1 mg NADH, 0.1  $\mu\text{g}$  enzyme, and 0.1 mM  $\text{MnSO}_4$  in acetate-phosphate buffer. The reaction was initiated by the addition of  $\text{H}_2\text{O}_2$  to a concentration of 100  $\mu\text{M}$  and followed at 340 nm. Non-enzymatic NADH oxidation has been subtracted. Enzymatic oxidation of ABTS (O): The reaction mixture (1 ml) contained 3 mg/ml BSA, 0.1  $\mu\text{g}$  enzyme, 0.1 mM  $\text{MnSO}_4$ , and 40  $\mu\text{g}$  ABTS in 50 mM Na lactate, 50 mM Na succinate. The reaction was initiated by the addition of  $\text{H}_2\text{O}_2$  to 100  $\mu\text{M}$  and followed at 415 nm. ABTS oxidation by Mn(III)-lactate ( $\Delta$ ) was performed in 1 ml acetate-phosphate buffer containing 40  $\mu\text{g}$  ABTS. The reaction was initiated by the addition of concentrated Mn(III)-lactate (Methods and Materials) to a final concentration of 10  $\mu\text{M}$  Mn(III).

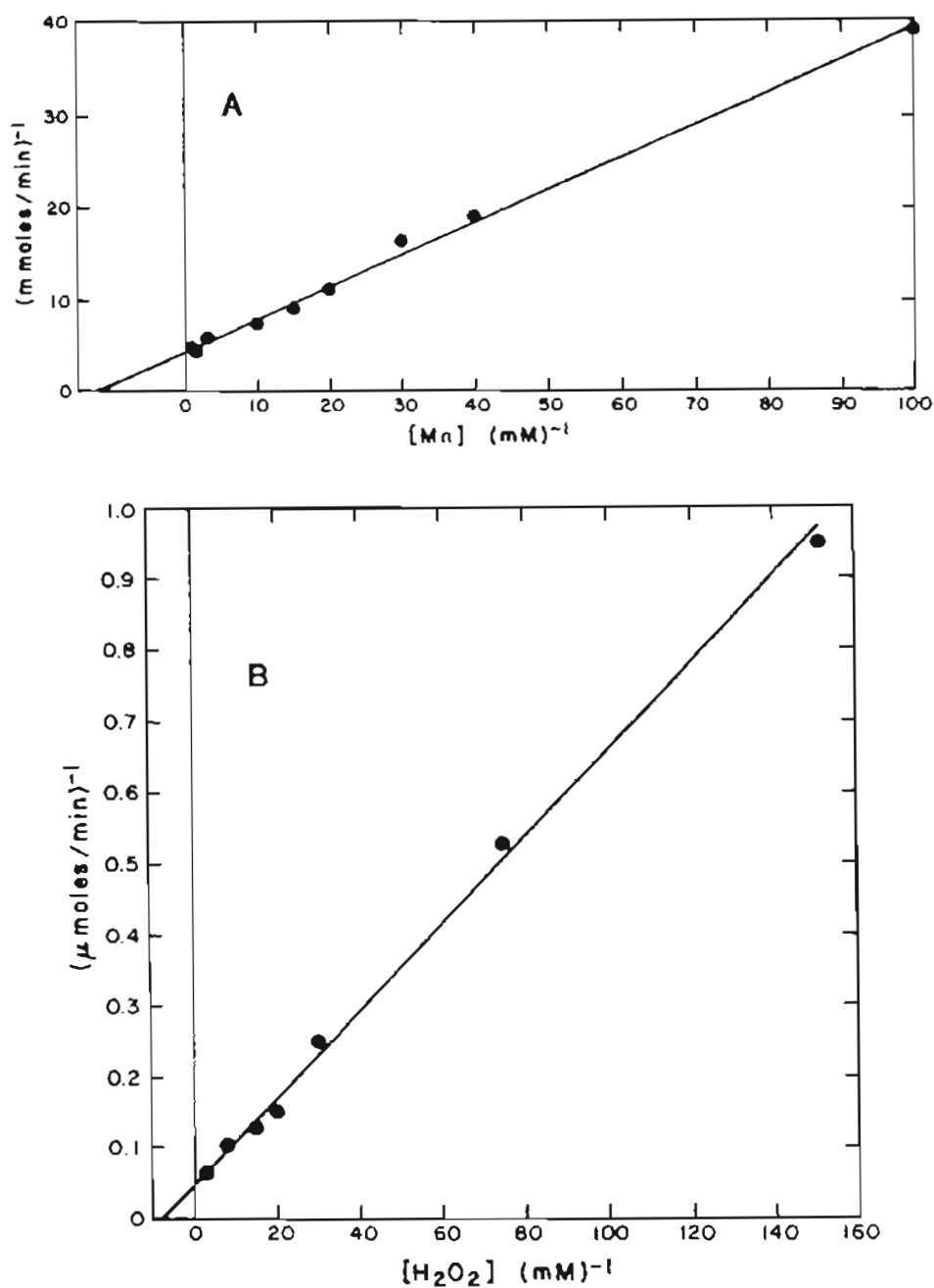
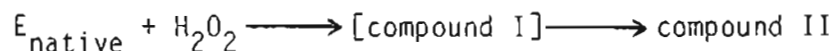


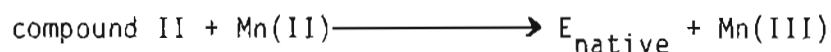
Fig. 6-3. Lineweaver-Burk plots for H<sub>2</sub>O<sub>2</sub> and Mn(II). (A) Manganese(III)-lactate formation followed by absorbance change at 240 nm. Reaction mixture (1 ml) contained 0.1 μg Mn-peroxidase and variable concentrations of MnSO<sub>4</sub> in 50 mM Na lactate, pH 4.5. The reaction was initiated by the addition of H<sub>2</sub>O<sub>2</sub> to 100 μM. Rate was calculated for an enzyme concentration of 1 mg/ml. (B) Manganese(III)-pyrophosphate formation followed by absorbance change at 256 nm. Reaction mixture (1 ml) contained 1 μg Mn-peroxidase, 0.1 mM MnSO<sub>4</sub> and 1 mM pyrophosphate in 50 mM Na acetate, pH 4.5. The reaction was initiated by variable amounts of H<sub>2</sub>O<sub>2</sub>.

extrapolated to a negative value. However, when pyrophosphate complex formation was measured against  $H_2O_2$  concentration, the double reciprocal plot was linear, giving a  $K_m$  of 140  $\mu M$  (Fig. 6-3B).

A further demonstration of the efficient oxidation of Mn(II) by this enzyme was the conversion of an oxidized form of the enzyme back to the native form by the addition of stoichiometric amounts of Mn(II). The Soret peak of the native enzyme, at 406 nm, was shifted to 412 nm after the addition of an equimolar amount of  $H_2O_2$ . We believe this absorbance shift is caused by the formation of compound II.<sup>130</sup>



A further addition of  $H_2O_2$  to 20  $\mu M$  (24-fold excess) caused the Soret peak to shift to 420 nm (data not shown), which is the value reported previously.<sup>130</sup> When an equimolar concentration of Mn(II) was added to the oxidized enzyme there was a slow reduction back to native enzyme (Fig. 6-4).



This reduction was not stoichiometric, however, because as little as one-fourth this equimolar concentration of Mn(II) allowed complete conversion to the native form.

The addition of equimolar amounts of guaiacol, NADH, or Co(II) (0.84  $\mu M$ ) to the oxidized enzyme produced no observable reduction. However at 10-fold greater concentrations, these compounds did lead to reduction (Table 6-1). At 1 mM concentrations, Co(II) reduced the peroxidase at a rate fifty-fold slower than Mn(II). Nickel(II) did not reduce the enzyme.

In the absence of Mn(II), pinacyanol, the most easily oxidized dye

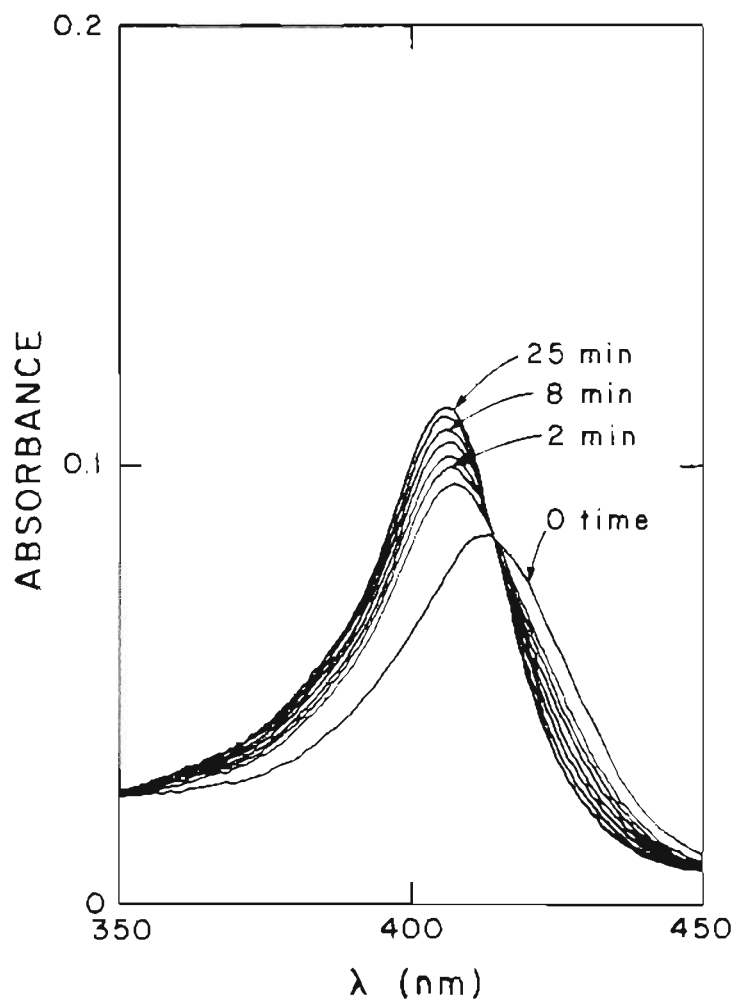


Fig. 6-4. Reduction of the oxidized Mn-peroxidase by Mn(II). Enzyme (0.85 nmole) in 1 ml of 20 mM Na succinate, pH 4.5, was oxidized by the addition of 0.85 nmole  $H_2O_2$ . The reduction of the enzyme was then followed after the addition of 0.85 nmole  $MnSO_4$ . Scans were taken at 1, 2, 3, 5, 8, 15, and 25 min.

TABLE 6-1  
 Rate of reduction of oxidized Mn-peroxidase by Mn(II)  
 and other substrates<sup>a</sup>

Substrate	Concentration	Molar Ratio Reductant:Enzyme	Reduction Rate Compared to Mn(II)
Mn(II)	0.85 $\mu$ M	1 : 1	100% (.080/min/ $\mu$ g) <sup>b</sup>
NADH	"	1 : 1 10 : 1	0 38
Guaiacol	"	1 : 1 10 : 1	0 2
Co(II)	"	1 : 1 500 : 1 1000 : 1	0 10 24
Ni(II)		1000 : 1	0
Mn(II)	850 $\mu$ M	1000 : 1	100% (.820/min/ $\mu$ g) <sup>b</sup>
Co(II)	"	1000 : 1	2

<sup>a</sup>The procedure was as described in Fig. 6-4.

<sup>b</sup>Rate of absorbance change at 405 nm calculated for 1  $\mu$ g enzyme.



we tested, was oxidized by the fungal peroxidase at a rate of 2  $\mu\text{moles}/\text{min}/\text{mg}$  enzyme at pH 4.5. Comparing this value to a rate of 114  $\mu\text{moles}/\text{min}/\text{mg}$  enzyme for the oxidation of Mn(II) in lactate (Fig.6-2A), we calculate that this enzyme catalyzes the oxidation of Mn(II) fifty times faster than pinacyanol. In contrast, horseradish peroxidase oxidizes pinacyanol approximately 300-fold faster than Mn(II).

To show that dye oxidation could be achieved by fungal peroxidase without direct contact between the enzyme and dye, a Poly B solution was placed in the bottom of a centrifugal ultrafiltration concentrator. Enzyme, Mn(II), and a  $\text{H}_2\text{O}_2$  generating system in Na lactate buffer was added to the top of the device. In control experiments, in which Mn(II) or enzyme was absent, absorbance of the poly B solution at 597 nm was 1.18 after one hour centrifugation at 25°C. With 1  $\mu\text{g}/\text{ml}$  Mn-peroxidase and Mn(II) present, the absorbance was reduced to 0.65. If ten-fold more horseradish peroxidase was used instead, the absorbance only dropped to 1.06.

The Mn-peroxidase rapidly oxidized NADH, as measured by the decrease in absorbance at 340 nm (Fig. 6-5). To demonstrate that the decrease in absorbance was due to the oxidation of NADH to  $\text{NAD}^+$ , we reversed the reaction. After a change in pH,  $\text{NAD}^+$  was specifically converted to NADH by glycerol dehydrogenase (Fig. 6-5). Taking into account the increase in volume from 1 ml to 1.14 ml, we could estimate that 94% of the initial NADH was reformed.

Figure 6-6 shows the enzymatic oxidation of ABTS by Mn-peroxidase in the presence of NADH but in the absence of exogenous  $\text{H}_2\text{O}_2$ . There was no net ABTS oxidation until about 90% of the NADH was oxidized.

