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THE INVOLVEMENT OF GLUCOSE OXIDASE IN LIGNIN DEGRADATION BY THE WHITE-ROT FUNGUS *PHANEROCHAETE CHRYSOSPORIUM*

By

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ABSTRACT

THE INVOLVEMENT OF GLUCOSE OXIDASE IN LIGNIN DEGRADATION BY THE WHITE-ROT FUNGUS PHANEROCHAETE CHRYSOSPORIUM

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Previous studies have shown that the production of hydrogen peroxide (H_2O_2) plays an important role in lignin degradation by the white-rot fungus, Phanerochaete chrysosporium. However, the metabolic source of H_2O_2 in ligninolytic cultures of this fungus was not known. In this study, glucose oxidase was identified as the primary physiological source of H_2O_2 in cell extracts of ligninolytic cultures of P. chrysosporium. Polyacrylamide gel electrophoresis of extracts from ligninolytic cultures followed by the diaminobenzidine/horseradish peroxidase staining procedure showed the presence of a single protein band that exhibited glucose-dependent H_2O_2 production. Both glucose oxidase activity and lignin degradation were triggered in response to nitrogen (N) or carbohydrate starvation, and were repressed in media containing high levels of N (24 mM) and carbohydrate (56 mM) or on the addition of exogenous N sources, such as glutamate, to the low N medium (2.4 mM N). The results indicated that glucose oxidase activity was the primary source of H_2O_2

production in ligninolytic cultures of P. chrysosporium and that nutritional parameters which affected lignin degradation had a parallel affect on glucose oxidase activity.

Glucose oxidase from ligninolytic cultures of P. chrysosporium was purified to homogeneity and was shown to be a flavoprotein with an apparent native molecular weight of 180,000 daltons and a denatured molecular weight of 80,000 daltons. It had optimal activity with D-glucose, which was stoichiometrically oxidized to D-gluconate. The Km values for glucose and O_2 were 38 mM and 0.95 mM, respectively. The enzyme had a pH optimum of 4.5. It was inhibited by Ag+ and o-phthalate but not by Cu++, NaF or KCN.

In an effort to better understand the involvement of glucose oxidase in lignin degradation, mutants deficient in this activity (gox-) were isolated and characterized. The mutant strains had little ligninolytic activity (2-(^{14}C) synthetic lignin ==> $^{14}CO_2$, were unable to decolorize poly R dye 481, and were also deficient in "ligninase" (a lignin-degrading enzyme) and peroxidase activity. Gox+ revertants regained most of the lost activities including the ability to degrade lignin. The results suggest that the genetic lesion in gox$^-$ mutants affects the regulation of a set of secondary metabolic characteristics.

To my parents, Erin, Jacki and Karin

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Lignin is one of the most abundant biopolymers in nature constituting approximately 20-30% of the dry weight of all vascular plants (23,94,136). Hence, lignin biodegradation and its utilization as a renewable resource for the production of chemical feedstocks and other useful products is of great interest. Previous studies have shown that lignin biodegradation is an oxidative process (101) and that hydrogen peroxide (H_2O_2) plays an important role in this degradation (136,137). A temporal correlation between ligninolytic activity and H_2O_2 production by *Phanerochaete chrysosporium* has been demonstrated (45,46). Growth under 100% O_2 markedly increased both H_2O_2 production and ligninolytic activity, and lignin degradation was inhibited by catalase and a scavenger of H_2O_2 (38). Cytochemical diaminobenzidine (DAB) staining techniques revealed that H_2O_2 production in ligninolytic cultures of *P. chrysosporium*, is localized in ovoid periplasmic microbody-like structures which are not found in non-ligninolytic

cells (46). Several H_2O_2-dependent, lignin degrading enzymes, have been isolated from the extracellular fluid of P. chrysosporium cultures. These enzymes were only found in ligninolytic cultures and have been shown to oxidize a variety of lignin model compounds (54,62,152,153), depolymerize lignin and decolorize the polymeric dye poly R481 (62,113). It became obvious from these studies that H_2O_2 plays an important role in lignin degradation by P. chrysosporium, however, the physiological source of H_2O_2 in ligninolytic cultures of this organism was not known.

The objective of this research is to determine the physiological source of H_2O_2 in lignin-degrading cultures of P. chryosoporium, study the nutritional and physiological parameters regulating this activity. Also, the enzyme(s) involved in H_2O_2 production will be purified and characterized.

Literature Review

Lignin is a three-dimensional, highly-branched, amorphous, aromatic polymer present in all vascular plants. Unlike other natural polymers, such as cellulose, starch and proteins, it has no precise structure and contains no readily hydrolyzable linkages that repeat at regular intervals (129,165).

Instead, lignin is an irregular polymer with many intermonomeric linkages recurring randomly throughout the molecule and thus, is one of the most resistant compounds to biological degradation.

Lignin is one of the most abundant and widely distributed renewable organic polymers on earth. It constitutes 25% of the dry weight of the estimated 100 billion metric tons of biomass produced annually in the biosphere, and is second only to cellulose in its abundance (136). However, lignin contains 60% of the carbon and 40% of the fuel value of total biomass (88) and thus is more important than its weight would indicate. Furthermore, it has been estimated that 1-3 x 10^{12} metric tons of lignineous material, primarily as peat and humus (161), have accumulated in the soil. Because of its great abundance and recalcitrance to microbial attack, lignin biodegradation plays an important role in the terrestrial carbon cycle.

In the past decade, the possible industrial uses of lignocellulosic materials for the production of various fuels, animal feeds and chemical feedstocks have been discussed in numerous reviews (20,28,34,87,95,107,136). A recent review by Janshekar and Fiechter (87) discussed in detail the possible applications and products which can be made from lignin (Table 1). It is estimated that 900-3,000

Table 1: Industrial Applications for Lignin.

1. Energy
2. Polymers and modified polymers

   filters, rubber reinforcements, carriers for controlled release in fertilizers and pesticides, dispersants, emulsion stabilizers, complexing agents
3. Prepolymers

   polyphenolics, resins and extenders, foams, adhesives
4. Fragmentation and chemical conversion

   a. hydrogenation---phenols, hydrocarbons

   b. hydrolysis---phenols, catechols, substitued phenols

   c. oxidation---vanillin, dimethyl sulfide

   d. alkali fission---phenolic acids, catechols

   e. pyrolysis---acetic acid, phenols, CO, CO_2, CH_4

   f. fast thermolysis---acetylene, ethylene

Adapted from reference 87.

million tons (87) of lignocellulosic by-products are generated annually by forestry, agriculture, paper-making and lumbering industries in the U.S. alone. Lignin occurs in close chemical and physical association with cellulose and hemicellulose in these materials and limits the efficient utilization of these carbohydrate polymers into useful products. Numerous pretreatments which depolymerize, solubilize or otherwise remove lignin from lignocellulosic materials, have been developed (17,39); however, most of these processes are either too energy intensive, create large quantities of chemical wastes or cannot be used on an industrial scale (95). Thus, in recent years lignin biotransformation by microbes has been an area of great interest (17,95,98,121,154). It is hoped that controlled microbial treatment of lignocellulosic materials might be a cost effective and energy efficient pretreatment process for the optimum utilization of biomass as useful products. As fossil fuel feedstocks become more scarce, chemical feedstocks, fuels and solvents made from biomass are likely to become economically competitive with those currently produced from petroleum (73).

In this review, I will discuss briefly the available literature on the biodegradation of lignin. I will focus on recent evidence for the involvement of

reduced oxygen species, in particular H_2O_2, in lignin degradation by certain wood-rotting fungi.

Structure and Chemistry of Lignin

A number of recent reviews (1,65,72,134) and books (13,28,105,132,147) have covered the accumulated knowledge on the chemistry and biosynthesis of lignin and should be consulted for a more complete treatment of the subject.

Biosynthesis and Structure. Lignin is a complex and variable biopolymer made of three cinnamyl alcohol derivatives; p-coumaryl, coniferyl and sinapyl alcohols (Figure 1). These precursors are synthesized by the general path: CO_2 ==> carbohydrates ==> phenyl propanoid amino acids ==> cinnamic acid derivatives ==> cinnamyl alcohol derivatives (28,77). The ratio of these three alcohols are known to vary between lignins from different plant species and with the age and type of tissue within a single species (145). Higuchi et al. (77) defined three major groups of lignins based on the relative ratios of these derivatives: Softwood lignin or guaiacyl lignin (found in most conifers, lycopods and ferns) is composed mostly of coniferyl alcohol units with a small amount of coumaryl and synapyl alcohol units. The relative proportions in spruce lignin are 80:14:6,

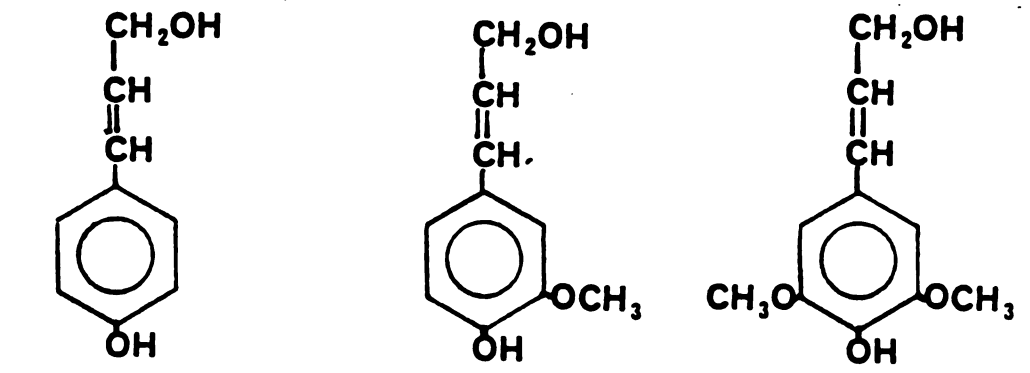


Figure 1. The structure of the immediate precursors used in the biosynthesis of lignin.

respectively. Hardwood lignin or guaiacyl-syringyl lignin, found mostly in angiosperms, contains approximately equal amounts of coniferyl and synapyl alcohol units with only minor amounts of coumaryl alcohol units. The proportions in beechwood are 49:46:5, respectively. Grass and bamboo lignin (guaiacyl-syringyl-p-hydroxyphenyl lignin) are thought to be composed of approximately equal amounts of all three cinnamyl alcohols; however, the exact ratios are not known because a considerable amount of p-courmaric acid is bound as esters to grass lignins (80).

Lignin is formed by the dehydrogenative polymerization of the above-mentioned cinnamyl alcohol derivatives (48,49,72,79,145). At the site of lignification these alcohols are oxidized by phenol-oxidases to yield phenoxy radicals which, because of their extended electron systems, are stabilized through equilibrium with several mesomeric forms. These different radical species derived from the three cinnamyl alcohols randomly condense with each other and with the radicals in the growing lignin polymer. A variety of intermonomeric linkages result (Table 2), including a number of C-C and C-O-C linkages, forming a complex, three-dimensional polymer. Several models of hard- and soft-wood lignin have been proposed (1,72,124), including a model by Harkin (Figure 2) of

Table 2: Major intermomer linkages and their frequencies in gymnosperm (spruce) and angiosperm (birch) lignins.

Linkage type	Units in Figure 3	% of total C9 units	
		spruce	birch
β-Aryl ether (β-O-4)	1-4, 5-4 5-6, 7-8, 13b-14b, 15-16	48	60
Phenylcourmaran (β-5)	17-18	9-12	6
Biphenyl (biaryl or 5-5)	12-13a	9.5-11	4.5
1,2-Diarylpropane (β-1)	16-20	7	7
Diphenyl ether (diaryl or 4-O-5	6-7	3.5-4	6.5
α-Aryl ether (α-O-4)	11-12	6-8	6-8
Pinoresinol (β-β)	8-9	2	

From reference 136.

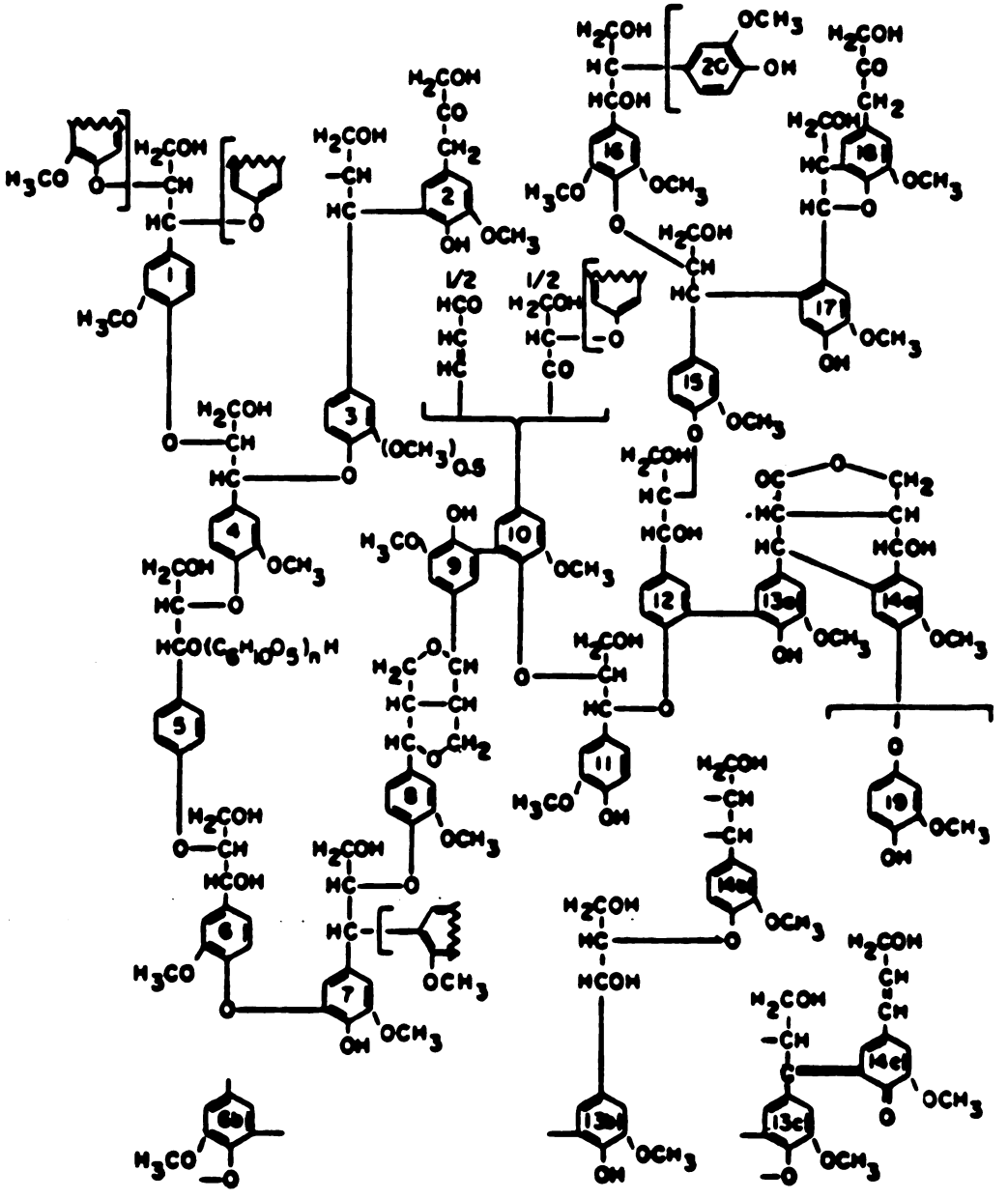


Figure 2. Schematic representation of spruce lignin (from reference 136).

a spruce lignin. The molecular weight of purified lignin varies from a few thousand to more than 10^6 daltons, depending on the isolation method. Therefore, the polymer illustrated in Figure 2 and most other lignin models published represent only a portion of the total lignin molecule (28).

Occurrence and Function of Lignin In Nature. Approximately 80% of lignin in plants is found within the secondary cell walls, closely associated with hemicellulosic and cellulosic components (145). However, the highest concentration of lignin is present in middle lamella, serving as an intercellular cementing substance that binds the cells together (44). Lignin is a recalcitrant polymer and thus protects plant cells against infection by plant pathogens. Lignin also minimizes water permeation across the cell wall of xylem tissue, imparts structural rigidity, and confers resistance to impact, compression and bending (145,146).

A major portion of the carbohydrate components of the cell wall are quickly metabolized when the plant dies, but lignin, although structurally modified is degraded only to a limited extent and forms a major constituent of humus and peat (164,165), which increase the aeration and moisture holding capacity of the soil and serves as an ion exchange resin

sequestering important inorganic molecules that increase the general soil fertility (82).

Biodegradation of Lignin

Quantification and Chemical Characterization of Lignin Degradation. Major advances have been made in understanding the biological decomposition of lignin because of the development of specific and sensitive radioisotopic methods for assaying metabolism of lignin. These assays are based on the oxidative metabolism of ^{14}C-[lignin]-lignocelluloses or $^{14}CO_2$. Both the rate and extent of lignin biodegradation can be determined in these radioisotopic assays by trapping and quantifying the $^{14}CO_2$ produced from these substrates.

^{14}C-[lignin]-lignocellulose can be selectively extracted from plants which have been fed labeled precursors of lignin biosynthesis (21,25,30,31,69), such as ^{14}C-phenylalanine. On the other hand, ^{14}C-DHPs are prepared by the dehydrogenative polymerization of chemically synthesized ^{14}C-coniferyl alcohol in a peroxidase/H_2O_2 catalyzed reaction (70,103). The advantages of using DHPs are: 1) they do not contain extraneous materials, such as carbohydrate; 2) they can be specifically labeled in

the side chain, aromatic ring or methoxyl groups and, therefore, the specific (structural/ chemical) nature of the attack on the lignin polymer by a particular microorganism can be determined. It is important to note that there may be considerable differences between the amount of $^{14}CO_2$ detected when ^{14}C-[lignin]-lignocellulose and ^{14}C-DHP are used as substrates. For example, Robinson et al. (144) found that a Bacillus sp. could degrade approximately 12% of ^{14}C-[lignin]-lignocellulose, but only 0.3% of ^{14}C-DHPs to $^{14}CO_2$ in 20 days. Also, failure to detect $^{14}CO_2$ from ^{14}C-lignins does not exclude the possibility of significant but partial degradation of the lignin polymer.

Recently, Glenn and Gold (54) showed that ligninolytic activity by P. chrysosporium is closely correlated with its ability to decolorize of polymeric dyes (e.g. Poly B-411, Poly R-481 and Poly Y-606) and have suggested that decolorization of these readily soluble, stable, inexpensive substrates could be used as a simple screening test for the selection of non-lignin degrading mutants of P. chrysosporium. Kelley and Reddy (91) showed a similar correlation between the production of ethylene from α-oxo-q-methylthiobutyric acid (KTBA) and ligninolytic activity in P. chrysosporium and suggested that

ethylene production from KTBA can be used as a sensitive measure of ligninolytic activity by wild-type P. chrysosporium.

A variety of other chemical and physical methods of varying degrees of reliability have also been developed for the study of lignin degradation (28, 87). Spectroscopic methods, including UV, IR and NMR, have been used to monitor lignin degradation (15,89,115,118). Chromatographic techniques, such as gel permeation chromatography, high performance liquid chromatography, and gas chromatography have been used to determine changes in molecular weight distribution of the lignin polymer and to identify aromatic acids produced during its degradation (81,85,100,102,117,118). Electron microscopic studies have also been used to understand the morphological changes in wood components that have been subjected to attack by wood-rotting fungi (4,11,36,44). Functional group analysis and elemental composition have provided useful information about the chemistry of lignin degradation (101,102). However, because of the complexity and diversity of the lignin polymer, a combination of chemical and/or physical methods mentioned above should be used to obtain complete information about the wide variety of modifications that occur during lignin degradation (87).

Lignin Degrading Organisms. Previous workers have shown that several different genera of bacteria and fungi are capable of metabolizing lignin and several reviews have been published on this subject (6,14,23,29,94,96,98,135, 136,164). Of the ligninolytic microorgansisms described to date, a group of mostly basidiomycetes and a few ascomycetes, called white-rot fungi, are known to degrade lignin more rapidly and more extensively than other micro-organisms. Consequently, this group of microorganisms has been studied most thoroughly. White-rot fungi are recognized by their ability to deplete all the major wood components: cellulose, hemicellulose and lignin and cause a whitish coloration in the decayed wood (4,28,98). These organisms can be separated into two primary groups: 1) simultaneous rot fungi which degrade all wood components at approximately the same rate and 2) white-pocket rot fungi which preferentially degrade lignin compared to cellulose and hemicellulose (11,87). P. chrysosporium, Coriolus versicolor, and Pleurotus ostreatus, which are simultaneous rot fungi, have all been shown to degrade ^{14}C-DHPs labeled in the ring, side chain or methoxyl group to $^{14}CO_2$ (67,68). Chemical analyses, spectroscopic analyses and chemical degradation studies, of spruce lignin attached by P.

chrysosporium, Polyporous anceps, Coriolus versicolor and Poria subacida showed that lignin degradation by these fungi is largely oxidative as evidenced by the high oxygen content, and the low hydrogen and methoxyl content of the decayed polymers in comparison to sound lignin (101,102). Electron microscopic studies of white-rot decay have shown that erosion troughs are formed in the immediate vicinity of the fungal hyphae (4,6,115) which are believed to be caused by extracellular catalysts. SEM studies revealed selective removal of lignin, but not cellulose and hemicellulose, by Xylobolus frustulans, Phellinus pini and Ionotus dryophilus (11,130).

Brown-rot fungi, which include numerous genera (53) that are taxonomically similar to white-rot fungi, characteristically attack the carbohydrate components of wood in preference to the lignin components and leave a brown residue in the decayed wood (8,97). Kirk and Adler (99) showed, by chemical analyses of sweetgum decayed by Lenzites trabea, that brown-rot fungi cause considerable alteration of the lignin polymer, including demethoxylation and ring hydroxylation, but little aromatic ring cleavage was detected. Thus, the principal difference between brown-rot and white-rot fungi appears to be the ability of the latter to metabolize aromatic rings

(97).

The soft-rot fungi, which include a variety of ascomycetes and fungi imperfecti (37,67,68), principally attack the carbohydrate components of wood, and the decay is usually accompanied by a softening of the surfaces of the woody tissue. Eslyn et al. (37) examined the degradation of specific wood components from various woods decayed by strains of Graphium, Monodicyls, Paccilomyces, Papulspora, Thielavia and Allescheria, and found that in alder and poplar wood the carbohydrate moieties were depleted faster than lignin by most of the species examined. Haider and Trojanowski (67) found that soft-rot species, Preussia, Chaetomium and Stachybotrys, were capable of releasing substantial amounts of $^{14}CO_2$ from ^{14}C-ring-, side chain-, and methoxyl-labeled DHP and from ^{14}C-[lignin] lignocellulose. These studies show that soft-rot fungi can metabolize ^{14}C-lignin to $^{14}CO_2$, but the detailed chemical nature of soft-rotted lignin remains to be elucidated.

Bacterial degradation of lignin has recently been reviewed by Crawford and Crawford (23). A number of bacteria including Nocardia sp. (35), Pseudomonads sp. (47), Aeromonas sp. (126), Xanthomonas sp. (128), Bacillus megaterium (144) and Streptomyces sp. (22-27), have been shown to degrade ^{14}C-DHP and ^{14}C-

[lignin]-lignocellulose to $^{14}CO_2$. For example, Nocardia sp. has been shown to convert ^{14}C-ring-, side chain-, and methoxyl-labeled DHP (5, 15 and 13%, respectively) to $^{14}CO_2$ (153). A number of strains of streptomyces were shown to degrade lignin but apparently cannot use the latter as a sole source of carbon and energy (27). Streptomyces badius was shown to degrade 13% of ^{14}C-[lignin]-lignocellulose in 42 days and SEM studies showed that it can degrade lignified cell walls in the inner bark of Douglas fir (27,150). A strain of Xanthomonas sp. has been reported to degrade as much as 77% of dioxane lignin in 15 days when it is provided as the sole carbon and energy source (128); however, dioxane lignin is not considered to be reliable substrate for studies on lignin biodegradation (28). Pseudomonas sp. were shown to be capable of utilizing kraft lignin as a sole source of carbon and energy; in fact, Forney et al. (47) found that addition of 0.01% (w/v) glucose to mixed cultures of pseudomonads actually decreases the extent of degradation of kraft lignin. Information about the biochemical nature of lignin degradation by bacteria is sparse; however, Crawford et al. (12,27,29) found that decay by Streptomyces viridosporus T7A was oxidative and involved demethylations, ring cleavage reactions, and oxidative

attack on phenylpropanoid side chains, which is similar to what is observed during white-rot degradation of lignin.

Until recently, anaerobic degradation of lignin was thought not to occur to any great extent (126,127,164). Brenner et al. (10) recently reported methanogenic degradation of ^{14}C-lignins incubated with anaerobic lake sediments (10). They have shown significant (17% in 294 days) anaerobic degradation or of ^{14}C-[lignin]-lignocellulose in aquatic sediments has been reported. However, the chemical nature of this degradation is not known. Also, a number of lignin model compounds, including 3,4,5 trimethoxybenzoic, syringic and 3-O-methylgallic acid as well as methoxylated and hydroxylated benzoate have been shown to be degraded by pure cultures of obligate anaerobic bacteria (74,163).

Physiology of Lignin Biodegradation

Most of the recent investigations on the physiology and biochemistry of lignin involved a single species, P. chrysosporium Burds (ATCC 34541; 16). The attractiveness of this organism lies in its rapid growth, extensive degradation of lignin, prolific conidiation, relatively high temperature optimum (40°C) and relatively low phenol oxidase

activity (which can cause repolymerization of degraded lignin fragments; 94,96). Also, optimal culture conditions for lignin degradation by this organism are known. These studies showed that lignin degradation is optimal at a pH of 4 to 4.5. (42,108). Sodium 2,2-dimethylsuccinate is the preferred buffer, whereas o-phthalate inhibits lignin degradation (42). A mixture of inorganic nitrogen source (e.g. NH_4NO_3) and an organic nitrogen source (e.g. asparagine) promotes optimal growth and lignin degradation (93,108). Thiamine is required for growth and a mixture of trace metals stimulate growth and lignin degradation (88,103,140).

In the past decade a wealth of information has become available on the physiology of lignin degradation by P. chrysosporium. These studies show that liginolytic activity by P. chrysosporium is a secondary metabolic event which is derepressed following the cessation of primary growth in response to either nitrogen, carbohydrate, or sulfur starvation (88,108). Synthesis of lignin degrading system does not require the presence of lignin in the growth medium (93). Lignin does not serve as a sole source of carbon/energy for P. chrysosporium or for a number of other white-rot fungi and that a co-substrate, such as glucose, xylose, cellulose, cellobiose or

succinate, is required for lignin degradation (3,104,108). Although the ligninolytic system is known to be synthesized in the absence of lignin, recent studies show that ligninolytic activity is several fold higher when P. chrysosporium is grown in the presence of lignin (155) or veratryl alcohol (38).

Nitrogen Effect: Lignin degradation by P. chrysosporium is profoundly affected by the level of nutrient nitrogen provided. Cultures grown with limiting amounts of nitrogen (2.4 mM) rapidly deplete the N source and enter the stationary phase of growth (108,114,138,140,162) during which the ligninolytic system appears and secondary metabolites, such as veratryl alcohol (119,148), are produced. Furthermore, the addition of exogenous nitrogen, such as NH_4+ or glutamate, to ligninolytic cultures inhibits lignin degradation (41,43). Glutamate, glutamine and histidine suppress ligninolytic activity, (83, 76 and 76%, respectively), compared to a control with no additions (41). The addition of an N source to ligninolytic, idiophasic cultures appears to cause the transition to primary growth, stimulating not only glucose and succinate metabolism but growth in general, and stopping secondary metabolic events, such as synthesis of veratryl alcohol and lignin degradation (43). Kirk et al. (94) hypothesized that

(nitrogen metabolism via) glutamate (metabolism) plays an important role in the initiation of the repression of secondary metabolism and in turn ligninolytic activity. In addition, Reid (140) has suggested that C/N ratio maybe more important to the control of lignin degradation than the absolute levels of nitrogen because abundant carbon causes the available nitrogen to be used in cell synthesis so that it does not repress ligninolytic activity. Recently, cyclic AMP levels have been shown to rise 10-fold in response to nitrogen starvation, and it has been speculated that cyclic AMP levels may be important in the regulation of secondary metabolism, including ligninolytic activity (120).

Degradation of a lignin model compound, 4-hydroxy-3-methoxyacetophenone as well as other lignin model compounds with b-guaiacyl ether-linkages has also been shown to occur in nitrogen starved cultures and was inhibited in high N cultures or by the addition of exogenous NH_4+ to nitrogen starved cultures (159). Interestingly, Odier and Roch (129) found that some white-rot fungi such as Dichomitus squalens, showed no suppression of the ligninolytic system by high levels of nitrogen.

Carbohydrate and Sulfur Effect: Recently, Jeffries et al. (88) have shown that ligninolytic

activity (^{14}C-synthetic lignin ==> $^{14}CO_2$) occurs in fungal cultures containing nonlimiting nitrogen (24 mM), but limiting glucose (8.8 mM). They have also shown that these culture conditions cause autolysis of the fungal cells and that autolysis ceases when ligninolytic activity does, suggesting that autolysis of some cells provides carbon and energy sources for other remaining cells to cause lignin degradation. Ligninolytic activity has also been reported to be triggered by limiting sulfur (20 mM) (88). However, Reid (139) in similar studies did not observe any stimulation of ligninolytic activity by P. chrysosporium under sulfur limiting conditions using ^{14}C-(lignin) lignocellulose as the substrate.