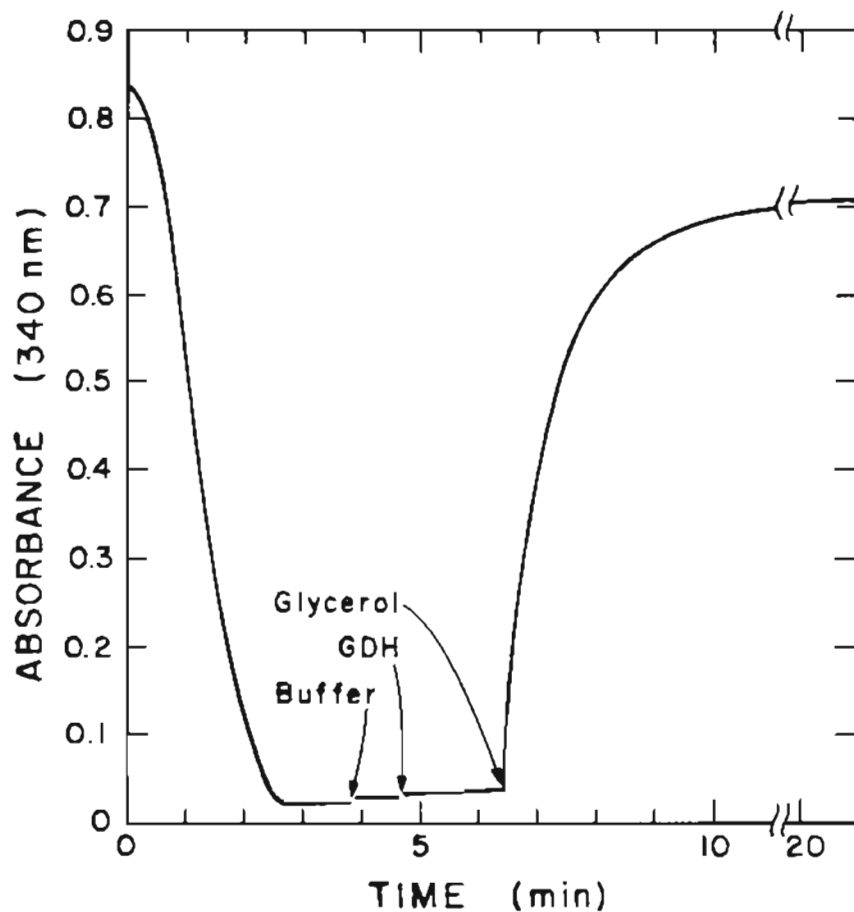


Fig. 6-5. Enzymatic oxidation of NADH to NAD<sup>+</sup> in the absence of H<sub>2</sub>O<sub>2</sub>. Reaction mixture (1 ml) contained 0.1 mM MnSO<sub>4</sub>, 3 mg/ml BSA, and 0.1 μg Mn-peroxidase in 50 mM Na succinate, 50 mM Na lactate, pH 4.5. The reaction was initiated by the addition of 0.1 mg NADH and the reaction was followed at 340 nm. After the reaction had stopped, the pH was changed to 10.1 by the addition of 0.1 ml of Na<sub>2</sub> carbonate containing 0.1 M EDTA and 0.8 M ammonium hydroxide. Glycerol dehydrogenase (GDH) (0.25 units) was then added and the reduction of NAD<sup>+</sup> was initiated by the addition of glycerol to a final concentration of 0.15 M.

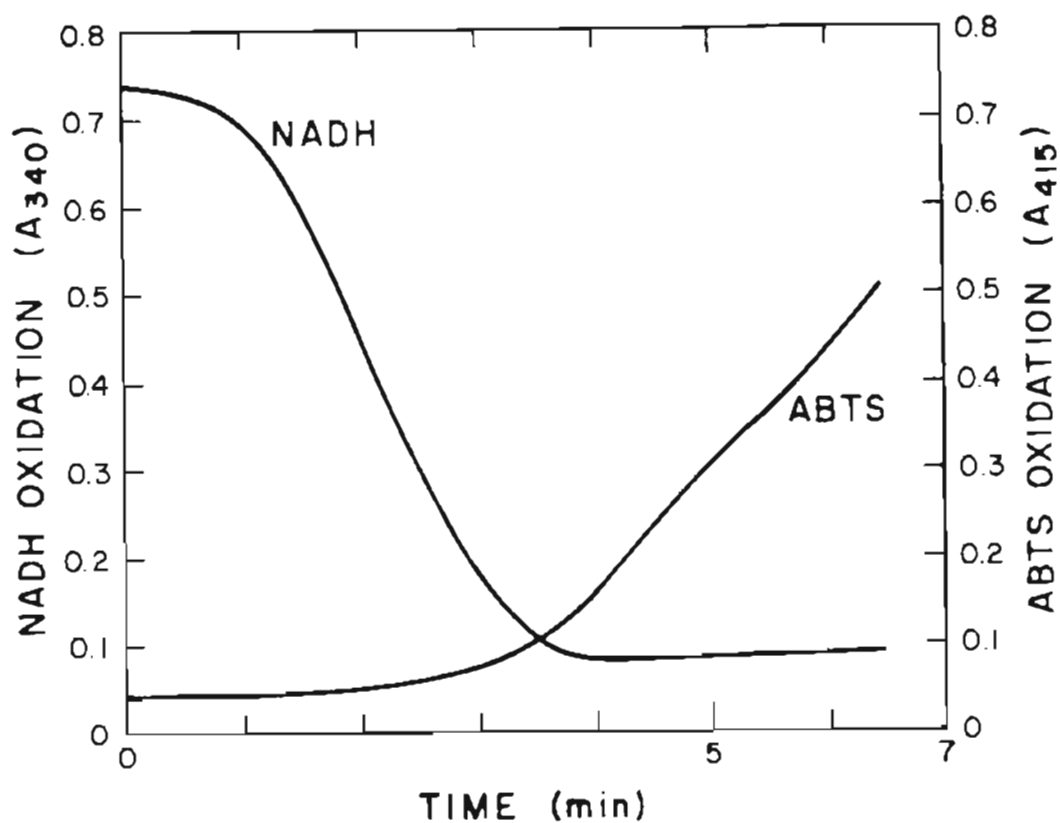


Fig. 6-6. Production of oxidizing agents during the oxidation of NADH by Mn-peroxidase. Reaction mixture contained protein, lactate, succinate, enzyme, and Mn(II) as in Fig. 6-5 plus 40  $\mu\text{g/ml}$  ABTS. The reaction was initiated by the addition of 0.1 mg NADH and oxidation of NADH (340 nm) or the oxidation of ABTS (415 nm) was followed.

As with the enzymatic oxidation of ABTS,<sup>130</sup> protein stimulated ABTS oxidation by Mn(III)-lactate. Optimum oxidation occurred at 1 mg/ml BSA. Increasing the concentration of lactate inhibited the oxidation of ABTS at all protein concentrations (data not shown). Lactate concentrations above 10 mM also inhibited ABTS oxidation by solid Mn(III) acetate (data not shown). NADH oxidation by Mn(III)-lactate was stimulated slightly by lactate at high protein concentration (10 mg/ml), but a slight inhibition occurred when protein was not present (data not shown).

The Mn(III)-lactate complex was capable of oxidizing all of the dyes which were oxidized by the fungal enzyme in the presence of Mn(II) (Table 6-2).<sup>130</sup> However, the relative rates of oxidation differ somewhat for these two systems. In both cases pinacyanol was oxidized the fastest (based on rate of change in peak absorbance). NADH, phenol red, and ABTS were oxidized at about the same relative rates, but Poly R and guaiacol oxidation differed significantly between the two systems. The low rate of enzymatic Poly R oxidation may be caused by direct inhibition of the enzyme by this dye. As we previously reported, Poly R can interact with protein, producing a precipitate.<sup>130</sup>

When  $H_2O_2$  was allowed to diffuse slowly into a weakly buffered solution of fungal enzyme and Mn(II), a thin brown film formed on the inner surface of the chamber, which was not removed by gently rinsing with distilled  $H_2O$ . This was confirmed to be  $MnO_2$  by the characteristic reactions produced by the application of test reagents to the film.<sup>217</sup> These reactions were: bubbling in the presence of

TABLE 6-2  
 NADH and dye oxidation by Mn(III)-lactate and comparison to  
 oxidation by Mn-peroxidase<sup>a</sup>

Substrate	(nm)	A/min	Relative Rate <sup>b</sup> Mn(III)	Enzyme <sup>c</sup>
Pinacyanol	603	1.840	100.0	100.0
Indigotrisulfonate	603	0.600	32.6	3.7
Poly B-411	597	0.368	20.0	2.0
NADH	340	0.253	13.8	13.9
Phenol Red	610	0.140	7.6	5.6
ABTS	415	0.110	6.0	9.1
Poly R-481	517	0.055	3.0	0.008
Variamine Blue RT Salt	376	0.0048	0.3	5.2
Guaiacol	450	0.0001	0.005	0.6

<sup>a</sup>Reaction mixture contained the substrates in 50 mM Na succinate, pH 4.5, in the following concentrations: Poly B, Poly R, phenol red, guaiacol and NADH, 100 µg/ml; ABTS, 40 µg/ml; indigotrisulfonate, 25 µg/ml; variamine blue RT salt, 10 µg/ml; pinacyanol chloride, 5 µg/ml. Reactions were initiated by the addition of concentrated Mn(III)-lactate (Methods and Materials) to a final concentration of 10 µM, and were followed spectrophotometrically at the wavelengths indicated in the table. Initial rate of oxidation was used in all cases except phenol red. In this case, the reaction was allowed to continue for 30 sec before the addition of 20 µl of 5 N NaOH. Absorbance was compared to mixture without Mn(III).

<sup>b</sup>Relative rate of change in absorbance compared to fastest rate.

<sup>c</sup>Based on enzymatic rate of oxidation from Reference 130.

10%  $H_2O_2$  solution, turning benzidine solution blue, and the disappearance of the film in hydroquinone solution.

#### Discussion

In a previous paper<sup>130</sup> we demonstrated that the extracellular manganese-dependent peroxidase from Phanerochaete chrysosporium was capable of rapidly oxidizing Mn(II), as measured by the formation of an Mn(III)-pyrophosphate complex. In this paper we show that a Mn(III)-lactate complex can also be produced by the enzyme in the presence of Mn(II) and  $H_2O_2$  in lactate buffer. We chose to examine the lactate complex because lactate was found to stimulate the enzymatic oxidation of dyes<sup>130</sup> and because this complex has been previously described.<sup>176</sup>

Since a published ultraviolet spectrum of Mn(III)-lactate was not available, we prepared the complex as described in Methods and Materials. The lactate complex exhibits an absorbance maximum at 240 nm with an extinction coefficient of 7800/M/cm. This is similar to the spectrum for the Mn(III)-gluconate complex previously reported.<sup>218</sup> The Mn(III)-lactate stock solution (4 mM Mn(III) in 2 M Na lactate, pH 4.5) had a half-life significantly shorter than the Mn(III)-pyrophosphate stock solution. This is consistent with the previously reported comparison of the relative reactivity of the two complexes.<sup>176</sup> The spectrum of the product produced by the enzyme in the presence of Mn(II) and  $H_2O_2$  was essentially identical with the synthesized Mn(III)-lactate (Fig. 6-1). However, at first a transient spectrum was produced (Fig. 6-1), which suggests an intermediate

complex of Mn(III) containing both lactate and water ligands.

The enzyme was an efficient oxidizer of manganese. At pH 4.5, 75% of the oxidizing capacity of  $H_2O_2$  was converted to Mn(III)-lactate, and 90% of the oxidizing capacity of  $H_2O_2$  was utilized in the formation of Mn(III)-pyrophosphate at both pH 4.5 and 7.5. The lactate complex was less stable than pyrophosphate complex.<sup>176</sup> It also reacted more rapidly with  $H_2O_2$  than Mn(III)-pyrophosphate.<sup>176</sup> The lower efficiency of Mn(III)-lactate formation is consistent with these observations. If the Mn(III) could be stabilized as it was produced by the enzyme, an efficiency of greater than 90% might be observed. In the case of horseradish peroxidase (HRP), the reaction cycle involves a) a two-electron oxidation of the native enzyme by  $H_2O_2$  to form compound I; b) subsequent one-electron reduction by the substrate to form compound II; and c) regeneration of the native enzyme by an additional one-electron reduction.<sup>211</sup> It is likely that the Mn-peroxidase undergoes the same catalytic cycle as HRP. We have only observed the compound II form of the Mn-peroxidase by spectroscopic analysis,<sup>130</sup> but compound I of HRP is unstable<sup>211</sup> and this may also be the case for the fungal enzyme. The high efficiency of conversion with Mn(II) to Mn(III) suggests that the oxidized enzyme can undergo two one-electron reductions. Although superoxide is capable of oxidizing Mn(II),<sup>176</sup> it is unlikely to be involved in the mechanism of this enzyme because high levels of superoxide dismutase produced negligible inhibition.

The rate of Mn(II) oxidation was strongly dependent on pH and the buffer employed. In lactate-phosphate buffer, the Mn(III)-lactate

complex was optimally produced at pH 5.0 (180  $\mu$ moles/min/mg enzyme), while the pyrophosphate complex produced in acetate-phosphate buffer containing 1 mM pyrophosphate has pH optima at 4.0 and 7.5 (6.8 and 6.6  $\mu$ moles/min/mg enzyme, respectively) (Fig. 6-2A). The peak rate of Mn(II) oxidation in lactate buffer was about 25-fold higher than that observed with pyrophosphate. The low rate of oxidation of Mn(II) in the presence of pyrophosphate was probably caused by the formation of a Mn(II)-pyrophosphate complex which may limit the accessibility of the metal ion to the enzyme. Pyrophosphate is fully ionized, and therefore should be the best chelator of Mn(III), above pH 9. The increased stability of the pyrophosphate complex or the formation of a hydroxy complex at higher pH may be a factor in the production of the second optimum at pH 7.5.

Other pH profiles for this enzyme can be obtained by assaying the oxidation of NADH or the dye ABTS. ABTS oxidation was carried out in buffer containing lactate and protein to optimize the rate of oxidation.<sup>130</sup> An optimum at pH 4.5 was obtained. Assuming that Mn(III) rather than the enzyme is the true oxidant, the rate of ABTS oxidation may reflect the rate of formation of Mn(III) and the rate at which Mn(III)-lactate (or other complexes) can react with ABTS. At pH values above 6, Mn(III)-lactate was a poor oxidant of ABTS (Fig. 6-2B). This is consistent with the less positive reduction potential observed for Mn(III)-gluconate at higher pH.<sup>219</sup> The enzyme would be expected to oxidize ABTS slowly above pH 6 even though a significant amount of Mn(III)-lactate was being generated, as observed. NADH was also used to measure rate of Mn(III) production because it was hoped



that, in the absence of a strong chelator, NADH would be rapidly oxidized by Mn(III) at any pH. The rate of NADH oxidation in the presence of  $H_2O_2$  was significantly higher than the rate of Mn(III)-lactate formation below pH 5.5. Optimum NADH oxidation occurred at pH 4.0 (472  $\mu$ moles/min/mg enzyme). This may represent the peak rate of Mn(III) production in the absence of a strong chelator.