Oxygen Effect: Kirk et al. (108) observed a 2-fold increase in ligninolytic activity by P. chrysosorium when grown under 100% O_2 as opposed to air. Bar-Lev and Kirk (9) showed that O_2 did not induce the ligninolytic system, but enhanced lignin metabolism after the system was formed. These results are consistent with the finding that lignin degradation is a highly oxidative process. However, O_2 pressure above 1 atm inhibits the growth of P. chrysosporium and does not increase the rate of lignin metabolism (142,143).

Lignin degradation by P. chrysosporium as well as

Coriolus versicolor has been shown to be strongly inhibited by agitation of the cultures (108,162). It is believed that agitation causes pellet formation which restricts not only the cell surface area exposed to the lignin polymer, but also limits the amount of O_2 diffusion into the interior of the pellet. However, recent work by Reid (141) seems to contradict these findings. In these studies, agitation of ligninolytic cultures did not significantly affect the rate of extent of ^{14}C-DHP degradation and in fact increased the degradation of ^{14}C-[lignin]-lignocellulose.

Biochemistry of Lignin Degradation

Our knowledge of the biochemical mechanism of lignin degradation has been advanced significantly in the past 5 years, and this knowledge and associated methodologies should contribute greatly to our understanding of lignin biodegradation in the coming few years. Recent studies have extended our knowledge on degradative pathways for several low molecular weight lignin model compounds used by white-rot fungi (87). The involvement of oxygen radicals (2,38,45,112) and extracellular lignin degrading enzymes (50,113, 151,152) in lignin degradation have been perhaps the most significant recent discoveries

in this area. Techniques for selecting mutants (58,59) and complementation analysis (56,59,60,61) have been developed. Finally, an autonomous replication sequence from P. chrysosporium has been characterized (133) and construction of lambda genomic library of this organism has been described and is being used for obtaining a better understanding of the genetics of lignin degradation (158).

Lignin biodegradation, based on cumulative evidence in the past two decades, appears to be an extracellular, oxidative and nonspecific process. Because of the size of the lignin polymer, direct uptake by microbial cells seems unlikely, and therefore, at least the initial attack on the lignin polymer must be extracellular. Analyses of white-rotted lignins and the solubilized products have shown a substantial increase in carboxyl and carbonyl groups and a decrease in hydrogen content suggest the oxidative nature of lignin degradation (84,85,101,102). Oxidation of the α-carbon of the propyl side chain of lignin monomers to a carbonyl group and hydroxylation and oxidative cleavage of the aromatic rings (94) offers further support to this conclusion. The fact that lignin is extensively degraded despite the variety of intermonomeric linkages (94) and the racemic nature of the asymmetric

carbons in the polymer indicates that the attack on lignin is nonspecific and nonstereoselective (122,125,149). Also, a variety of polymers which are structurally different, such as polyguaiacol (32) and Poly-R dyes (54), are metabolized efficiently by white-rot fungi. Therefore, given the specific nature of most enzymes, it was first thought that there would be a wide variety of enzymes involved in lignin degradation.

Enzymology of Lignin Biodegradation. While most of the enzymes responsible for lignin degradation have not been determined, several types of enzymes from various microorganisms which are thought to participate in lignin degradation have been described: 1) phenoloxidases (laccase, peroxidase and tyrosinase), 2) mono- and di-oxygenases, 3) aromatic alcohol oxidase and moist recently, 4) "ligninase", a new class of lignin-degrading enzymes. All these enzymes could account for the oxidative nature of lignin degradation, were extracellular and were relatively non-specific as evidenced by their reactivity with several types of lignin model compounds.

The phenol-oxidizing enzymes known collectively as phenol oxidases includes three distinct types of enzymes: laccase (O_2: p-diphenol oxidoreductase),

peroxidase (donor: H_2O_2 oxidoreductase). Reviews by Ander and Eriksson (7,8) present a detailed description of the reactions catalyzed by these enzymes. Phenol oxidases can cause limited structural changes in lignin, but their involvement in total degradation of lignin to CO_2 is unlikely (28,52,66,87,90,125). The functional roles attributed to phenol oxidases include: 1) detoxifying low molecular weight phenols released during lignin degradation (52), 2) performing reactions critical to the preparation of the lignin polymer for degradation, such as demethylation or removal of asymmetric centers making the polymer more desirable to stereospecific attack by enzymes (102,149) 3) limited depolymerization of lignin polymer and 4) regulation both lignin-degrading and polysaccharide-degrading enzymes (5). Mutants of P. chrysosporium lacking phenol oxidase were unable to degrade lignin; however, these mutants were later shown to be pleiotropic for a variety of other enzymes (5,59). Furthermore, at least one of these mutants (phe3) was shown recently to extensively metabolize certain lignin preparations (Ander, personal communication). Thus, the actual function of phenol oxidases in lignin degradation is yet to be established.

Both mono- and di-oxygenases can catalyze

reactions which would aid in the biodegradation of lignin. Mono-oxygenases by incorporating one atom of oxygen cause of hydroxylation of methylated or demethylated aromatic rings (18) which results in the formation of ortho-diphenols and an increase the solubility of lignin substrates. Recently, lignin-degrading oxygenases have been isolated (see below) which actually appear to be peroxidases. Dioxygenases incorporate both atoms of oxygen into the ring structure which helps in ring cleavage (78). Both mono- and di-oxygenases have been reported in a variety of white- and brown-rot fungi (78), but their exact involvement in lignin degradation is not known.

An aromatic alcohol oxidase, isolated in culture filtrates of Fusarium solani (86) and Polystictus versicolor (40), oxidizes a wide variety of aromatic monomers and dimers as well as various lignin preparations having α, ß-unsaturated alcohol groups in their side chains. This enzyme is extracellular, nonspecific and reacts with the lignin polymer and could be important in lignin degradation by this organism in preparing the phenylpropanoid side chains for further oxidation (28).

Several other enzymes have been thought to be important in lignin degradation. Ander and Eriksson (8,160) proposed that cellobiose:quinone

oxidoreductase (CBQase) is involved in the degradation of both cellulose and lignin by Sporotrichum pulverulentum, but its requirement for lignin degradation has not been shown. Greene (63) has postulated the involvement of glucose oxidase in lignin degradation by Polyporous versicolor based on the rationale that it can: 1) prevent the toxic accumulation of quinoid intermediates and 2) improve the efficiency of lignol oxidations. He has suggested that glucose oxidase preferentially reacts with quinoid than O_2. This suggestion would lead to the prediction that reduced oxygen tension should increase lignin degradation, when in fact the opposite is true. Also, experimental support for the proposed reactions with the lignin polymer is lacking. And in fact, no enzyme has been isolated by Greene that could catalyze many of the complex reactions that occur during lignin degradation by white-rot fungi, such as cleage of ß-O-4 linkages, cleavage of $C\alpha^{-C\beta}$ side-chain bonds, oxidative fission of aromatic ring and demethylations (29). This led to the belief that the initial attack on lignin may not be enzymatic, but due to the action of oxygen radicals (71).

Involvement of Reduced Oxygen Species in Lignin Degradation. Amer and Drew (2) reported that superoxide anion (O_2^-) is produced by whole cells of

Coriolus versicolor and speculated that this anion may be involved in lignin degradation by this fungi. The observation that the addition of superoxide dismutase to cultures of P. chrysosporium inhibits ligninolytic activity supports this idea (38). However, the relative non-reactivity of O_2^- and the growing belief that essentially all the manifestations of O_2^- can be explained by its participation in generating hydroxyl radicals (·OH) in aqueous systems has led to the suggestion that O_2^- may not be directly involved in lignin degradation, but may serve as a reductant in the iron-catalyzed Haber-Weiss reaction (see below) for the production of ·OH (146).

Nakatsubo et al. (123) reported inhibition of lignin degradation by P. chrysosporium using anthracene-9,10-bis ethanesulfonic acid (AES) as a scavenger of singlet oxygen ($'O_2$). However, Kirk et al. (106) questioned the involvement of $'O_2$ in lignin degradation, after finding that artificial $'O_2$ generating systems produce different degradative produces from ß-1-lignin model compounds compared to those produced by P. chrysosporium.

Forney et al (45) proposed that H_2O_2-derived, ·OH may be involved in the attack on the lignin polymer by P. chrysosporium. This proposed was supported by the following observations: i) ·OH is highly reactive

with a variety of organic compounds (33,71), including rapid degradation/depolymerization of the lignin polymer (T. McFadden, L. Forney and C. Reddy, unpublished data); ii) its attack is nonspecific and nonstereoselective (157); and iii) it is generated in biological systems by a one-electron reduction of H_2O_2 (19,33,51; See Reaction 1 and 2 below) which is known to be produced by numerous wood rotting fungi (75,76,109-111).

Fenton Reaction

1) $H_2O_2 + Fe\ (II) \Longrightarrow \cdot OH^- + Fe\ (III)$

Iron-Catalyzed Haber-Weiss Reaction

$$Fe(III)\ chelate + O_2^- \Longrightarrow Fe(II)\ chelate + O_2$$

$$Fe(II)\ chelate + H_2O_2 \Longrightarrow \cdot OH + OH^- + Fe(III)\ chelate$$

---

2) Sum: $H_2O_2 + O_2^- \Longrightarrow \cdot OH + O_2$

The involvement of H_2O_2 and $\cdot OH$ in lignin degradation by P. chrysosporium has been examined in several studies. The production of H_2O_2 and $\cdot OH$ (as measured by ethylene production from α-oxo-q-methylthiobutyric acid) was shown to coincide with the appearance of ligninolytic activity (45,137). H_2O_2 production was markedly enhanced by growing the fungus under 100% O_2, mimicking what is seen with

ligninolytic activity (38). The addition of either •OH scavenging agents (e.g. mannitol and benzoate) or catalase, a H_2O_2 scavenger, to ligninolytic cultures of P. chrysosporium not only inhibited •OH and H_2O_2 production, but also, inhibited lignin degradation (38,45). Hydroxyl radical was demonstrated in cell extracts of ligninolytic cultures by showing the •OH-dependent hydroxylation of p-hydroxybenzoic acid to form protocatechuic acid, and by detection of the nitroxide radical of 5,5-dimethyl-1-pyroline-N-oxide by EPR (45). Both the hydroxylation reaction and the formation of the nitroxide radicals were markedly stimulated by azide which is known to inhibit catalase (45). Electron microscopic studies showed that H_2O_2 production by ligninolytic cell of P. chrysosporium is located in unique periplasmic microbodies which are not found in non-ligninolytic cells (46).

The involvement of H_2O_2 and •OH in lignin degradation is in keeping with the earlier findings that lignin degradation is oxidative in nature and is mediated by non-specific, non-stereoselective extracellular agents (54,122). The involvement of •OH is also in agreement with reports that the aromatic rings of lignin are extensively hydroxylated during degradation by white-rot fungi (102). Several enzymatic activities which produce H_2O_2 in fungi are

known, including glucose oxidase, galactose oxidase, sorbose oxidase and carbohydrate oxides (83). Greene and Gould (64) have suggested that a peroxisomal fatty acyl coenzyme A oxidase activity may be an important source H_2O_2 in ligninolytic cultures of P. chrysosporium. However, the specific activity for this enzyme is very low and a temporal correlation between the onset of lignin degradation and fatty acyl CoA oxidase activity has not been shown.

While many researchers were trying to determine the involvement of oxygen radicals in lignin degradation, a new class of enzymes have recently been isolated from P. chrysosporium which can account for many of the reactions involved in lignin degradation. Lignin-degrading enzymes ("ligninases") from concentrated extracellular fluid of ligninolytic cultures of P. chrysosporium have been reported by several investigators (50,55,62,113,151,152). These enzymes have been purified and characterized. They appear to be similar if not identical. Typical ligninase has a molecular weight of approximately 41,000 daltons and a heme IX prosthetic group. It has an obligatory requirement for H_2O_2 for activity, and shows little specificity or stereoselectivity (113,152). Tien and Kirk (152) reported Cα-Cß cleavage of side chains of non-phenolic ß-1 lignin

model compounds (See Figure 3A) as well as oxidation of various ß-O-4 model compounds (See Figure 3B) by their ligninase. They concluded that their enzyme has oxygenate activity (152). Kuwahara et al. (113) showed incorporation of ^{18}O from $^{18}O_2$ during a diarylypropane cleavage, suggesting that this enzyme is an oxygenase. Ligninase also has peroxidase activity and generate •OH (as evidenced by ethylene production from KTBA) in the presence of H_2O_2. These enzymes do oxidize spruce and birch lignin as well as the polymeric dye, Poly R. Kersten et al. (92) have proposed a mechanism for this enzyme involving a cation radical intermediate which has been detected during the conversion of 1,4-dimethoxybenzene to p-benzoquinone and methanol. However, the extent of the modification of the lignin polymer by ligninase(s) has not been characterized in detail.

As summarized in the scheme below, evidence to date clearly shows that H_2O_2 plays an integral role in lignin degradation and that •OH or equivalent reduced oxygen species is involved in lignin degradation. However, it is not clear whether •OH is produced chemically by Fenton-type reaction (45) or by peroxidases, such as ligninases, as shown by Palmer and Evans (131). Also, it remains to be seen whether the •OH or an equivalent species is bound to the

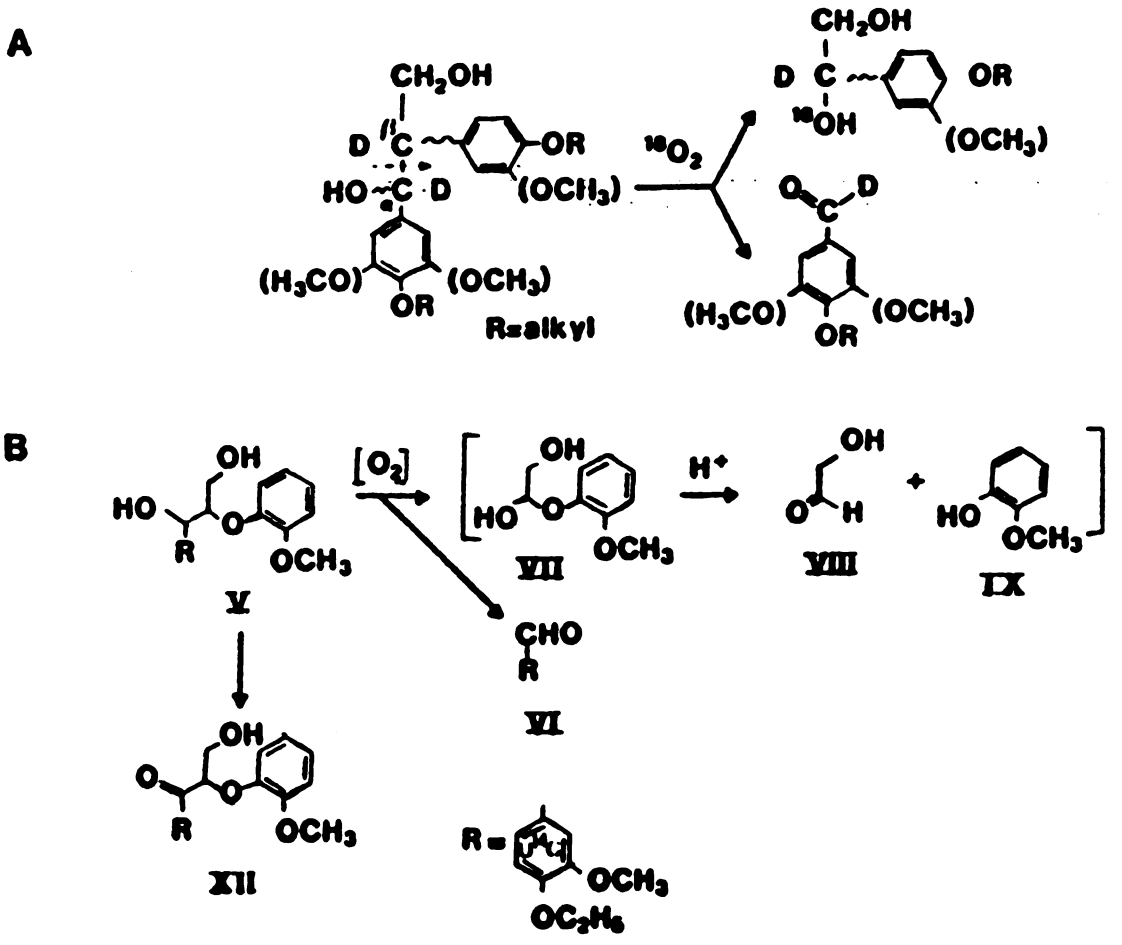
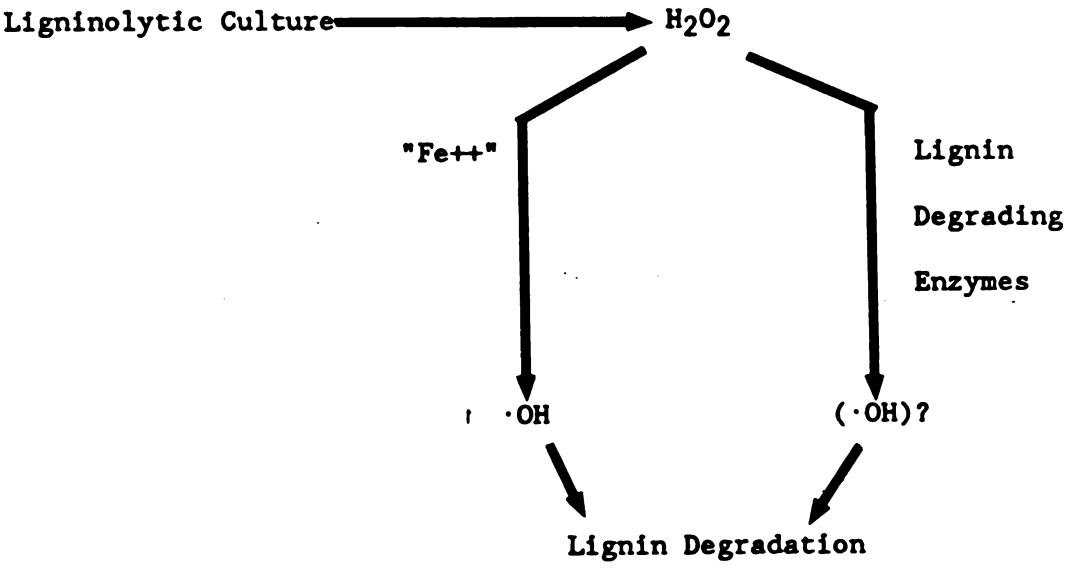


Figure 3. Degradation of lignin model compounds by an enzyme from Phanerochaete chyrysosporium (from ref. 29)

active site of ligninase(s) or not. Research in progress in several laboratories should be able to answer these questions in the near future.



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21WP/RPP/glucose

Chapter one:

Identification of Glucose Oxidase Activity as the Primary Source of Hydrogen Peroxide Production in Ligninolytic Cultures of *Phanerochaete chrysosporium*

Abstract

The primary enzymatic activity involved in H_2O_2 production by extracts of ligninolytic cultures of Phanerochaete chrysosporium was investigated. Glucose supported the highest level of oxygen-dependent H_2O_2 production by cell extracts compared to a number of other substrates tested. No H_2O_2 production was observed anaerobically under N_2. Polyacrylamide gel electrophoresis of extracts from ligninolytic cultures followed by diaminobenzidine/horseradish peroxidase staining procedure showed that only one protein band exhibited glucose-dependent H_2O_2 production. This protein band was not seen in extracts of non-ligninolytic cultures or in the extracellular fluid of ligninolytic cultures. Extracts of cells grown with either xylose, succinate or cellobiose showed the presence of only one glucose-dependent H_2O_2-producing band, with electrophoretic mobility similar to that observed in extracts of glucose-grown cells. Both glucose oxidase activity and lignin degradation were triggered in response to nitrogen (N) or carbohydrate starvation, and were repressed in media containing high levels of N (24 mM) or carbohydrate (56 mM), or on addition of exogenous N sources such as glutamate to the low N medium (2.4 mM N). The results indicate that glucose oxidase activity is the primary source of H_2O_2 in ligninolytic cultures of P. chrysosporium and that nutritional parameters which affect lignin degradation have a parallel effect on glucose oxidase activity.

Keywords: Phanerochaete chrysosporium--lignin degradation--glucose oxidase--nutritional parameters--hydrogen peroxide production--white-rot basidiomycete.

Lignin is one of the most abundant biopolymers in nature constituting approximately 20-30% of the dry weight of all vascular plants (Crawford 1981, Kirk 1984, Reddy 1984). Hence, lignin biodegradation and its utilization as a renewable resource for the production of chemical feedstocks and other useful products is of great interest. Previous studies have shown that lignin biodegradation is an oxidative process (Kirk and Chang 1974) and that H_2O_2 plays an important role in this degradation (Crawford and Crawford 1984, Reddy 1984, Reddy et al. 1983; Patterson and Lundquist 1984). A temporal correlation between ligninolytic activity and H_2O_2 production by P. chrysosporium has been demonstrated (Forney et al. 1982a,b). Growth under 100% O_2 markedly increased both H_2O_2 production and ligninolytic activity, and lignin degradation was inhibited by catalase, a scavenger of H_2O_2 (Faison and Kirk 1983). Cytochemical diaminobenzidine (DAB) staining techniques revealed that H_2O_2 production in ligninolytic cultures of P. chrysosporium, is localized in ovoid periplasmic microbody-like structures which are not found in non-ligninolytic cells (Forney et al. 1982b). Several H_2O_2-dependent, lignin degrading enzymes, have been

isolated from the extracellular fluid of P. chrysosporium cultures. These enzymes were only found in ligninolytic cultures and have been shown to oxidize a variety of lignin model compounds (Tien and Kirk 1983, Tien and Kirk 1984, Glenn et al 1983, Gold et al. 1984), depolymerize lignin (Tien and Kirk 1984) and decolorize the polymeric dye poly R-481 (Gold et al. 1984; Kuwahara et al. 1984). Also, an H_2O_2-dependent lignin demethylase has recently been described by Frick and Crawford (1983). These results indicated that H_2O_2 plays an important role in lignin degradation by P. chrysosporium; however, the physiological source of H_2O_2 in ligninolytic cultures of this organism was not known. The results presented here show that glucose oxidase (E.C. 1.1.3.4) is the primary source of H_2O_2 in ligninolytic cultures of P. chrysosporium.

METHODS AND MATERIALS

Organism, culture conditions and media. P. chrysosporium (ATCC 34541) was maintained and conidial inoculum was prepared and used as previously described (Kirk et al. 1978). The composition of the low and high N media as well as the low and high carbohydrate media was previously described (Kelley and Reddy 1982). Inoculated media in foam-stoppered flasks were covered with plastic bags to minimize evaporation and were incubated at 37°C without agitation.

Assay for ligninolytic activity. Ligninolytic activity was assayed as previously described (Fòrney et al. 1982a) by measuring the evolution of $^{14}CO_2$ from 2'-(^{14}C)-

synthetic lignin (30,000 dpm/flask, unless otherwise mentioned).

Assay for H_2O_2. Three 50 ml cultures were harvested by centrifugation and washed 3X with 50 ml Na 2',2'-dimethylsuccinate buffer (DMS, pH 4.5). The washed mycelial pellet was resuspended in 10 ml DMS buffer, mixed with 0.1 mm size glass beeds in a 1:1 ratio (mycelial wet weight:glass beads), blended for 3 min at 4°C using an Omni-mixer (Sorval, Inc., Newton, CT) and centrifuged at 27,000 x g for 15 min at 4°C. The supernatant cell extract was used for the assay. H_2O_2 production was assayed spectrophotometrically by measuring the peroxidative oxidation of o-dianisidine (Decker 1977). The reaction mixture consisted of 1.0 ml of oxygen-saturated DMS buffer (10 mM, pH 4.5), 1.0 ml of 0.31 mM o-dianisidine, 0.3 ml of a 1.0 M solution of D-glucose or other substrates (0.1 M final concn.), 0.1 ml peroxidase (60 U/ml; E.C. 1.11.1.7; Sigma, St. Louis, MO), and 0.5 ml cell extract. After 5 min of incubation at 37°C, the change in absorbance at 460 nm, caused by peroxidative oxidation of o-diansidine was measured using a CARY 219 spectrophotometer (Varian Associated, Palo Alto, CA, USA) and the units of glucose oxidase activity were calculated as previously described (Decker 1977). In one experiment, the extracellular fluid from 6-day-old ligninolytic cultures of P. chrysosporium, (Tien and Kirk 1984), concentrated 20-fold by an Amicon ultrafiltration unit equipped with a PM-10 filter (Amicon

Co., Lexington, MA, USA), was used to assay for H_2O_2 production, to determine if glucose oxidase activity is extracellular or not.

Electrophoresis of extracts. Polyacrylamide gel electrophoresis of cell extracts was performed on an 8% native gel with a 5% stacking gel as previously described (Melachouris 1968). Samples (50 ul) were placed on a 1.5 mm slab gel (16 x 18 cm) or a 0.5 cm tube gel, and electrophoresed at constant current (30mA for slab gel and 2.5 mA/tube for tube gels) for 3.5 h at 4°C using a Buchler Model 3-1014A power supply (Buchler Instruments, NJ). The gels were stained for H_2O_2-generating enzymes with a diaminobenzidine (DAB)/horseradish peroxidase (HRP) system (Cohen 1972). In certain experiments, parallel gels were stained for protein by the silver staining method of Morrissey (1981).

Chemical assays. Protein content of the extracts was determined by the procedure of Lowry et al. (1951) using bovine serum albumin (IV, Sigma Chemical Co., St. Louis, MO, USA) as the standard. Reducing sugar content in the crude cell extract was determined using the dinitrosalicylic acid procedure (Miller 1957) and the extent of glucose contamination in various assay substrates was determined using the procedure described by Raabo and Terkildsen (1960).

RESULTS

Substrate specificity. To identify the enzymatic activity responsible for hydrogen peroxide (H_2O_2)

production in ligninolytic cultures of P. chrysosporium, we first investigated the ability of different substrates to support H_2O_2 production by cell extracts of 6-day-old cultures grown in low N medium with glucose as the growth substrate (Table 1). The highest specific activity for H_2O_2 production was observed with D-glucose as the substrate. L-sorbose, D-cellobiose, D-mannose, and D-maltose supported lower levels of H_2O_2 production as compared to glucose.

Glucose-dependent H_2O_2 production was not observed when the reaction mixture was made anaerobic by sparging it with O_2-free N_2 for 10 min (Table 1B). H_2O_2 production activity reappeared and was comparable to that observed before (Table 1A) when the reaction mixture was replaced with air.

The above results show that glucose is the primary substrate for H_2O_2 production and that oxygen is required for H_2O_2 production in extracts of ligninolytic cultures of P. chrysosporium. These results further suggest that either glucose oxidase (Decker 1977) and/or a carbohydrate oxidase (Janssen and Ruelius 1968) may be involved in H_2O_2 production in these extracts.

Gel electrophoresis and identification of H_2O_2-producing activity. To determine if one or more enzymes were involved in H_2O_2 production, extracts of glucose-grown ligninolytic cultures were electrophoresed on a native polyacrylamide gel, and the protein bands positive for H_2O_2

generating activity were visualized by the diaminobenzidine (DAB)/horseradish peroxidase (HRP) staining procedure using D-glucose as the substrate (Fig. 1, gel 1). A single, intensely stained band of activity was observed. When parallel gels were stained separately for H_2O_2 generating activity with different substrates, L-sorbose, D-xylose, D-cellobiose, D-maltose and D-mannose were the only other substrates with which an H_2O_2-positive protein band was observed (Fig. 1, gels 2-6). The protein band observed with each of these substrates had the same electrophoretic mobility as that of the glucose-dependent activity band (gel 1). The results presented in Table 1 and Fig. 1 together suggest that a single enzyme is primarily involved in H_2O_2 production in ligninolytic cells and that the enzyme either has a broad substrate specificity and/or some of the substrates have glucose contamination. Further studies showed no detectable glucose contamination in sorbose, but a low level of glucose contamination (0.8-1.4 mg/g) in xylose, cellobiose, maltose and mannose. These low levels of glucose contamination could explain the reactivity of the protein band in gels 2 to 6 (Fig. 1). These results support the view that glucose-dependent H_2O_2 production mediated by glucose oxidase is the primary source of H_2O_2 in ligninolytic cells of P. chrysosporium.

Demonstration of a novel, extracellular, H_2O_2-dependent mono-oxygenases in ligninolytic cultures of P. chrysosporium is an exciting recent discovery (Tien and Kirk 1984). Hence, we investigated the possibility that

glucose-dependent, H_2O_2-producing enzymatic activity occurs extracellularly in lignin-degrading cultures. Also, previous demonstration of an extracellular glucose oxidase in cultures of Penicillium purpurogenum was consistent with this view (Nakamatsu 1975). Our results showed no detectable glucose-dependent H_2O_2-producing protein band in 20x concentrated extracellular culture fluid from 6-day-old, glucose-grown ligninolytic cultures (Fig. 2A, lane 2), whereas a distinct DAB-positive band was seen in cell extracts of the same culture (Fig. 2A, lane 1). No glucose oxidase activity was detectable in the concentrated extracellular culture fluid even by the spectrophotometric assay; however, ligninase activity was readily detectable in the same preparation using the assay procedure of Tien and Kirk (1984) suggesting that lack of sufficient protein is not the problem.