Using the double reciprocal plots we obtained a  $K_m$  for Mn(II) of 80  $\mu$ M and a  $K_m$  for  $H_2O_2$  of 140  $\mu$ M. The  $K_m$  for  $H_2O_2$  was based on Mn(III)-pyrophosphate formation because of the Mn(III)-lactate plot was non-linear. This non-linearity was probably due to the greater reactivity of Mn(III)-lactate compared to Mn(III)-pyrophosphate.<sup>176</sup> At higher  $H_2O_2$  concentration, more of the Mn(III)-lactate may be reduced by the  $H_2O_2$ ,<sup>176</sup> giving the appearance of a lower than expected rate of Mn(III)-lactate production.

By measuring the rate at which the compound II form of the Mn-peroxidase was converted to the native form, we estimated the relative rates of oxidation of various compounds by the enzyme. Only Mn(II) was capable of being oxidized at concentrations equimolar with the enzyme (0.85  $\mu$ M). The  $H_2O_2$  complex was totally reduced by much less Mn(II) than could be explained by stoichiometric considerations. This is probably caused by the reduction of Mn(III) to Mn(II) by  $H_2O$ , buffer, or a contaminant. At 10-fold greater concentrations, NADH, guaiacol and cobalt(II) were oxidized slowly. At 1 mM concentrations, Mn(II) was oxidized approximately 50 times more rapidly than Co(II). The slower rate of oxidation of Co(II) may be due to a greater specificity of the enzyme for Mn(II) or a greater oxidation potential

of Co(II).<sup>220</sup> Nickel was not oxidized at any concentration.

As with HRP,<sup>221</sup> the fungal peroxidase converted NADH in the absence of exogenous  $H_2O_2$  to enzymatically active  $NAD^+$ . By reversing the reaction with glycerol dehydrogenase, a 94% recovery of NADH was obtained. If ABTS was present along with NADH, it was not oxidized until 90% of the NADH was consumed. This indicates that either  $H_2O_2$  or Mn(III) accumulated during NADH oxidation. Although it is well established that P. chrysosporium produces  $H_2O_2$  during the time when it is competent to degrade lignin,<sup>134,136,200</sup> the mechanism of  $H_2O_2$  production has not been determined. Recently it has been suggested that sea urchin ovoperoxidase is responsible for the production of extracellular  $H_2O_2$  by functioning as an NAD(P)H oxidase.<sup>222</sup> Excretion of NAD(P)H by sea urchin eggs has not been observed. However, ovoperoxidase is a membrane bound enzyme, and NAD(P)H may be released into the perivitelline space and quickly resequesered.<sup>222</sup> The Phanerochaete Mn-peroxidase may also serve as an NAD(P)H oxidase. Because the Mn-peroxidase is an extracellular enzyme, the production of  $H_2O_2$  by NADH oxidation would probably require extracellular NADH. This may explain the presence of NADH in the medium of P. chrysosporium, and the increase in NADH concentration that occurs during secondary metabolism.<sup>201</sup> Any  $H_2O_2$  produced by this mechanism could be used by other extracellular peroxidases to degrade lignin.<sup>147,171</sup>

A comparison of the capacities of Mn-peroxidase and HRP for manganese and dye oxidation indicates that the two enzymes have strikingly different specificities. Pinacyanol, an easily oxidized

dye,<sup>130</sup> was oxidized at least fifty-fold more slowly than Mn(II) by the Mn-peroxidase, but it was oxidized at least 300-fold faster than Mn(II) by HRP. This suggests that unlike HRP, a true phenol oxidase, the fungal enzyme is principally a manganese oxidase. The use of other dyes indicates this even more strongly. Because manganese greatly stimulated the oxidation of all dyes by the fungal peroxidase,<sup>130</sup> it is likely that the enzyme directly oxidizes all of these compounds at rates considerably slower than for the oxidation of Mn(II). The stimulation by manganese suggests the rapid production of Mn(III) and the rapid reaction of Mn(III) (or its complex) with dyes. The physical characteristics of the fungal enzyme and HRP are similar,<sup>130</sup> and even the substrate specificities of the two enzymes suggests a quantitative rather than qualitative difference between them. In terms of a recent model of oxygen-binding proteins,<sup>223</sup> this comparison suggests that the substrate binding domains of the two enzymes are somewhat different from each other. The site of substrate binding may be much smaller for the Mn-peroxidase, or may contain ligands which favor the binding of manganese over organic compounds.

When Mn(II) was present, the Mn-peroxidase was effective at dye oxidation even though it was separated from the dye by a semipermeable membrane. Horseradish peroxidase also catalyzed dye oxidation in this manner, but at a much slower rate. The dye oxidation is undoubtedly accomplished by Mn(III)-lactate which can cross the membrane (Fig. 6-7). This mechanism may be used by the *P. chrysosporium* to oxidize structures within wood which are inaccessible to direct enzymic attack.

A wide variety of dyes were oxidized by Mn(III)-lactate (Table 6-2).

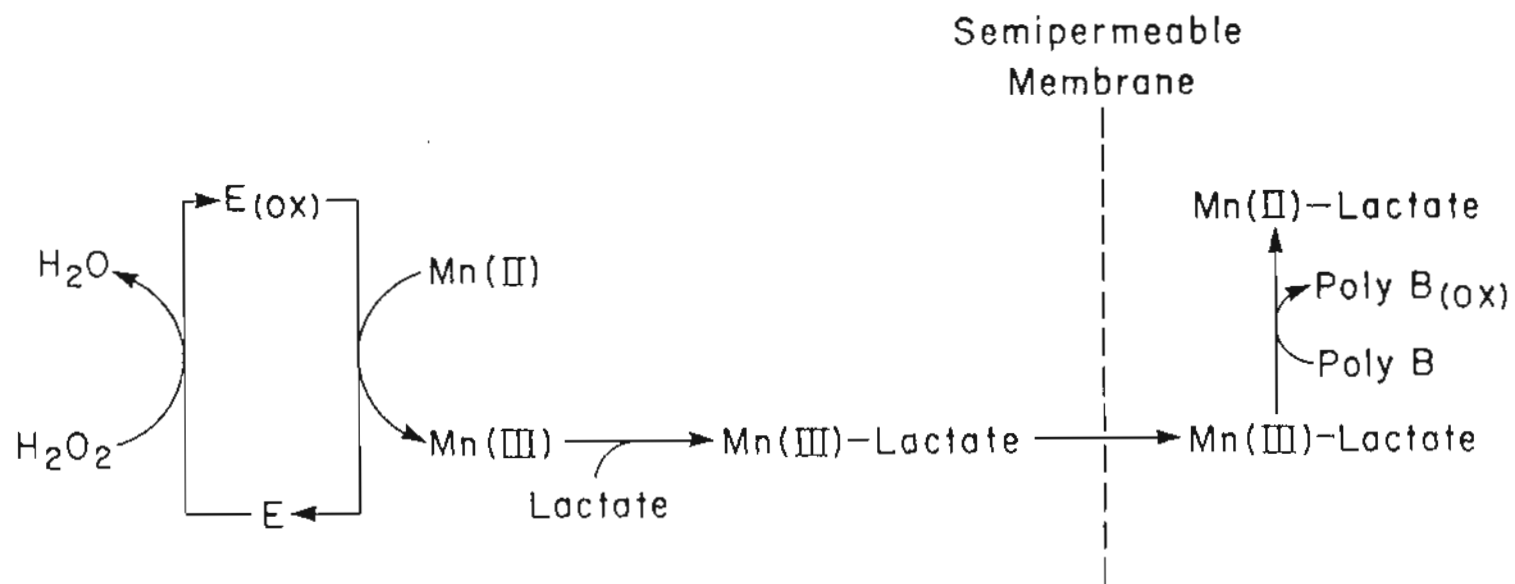


Fig. 6-7. Scheme for the oxidation of the dye Poly B by Mn-peroxidase without direct contact.

Because the enzymatic oxidation of dyes varied with the concentration of lactate and protein,<sup>130</sup> we tested their effect on the oxidation of ABTS and NADH by Mn(III)-lactate. These results had features both similar and different from that observed with enzymatic oxidation. Increasing concentrations of lactate produced increasing inhibition of ABTS oxidation by Mn(III)-lactate. This is in contrast to the marked stimulation of enzymatic oxidation produced by up to 60 mM lactate.<sup>130</sup> Some stimulation of ABTS oxidation by Mn(III)-lactate was produced by 1 mg/ml BSA, but it was not as large as that produced with the enzyme. These results may indicate that Mn(III)-lactate is not the most active species involved in dye oxidation. Perhaps a complex containing both water and lactate ligands is the most effective oxidant. The enzyme produces a continuous supply of Mn(III), and as Fig. 6-1 shows, an intermediate complex of Mn(III) appeared to form before Mn(III)-lactate was observed. Perhaps this intermediate complex is responsible for most of the dye oxidation.

It has been known for many years that small black spots within decaying wood are associated with certain white rot fungi.<sup>224</sup> A strong correspondence has been found between these spots, identified as manganese oxides, and regions of delignified wood.<sup>225</sup> Manganese concentrations in white-rotted wood can be over 100-fold higher than sound wood. This condition is believed to be caused by the oxidation of Mn(II), which leads to the precipitation of manganese oxides. The lower concentration of soluble Mn creates a gradient which results in further manganese transport from the soil into the wood. Numerous examples are known of microorganisms oxidizing Mn(II).<sup>226</sup> They are

found in soil<sup>227</sup> and marine sediments,<sup>228</sup> as well as decaying wood.<sup>225</sup> Normally this biological manganese oxidation occurs around neutral pH, and may provide energy for the organism.<sup>229,230</sup> However, Mn oxidation has also been observed below pH 5.0.<sup>231,232</sup> This is well below the normal pH range for biological oxidation<sup>233</sup> and the pH limit of pH 8.6 for oxidation in inorganic systems.<sup>234</sup> It is unlikely that manganese oxidation at low pH is a source of energy for microorganisms, but the production of insoluble manganese oxides may represent a mechanism for detoxifying high levels of manganese. This may be one of the functions of the fungal Mn-peroxidase.

The presence of Mn(II) in liquid cultures of P. chrysosporium leads to the formation of yellow or brown regions clearly discernible on the mycelium. The higher the initial concentration of Mn(II) in the medium, the larger and more intense these areas become. As with MnO<sub>2</sub>, hydroquinone solutions bleach this color (unpublished data). It is therefore likely that this color is caused by oxides or other insoluble complexes of manganese. Since, as indicated in this paper, the fungal peroxidase was capable of producing MnO<sub>2</sub> under certain conditions, it may be involved in the production of this colored substance.

Although it is known that an extracellular macromolecular substance from Streptomyces<sup>217</sup> is capable of oxidizing Mn(II), to our knowledge, the P. chrysosporium peroxidase is the first purified enzyme with the specific function of manganese oxidation. Manganese oxides were not normally observed in in vitro reactions with Mn-peroxidase. This may be due to the stabilization of Mn(III) by the complexing

agents (lactate, pyrophosphate) or because the  $H_2O_2$  needed to enzymatically oxidize the Mn(II) can also reduce the Mn(III) and Mn(IV) as they are formed.<sup>176</sup>

At least one enzyme from P. chrysosporium has been discovered which may play a direct role in lignin degradation.<sup>147,167</sup> Recent publications describe an extracellular manganese-stimulated enzyme from this organism which can oxidize certain lignin model compounds.<sup>235,236</sup> This appears to be the same enzyme that we discovered and isolated,<sup>130,146</sup> and now describe further in this paper. It is therefore likely that there is a single extracellular manganese-stimulated peroxidase from P. chrysosporium which is capable of oxidizing both dyes and lignin model compounds through the formation of Mn(III). Manganese(III) is a strong oxidant, capable of oxidizing a wide variety of compounds including olefins,<sup>237</sup> organic acids,<sup>238</sup> and sugars.<sup>196</sup> While P. chrysosporium degrades lignin in the absence of manganese,<sup>112</sup> it appears that within decaying wood, Mn in its higher oxidation states may play an important role in the degradation of lignin.

## CHAPTER 7

Investigation of the Physiological Role of the  
Manganese-Peroxidase from Phanerochaete chrysosporium

## Introduction

Only white rot fungi are known to efficiently degrade lignin to carbon dioxide and water.<sup>20</sup> Of this group of organisms, Phanerochaete chrysosporium is the best studied. In P. Chrysosporium, the ability to degrade lignin occurs during the secondary metabolic stage of growth, which develops after the culture medium has been largely depleted of nutrient carbon or nitrogen.<sup>114</sup> In nitrogen limited cultures growth resumes and ligninolytic activity is suppressed upon the addition of ammonium ion or glutamate.<sup>114</sup>

A close correlation has been found between lignin degradation and the decolorization of polymeric dyes by P. chrysosporium.<sup>131</sup> Like ligninolytic activity, decolorization occurs during secondary metabolism and is suppressed by high levels of nutrient nitrogen. High oxygen tension stimulates both activities, and a variety of inhibitors of lignin degradation also inhibit dye decolorization. In addition, a mutant of P. chrysosporium lacking phenol oxidase and ligninolytic activity<sup>125</sup> was also unable to decolorize these dyes, while a revertant regained all three activities (lignin degradation, dye decolorization and phenol oxidase activity).<sup>125,131</sup>

It is now known that during secondary metabolism, several extracellular peroxidases are produced by P. chrysosporium.<sup>102,130,146</sup> These enzymes are believed to be



involved in lignin degradation.<sup>102,145,147</sup> The production of extracellular hydrogen peroxide by white rot fungi during lignin degradation also suggests that peroxidases play a role in ligninolytic activity.<sup>135,136,200</sup> One of the extracellular *P. chrysosporium* enzymes, a manganese oxidizing peroxidase (Mn-peroxidase), is capable of oxidizing many dyes in the presence of Mn(II), including Poly B and Poly R,<sup>130</sup> and is also able to oxidize several lignin model compounds.<sup>236</sup> The principal activity of this enzyme is Mn(II) oxidation.<sup>130</sup> All other oxidations appear to be the result of the enzymatic production of Mn(III), which can then oxidize other compounds<sup>130</sup> (and see Chapter 6).