Both nitrogen and carbohydrate concentration in the medium are known to have a profound effect on lignin degradation by P. chyrsosporium. Cultures of this fungus grown in low N (2.4 mM) medium exhibit high levels of both ligninolytic activity and H_2O_2 production, whereas cultures grown under identical conditions in high N (24 mM) medium show little of these activities (Kirk et al. 1978; Forney et al. 1982b; Kutsuki and Gold 1982; Greene and Gould 1983). Consistent with these earlier studies, the DAB-positive protein band (lane 1) was also not seen in extracts of non-ligninolytic cultures grown for 4 days in

low N medium (Fig 2A, lane 3) or for 14 days in high N medium (Fig. 2A, lane 4). In a parallel gel stained for protein (Fig. 2B), a band corresponding to the glucose-dependent, H_2O_2-generating band (Fig 2A, lane 1) was seen only in extracts of non-ligninolytic cells (Fig. 2B, lanes 3 & 4) indicating that the H_2O_2-producing activity is attributable to a specific protein band (Fig. 2B, lane 1). These results indicate that the glucose oxidase activity is correlated with lignin degradation.

Effect of different co-substrates. It has been known for some time that lignin does not serve as a sole source of carbon/energy for P. chrysosporium and that a co-substrate, such as glucose, xylose or succinate, is required for growth and lignin degradation (Kirk et al. 1976, 1978). Each co-substrate is known to support different levels of lignin degradation and it is possible that each of the co-substrates induces different H_2O_2-producing oxidase. To test this possibility, glucose, xylose or succinate were added to parallel cultures to give equi-molar carbon concentrations, and were assayed for ligninolytic activity, and H_2O_2 production (Table 2). Lignin degradation with different co-substrates decreased in the order: D-glucose > D-xylose > succinate. Hydrogen peroxide production by cell extracts of cultures grown with each of the co-substrates was then determined using the respective growth substrate (D-glucose, D-xylose and succinate) or glucose in the assay (Table 2). Hydrogen peroxide producing activity was maximal when extracts of

glucose-grown cells were assayed with glucose as the substrate. In contrast, little or no H_2O_2 production was seen when extracts of cells grown with succinate or xylose were assayed, respectively, with succinate or xylose as the substrate. It was of interest that even the extracts of cells grown with xylose or succinate as the growth substrate displayed high levels of specific activity for H_2O_2 production when glucose was employed as the assay substrate. These results suggest that regardless of the co-substrate used, glucose oxidase was the predominant activity generating H_2O_2.

To further demonstrate that glucose oxidase is the primary source of H_2O_2 production in ligninolytic cultures of this organism, cell extracts from cultures grown with either D-glucose, D-xylose, succinate or D-cellobiose, as the co-substrate (growth substrate) were subjected to polyacrylamide gel electrophoresis. Each gel was then stained for H_2O_2-generating activity, with D-glucose as the substrate. A single H_2O_2-generating protein band with a slightly different electrophoretic mobility was seen in each case (Fig. 3A; see discussion below). When parallel gels were stained for H_2O_2-generating activity with D-xylose, D-cellobiose or succinate as the substrate, instead of D-glucose, no additional band of H_2O_2-producing activity was seen (data not shown). Parallel gels stained for protein showed one protein band each corresponding to the respective H_2O_2-producing band (Fig.3B). It is not clear

whether the minor differences in mobility of the protein bands in lanes 1 to 4 (Fig. 3A) represent slight differences in physical characteristics of the protein in the four extracts or are a reflection of unknown differences in the four cell extracts which are affecting the mobility of the H_2O_2-producing protein. The answer to this question should await purification of the H_2O_2-producing enzyme from cultures grown on each of the co-substrates. Regardless, glucose oxidase appears to be the predominant H_2O_2-producing activity in ligninolytic cultures of P. chrysosporium, irrespective of the growth substrate used.

Effect of various culture parameters on glucose oxidase activity. Culture parameters such as nitrogen and carbohydrate concentrations are known to influence ligninolytic activity of P. chrysosporium (Keyser et al. 1978; Kirk et al., 1978; Fenn and Kirk 1980; Fenn et al. 1981). Addition of either $(NH_4)_2SO_4$, L-glutamate, or certain other aminoacids to nitrogen-straved lignin-degrading cultures of P. chrysosporium suppressed ligninolytic activity. Given the above evidence that glucose oxidase activity is the primary source of H_2O_2 in ligninolytic cultures of P. chrysosporium, we studied the effect of nitrogen concentration on the specific activity of this enzyme. The results of this study confirm and extend the results of our preliminary studies (Reddy et al. 1983) in demonstrating an inverse correlation between the nitrogen concentration in the medium and the specific

activity for glucose oxidase as well as ligninolytic activity in cultures of P. chrysosporium (Table 3A). Furthermore, the addition of exogenous N source, such as $(NH_4)_2SO_4$ or L-glutamate resulted in parallel suppression of both glucose oxidase and lignin degradation activities (Table 3B).

Jeffries et al. (1981) reported that lignin degradation is repressed in a high N medium (24 mM N) containing high levels (56 mM) of glucose, but not in an identical medium containing low levels (8.8 mM) of glucose. The results of this study show that glucose oxidase activity, akin to ligninolytic activity, was higher in the low carbohydrate medium compared to that observed in the high carbohydrate medium (Table 3C). Even so, glucose oxidase activity in low carbohydrate medium was considerably lower than that seen in the low nitrogen medium. This may perhaps reflect the fact that cell yield is extremely low in low carbohydrate medium and apparently glucose oxidase accounts for only a small portion of the total cellular protein under these conditions.

Discussion

Recent studies show that H_2O_2 plays an integral role in lignin degradation (Forney et al., 1982a, Faison and Kirk 1983, Crawford and Crawford 1984). The results of the present study indicate that the primary source of H_2O_2 is glucose oxidase. We have shown that extracts of glucose-grown, ligninolytic cultures of P. chrysosporium can

produce H_2O_2 by oxidizing D-glucose in the presence of O_2. Polyacrylamide gel electrophoresis of the cell extract and visualization of H_2O_2-producing protein band using DAB/HRP staining procedure revealed the presence of a single, glucose-dependent band of activity. This protein band was seen only in extracts of ligninolytic cultures. Substrates which supported low levels of H_2O_2 production by this protein include sorbose, maltose, mannose, xylose and cellobiose. Although we have shown that the latter four substrates were contaminated with glucose, recent data with the purified enzyme have shown that glucose oxidase from P. chrysosporium does exhibit low levels of activity with L-sorbose, D-xylose, and D-maltose.

The results of this study show D-glucose supported the higher levels of H_2O_2 production and ligninolytic activity than the other co-substrates tested (Table 2). Xylose-grown and succinate-grown cultures degraded less lignin, and cell extracts of these cultures produced significant amounts of H_2O_2 only when glucose was supplied. When cell extracts from glucose-, xylose-, succinate- or cellobiose-grown cultures were electrophoresed and stained for H_2O_2-generating activity, a single band of glucose-dependent activity was seen (Fig. 3). However, the electrophoretic mobility of the band in individual extracts was slightly different; the band in extracts of succinate grown cells showed the more pronounced change. Whether these differences in mobilities represent a significant difference in physical characteristics of the protein in a

given extract or a reflection of differences in the cell extracts which are affecting the protein mobility is not known. The answer to this question should await purification of the enzyme from cultures grown on each of the co-substrates. Regardless, glucose oxidase appears to be the predominant H_2O_2-producing activity in ligninolytic cultures of P. chrysosporium, irrespective of the growth substrate used.

Both nitrogen and carbohydrate concentration in the medium are known to have a profound effect on lignin degradation by P. chrysosporium. Cultures of this fungus grown in low N (2.4 mM) medium exhibit high levels of not only ligninolytic activity but also H_2O_2 and hydroxyl radical ($^{\cdot}OH$) production, whereas cultures grown under identical conditions in high N (24 mM) medium show little or none of these activities (Kirk et al. 1978, Forney et al. 1982, Kelley and Reddy 1982, Kutsuki and Gold 1982, Greene and Gold 1983). Consistent with these earlier studies, the results presented here show that nitrogen starvation also triggers glucose oxidase activity. Jeffries et al. (1981) showed that ligninolytic activity is triggered by carbohydrate-starvation even in the presence of high levels of N (24 mM). Our results show that glucose oxidase activity is detectable in carbohydrate-starved cultures, albeit at much lower levels compared to that seen in cultures grown in low N medium which contains non-limiting amounts of glucose. This is in agreement with the

previous finding that the extent of lignin degradation in low N medium depends on the amount of carbohydrate supplied (Jeffries et al., 1981).

Fenn et al. (1981, 1980) showed that the addition of either $(NH_4)_2SO_4$, L-glutamate or certain other amino acids to nitrogen-starved ligninolytic cultures of P. chrysosporium suppressed ligninolytic activity. Similarly, we found that the addition of exogenous N sources suppresses not only ligninolytic activity, but also glucose oxidase activity. Consistent with previous results, glutamate was a more effective suppressor of both these activities than $(NH_4)_2SO_4$.

Demonstration of glucose oxidase activity in P. chrysosporium is consistent with the fact that in nature glucose derived from cellulose hydrolysis is beleived to be the major sugar substrate available to wood-degrading fungi. Green (1977) demonstrated high levels of glucose oxidase in crude extracts of another white-rot fungus, Polyporus versicolor and postulated that glucose oxidase in concert with laccase may play a role in lignin degradation; however, conclusive evidence in support of this hypothesis is lacking. Our results are also consistent with previous reports documenting the wide distribution of glucose oxidase in other fungi (Gancedo et al. 1967).

Our results show that glucose oxidase plays an important role in H_2O_2 production in ligninolytic cells of P. chrysosporium. However, H_2O_2-generating oxidases for a variety of other substrates are known in eukaryotes

(Tolbert 1981) and contribution of one or more of these enzymes to H_2O_2 production in P. chrysosporium cannot be ruled out. Greene and Gould (1984) reported fatty acyl-CoA oxidase activity in P. chrysosporium which catalyzed oxygen-dependent H_2O_2 production, by starved mycelia of P. chrysosporium. However, the H_2O_2-producing activity observed by these authors was about three orders of magnitude less than the glucose-dependent H_2O_2 producing activity observed by us in this study. Furthermore, we did not detect fatty acyl CoA-dependent H_2O_2 production with the cell extracts. Therefore, as Greene and Gould (1984) pointed out, it is difficult to determine the role of fatty acyl CoA oxidase in lignin degradation by P. chrysosporium.

In conclusion, glucose oxidase activity appears to be the primary physiological source of H_2O_2 in cell extracts of ligninolytic cultures of P. chrysosporium. Polyacrylamide gel electrophoresis data show that a single protein band, found only in extracts of ligninolytic cultures and not in the extracellular fluid is responsible for this activity. Glucose oxidase activity, similar to ligninolytic activity, appears to be a secondary metabolic event and is triggered in response to nitrogen or carbohydrate starvation. Glucose oxidase from P. chrysosporium has recently been purified to homogeneity (Kelley RL and Reddy CA, unpublished data). In support of the results of this study, the purified enzyme appears to have a relatively low substrate specificity compared to that from other fungi.

It gave optimal activity with glucose, but exhibited only low levels of activity with L-sorbose, D-xylose and D-maltose. A recent study from our laboratory (Ramasamy et al., 1985) also showed that glucose oxidase-negative mutants of P. chrysosporium are deficient in both glucose oxidase activity and lignin degradation, whereas both these activities reappeared in glucose oxidase positive revertants. These results provide additional support to the view that glucose oxidase activity plays an important role in lignin degradation by P. chrysosporium.

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Table 1. The effect of different substrates on hydrogen peroxide (H_2O_2) production by cell extracts of glucose-grown ligninolytic cultures of *Phanerochaete chrysosporium*

	Substrate	H_2O_2 production (nmol/min · mg)
A	D-Glucose	61.5 ± 3.9
	L-Sorbose	28.2 ± 2.7
	D-Cellobiose	12.5 ± 3.4
	D-Maltose	10.3 ± 1.8
	D-Mannose	3.5 ± 1.0
	D-Xylose	0.5 ± 0.26
B	i. Glucose-(anaerobically under N_2 gas)	trace
	ii. Glucose-(after replacing N_2 in B.i. above with oxygen)	63.5 ± 14.5

Cultures grown for 6d in low N basal medium were used for preparing the cell extracts. All substrates were used at 0.1 M final concentration. Values given are mean ± standard deviation and have been corrected for the "no substrate" controls

Table 2. The effects of different co-substrates on ligninolytic activity and on hydrogen peroxide (H_2O_2) production by cultures of *P. chrysosporium*

Co-Substrate	Ligninolytic activity (dpm/day · flask)	Assay substrate	Specific activity for H_2O_2 production (nmol/min · mg)
D-Glucose	604.2 ± 149.1	glucose	80.7 ± 0.2
D-Xylose	291.7 ± 153.9	xylose	14.5 ± 2.0
		glucose	113.5 ± 7.0
Succinate	216.4 ± 141.1	succinate	0.0
		glucose	57.0 ± 9.5

Cultures were grown separately in a low nitrogen (2.4 mM N) medium containing equimolar carbon concentrations of one of the following substrates: 56 mM glucose, 66.7 mM xylose, or 83.0 mM dissodium succinate for 14 d at 39°C. All values given represent the mean ± standard deviation and have been corrected for the "no substrate" controls

Table 3. The effect of nitrogen and carbohydrate concentrations on the specific activity for lignin degradation and glucose oxidase activity

	Medium	Ligninolytic activity (dpm/day · flask)	Glucose oxidase activity (nmol/min · mg)
A.	Low nitrogen	330.1 ± 164.0	118.4 ± 1.3
	High nitrogen	25.0 ± 13.4	0.6 ± 0.2
B.	Low nitrogen	466.1 ± 134.8	84.5 ± 2.4
	Low nitrogen + $(NH_4)_2SO_4$ (2.8 mM)	141.7 ± 103.7	1.6 ± 0.02
	Low nitrogen + glutamate (2.6 mM)	55.4 ± 48.2	1.1 ± 0.3
C.	Low carbohydrate	54.6 ± 9.3	2.1 ± 1.5
	High carbohydrate	14.4 ± 0.5	0.9 ± 0.4

Low nitrogen (2.4 mM N), high nitrogen (24 mM N), low carbohydrate (8.8 mM glucose) and high carbohydrate (56 mM glucose) media were described previously (Kelley and Reddy 1982). See methods for other details. Ligninolytic activity is based on the amount of $^{14}CO_2$ released by 6-day-old cultures. Cell extracts of 6 day old cultures was used for determining glucose oxidase activity. Additional N sources were added to cultures on the fourth day of incubation.

Figure 1. Polyacrylamide gel electrophoresis of cell extracts from 6-day-old cultures of P. chrysosporium grown in low N medium (containing glucose as the substrate). Tube gels loaded with 1.4 mg protein were stained overnight for H_2O_2 generating activity (Cohen 1972) using the diaminobenzidine/horseradish peroxidase staining procedure, in the presence of D-glucose (1), L-sorbose (2), D-cellobiose (3), D-xylose (4), D-maltose (5), and D-mannose (6). In this procedure, H_2O_2-generating protein bands will stain dark brown (i.e. DAB-positive) due to the peroxidative oxidation of diaminobenzidine.