In this paper we investigate further the role of Mn-peroxidase in ligninolytic activity and dye decolorization. We find that both lignin degradation and dye decolorization can occur in the absence of the Mn-peroxidase, although this enzyme probably plays a role in dye degradation during the first few days of secondary metabolism. While lignin and dye degradation were observed at the same time, the rate of dye degradation does not always appear to be a quantitative measure of ligninolytic activity.

#### Methods and Materials

Cultures of *P. chrysosporium* were maintained on slants as previously described.<sup>150</sup> The strain of *P. chrysosporium* used in this paper, derived from the wild-type strain ME-446, was isolated in this lab (Gold, M.H., unpublished data) and has been used previously.<sup>130,147</sup> For cultures grown in the absence of manganese, slants were identical

except that no manganese was added. Erlenmeyer flasks (250 ml) containing 25 ml of medium were inoculated with  $\sim 5 \times 10^7$  conidia and incubated in high humidity to reduce evaporation from cultures grown in air. The medium, containing 2% glucose and 1.2 mM ammonium tartrate, nutrient salts, and buffered with 20 mM sodium 2,2-dimethylsuccinate, pH 4.5, was as previously described.<sup>118</sup> For the time dependence of peroxidase production, cultures grown under oxygen were stoppered and purged with 100% O<sub>2</sub> immediately after inoculation and at two-day intervals or after samples were removed. Alternatively, and in all other experiments, cultures described as grown under oxygen were grown in air for two days, then stoppered and purged with O<sub>2</sub> every other day as described previously.<sup>59,120</sup> All data presented in this paper represents the average of two cultures.

Ligninolytic activity was measured as the release of <sup>14</sup>C<sub>2</sub>O from <sup>14</sup>C-ring labeled synthetic lignin.<sup>59,125</sup> Lignin (50,000 cpm, specific activity =  $3 \times 10^5$  cpm/mg) was added to five-day cultures grown under O<sub>2</sub>. The flasks were then stoppered and were purged with O<sub>2</sub> at intervals as indicated in the figure. Basic scintillation fluid was used during purging to trap CO<sub>2</sub> as described previously.<sup>59,125</sup> Radioactivity was measured in a Beckman LS-3133P scintillation spectrometer.

Peroxidase activity was assayed by diluting aliquots of culture medium or buffer containing mycelium, 100-fold into 50 mM Na succinate, 50 mM Na lactate, pH 4.5, containing 0.1 mM MnSO<sub>4</sub>, 3 mg/ml gelatin, and 40 µg/ml ABTS [diammonium 2,2-azino-bis(3-ethyl-6 benzothiazoline-sulfonate)]. The reaction was initiated by the addition of H<sub>2</sub>O<sub>2</sub> to

a final concentration of 50  $\mu\text{M}$  and the reaction was followed at 415 nm in a Shimadzu UV-260 spectrophotometer at room temperature, as previously described.<sup>130</sup> No peroxidase activity was observed in the absence of Mn(II), an indication that all peroxidase activity that was being measured was produced by the Mn-peroxidase.

Decolorization of Poly R was measured as described previously.<sup>131</sup> The dyes were added to cultures to a final concentration of 0.02%. Immediately after addition and at three hour intervals, 0.1 ml aliquots were removed and diluted 10-fold into distilled water. Absorbance at 517 and 362 nm was measured, and an absorbance ratio ( $A_{517}/A_{362}$ ) was computed as previously described.<sup>131</sup> Absorbance ratio rather than absorbance was used because it is not affected by dilution of the dye or adsorption of the dye to mycelium.<sup>131</sup> The rate of change in absorbance ratio was computed as the difference in absorbance ratio divided by the time interval between samples (3 hrs). Flasks were purged with oxygen after addition of Poly R.

Chemicals. The  $^{14}\text{C}$ -labeled lignin (DHP, dehydrogenated polymerizate) was synthesized as previously described<sup>125</sup> and dissolved in dimethylformamide. Poly R-481 was purchased from Sigma Chemical Company. ABTS was purchased from Boehringer Mannheim Company. Bactogelatin was purchased from Difco. All other compounds were reagent grade.

## Results

Figure 7-1 shows the levels of extracellular Mn-peroxidase from cultures of P. chrysosporium grown in air (i.e. 21%  $\text{O}_2$ ) and 100%

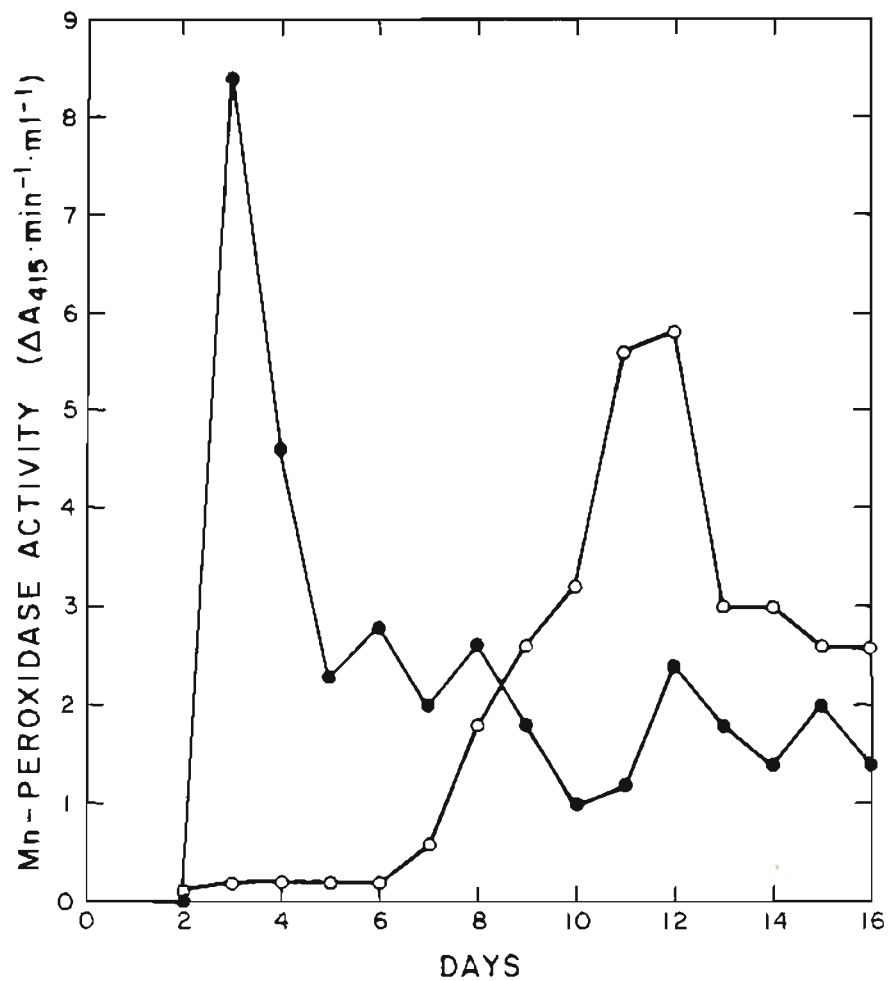


Fig. 7-1. Effect of air and oxygen on the time dependence of Mn-peroxidase activity. Beginning on day 2, aliquots of medium from air-grown (O) or oxygen-grown (●) cultures were removed and assayed for Mn-peroxidase activity as described in the text.

oxygen. In 100% O<sub>2</sub> the peroxidase level rose from 0 to 8.4 units/ml of medium between days two and three. The peroxidase level fell almost as rapidly after day three. By day five the medium contained only about 2.2 units/ml. The average peroxidase level decreased slowly after day five; about 30% further loss by day 16. Similar results were obtained if cultures were grown in air for the first two days before purging with O<sub>2</sub>. In air, a significant increase in Mn-peroxidase did not occur until day seven, and the peak peroxidase concentration, 5.8 units/ml, was not attained until day 12. After the peak, peroxidase levels in air-grown cultures also decreased rapidly.

Normal growth of *P. chrysosporium* appeared to occur in media prepared without manganese. The only visible difference between cultures grown in standard media (which contained about 35 μM Mn) and cultures grown without Mn was the color of the fungal mat. Growth in medium containing Mn led to the development of yellow or brown spots. If Mn is not present, the fungal mat remained white. In order to keep Mn levels as low as possible, conidia which were used to inoculate the liquid cultures were obtained from slants lacking Mn. However no attempts were made to remove trace levels of Mn from glassware or water other than the standard practice of using distilled water and rinsing all glassware before use.

Ligninolytic activity, as measured by the release of <sup>14</sup>C<sub>2</sub>O from <sup>14</sup>C-ring-labeled DHP, was approximately the same in cultures grown without Mn as in standard cultures (Fig. 7-2). This confirms an earlier report which also found that lignin degradation was not dependent on Mn.<sup>112</sup>

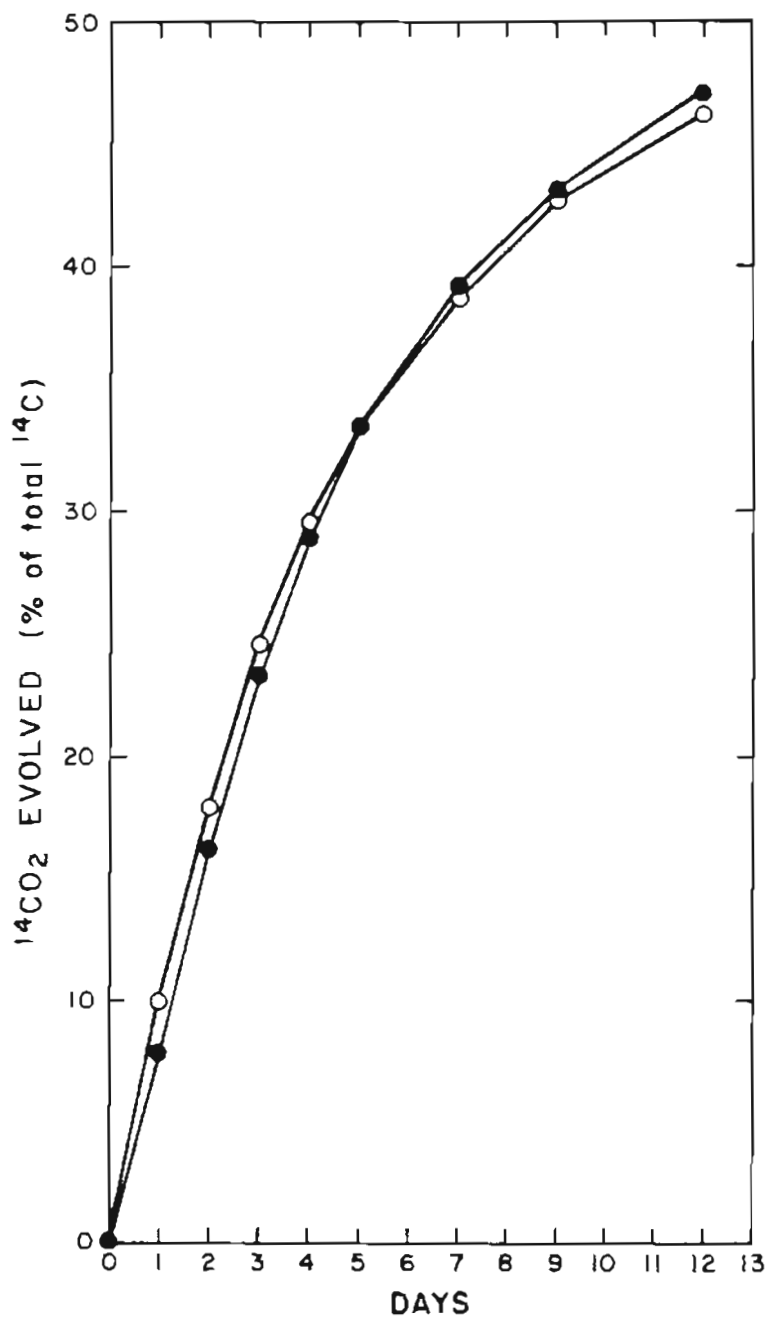


Fig. 7-2. Ligninolytic activity in cultures of *P. chrysosporium* grown with and without manganese.  $^{14}\text{C}$ -ring labeled lignin was added to 5-day-old cultures grown in media containing 0  $\mu\text{M}$  Mn (O) or 35  $\mu\text{M}$  Mn (●). Cultures were purged with 100%  $\text{O}_2$  immediately after addition of the lignin and on the days indicated in the figure. Evolved  $^{14}\text{CO}_2$  was measured as described in the text.

The Mn-peroxidase was never detected in medium from cultures grown without Mn. Cell extracts of these cultures also lacked peroxidase activity. In addition, if the medium was removed from the fungal mat and incubated with  $\text{MnSO}_4$  for up to 24 hrs at 25°C, no peroxidase activity was observed. If  $\text{MnSO}_4$  was added to cultures grown in the absence of Mn, the Mn-peroxidase was quickly produced (Fig. 7-3A). In four-day-old cultures grown without Mn, peak peroxidase levels occurred 24 hrs after addition of  $\text{MnSO}_4$ . At this time, 9.0 units/ml were produced when Mn concentration was 100  $\mu\text{M}$ , but only 2.4 units/ml were produced when Mn concentration was 10  $\mu\text{M}$ . If 100  $\mu\text{M}$  Mn was added to 5-day-old cultures, peroxidase levels reached the peak activity of 18.5 units/ml after 12 hrs (Fig. 7-3A). The addition of the RNA polymerase inhibitor 5-flourouracil, reduced the production of peroxidase about 60% if added simultaneously with manganese. Later addition produced no significant effect (Fig. 7-3B). Inhibition of protein synthesis with cycloheximide completely prevented the appearance of peroxidase when it was added to cultures along with Mn (Fig. 7-3C). When cycloheximide was added six hrs and 12 hrs after the addition of Mn, peroxidase activity was inhibited 60% and 30% respectively.