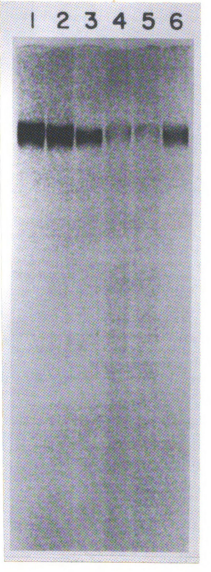


Figure 1.

Figure 2. Polyacrylamide gel electrophoresis of cell extracts of *P. chrysosporium*. Slab gel in panel A were stained for glucose oxidase activity using the procedure described in the legend for Fig. 1. Glucose was used as the substrate. Gels in panel B were stained for protein by the silver staining procedure (Morrissey 1981). In each panel, lanes 1, 2, 3 and 4 represent cell extract from ligninolytic culture (grown for 6 days in low N medium; 70 ug protein), extracellular culture fluid (20X) from ligninolytic cultures (20ug protein), extracts of non-ligninolytic cultures grown in low N medium for 4 days (40 ug protein) or in high N medium from 14 days (40 ug protein), respectively.

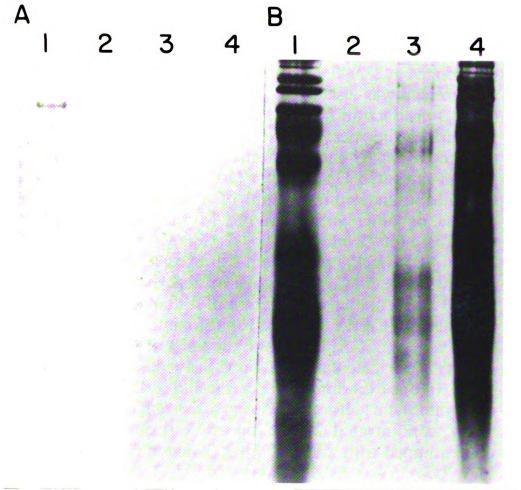


Figure 2.

Figure 3. Polyacrylamide gel electrophoresis of cell extracts of ligninolytic cultures grown on various growth substrates. Gels were stained for H_2O_2-generating activity (panel A) or protein (panel B) using the procedures described in the legend for Fig. 1. Lanes 1, 2, 3 and 4, respectively, contained cell extracts from cultures grown in low N medium with glucose (70 ug protein), xylose (60 ug protein), succinate (50 ug protein), or cellobiose (60 ug protein) as the growth substrate for 9 d.

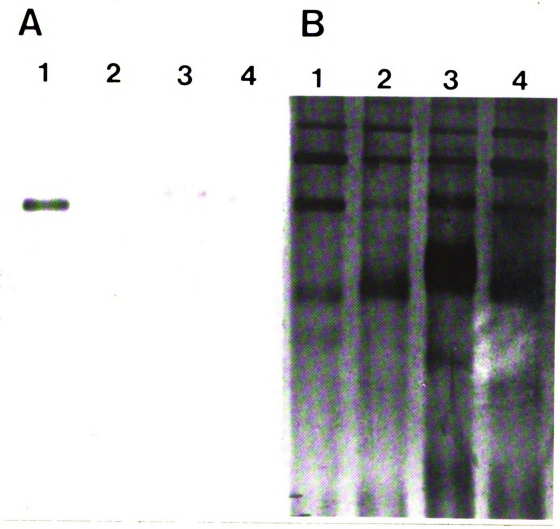


Figure 3.

Chapter two:

Purification and Characterization of Glucose Oxidase from Ligninolytic Cultures of *Phanerochaete chrysosporium*

ABSTRACT

Glucose oxidase, an important source of hydrogen peroxide in lignin degrading cultures of Phanerochaete chrysosporium, was purified to electrophoretic homogeneity by a combination of ion-exchange and molecular sieve chromatography. The enzyme is a flavoprotein with an apparent native molecular weight of 180,000 daltons and a denatured molecular weight of 80,000 daltons. This enzyme does not appear to be a glycoprotein unlike glucose oxidases reported from other fungi. It gives optimal activity with D-glucose, which is stoichiometrically oxidized to D-gluconate; however, it also exhibits some activity with L-sorbose, D-xylose and D-maltose. The enzyme has a relatively broad pH optimum of 4 to 5. It is inhibited by Ag^{+} and o-phthalate, but not by Cu^{++}, NaF or KCN (each 10 mM).

INTRODUCTION

Hydrogen peroxide (H_2O_2) plays an important role in the ligninolytic system of Phanerochaete chrysosporium, a basidiomycete extensively used in studies of lignin biodegradation (16,21). Forney et al. (9,10) showed a temporal correlation between ligninolytic activity and H_2O_2 production. Both H_2O_2 production and ligninolytic activity increased when cultures were incubated under 100% O_2, and lignin degradation was inhibited by catalase, which metabolizes H_2O_2 to yield O_2 and H_2O (7). Nutritional

parameters, which are known to affect ligninolytic activity, such as nitrogen and carbohydrate concentration, were shown to have a similar affect on H_2O_2 production (12,15,18; C. A. Reddy and R. L. Kelley, in C. O'Rear and G. C. Llewellyn, ed, Biodegradation 6, in press). H_2O_2-producing periplasmic microbodies were seen only in lignin degrading cultures but not in non-ligninolytic cultures (10). H_2O_2-dependent extracellular, lignin-degrading oxygenases (ligninases) have been demonstrated in ligninolytic cultures of P. chrysosporium (2,11,15,24).

Glucose oxidase (EC 1.1.3.4) has been recently identified as the predominant source of H_2O_2 production in ligninolytic cultures of P. chrysosporium (Reddy and Kelley, in press) as evidenced by the following observations: Glucose supported the highest level of H_2O_2 production in cell extracts. Polyacrylamide gel electrophoresis of these extracts showed the presence of a single protein band that supported glucose-dependent H_2O_2 production; this protein band was missing in extracts of non-ligninolytic cultures. An H_2O_2-producing protein band with similar electrophoretic mobility was observed in cell extracts regardless of the fact whether glucose, cellobiose, xylose, or succinate was employed as the growth substrate. Both glucose oxidase activity and ligninolytic activity are secondary metabolic events and are triggered in response to nitrogen or carbohydrate starvation (Reddy and Kelley, in press). In this report, we have described

the characteristics of glucose oxidase purified to electrophoretic homogeneity from ligninolytic cultures of P. chrysosporium.

MATERIALS AND METHODS

Organism and culture conditions. P. chrysosporium (ATCC 34541) was maintained and conidial inoculum was prepared as previously described (17). Sterile media in foam-stoppered flasks were inoculated with conidial suspensions in water (1.25×10^6 conidia/ml, 0.5 ml inoculum/10 ml medium) as previously described (17). The flasks were incubated at 37°C without agitation for 6 days.

Assay for glucose oxidase. Glucose oxidase activity was determined at 37°C by monitoring the change in absorbance at 460 nm due to the peroxidative oxidation of o-dianisidine by horseradish peroxidase and using a molar extinction coefficient of 8.3 (5). The reaction mixture consisted of 1.5 ml of citrate-Na phosphate buffer (0.1 M, pH 4.5), 1.0 ml of o-dianisidine (0.3 mM), 0.3 ml of a 1 M solution of the substrate (such as D-glucose, L-sorbose, D-xylose or D-maltose) in water, 0.1 horseradish peroxidase (HRP; 60 U/ml; EC 1.11.1.7; Sigma Chemical Co., St. Louis, MO), and 0.1 ml glucose oxidase solution. The reaction mixture was bubbled with 100% O_2 for 10 min prior to the addition of the glucose oxidase.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of different glucose oxidase preparations was done according to Laemmli (19) using gel slabs (14 x 18 cm x 1.5 mm) with a 4%

stacking gel and a 10% running gel. Samples (50 ul) were placed on the slab gel in 0.05 M tris-HCL buffer (pH 6.7) containing 20% w/v glycerol, 4% SDS, and 10% 2-mercaptoethanol. Electrophoresis was performed at 30 mA for 3.5 h using a Buchler Model 3-1014A power supply (Buchler Instruments Div., Fort Lee, N.J.) set at constant amperage. Gels were stained for protein with Coomassie brillant blue R250 as previously described (27). Gels were stained for protein-bound carbohydrate by using the dansyl hydrazine staining procedure as described by Eckhardt et al. (6).

Protein and flavin assays. Protein content was determined by the procedure of Lowry et al. (20) using Bovine serum albumin (IV, Sigma Co., St. Louis, MO) as the standard. Flavin content was determined by the method of Cerletti et al. (4).

Purfication of glucose oxidase. All purfication steps were done at 4°C. The pH of the phosphate buffer used was 6.8. Protein solutions were concentrated as needed by ultrafiltration using an Amicon ultrafiltration unit (Amicon Corp., Lexington, MA) equipped with a PM-10 filter (10,000 daltons pore size).

1. Preparation of cell extracts. Cultures of P. chrysosporium grown in low nitrogen medium for 6 d at 37°C, were collected by filtration of six layers of gauze in a Buchner funnel. The mycelial mat (~15 g dry wt.) was washed 3 times with 200 ml of 0.1 Na phosphate buffer (PO_4

buffer). The washed mycelium was resuspended in 100 ml of the same buffer, mixed with glass beads (0.1 mm) in a 1:1 ratio (glass beads:mycelial wet wt.), and blended at 4°C using an Omni-mixer (Sorvall Inc., Norwalk, CT) for 15 min. The glass beads and unbroken mycelium were removed by centrifugation at 4080 x g for 10 min. The supernatant was saved and the pellet was resuspended in 10 ml of PO_4 buffer and blended for an additional 15 min. The supernatants were combined and frozen until needed.

ii. DEAE-Sephadex Chromatography. The frozen cell extracts were thawed, clarified by centrifugation at 27,000 x g for 15 min at 4°C, and diluted 5-fold with distilled water. This protein solution was applied to a DEAE-Sephadex (A50; Pharmacia Fine Chemicals, Piscataway, NJ) column (50 ml of gel, 2.4 x 16 cm gel bed) previously equilibrated with 0.01 M PO_4 buffer. The column was washed with 50 ml of the same buffer, and the protein was eluted stepwise from the column with 100 ml volumes of 10 mM PO_4 buffer containing 0.05, 0.10 and 0.25 M NaCl. Fractions (2.25 ml) were collected and tested for glucose oxidase activity as described above. Fractions with the highest activity were pooled and concentrated by ultrafiltration.

iii. Sephacryl chromatography. The concentration protein from the DEAE-Sephadex step was loaded onto a Sephacryl S-300 column (Pharmacia, 170 ml of gel, 2.4 cm x 46 cm gel bed) equilibrated with 0.1 M PO_4 buffer. The column was eluted with the same buffer at a flow rate of 0.25 ml/min. Fractions (1.5 ml) were collected and those

with the highest activity were pooled.

iv. DEAE-Sepharose chromatography. The pooled fractions were applied to a DEAE-Sepharose CL-6B column (Pharmacia, 20 ml of gel, 1.6 x 20 cm gel bed) previously equilibrated with 0.01 M PO_4 buffer. Protein was eluted from the column with a linear salt gradient (440 ml total vol., 0 to 100 mM NaCl) in 0.01 M PO_4 buffer. The NaCl concentration in each fraction was calculated by using conductivity values as compared to those of NaCl standards. The flow was 0.25 ml/min and fractions (1.5) were collected and tested for activity.

Molecular weight determination. The molecular weight of the purified glucose oxidase was determined by gel filtration chromatography, using a Sephacryl S-300 column. The column was calibrated with Biorad gel filtration standard containing thryoglobulin (670,000), gammaglobulin (158,000), ovalbumin (44,000), myoglobin (17,000) and vitamin B_{12} (1,350) obtained from BioRad Laboratories, Richmond, CA. Molecular weight marker kit for SDS gel electrophoresis (MW-SDS-200) was purchased from Sigma Chemical Co. St. Louis, MO.

Effect of pH. A citrate-Na phosphate buffer adjusted to pH 3 to 6 was used to determine the optimal pH for glucose oxidase activity. Because pH could affect both glucose oxidase activity as well as the peroxidatic oxidation of o-dianisidine, glucose oxidase activity in this experiment was determined by monitoring the production

of H_2O_2 directly as measured by the change in absorbance at 240 nm at 37°C. The reaction mixture consisted of 0.5 ml of citrate-PO_4 buffer (0.1 M. pH 3 to 6), 0.1 ml glucose solution (1 M) and 0.4 ml of glucose oxidase (0.1 mg protein/ml).

Enzyme inhibition. Different metal ions or o-phthalate citrate-PO_4 buffer (0.1 M, pH 4.5) were added to the glucose oxidase assay mixture described above. Since KCN is known to inhibit HRP, activity of purified glucose oxidase in the presence of KCN was determined by measuring the anaerobic reduction of 2,6-dichlorophenol-indophenol (25).

Determination of apparent Km and Vmax. The apparent Km for glucose of the purified glucose oxidase was determined by measuring the initial velocities over a range of glucose concentrations (2 to 125 mM) at an O_2 concentration of 1.6 mM. For determining the Km for oxygen, the reaction mixtures, containing 0.1 M glucose, in stoppered cuvettes were bubbled with 100, 80, 60, 40, 20, or 10% O_2 in N_2, which was obtained by mixing the gases through a pair of calibrated flow-meters (model 7322, Matheson, East Rutherford, NJ). After bubbling for 10 min at 37°C, the reaction mixtures were then allowed to equilibrate for 15 min at 37°C. The initial oxygen concentration in each reaction mixture was determined by measuring the amount of dissolved O_2 present in an identically treated parallel cuvette using a biological oxygen monitor (model 5331, Yellow Springs Instrument, Co.,

Yellow Springs, Ohio) equipped with a Clark-type electrode (22). Apparent Km values were calculated from Lineweaver-Burk plots (Fig. 5 A & B).

Quantification of glucose and gluconate. D-glucose and D-gluconate were identified and quantified by gas-liquid chromatography. Purified enzyme (0.5 ml containing 0.35 U/ml) was added to a reaction mixture containing 200 ul catalase solution (1 mg/ml) and 0.5 ml glucose solution (0.1 M in 0.1 M citrate-phosphate buffer) and incubated for 16 h at 37°C. An internal standard of L-erythritol was added to the reaction mixture prior to sialylation. The reaction mixture was evaporated to dryness and dissolved in methanol (acidified with 50 ul trifluoroacetic acid/ml). Undissolved material was removed by centrifugation and the supernatant was evaporated to dryness, dissolved in 0.5 ml acetonitrile plus 0.5 ml N,O-bis-(trimethylsilyl) trifluoroacetamide (Pierce Chemical Co., Rockford, IL), and was heated for 30 min at 70°C. Samples were analyzed using a Varian 3700 gas chromatograph equipped with a flame ionization detector, a Hewlett-Packard 3390A digital integrator and a glass column (2 m x 0.3 cm) packed with 3% SE-30 on 80/100 mesh Chromosorb W(HP). The carrier gas was He at 25 ml/min and the chromatograph oven was temperature programmed to hold at 140°C for 10 min and then to increase at 2°C/min to 220°C. D-glucose and D-gluconate were quantified from peak areas as compared to those of the standards.