In order to determine if other nutritional requirements were necessary for peroxidase production, fungal mats were washed with 20 mM Na succinate buffer, pH 4.5, and transferred into buffer containing glucose and other salts as indicated in Figure 7-4. Cultures grown with Mn and transferred into buffer containing no other salts or only  $\text{MnSO}_4$  produce low levels of Mn-peroxidase. The original enzyme level of 3.1 units/ml was reduced to 0.2 units/ml immediately after washing and

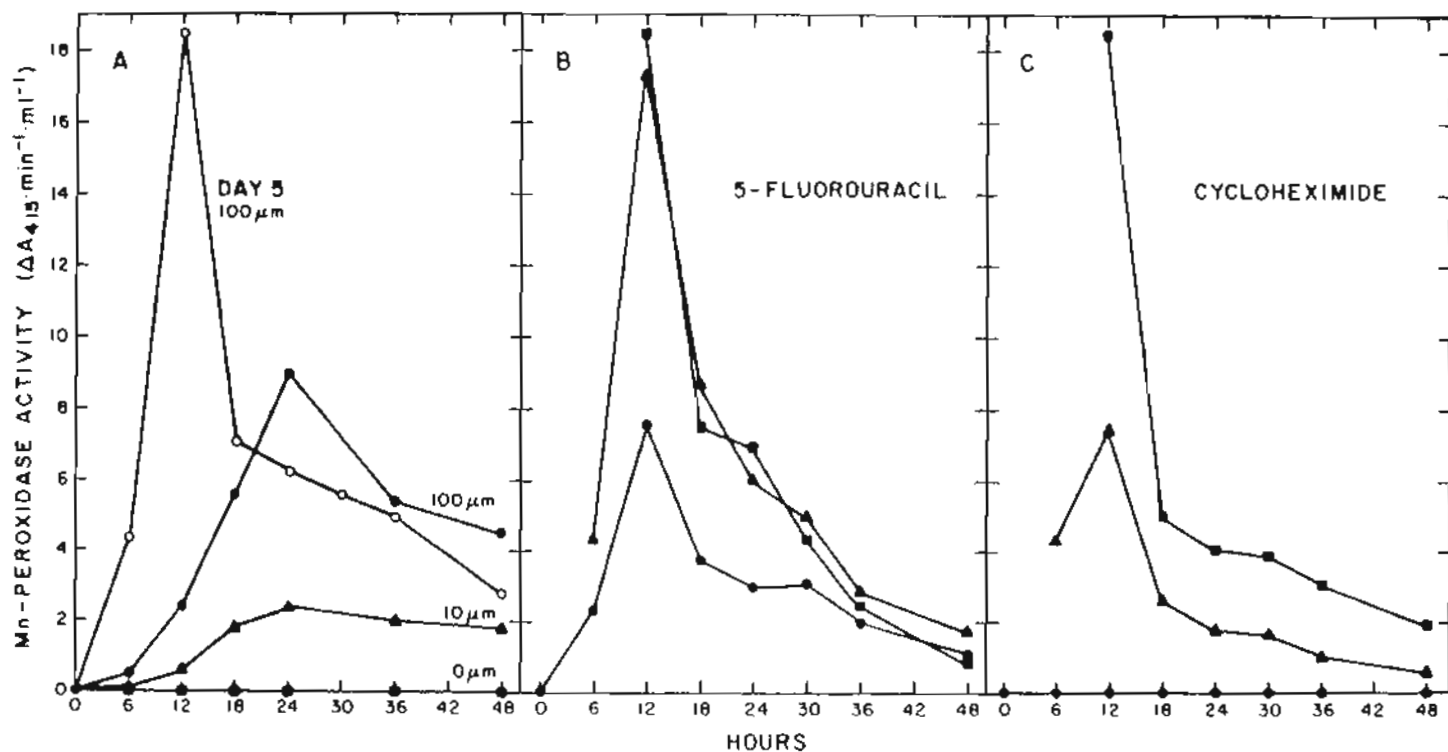


Fig. 7-3. Effect of culture age, manganese concentration, and inhibitors on the production of Mn-peroxidase. (A) At 0 hrs,  $\text{MnSO}_4$  was added to cultures grown in the absence of Mn. To 4-day-old cultures, Mn was added to a final concentration of 0  $\mu\text{M}$  ( $\blacksquare$ ), 10  $\mu\text{M}$  ( $\blacktriangle$ ), or 100  $\mu\text{M}$  ( $\bullet$ ). To 5-day-old cultures, Mn was added to a final concentration of 100  $\mu\text{M}$ . At the times indicated, aliquots of the medium were removed and assayed for Mn-peroxidase activity as described in the text. (B) At 0 hrs ( $\bullet$ ), 6 hrs ( $\blacktriangle$ ), or 12 hrs ( $\blacksquare$ ) after the addition of  $\text{MnSO}_4$  to 5-day-old cultures, 5-fluorouracil was added to a final concentration of 50  $\mu\text{g/ml}$ . Aliquots of the medium were removed at the times indicated and assayed for Mn-peroxidase activity. (C) The same procedure as in part B except cycloheximide was added to a final concentration of 50  $\mu\text{g/ml}$  instead of 5-fluorouracil.



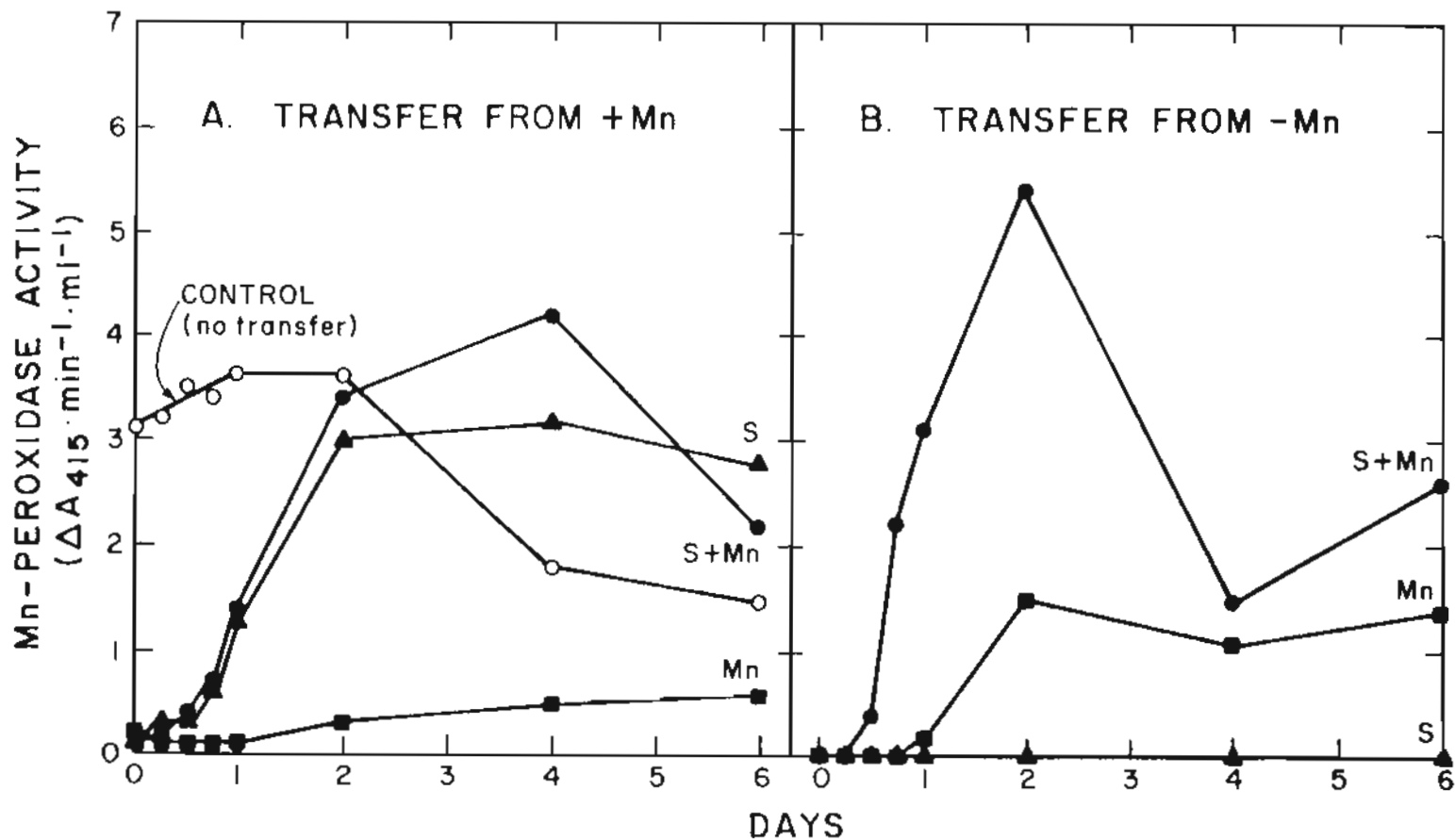


Fig. 7-4. Effect of manganese and nutrient salts on the production of Mn-peroxidase from transferred fungal mats. (A) Five-day-old cultures grown in the presence of 35  $\mu\text{M}$  Mn were washed with 20 mM Na 2,2-dimethylsuccinate, pH 4.5, and transferred into the same buffer containing glucose plus 100  $\mu\text{M}$  Mn (■), glucose plus nutrient salts (S) (▲), or glucose, nutrient salts, and 100  $\mu\text{M}$  Mn (●). Aliquots of the medium were removed at the times indicated and assayed for Mn-peroxidase. (B) The same procedure as in part A except cultures were grown in the absence of Mn.

transfer. After six days the level reached 0.6 units/ml (Fig. 7-4A). If all of the nutrient salts contained in complete medium except Mn were present in the transfer buffer, approximately 3 units/ml peroxidase were produced two days after transfer. Complete nutrient salts (containing Mn) led to the production of only slightly higher levels of peroxidase. Cultures grown without Mn produced no Mn-peroxidase unless Mn was present in the transfer buffer (Fig. 7-4B). If only  $\text{MnSO}_4$  was present, about 1.3 units/ml of enzyme was produced. The highest levels of peroxidase occurred when complete nutrient salts were present; producing a peak of 5.4 units/ml on day two.

To estimate the importance of the Mn-peroxidase in fungal dye degradation, the decolorization of the dye Poly R was followed in cultures grown with and without Mn (Fig. 7-5). Like peroxidase activity, dye decolorization increased rapidly between days two and three in cultures grown with Mn. On day two the rate of decolorization was about 0.001/hr. The rate of decolorization increased to 0.155/hr on day three, and continued to increase slightly over the next few days. Cultures grown without Mn produced almost no decolorization for three days, then the rate of decolorization increased linearly until the experiment was stopped on day eight. Although the rate of decolorization on day eight (0.116) was still well below that from cultures grown with Mn (0.174/hr), the curves suggest that Poly R decolorization would be about the same for both types of cultures around day 10.

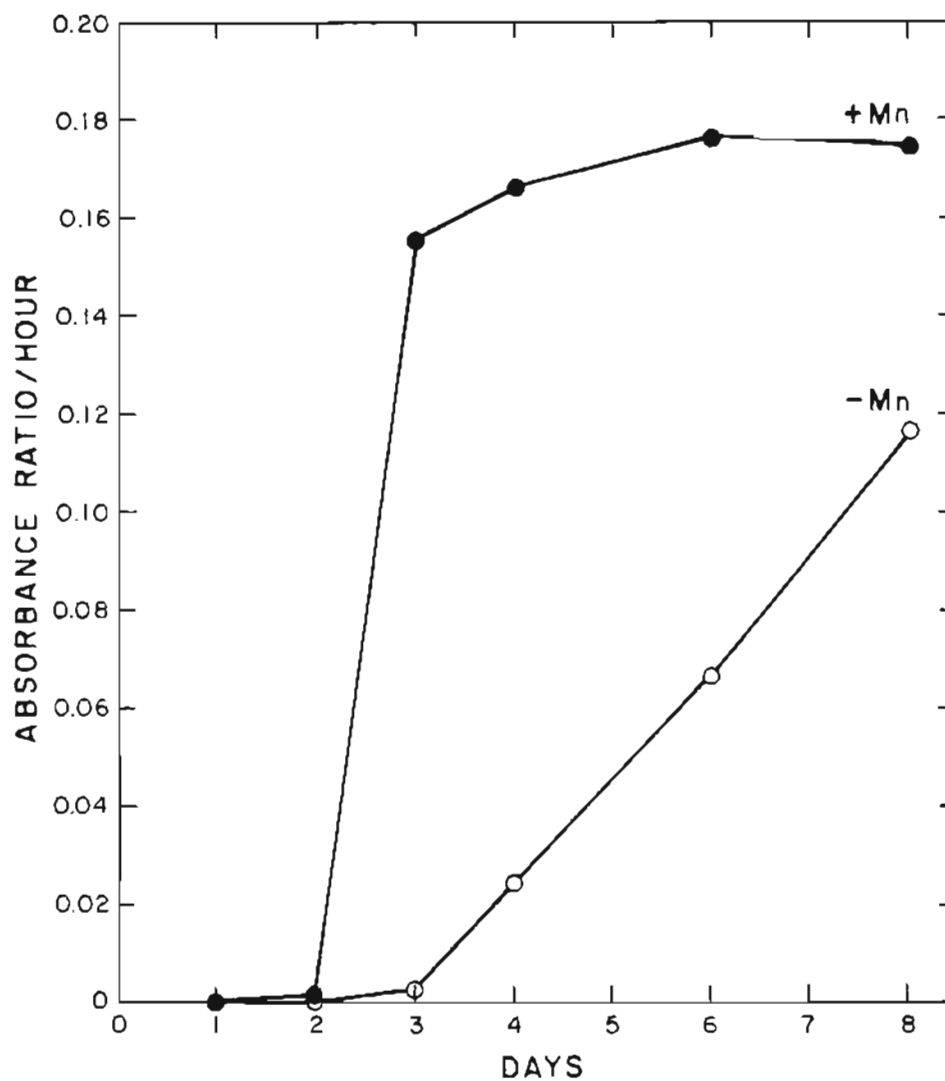


Fig. 7-5. Time dependence of Poly R decolorization in cultures grown with and without manganese. Poly R was added to cultures to a final concentration of 0.02% on the days indicated and the initial rate of decolorization was determined as described in the text.