RESULTS

Purification of glucose oxidase. The purification of glucose oxidase from P. chrysosporium is summarized in Table 1. A purification of about 90-fold was routinely achieved. In the DEAE-Sephadex step, about 60% of the total glucose oxidase activity present in crude cell extracts was recovered in the 0.25 NaCl eluate, giving about a five-fold increase in specific activity. The subsequent Sephacryl S-300 step (Fig. 1A) produced a 38-fold enrichment in specific activity and a 41% recovery of total activity. The elution profile from the DEAE-Sepharose column (Fig. 1B) showed that a single protein peak had all the glucose oxidase activity with approximately 90-fold enrichment in specific activity and an enzyme recovery of 9%. The DEAE-Sepharose protein fraction was found to be homogeneous based on SDS-PAGE analysis (Fig 2).

Molecular weight. Based on gel filtration chromatography on Sephacryl S-300 column, the apparent molecular weight of purified glucose oxidase was estimated to be 180,000 daltons (Fig. 3). The denatured molecular weight, determined by SDS-PAGE, was estimated to be 80,000 daltons (Fig. 4A).

Carbohydrate and flavin content. Staining of the purified glucose oxidase from P. chrysosporium for protein bound carbohydrate by the dansyl hydrazine method showed no detectable carbohydrate (Fig. 4B, lane 2), whereas an equal amount of commercially prepared glucose oxidase from

Aspergillus niger, which is known to be a glycoprotein (5,23), stained positive (Fig. 4B, lane 1). Flavin analysis indicated that the purified enzyme contains 1.5 moles of flavin per mole of protein.

pH optimum. The purified enzyme had a pH optimum between 4.6 and 5.0. In comparison, glucose oxidase from P. notatum and A. niger were reported to have a pH optima of 5.5 and 5.6, respectively (1,3).

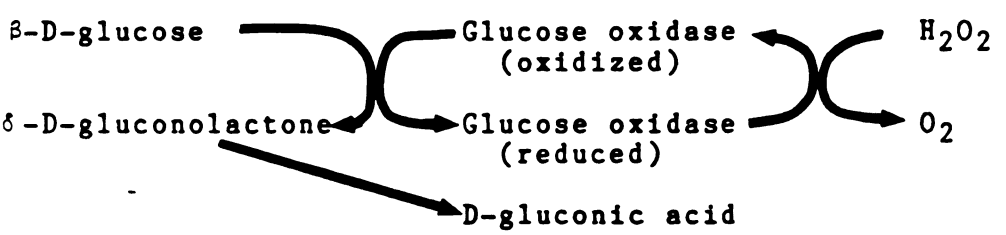
Enzyme inhibition. Glucose oxidase from P. chrysosporium, similar to that from A. niger, was inhibited by Ag^{+}, but was not inhibited by Cu^{++}, KCN, or NaF; in fact, there was substantial stimulation of activity in the presence of the latter (Table 2). Also, glucose oxidase from P. chrysosporium was severely inhibited by o-phthalate, whereas the commercial glucose oxidase from A. niger showed only a limited inhibition of activity.

Kinetic properties and substrate specificity. The apparent K_m values for glucose and O_2 for this enzyme were 38 mM and 0.95 mM respectively (Fig. 5A and B). The V_{max}/K_m data (Table 3) show that glucose is the primary substrate for the enzyme whereas the others are relatively minor substrates at best. Furthermore, compared to a specific activity of 12.27 (unol min/ min per mg) with glucose as the substrate, the following substrates had $\leq$ 1% activity: cellobiose, glycolate, mannose, gluconate, ethanol, acetate, lactate, succinate, pyruvate, D-galactose and D-gluconolactone.

D-glucose was stoichiometrically oxidized to D-gluconate: from 28.6 moles of D-glucose oxidized, we obtained 26.1 moles of gluconate which amounts to 91.2% recovery. These results are in agreement with the results obtained with glucose oxidases from other fungi (1,3,5).

DISCUSSION

Fungal glucose oxidase (β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) catalyzes the oxidation of D-glucose to δ-D-gluconolactone and H_2O_2 in the presence of molecular oxygen (1,3,4,25; see the reaction sequence below). In a subsequent step δ-D-gluconolactone is nonenzymatically hydrolyzed to D-gluconic acid. This enzyme has been demonstrated in various Aspergillus and Penicillum species (3,23,28).



Certain enzymes in animal tissues also catalyze oxidation of D-glucose (or derivatives) to δ-D-gluconolactone, but these are readily differentiated from glucose oxidase because they do not require molecular oxygen and H_2O_2 is not a product (28). Glucose oxidase from different fungi has a molecular weight range from 150,000-186,000 daltons and normally consists of two identical polypeptide chain subunits covalently linked by disulfide bonds (3,5,28).

The glucose oxidase that we have isolated from P. chrysosporium is a flavoprotein with a native molecular

weight of 180,000 daltons and a denatured molecular weight of 80,000 daltons. Presumably this enzyme, similar to other glucose oxidases, consists of two identical polypeptides (MW of 80,000 daltons each). The overestimation of the native molecular weight may perhaps be due to hydrodynamic properties of this enzyme different from other glucose oxidases. Our flavin analysis data revealed 1.5 mol of flavins per mol of purified glucose oxidase from P. chrysosporium. Using identical procedures, we showed that A. niger glucose oxidase has 1.6 flavins per mole of protein. Since A. niger enzyme has been shown to have two flavins per mole of protein by a number of earlier investigators (3,23,25), we believe that both P. chrysosporium and A. niger glucose oxidases actually contain two mol of flavins per mol of protein and the lower value of 1.5 to 1.6 we obtained experimentally is apparently due to a limitation of the analytical procedure employed by us.

No carbohydrate was detectable in glucose oxidase from P. chrysosporium based on the dansyl hydrazine method (6) used in this study (Fig. 4B). Under identical conditions, an equal amount of the enzyme from A. niger, which has been reported to contain approximately 18% sugar residues (5), stained strongly positive. These results suggest either that P. chrysosporium glucose oxidase is not a glycoprotein or that it contains a very low level of carbohydrate which is not detectable by the procedure used (6). This finding

is of interest in light of the previous observations that a majority of peroxisomal proteins appear not to be glycosylated (25,26) and that H_2O_2 production in ligninolytic cultures of P. chrysosporium, presumed to be due to glucose oxidase activity, has been shown to be localized in periplasmic, peroxisome-like structures (10).

Glucose oxidases from other fungal sources have been shown to possess a relatively low affinity for glucose with K_m values ranging from 0.11 mM to 33 mM and a slightly higher affinity for O_2 with K_m values from 0.2 to 0.83 mM (3,24). The K_m values for glucose and O_2 (38 mM and 0.95 mM, respectively) for the enzyme isolated from P. chrysosporium fall within the range of the values reported for previously described glucose oxidases.

Glucose oxidase from Aspergillus and Penicillim was shown to be highly specific for β-D-glucose. Although D-mannose, D-galactose, 2-deoxy-D-glucose and D-xylose have been shown to exhibit low activities as substrates, no greater than 2% of the activity found with glucose was found with these or 50 other carbohydrates tested (1,3). The enzyme from P. chrysosporium on the other hand appears to be less specific in that it gave 33%, 13% and 7% specific activity, respectively, with sorbose, xylose and maltose compared with that seen with glucose as the substrate. However, a comparison of the V_{max}/K_m ratios for the different substrates clearly shows that glucose is the primary substrate for this enzyme. Another H_2O_2-producing enzyme, designated carbohydrate oxidase, has been partially

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purified from extracts of a white-rot fungus, Polyporus obtusus (13). This enzyme exhibited 59% and 38% actively, respectively, with L-sorbose and D-xylose compared to that observed with glucose. P. obtusus enzyme was however, different from P. chrysosporium glucose oxidase in that it could utilize D-gluconate as the substrate (14% of the activity observed with glucose) and showed no activity with D-maltose. A third type of H_2O_2-generating oxidase, L-sorbose oxidase from Trametes sanguinea, which catalyzed the oxidation of L-sorbose, D-glucose, D-galactose, D-xylose and D-maltose, has been described (29). It has been suggested that this enzyme is similar to the P. obtusus carbohydrate oxidase (13). The apparent low substrate specificity of P. chrysosporium glucose oxidase described here may allow the organism to utilize sugars derived not only from cellulose but also from hemicelluloses found in woody material, its natural habitat, to produce H_2O_2 which is known to be important to the ligninolytic system.

Inhibition studies showed that glucose oxidase from P. chrysosporium, similar to glucose oxidase from A. niger, is inhibited by Ag^+ but not by Cu^{++}, NaF, or KCN (Table 2). Earlier results showed inhibition of lignin degradation when o-phthalate was used as a buffer in the growth medium (8). The results of this study show that glucose oxidase from P. chrysosporium is severely inhibited by o-phthalate suggesting that the inhibition of lignin degradation by this compound may at least partially be due to its effect

on H_2O_2 production by glucose oxidase.

In this report we have described the purification, characterization and kinetic properties of glucose oxidase from P. chrysosporium. This enzyme is similar in its physical and kinetic properties to glucose oxidases isolated from other fungal sources, except that we were unable to demonstrate the presence of carbohydrate in this protein. The enzyme is severely inhibited by o-phthalate and Ag^+.

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TABLE 1. Purification data for glucose oxidase from ligninolytic cultures of *P. chrysosporium*

Fraction	Sp act (U/mg)[a]	Total protein (mg)	Total activity (U)	Yield (%)	Purification (fold)
Crude cell extract	0.17	285	48.4	100	1.0
DEAE-Sephadex	0.91	32	29.1	60	5.4
Sephacryl S-300	6.39	3.1	19.8	41	37.8
DEAE-Sepharose	15.1	0.28	4.2	9	89.3

[a] Specific activity is defined in units per milligram of protein. One unit of activity represents the oxidation of 1 μmol of *o*-dianisidine per min at 37°C and pH 4.5.

TABLE 2. Comparison of the effects of *o*-phthalate, KCN, NaF, and different metal ions on glucose oxidase from *P. chrysosporium* and commercially prepared *A. niger* glucose oxidase

Addition	Final concn (mM)	% Activity of glucose oxidase from:	
		A. niger[a]	*P. chrysosporium*
None		100	100
KCN	10	128	135
NaF	10	101	111
$CuCl_2$	10	133	123
$AgSO_4$	10	36	25
o-Phthalate	50	86	12

[a] Commercial *A. niger* glucose oxidase was obtained from Sigma. The concentration of each enzyme preparation was adjusted to give 0.015 U of activity per ml.

TABLE 3. Substrate specificity of purified glucose oxidase[a]

Substrate	V_{max} (μmol/min per ml)	K_m (mM)	V_{max}/K_m	% Sp act
D-Glucose	15	38.0	0.395	100
L-Sorbose	5	217.4	0.023	5.8
D-Xylose	2	105.2	0.019	4.8
D-Maltose	1	55.5	0.018	4.5

[a] Initial velocity was determined by the *o*-dianisidine–horseradish peroxidase assay described in Materials and Methods.

Fig. 1.A. Elution profile of protein and glucose oxidase activity (nmol/min/mg protein) on Sephacryl S-300 column. Fractions of 1.5 ml were collected and assayed for activity and protein. B. Elution profile of protein and glucose oxidase activity from a column of DEAE-Sepharose. Protein was eluted from the column with a linear salt gradient (see Material and Methods). Each datum point for salt concentration) was determined by conductivity measurements. Fractions (1.5 ml) were assayed for glucose oxidase activity and for protein content.

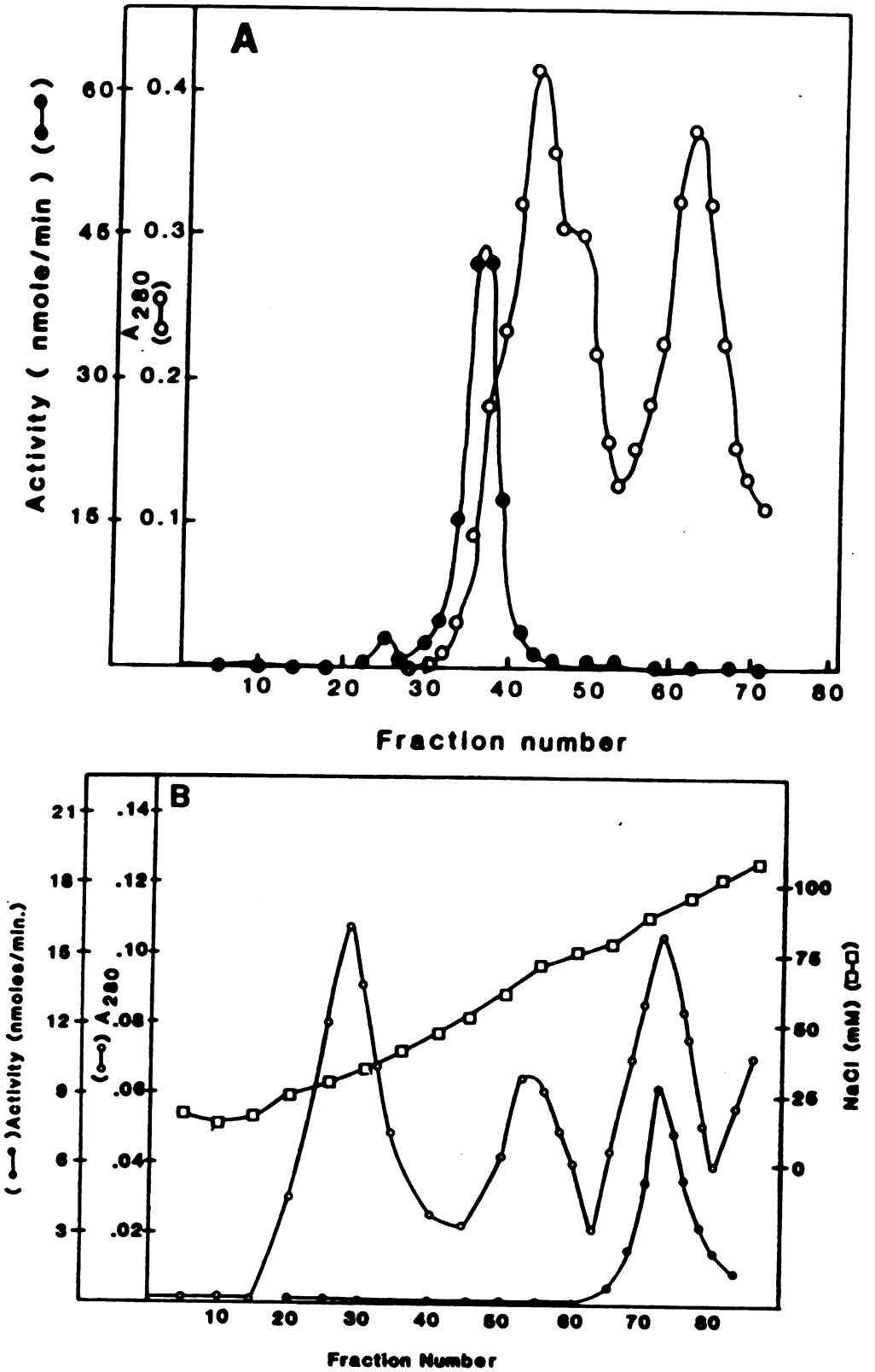

A
Activity (nmole/min) (●—●)
A280 (○—○)
60
45
30
15
0.4
0.3
0.2
0.1
10
20
30
40
50
60
70
80
Fraction number
B
(●—●)Activity (nmoles/min.)
(○—○) A280
NaCl (mM) (□—□)
21
18
15
12
9
6
3
.14
.12
.10
.08
.06
.04
.02
100
75
50
25
0
10
20
30
40
50
60
70
80
Fraction Number

Figure 1.