## Discussion

In a previous paper we showed a close correlation between the ability of P. chrysosporium to degrade lignin and the ability to decolorize several polymeric dyes.<sup>131</sup> Since that time, several extracellular peroxidases from P. chrysosporium have been purified.<sup>130,147,171,236</sup> One of these enzymes, the Mn-peroxidase was capable of decolorizing Poly B and Poly R in vitro. However, the physiological importance of this enzyme in cultures had not been evaluated. The effects of manganese were investigated because previous research showed that the principal substrates for the Mn-peroxidase are  $H_2O_2$  and Mn(II).<sup>130</sup> It was worthwhile to determine whether the elimination of manganese from the growth medium would lead to a decrease of dye decolorization or ligninolytic activity.

The presence of Mn in the culture medium was found to be required for the production of the manganese peroxidase, a further implication that Mn(II) is the true substrate of this enzyme. When Mn was not present during growth, no extracellular peroxidase was detected. Under these conditions, transferring mycelium to buffer containing Mn(II) led to the production of some peroxidase, but much higher levels were produced if transfer was made into buffer containing both Mn(II) and nutrient salts. If the fungus was grown with Mn(II) and then transferred to buffer containing only glucose, peroxidase levels initially dropped to 7% of control levels and rose very slowly. However, if nutrient salts were also present, high levels of peroxidase were produced by 48 hrs. The other components of nutrient salts which stimulate peroxidase production and accumulation have not yet been

determined. The transfer of fungus to nutrient salts and glucose containing Mn produced only a slightly greater stimulation. This suggests that a significant amount of manganese has been accumulated by the fungus by the fifth day of growth, and it is not removed by brief washing. However, it is also possible that the Mn-peroxidase mRNA or a precursor of the enzyme is synthesized before transfer and leads to production of the peroxidase without a requirement for manganese. The other factor(s) may be involved in enzyme secretion, stability, or induction.

After the addition of Mn(II), the enzyme levels in the medium are higher and reach peak values earlier in 5-day cultures than in 4-day cultures. Extraction of the fungus failed to reveal the presence of intracellular peroxidase during this period, so there is probably no accumulation of active enzyme within the cell. An inactive precursor may be present which can be quickly processed to active Mn-peroxidase. Another possibility is that messenger RNA for the peroxidase is accumulating during this period. Cycloheximide, an inhibitor of protein synthesis, was much more potent at blocking the production of peroxidase than 5-fluorouracil (5-FU), an inhibitor of messenger RNA synthesis. In the case of 5-FU, inhibition was only observed when it was added along with Mn, but cycloheximide exerted an effect up to 12 hrs after the addition of Mn. An inhibitor of mRNA synthesis might be expected to produce almost no inhibition of peroxidase production if control was at the translational level. However, if the fungus had a high rate of mRNA turnover, some reduction of peroxidase synthesis might have occurred soon after the addition of 5-FU. If this was the case, greater

inhibition may occur when 5-FU is added before Mn. Either cycloheximide or 5-FU may have inhibited the uptake of Mn(II) by the fungus, and thereby reduced Mn-peroxidase synthesis. A direct examination of Mn-peroxidase mRNA synthesis will be required to better understand the regulation of this enzyme by Mn, nitrogen, and oxygen levels.

The amount of peroxidase produced was related to the concentration of Mn(II) in the medium. In four-day cultures there was about four times more peroxidase produced when 100  $\mu$ M rather than 10  $\mu$ M Mn(II) was used. The rapid increase of Mn-peroxidase observed during the initial production of the enzyme was followed by an equally rapid fall of activity to about one-third peak levels. After this initial decrease, loss of activity was much slower. The rate of loss of peroxidase activity was not significantly affected by the presence of 5-fluorouracil or cycloheximide. With enzyme production inhibited, the rate of enzyme degradation can be measured directly. A half-life for the peroxidase of as long as 18-24 hrs at 37°C is estimated. Thus, the early loss of activity may be due to a specialized process, such as proteolytic hydrolysis of the enzyme.

A previous report found that the ligninolytic activity of P. chrysosporium was not affected by removing Mn from the growth medium.<sup>112</sup> We confirm that observation in this paper. The absence of Mn-peroxidase in these cultures indicates that in liquid medium using low levels of lignin, the Mn-peroxidase is not required for lignin degradation.

In three-day-old cultures grown with Mn, there was a rapid increase in both the rate of Poly R decolorization and the level of

Mn-peroxidase. However, the level of peroxidase quickly declined while the rate of dye decolorization remained high. Cultures grown without Mn did not decolorize Poly R until the fourth day, and the rate of decolorization increased slowly after that. These data suggests that Mn-peroxidase is involved in the decolorization of Poly R at early stages, but at least one more enzyme is produced which can also decolorize Poly R. This may be the lignin peroxidase (ligninase)<sup>102,147</sup> or yet another enzyme.

Comparing five-day-old cultures grown with and without Mn, we found that cultures grown without Mn had slightly higher ligninolytic activity, but only one-third the rate of Poly R decolorization. The lack of quantitative correlation between lignin degradation and dye decolorization suggests that these two activities are not carried out by an identical complement of enzymes. However, so far, we have been unable to find mutants of P. chrysosporium or produce conditions in which ligninolytic activity is observed but no dye decolorization, or in which dye decolorization but not ligninolytic activity occurs. This suggests that there is at least one enzyme involved in both activities. The lignin peroxidase may be an example of this. It is likely to be involved in lignin degradation, and is also capable of slowly decolorizing Poly R (Mayfield, M.B. and Gold, M.H., unpublished data).

Although we have found no evidence to indicate that the Mn-peroxidase is required for lignin degradation in our experiments, the conditions we have used are far from those which exist in the natural environment of P. chrysosporium. In our experiments we use high specific activity lignin; therefore we introduce very little mass of

lignin into our cultures. However, lignin represents a significant proportion of the total biomass of wood.<sup>20</sup> The degradation of large amounts of lignin by white rot fungi may produce significant quantities of toxic phenolic compounds which are known to inhibit the lignin peroxidase (Renganathan, V. and Gold, M.H., unpublished data). These phenolics would be polymerized and therefore detoxified by the Mn-peroxidase. The rapid rise in Mn-peroxidase levels early in secondary metabolism may represent the method used by the fungus to remove soluble phenolic compounds from the wood before the lignin peroxidase begins working. The liquid media which we employ allows the rapid diffusion of enzymes. However, wood may restrict the movement of macromolecules, thus preventing enzymes such as lignin peroxidase from reaching its site of action. The Mn-peroxidase functions by producing highly reactive Mn(III).<sup>130</sup> This oxidant may diffuse to sites inaccessible to enzymes. In wood rotted by white rot fungi, oxides of manganese are associated with regions from which the lignin has been removed.<sup>225</sup> The fact that Mn-peroxidase produced manganese oxides<sup>130</sup> also suggests that this enzyme plays a role in lignin degradation. Further research is planned to determine the involvement of Mn-peroxidase in lignin degradation under more natural conditions.



## CHAPTER 8

## Final Comments

Although lignin and dye degradation always appear to occur at the same time, we cannot consider dye decolorization to be a quantitative measure of ligninolytic activity. However, polymeric dye decolorization is perhaps the best quantitative measure of extracellular peroxidase activity in whole cultures. The decolorization of polymeric dyes was developed primarily as a means of screening for mutants of white rot fungi with altered ligninolytic activity and as a method of finding new ligninolytic organisms. So far, all of the white rot fungi we have tested have decolorized Poly B and Poly R, but none of the brown rot fungi have this ability. Recently other laboratories have used this dye assay developed in our laboratory as a means of distinguishing ligninolytic from non-ligninolytic organisms, but a comprehensive testing of microorganisms still needs to be carried out. At the present time, the biodegradation of lignin for papermaking or bleaching is not a commercial reality. This situation may be remedied by using the dye decolorization assay to find organisms or mutant strains with more active lignin degrading systems. In order to understand the control of secondary metabolism and ligninolytic activity in Phanerochaete chrysosporium, more research is needed with mutants defective for lignin degradation. Polymeric dyes have been used on plates in our laboratory to test large numbers of colonies for modified ligninolytic activity. Some mutants have been found with this method, but many more are needed.

Although it is likely that the lignin peroxidase (ligninase) is involved in lignin degradation by P. chrysosporium, the role of the Mn-peroxidase is unclear. Preliminary evidence suggests that the Mn-peroxidase is involved in the production of extracellular  $H_2O_2$ , but more research is necessary to support this. If these enzymes are both required for lignin degradation, then other white rot fungi should also possess enzymes with similar functions. More work on the purification of enzymes from other lignin degrading organisms would be beneficial. It is also likely that still other enzymes involved in lignin degradation will be isolated from P. chrysosporium.

The complete picture of the lignin degradation process is just beginning to emerge. The use of liquid cultures is an important method for analyzing lignin degradation, but the process of lignin degradation within wood may be much more complex. The role of manganese oxides in the depletion of lignin from wood must be studied further. The Mn-peroxidase can produce  $MnO_2$ , but further research is required to determine whether it performs this function under natural conditions and if the production of  $MnO_2$  is simply a method of detoxifying manganese or performs another function.

Even if the Mn-peroxidase does not play a major role in lignin degradation, it represents an excellent example of a novel secondary metabolic enzyme. It is also the only enzyme known with the specific function of oxidizing Mn(II). This enzyme is easy to study because it is an extracellular enzyme and because the assay is simple and sensitive. Although the level of activity changes dramatically with age of the culture and with levels of oxygen, this peroxidase is present

during secondary metabolism if manganese is included in the culture medium. Because enzyme production is sensitive to oxygen and Mn(II) concentrations, research into control of enzyme levels could lead to an understanding of how the fungus detects  $O_2$  and Mn. Finally, because the Mn-peroxidase can be produced quickly and in large quantities after the addition of Mn, the enzyme may be a good model for the study of secretion of enzymes by P. chrysosporium and will also be of value in the understanding of transcriptional and translational control of protein production during secondary metabolism.

## REFERENCES

1. Payen, A. (1838) *Compt. Rend.* 7:1052-1056.
2. Brauns, F.E. (1952) "The Chemistry of Lignin." Academic Press, New York.
3. Brauns, F.E. and Brauns, D.A. (1960) "The Chemistry of Lignin, Supplement Volume." Academic Press, New York.
4. Pearl, I.A. (1967) "The Chemistry of Lignin." Marcel Dekker, Inc., New York.
5. Adler, E. (1977) *Wood Sci. Technol.* 11:169-218.
6. Tilghman, B.C. (1866) *Brit. Pat.* 2924.
7. Watt, C. and Burgess, H. (1854) *U.S. Pats.* 1448, 1449.
8. Dahl, C.F. (1884) *U.S. Pat.* 296935.
9. Adler, E. (1966) in "Lignin Structure and Reactions" (Marton, J., ed.). American Chemical Society, Washington, D.C. p. ix.
10. Klason, P. *Cellulosechem.* (1923) 4:81-84.
11. Tiemann, F. and Mendelsohn, B. (1875) *Ber.* 88:1127-1133.
12. Klason, P. (1897) *Svensk. Kem. Tidskr.* 9:133-137.
13. Freudenberg, K., Lautsch, W. and Engler, K. (1940) *Ber.* 73B:167-171.
14. Gardner, J.A.F. and Hibbert, H. (1944) *J. Am. Chem. Soc.* 66:607-610.
15. Harris, E.E., D'Ianni, J. and Adkins, H. (1938) *J. Am. Chem. Soc.* 60:1467-1470.
16. Erdtman, H. (1933) *Biochem. Z.* 258:172-180.
17. Freudenberg, K. and Richtzenhain, H. (1943) *Ber.* 76B:997-1006.

18. Bjorkman, A. (1954) *Nature*. 174:1057-1058.
19. Adler, E. (1964) *Chim. Biochim. lignine, Cellulose Hemi-celluloses, Actes Symp. Intern., Grenoble, France.* pp. 73-83.
20. Crawford, R.L. (1981) "Lignin Biodegradation and Transformation." Wiley-Interscience, New York.
21. Wilson, C.L., Loomis, W.E. and Steeves, T.A. (1971) "Botany, 5th Edition." Holt, Rinehart and Winston, New York. pp. 62-64.
22. Cote, W.A. (1977) in "Recent Advances in Phytochemistry, Vol. 11" (Loewus, F.A. and Runeckles, V.C., eds.). Plenum Press, New York. pp. 1-44.
23. Higuchi, T. (1980) in "Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications Vol. 1" (Kirk, T.K., Higuchi, T. and Chang, H.-m., eds.). CRC Press, Inc., Boca Raton, FL. pp. 1-20.
24. Kosikova, B., Joniak, D. and Kosakova, L. (1979) *Holzforschung*. 33:11-14.
25. Vance, C.P., Kirk, T.K. and Sherwood, R.T. (1980) *Ann. Rev. Phytopath.* 18:259-288.
26. Higuchi, T., Shimada, M., Nakatsubo, F. and Tanahashi, M. (1977) *Wood Sci. Technol.* 11:153-167.
27. Gross, G.G. (1977) in "Recent Advances in Phytochemistry, Vol. 11" (Loewus, F.A. and Runeckles, V.C., eds.). Plenum Press, New York. pp. 141-184.
28. Neish, A.C. (1961) *Phytochemistry*. 1:1-24.
29. Harkin, J.M. and Obst, J.R. (1973) *Science* 180:296-298.
30. Robinson, T. (1975) "The Organic Constituents of Higher Plants,

- Their Chemistry and Interrelationships, 3rd Edition." Cordus Press, North Amherst, MA. pp. 68-81.
31. Harkin, J.M. (1967) in "Oxidative Coupling of Phenols" (Battersby, A.R. and Taylor, W.I., eds.). Marcel Dekker, Inc., New York. pp. 243-321.
  32. Freudenberg, K., Chen, C.L. and Cardinale, G. (1962) Chem. Ber. 95:2814-2828.
  33. Sarkanen, K.V. and Ludwig, C.H. (eds.) (1971) "Lignins: Occurrence, Formation, Structure, and Reactions." Wiley-Interscience, New York.
  34. Freudenberg, K. (1968) in "Constitution and Biosynthesis of Lignin" (Neish, A.C. and Freudenberg, K., eds.). Springer-Verlag, New York. pp. 47-122.
  35. Nakamura, Y. and Higuchi, T. (1976) Holzforschung. 30:187-191.
  36. Hackett, W.F., Connors, W.J., Kirk, T.K. and Zeikus, J.G. (1977) Appl. Environ. Microbiol. 33:43-51.
  37. Jensen, V. (1974) in "Biology of Plant Litter Decomposition, Vol. 1" (Dickinson, C.H. and Pugh, G.J.F., eds.). Academic Press, New York.
  38. Esenther, G.R. and Kirk, T.K. (1974) Ann. Entomol. Soc. Am. 67:989-999.
  39. Neuhauser, E.F., Hartenstein, R. and Connors, W.J. (1978) Soil Biol. Biochem. 10:431-435.
  40. Lee, K.E. and Wood, T.G. (1971) "Termites and Soils." Academic Press, New York.
  41. Bellamy, W.D. (1974) Biotechnol. Bioeng. 16:869-880.

42. Humphrey, A.E., Moreira, A., Armiger, W. and Zabriskie, D. (1977) *Biotechnol. Bioeng. Symp. No. 7.* pp. 45-64.
43. Kirk, T.K., Connors, W.J. and Zeikus, J.G. (1977) *Rev. Adv. Phytopathol.* 11:369-394.
44. Falkehag, S.I. (1975) *Applied Polymer Symp.* 28:247-257.
45. Han, Y.W. and Anderson, A.W. (1975) *Appl. Microbiol.* 30:930-934.
46. Ford, C.W. (1978) *Aust. J. Agric. Res.* 29:1157-1166.
47. Ander, P. and Eriksson, K.-E. (1978) *Prog. Ind. Microbiol.* 14:1-58.
48. Harkin, J.M., Crawford, D.L. and McCoy, E. (1974) *Tappi.* 57:131-134.
49. Dhawan, S. and Gubta, J.K. (1977) *J. Gen. Appl. Microbiol.* 23:155-161.
50. Hajny, G.J., Gardner, C.H. and Ritter, G.J. (1951) *Ind. Eng. Chem.* 43:1384-1389.
51. Goldstein, I.S. (1975) *Science.* 189:847-852.
52. Kirk, T.K. (1971) *Ann. Rev. Phytopathol.* 9:185-210.
53. Effland, M.J. (1977) *Tappi.* 60:143-144.
54. Brauns, F.E. (1962) *Holzforschung.* 16:97-102.
55. Kratzl, K. and Billek, G. (1957) *Tappi.* 40:269-285.
56. Bjorkman, A. and Person, B. (1957) *Sven. Papperstid.* 60:158-169.
57. Chang, H.-m., Cowling, E., Brown, W., Adler, E. and Miksche, G. (1975) *Holzforschung.* 29:153-159.
58. Pew, J.C. and Weyna, P. (1962) *Tappi.* 45:247-256.
59. Kirk, T.K., Connors, W.J., Blead, R.D., Hackett, W.F. and Zeikus, J.G. (1975) *Proc. Natl. Acad. Sci. USA.* 72:2515-2519.

60. Crawford, R.L., Robinson, L.E. and Foster, R.D. (1981) *Appl. Environ. Microbiol.* 41:1112-1116.
61. Lundquist, K., Kirk, T.K. and Connors, W.J. (1977) *Arch. Microbiol.* 112:291-296.
62. Lundquist, K. and Kirk, T.K. (1980) *Tappi.* 63:80-82.
63. Schubert, W.J. and Nord, F.F. (1950) *J. Am. Chem. Soc.* 72:3835-3838.
64. Brown, W., Cowling, E.B. and Falkehag, W.I. (1968) *Sven. Papperstid.* 22:811-821.
65. Ander, P. and Eriksson, K.-E. (1977) *Physiol. Plant.* 41:239-248.
66. Moore, W.E. and Johnson, D.B. (1967) "Procedures for the Analysis of Wood and Wood Products." USDA Forest Service, Forest Products Laboratory, Madison WI.
67. Beal, F.C., Merrill, W., Baldwin, R.C. and Wang, J.H. (1976) *Wood Fiber.* 8:159-167.
68. Kirk, T.K. (1975) *Holzforschung.* 29:99-107.
69. Blanchette, R.A. (1984) *Appl. Environ. Microbiol.* 48:647-653.
70. Odier, E. and Monties, B. (1977) *C.R. Acad. Sci. Paris, Series D.* 284:2175-2178.
71. Hiroi, T. and Eriksson, K.-E. (1976) *Sven. Papperstidn.* 79:157-161.
72. Kyrklund, B. and Strandell, G. (1967) *Papper och Tra.* 49:99-106.
73. Chang, H.-m. Chen, C.-L. and Kirk, T.K. (1980) in "Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications Vol. 1" (Kirk, T.K., Higuchi, T. and Chang H.-m., eds.). CRC Press, Inc., Boca Raton, FL. pp. 215-230.



74. Hiroi, T. and Tamai, A. (1983) in "Recent Advances in Lignin Biodegradation Research" (Higuchi, T., Chang, H.-m. and Kirk, T.K., eds.). Uni, Ltd., Tokyo. pp. 34-43.
75. Tai, D., Terasawa, M., Chen, C.-L., Chang, H.-m., and Kirk, T.K. (1983) in "Recent Advances in Lignin Biodegradation Research" (Higuchi, T., Chang, H.-m. and Kirk, T.D., eds.). Uni, Ltd., Tokyo. pp. 44-63.
76. Enoki, A., Goldsby, G.P. and Gold, M.H. (1981) Arch. Microbiol. 129:141-145.
77. Enoki, A. and Gold, M.H. (1982) Arch. Microbiol. 132:123-130.
78. Haider, K., Trojanowski, J. and Sundman, V. (1978) Arch. Microbiol. 119:103-106.
79. Dagley, S. (1977) Surv. Prog. Chem. 8:121-170.
80. Crawford, R.L. and Crawford, D.L. (1978) Dev. Ind. Microbiol. 19:35-49.
81. Ferry, J.G. and Wolfe, R.S. (1976) Arch. Microbiol. 107:33-40.
82. Keith, C.L., Bridges, R.L., Fina, L.R., Iverson, K.L. and Cloran, J.A. (1978) Arch. Microbiol. 118:173-176.
83. Phelan, M.B., Crawford, D.L. and Pometto, III, A.L. (1979) Can. J. Microbiol. 25:1270-1276.
84. Crawford, R.L., Robinson, L.E. and Cheh, A.M. (1980) in "Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications Vol. 1" (Kirk, T.K., Higuchi, T. and Chang, H.-m., eds.). CRC Press, Inc., Boca Raton, FL. pp. 62-76.
85. Norman, A.G. (1936) Science Progr. 30:442-456.
86. Waksman, S.A. (1944) in "Wood Chemistry" (Wise, L.E., ed.).

- Reinhold Press, New York. pp. 853-860.
87. Gottlieb, S. and Pelczar, M.J. (1951) *Bacteriol. Rev.* 15:55-76.
  88. Higuchi, T. (1954) *Bull. Nagoya Regional Forestry Office.* 6:134-148.
  89. Crawford, D.L. and Crawford, R.L. (1980) *Enzyme Microb. Technol.* 2:11-22.
  90. Higuchi, T., Chang, H.-m. and Kirk, T.K. (eds.) (1983) "Recent Advances in Lignin Biodegradation Research." Uni, Ltd. Tokyo.
  91. Gold, M.H., Glenn, J.K., Mayfield, M.B., Morgan, M.A. and Kutsuki, H. (1983) in "Recent Advances in Lignin Biodegradation Research" (Higuchi, T., Chang, H.-m. and Kirk, T.K., eds.). Uni, Ltd., Tokyo. pp. 219-232.
  92. Kaplan, D.L. and Hartenstein, R. (1980) *Soil Biol. Biochem.* 12:65-74.
  93. Levi, M.P. and Preston, R.D. (1965) *Holzforschung.* 19:183-190.
  94. Haider, K. and Trojanowski, J. (1975) *Arch. Microbiol.* 105:33-42.
  95. Iwahara, S. (1983) in "Recent Advances in Lignin Biodegradation Research" (Higuchi, T., Chang, H.-m. and Kirk, T.K., eds.). Uni, Ltd., Tokyo. pp. 96-111.
  96. Iwahara, S. (1980) in "Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications Vol. 1" (Kirk, T.K., Higuchi, T. and Chang, H.-m., eds.). CRC Press, Inc., Boca Raton, FL. pp. 151-170.
  97. Drew, S.W. and Kadam, K.L. (1979) *Dev. Ind. Microbiol.* 20:153-161.

98. Gulyas, F. (1967) *Agrokem. Talajtan.* 16:137-150.
99. Ledingham, G.A. and Adams, G.A. (1942) *Can. J. Res. Soc.* 20:13-27.
100. Gradziel, K., Haider, K., Kochmanska, J., Malarczyk, E. and Trojanowski, J. (1978) *Acta Microbiol. Pol.* 27:103-109.
101. Odier, E. and Monties, B. (1978) *Ann. Microbiol. (Inst. Pasteur).* 129A:361-377.
102. Kirk, T.K. and Shimada, M. (1985) in "Biosynthesis and Biodegradation Of Wood Components" (Higuchi, T. ed.). Academic Press, New York. pp. 579-605.
103. Crawford, D.L. and Sutherland, J.B. (1979) *Dev. Ind. Microbiol.* 20:143-151.
104. Haider, K. and Trojanowski, J. (1980) in "Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications Vol. 1" (Kirk, T.K., Higuchi, T. and Chang H.-m., eds.). CRC Press, Inc., Boca Raton, FL. pp. 111-134.
105. Kawase, K. (1962) *J. Agric. Hokkaido Imp. Univ.* 52:186-245.
106. Kirk, T.K. and Chang, H.-m. (1974) *Holzforshung.* 28:217-222.
107. Haider, K. and Grabbe, K. (1967) *Zentralbl. Bakteriol. Parasitenkd., Infektionskr., Hyg.* 205:91-96.
108. Yajima, Y., Enoki, A., Mayfield, M.B. and Gold, M.H. (1979) *Arch. Microbiol.* 123:319-321.
109. Pelczar, M.J. Jr., Gottlieb, S. and Day, W.C. (1950) *Arch. Biochem.* 25:449-451.
110. Ishikawa, H., Schubert, W.J. and Nord, F.F. (1963) *Arch. Biochem. Biophys.* 100:140-149.

111. Kirk, T.K., Connors, W.J. and Zeikus, J.G. (1976) *Appl. Environ. Microbiol.* 32:192-194.
112. Jeffries, T.W., Choi, S. and Kirk, T.K. (1981) *Appl. Environ. Microbiol.* 42:290-296.
113. Cowling, E.B. and Merrill, W. (1966) *Can. J. Bot.* 44:1539-1554.
114. Fenn, P., Choi, S. and Kirk, T.K. (1981) *Arch. Microbiol.* 130:66-71.
115. MacDonald, M.J., Paterson, A. and Broda, P. (1984) *J. Bacteriol.* 160:470-472.
116. Lundquist, K. and Kirk, T.K. (1978) *Phytochemistry.* 17:1676.
117. Shimada, M., Nakatsubo, F., Kirk, T.K., and Higuchi, T. (1981) *Arch. Microbiol.* 129:321-324.
118. Kirk, T.K., Schultz, E., Connors, W.J., Lorenz, L.F. and Zeikus, J.G. (1978) *Arch. Microbiol.* 117:277-285.
119. Bar-Lev, S.S. and Kirk, T.K. (1981) *Biochem. Biophys. Res. Commun.* 99:373-378.
120. Weinstein, D.A., Krisnangkura, K., Mayfield, M.B. and Gold, M.H. (1980) *Appl. Environ. Microbiol.* 39:535-540.
121. Keyser, P., Kirk, T.K. and Zeikus, J.G. (1978) *J. Bacteriol.* 135:790-797.
122. Ulmer, D.C., Leisola, M.S.A. and Feichter, A. (1984) *J. Biotechnol.* 1:13-24.
123. Davidson, R.W., Campbell, W.A. and Blaisdell, D.J. (1938) *J. Agric. Res.* 57:683-695.
124. Ander, P. and Eriksson, K.-E. (1976) *Arch. Microbiol.* 109:1-8.
125. Gold, M.H., Mayfield, M.B., Cheng, T.M., Krisnangkura, K.,

- Shimada, M., Enoki, A. and Glenn, J.K. (1982) *Arch. Microbiol.* 132:115-122.
126. Gierer, J. and Opara, A.E. (1973) *Acta. Chem. Scand.* 27:2909-2922.
127. Kaplan, D. (1979) *Phytochemistry.* 18:1917-1920.
128. Frick, T. and Crawford, R.L. (1983) in "Recent Advances in Lignin Biodegradation Research" (Higuchi, T., Chang, H.-m. and Kirk, T.K., eds.). Uni, Ltd., Tokyo. pp. 143-152.
129. Trojanowski, J. and Leonowicz, A. (1969) *Microbios.* 3:247-251.
130. Glenn, J.K. and Gold, M.H. (1985) *Arch. Biochem. Biophys.* 242:329-341.
131. Glenn, J.K. and Gold, M.H. (1983) *Appl. Environ. Microbiol.* 45:1741-1747.
132. Nakatsubo, F., Reid, I.D. and Kirk, T.K. (1981) *Biochem. Biophys. Res. Commun.* 102:484-491.
133. Amer, G.I. and Drew, S.W. (1980) *Dev. Ind. Microbiol.* 22:479-484.
134. Faison, B.D. and Kirk, T.K. (1983) *Appl. Environ. Microbiol.* 46:1140-1145.
135. Forney, L.J., Reddy, C.A., Tien, M., and Aust, S.D. (1982) *J. Biol. Chem.* 257:11455-11462.
136. Kutsuki, H. and Gold, M.H. (1982) *Biochem. Biophys. Res. Commun.* 109:320-327.
137. Kutsuki, H., Enoki, A. and Gold, M.H. (1983) *Photochem. Photobiol.* 37:1-7.
138. Kirk, T.K., Nakatsubo, F. and Reid, I.D. (1983) *Biochem. Biophys. Res. Commun.* 111:200-204.

139. Gold, M.H., Kutsuki, H. and Morgan, M.A. (1983) *Photochem. Photobiol.* 38:647-651.
140. Cohen, G. and Cederbaum, A. (1980) *Arch. Biochem. Biophys.* 199:438-447.
141. Pryor, W.A. and Tang, R.H. (1978) *Biochem. Biophys. Res. Commun.* 81:498-503.
142. Dorfman, L.M. and Adams, G.E. (1973) *Nat. Std. Ref. Data Service, Nat. Bur. Standards.* 46:1-59.
143. Halliwell, B. (1978) *FEBS Lett.* 92:321-326.
144. Anbar, M. and Neta, P. (1967) *Int. Appl. Radiat. Isot.* 18:493-523.
145. Glenn, J.K., Morgan, M.A., Mayfield, M.B., Kuwahara, M. and Gold, M.H. (1983) *Biochem. Biophys. Res. Commun.* 114:1077-1083.
146. Kuwahara, M., Glenn, J.K., Morgan, M.A. and Gold, M.H. (1984) *FEBS Lett.* 169:247-249.
147. Gold, M.H., Kuwahara, M., Chiu, A.A. and Glenn, J.K. (1984) *Arch. Biochem. Biophys.* 234:353-362.
148. Yamaguchi, K.S. and Sawyer, D.T. (1985) *Israel J. Chem.* 25:164-176.
149. Reid, I.D. (1979) *Can. J. Microbiol.* 57:2050-2058.
150. Gold, M.H. and Cheng, T.M. (1978) *Appl. Environ. Microbiol.* 35:1223-1225.
151. Dawson, D.J. (1981) *Aldrichimica Acta.* 14:23-29.
152. Eaton, D., Chang, H.-m. and Kirk, T.K. (1980) *Tappi.* 63:103-109.
153. Corso, C.R., deAngelis, D.F., deOliveira, J.E. and Kiyon, C. (1981) *Eur. J. Appl. Microbiol. Biotechnol.* 13:64-66.

154. Chua, M.G.S., Choi, S. and Kirk, T.K. (1983) *Holzforschung*. 37:55-61.
155. Nakatsubo, F., Kirk, T.K., Shimada, M. and Higuchi, T. (1981) *Arch. Microbiol.* 128:484-491.
156. Hata, K. (1966) *Holzforschung*. 20:142-147.
157. Ishikawa, H., Schubert, W.J. and Nord, F.F. (1963) *Arch. Biochem. Biophys.* 100:131-139.
158. Kirk, T.K. and Chang, H.-m. (1975) *Holzforschung*. 29:56-64.
159. Reid, I.D. and Seifert, K.A. (1980) *Can. J. Microbiol.* 26:1168-1171.
160. Hall, P. (1980) *Enzyme Microb. Technol.* 2:170-176.
161. Diguisseppi, J. and Fridovich, I. (1980) *Arch. Biochem. Biophys.* 205:323-329.
162. Enoki, A., Goldsby, G. and Gold, M.H. (1980) *Arch. Microbiol.* 125:227-232.
163. Gold, M.H., Kutsuki, H., Morgan, M.A. and Kuhn, R.M. (1983) *Proc. 1983 Internat. Symp. Wood Pulp. Chem.*, vol. 4, pp. 165-168.
164. Kirk, T.K. and Nakatsubo, F. (1983) *Biochim. Biophys. Acta.* 756: 376-384.
165. Farmer, V.C., Henderson, M.E.K. and Russel, J.D. (1959) *Biochim. Biophys. Acta.* 35:202-211.
166. Enoki, A., Yajima, Y. and Gold, M.H. (1981) *Phytochemistry* 20: 1543-1546.
167. Tien, M. and Kirk, T.K. (1983) *Science*. 221:661-663.
168. Pick, E. and Keisari, Y. (1980) *J. Immunol. Meth.* 38:161-170.
169. Andrews, P. (1964) *Biochem. J.* 96:595-605.

170. Nakatsubo, F., Reid, I.D. and Kirk, T.K. (1982) *Biochim. Biophys. Acta.* 719:284-291.
171. Tien, M. and Kirk, T.K. (1984) *Proc. Natl. Acad. Sci. USA.* 81:2280-2284.
172. Weber, K. and Osborne, M. (1969) *J. Biol. Chem.* 244:4406-4412.
173. Dunford, H.B. and Stillman, V.S. (1976) *Coord. Chem. Rev.* 19:187-251.
174. Putter, J. and Becker, R. (1981) in "Methods of Enzymatic Analysis, Vol. 3" (Bergmeyer, H. U., ed.). p. 286.
175. Machly, A.C. and Chance, B. (1954) in "Methods of Biochemical Analysis, Vol. 1" (Glick, D., Ed.). p. 357.
176. Archibald, F.S. and Fridovich, I. (1983) *Arch. Biochem. Biophys.* 214:452-463.
177. Appleby, C.A. and Morton, R.K. (1959) *Biochem. J.* 73:539-550.
178. Lowry, O.H., Rosebrough, J.J., Farr, A.L. and Randall, R.J. (1951) *J. Biol. Chem.* 193:265-275.
179. Krisnangkura, K. and Gold, M.H. (1979) *Holzforschung.* 33:174-176.
180. Sarkanen, K.V. (1971) in "Lignins: Occurrence, Formation, Structure and Reactions" (Sarkanen, K.V. and Ludwig, C.H., eds.). Wiley-Interscience, New York. pp. 95-195.
181. Bavendamm, W. (1928) *Z. Pflanzenkr.* 38:257-276.
182. Kirk, T.K. and Kelman, A. (1965) *Phytopathology.* 55:739-745.
183. Ishihara, I. and Miyazaki, M. (1974) *J. Japan. Wood Res. Soc.* 20:39-41.
184. Cotton, M.L., Dunford, H.B. and Raycheba, J.M.T. (1973) *Canad. J. Biochem.* 51:627-631.



185. Keele, B.B., Jr., McCord, J.M. and Fridovich, I. (1970) *J. Biol. Chem.* 245:6176-6181.
186. Vance, P.G., Keele, B.B., Jr. and Rajagopalan, K.V. (1972) *J. Biol. Chem.* 247:4782-4786.
187. Weisinger, R.A. and Fridovich, I. (1973) *J. Biol. Chem.* 248:3582-3592.
188. Que, L., Jr., Widom, J. and Crawford, R.L. (1981) *J. Biol. Chem.* 256:10941-10944.
189. Kono, Y. and Fridovich, I. (1983) *J. Biol. Chem.* 258:6015-6019.
190. Spector, M. and Winget, G.D. (1980) *Proc. Natl. Acad. Sci. USA.* 77:957-959.
191. Dismukes, G. C. and Siderer, Y. (1981) *Proc. Natl. Acad. Sci. USA.* 78:274-278.
192. Yokota, K. and Yamazaki, I. (1965) *Biochim. Biophys. Acta.* 105:301-312.
193. Halliwell, B. (1978) *Planta.* 140:81-88.
194. Homann, P. (1965) *Biochemistry.* 4:1902-1911.
195. Kenten, R.H. and Mann, P.J.G. (1952) *Biochem. J.* 52:125-130.
196. Bhatnagar, R.P. and Fadnis, A.G. (1978) *Monatsh. Chem.* 109:319-323.
197. Pelizzetti, E., Mentasti, E. and Pramauro, E. (1978) *J. Chem. Soc. Dalton Trans.*, 61-63.
198. Pelizzetti, E. (1980) *J. Chem. Soc. Dalton Trans.*, 484-486.
199. Abbot, M.T. and Udenfreund, S. (1974) in "Molecular Mechanisms of Oxygen Activation" (Hayaishi, O., ed.), Academic Press, New York. pp. 167-214.

200. Greene, R.V. and Gould, J.M. (1983) *Biochem. Biophys. Res. Commun.* 117:275-281.
201. Kuwahara, M., Ishida, Y., Miyagawa, Y. and Kawakami, C. (1984) *J. Ferment. Technol.* 62:237-242.
202. Gross, G.G., Jouse, C. and Elstner, E.F. (1977) *Planta.* 136:271-276.
203. Koenigs, J.W. (1972) *Phytopath.* 62:100-110.
204. Arora, D.S., and Sandhu, D.K. (1985) *Enzyme Microb. Technol.* 7:405-408.
205. Renganathan, V., Miki, K., and Gold, M.H. (1985) *Arch. Biochem. Biophys.* 241:304-314.
206. Kersten, P.J., Tien, M., Kalyanaraman, B. and Kirk, T.K. (1985) *J. Biol. Chem.* 260:2609-2612.
207. Andersson, L.A., Renganathan, V., Chiu, A.A., Loehr, T.M. and Gold, M.H. (1985) *J. Biol. Chem.* 260:6080-6087.
208. Hammel, K.E., Tien, M., Kalyanaraman, B., and Kirk, T.K. (1985) *J. Biol. Chem.* 260:8348-8353.
209. Schoemaker, H.E., Harvey, P.J., Bowen, R.M., and Palmer, J.M. (1985) *FEBS Lett.* 183:7-12.
210. Harvey, P.J., Schoemaker, H.E., Bowen, R.M., and Palmer, J.M. (1985) *FEBS Lett.* 183:13-16.
211. Dunford, H.B. (1982) in "Advances in Inorganic Biochemistry Vol.4" (Eichhorn, G.L., and Marzilli, L.G., eds.). Elsevier, New York. pp. 41-68.
212. Thomas, J.A., Morris, D.R., and Hager, L.P. (1970) *J. Biol. Chem.* 245:3129-3134.

213. Floris, G., Medda, R., and Rinaldi, A. (1984) *Phytochem.* 23:953-956.
214. Horecker, B.L., and Kornberg, A. (1948) *J. Biol. Chem.* 175:385-390.
215. Lin, E.C.C., and Magasanik, B. (1960) *J. Biol. Chem.* 235:1820-1823.
216. Abei, H. (1974) in "Methods of Enzymatic Analysis 2nd Ed. Vol. 2" (Bergmeyer, H.U., and Gawehn, K., eds.). Academic Press, New York. pp. 673-684.
217. Bromfield, S.M. (1979) *Soil Biol. Biochem.* 11:115-118.
218. Bodini, M.E., Willis, L.A., Riechel, T.L., and Sawyer, D.T. (1976) *Inorg. Chem.* 15:1538-1543.
219. Bodini, M.E., and Sawyer, D.T. (1976) *J. Am. Chem. Soc.* 98:8366-8371.
220. Cotton, F.A. and Wilkinson, G. (1972) "Advanced Inorganic Chemistry; A Comprehensive Text." Interscience, New York.
221. Klebanoff, S.J. (1959) *J. Biol. Chem.* 234:2480-2485.
222. Turner, E., Somers, C.E. and Shapiro, B.M. (1985) *J. Biol. Chem.* 260:13163-13171.
223. Mason, H.S. (1981) in "Invertebrate Oxygen-Binding Proteins: Structure, Active Site and Function" (Lamy, J. and Lamy, J., eds.), Marcel Dekker, New York, pp. 455-468.
224. Hubert, E.E. (1931) "An Outline of Forest Pathology." John Wiley and Sons, Inc., New York.
225. Blanchette, R.A. (1984) *Phytopath.* 74:725-730.

226. Hooper, A.B., and DiSpirito, A.A. (1985) *Microbiol. Rev.* 49:140-157.
227. Bromfield, S.M. (1956) *Aust. J. Biol. Sci.* 9:238-252.
228. Krumbein, W.E. (1971) *Naturwissenschaften* 58:56-57.
229. Erlich, H.L. (1975) *Soil Sci.* 119:36-41.
230. Arcuri, E.J., and Erlich, H.L. (1979) *Appl. Environ. Microbiol.* 37:916-923.
231. Ivarson, K.C., and Heringa, P.K. (1972) *Can. J. Soil Sci.* 52:401-416.
232. Bromfield, S.M. (1976) *Aust. J. Soil Res.* 14:95-102.
233. Leeper, G.W. and Swaby, R.J. (1940) *Soil Sci.* 49:163-169.
234. Morgan, J.J. and Stumm, W. (1965) in "Advances in Water Pollution Research 1964 Vol. 1" (Jagg, D., ed.). Pergamon Press, London.
235. Huynh, V.-B., and Crawford, R.L. (1985) *FEMS Microbiol. Lett.* 28:119-123.
236. Paszcynski, A., Huynh, V.-B., and Crawford, R. (1985) *FEMS Microbiol. Lett.* 29:37-41.
237. Gilmore, J.R., and Mellor, J.M. (1971) *J. Chem. Soc. (C)*. 2355-2357.
238. Barek, J., Berka, A., and Civišová, D. (1984) *Collect. Czech. Chem. Commun.* 49:954-962.

## VITA

Jeffrey K. Glenn was born in Chicago, Illinois on October 30, 1952. He received the B.A. degree in Biochemistry from Northwestern University, Evanston, Illinois in August, 1974. He immediately entered the Molecular Biology Program at the University of Wisconsin - Madison, and received the M.S. degree in Molecular Biology in June, 1978. He remained in Madison for an additional year as a student in the Library Science Department at the University of Wisconsin. During this time he worked in the Chemistry Department as a teaching assistant and tutored physics for the Minority Engineering Program of the School of Engineering. Mr. Glenn received the M.A. degree in Library Science in July, 1979.

In August, 1979 Mr. Glenn began working for the Science Departments at Kentucky State University in Frankfort, Kentucky. He set up the Science Learning Center, an audio-visual aids program, and taught introductory chemistry.

In August, 1980 he began his studies for the Ph.D. at the Oregon Graduate Center.