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of glucose oxidase preparation obtained from different purification steps (see Table 1). Molecular weight standards (lane MW), the crude cell extract (lane 1: 0.14 mg protein), the DEAE-Sephadex fraction (lane 2; 20 ug protein), the Sephacryl S-300 (lane 3; 9.5 ug protein), and DEAE-Sepharose fraction (lane 4; 1 ug protein) were stained with Coomassie blue as described in the text.

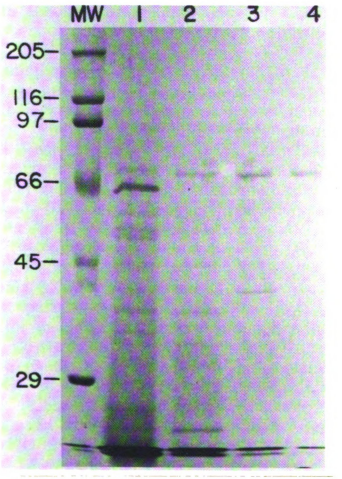


Figure 2.

Fig. 3 Molecular weight determination of purified glucose oxidase by gel filtration chromatography with a Sephacryl S-300 column. See the text for details. V_e, Elution volume.

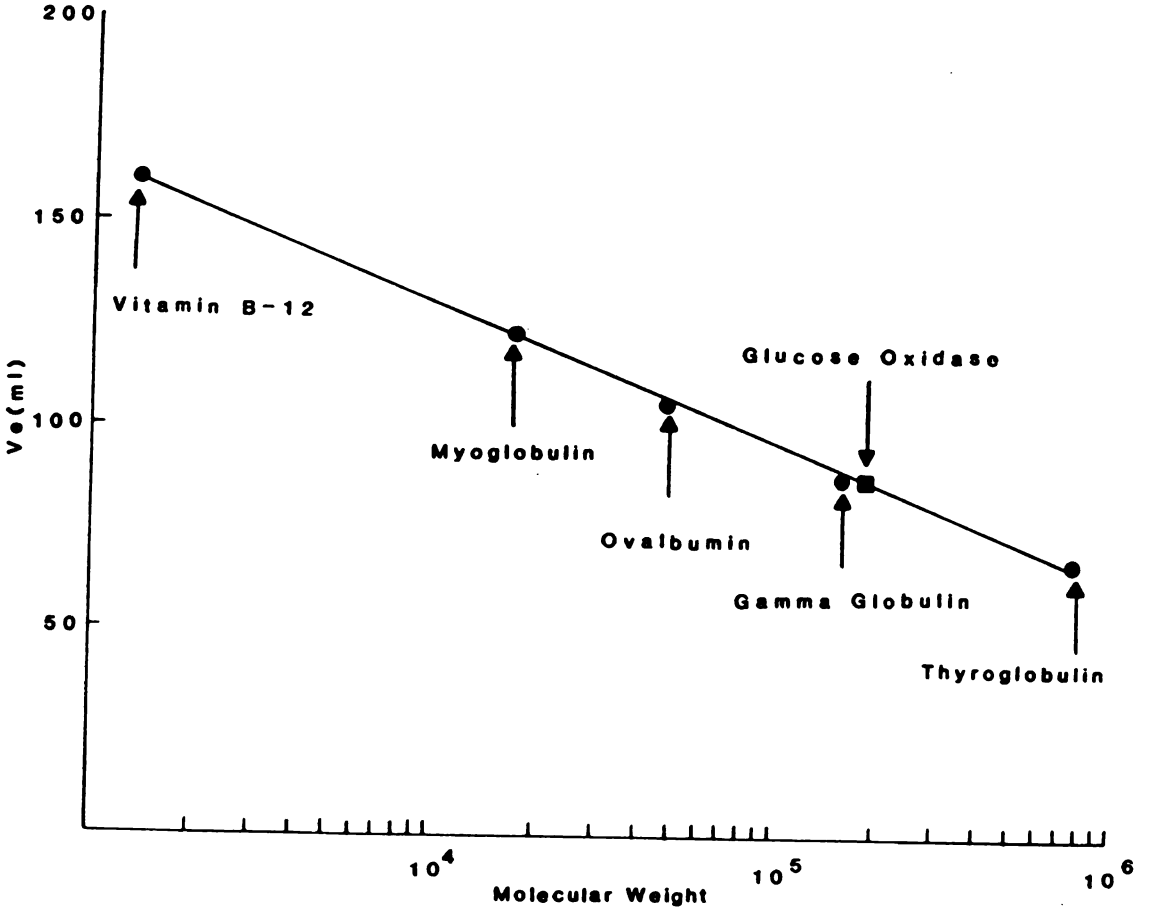

200
150
100
50
Ve(ml)
Vitamin B-12
Myoglobulin
Glucose Oxidase
Ovalbumin
Gamma Globulin
Thyroglobulin
10^4
10^5
10^6
Molecular Weight

Figure 3.

Fig. 4. Molecular weight determination and staining for carbohydrate and protein after SDS-PAGE of commercial glucose oxidase from A. niger and that from P. chrysosporium. In panel A, molecular weight standards (lane MW), A. niger glucose oxidase (lane 1) and glucose oxidase from P. chrysosporium (lane 2) were stained with Coomassie blue. In panel B, A. niger glucose oxidase (panel B, lane 1) and glucose oxidase from P. chrysosporium (lane 2) were stained for carbohydrate by the dansylhydrazine method as described in the text. In each panel, 1 ug of the respective enzyme was used.

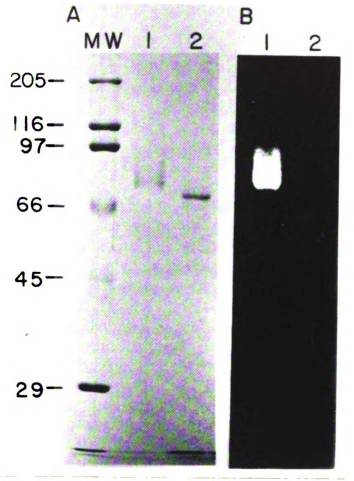
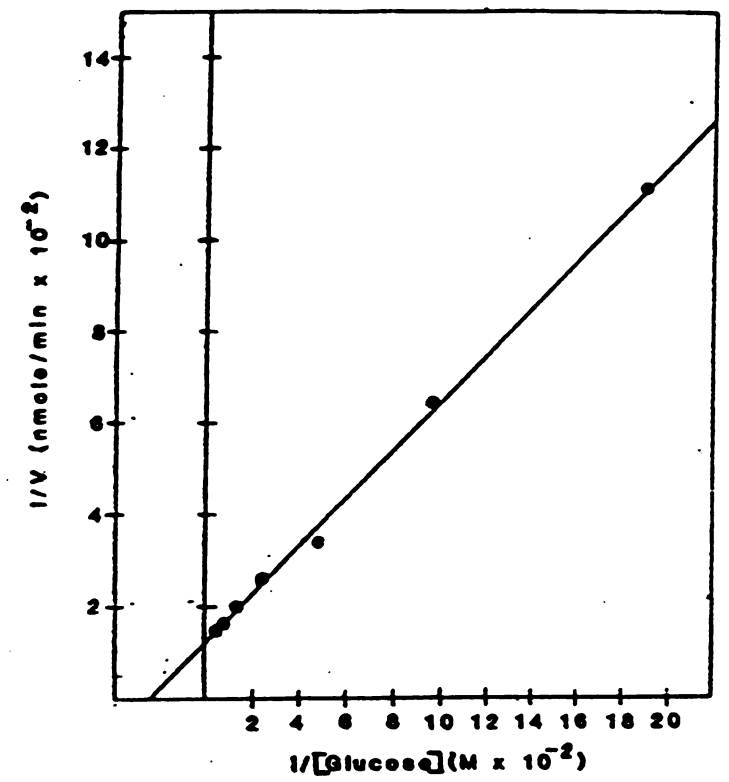


Figure 4.

Fig. 5. Lineweaver-Burk plots showing the effect of glucose and O_2 concentration on glucose oxidase activity. Glucose oxidase was assayed as described in methods. (A) Glucose concentration was varied at an O_2 concentration of 1.6 mM, (B) O_2 concentration was varied at glucose concentration of 0.1 M. Each point is the mean of these three values. Each reaction contained 1.6 ug of enzyme.


1/V (nmole/min x 10^{-2})
14
12
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20
1/[Glucose](M x 10^{-2})

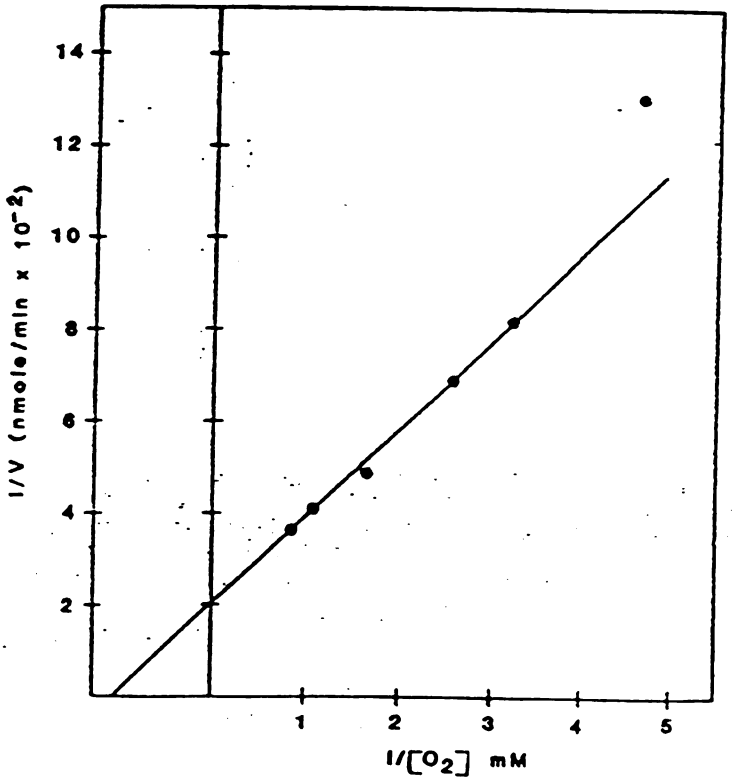

1/V (nmole/min x 10^{-2})
14
12
10
8
6
4
2
1 2 3 4 5
1/[O_2] mM

Figure 5.

Chapter three:

Characterization of Glucose Oxidase-Negative Mutants of a Lignin Degrading Basidiomycete *Phanerochaete chrysosporium*

ABSTRACT

Previous studies have shown that H_2O_2 plays an important role in lignin degradation by Phanerochaete chrysosporium, and that glucose oxidase (EC 1.1.3.4) is the primary physiological source of H_2O_2 in ligninolytic cultures of this organism. The isolation and characterization of glucose oxidase-negative (gox$^-$) mutants of P. chrysosporium is described. These mutants are deficient not only in their ability to produce H_2O_2 but also in lignin degradation (2'-^{14}C-synthetic lignin - $^{14}CO_2$), ligninase and peroxidase activities, decolorization of the dye poly-R 481, and production of ethylene from - oxy- -methylthiobutyric acid (KTBA). The gox$^-$ mutants retained, albeit at a lower level, the capacity to produce veratryl alcohol, a typical secondary metabolite, and produced conidia at a level comparable to that of the wild type. The addition of ligninase and/or glucose oxidase to a gox$^-$ mutant (GOX-10) did not enhance its capacity to degrade lignin. The Gox$^+$ revertant strains regained glucose oxidase activity, the ability to degrade lignin, as well as the other characteristics that were missing in the gox$^-$ mutants. The results suggest that the genetic lesion

in these mutants affects the regulation of a set of secondary metabolic characteristics.

INTRODUCTION

Lignin degradation by the white-rot fungus, Phanerochaete chrysosporium, has been studied extensively (Crawford and Crawford, 1984; Kirk, 1984; Reddy, 1984). Several recent studies have shown that H_2O_2 plays an important role in lignin degradation (Reddy and Kelley, 1985). Glucose oxidase (GOX) has been shown to be the predominant source of H_2O_2 in lignin degrading cultures of P. chrysosporium (Reddy and Kelley, 1985; Kelley and Reddy, 1985; Reddy et al. 1983). Preliminary studies have shown that glucose oxidase-negative (gox$^-$) mutants of this fungus are deficient in H_2O_2 production and in lignin degradation (Ramasamy et al. 1985). Lack of H_2O_2 production by the gox$^-$ mutants supports earlier evidence that glucose oxidase is the primary source of H_2O_2 in P. chrysosporium cultures grown under ligninolytic conditions. Native polyacrylamide gel electrophoresis of the extracts of wild type, gox$^-$ mutant and gox$^+$ revertant, followed by staining of the gels for GOX activity showed the presence of a single protein band capable of glucose-dependent H_2O_2 produciton in extracts of the wild type and gox$^+$ revertant but not in those of the gox$^-$ mutant. Parallel gels stained for protein showed a corresponding protein band in extracts of the wild type and the gox$^+$ revertant but not in extracts of

the gox⁻ mutant. We have presented here the metabolic features of a number of gox⁻ mutants and gox⁺ revertants, which should be useful for future studies on the genetics of lignin degradation by P. chrysosporium.

MATERIALS AND METHODS

Organism and culture conditions. P. chrysosporium (ATCC 34541) was maintained through periodic transfer on malt extract agar slants previously described (Kirk et al. 1978). Composition and preparation of the low N medium used in this study has been described (Kirk et al. 1978). Unless described otherwise, 0.5 cm mycelial disc from 7 d-old GOX plates (see below) served as the inoculum and cultures were incubated in air without agitation at 39°C.

GOX medium used for the isolation of gox⁻ mutants has been described (Ramasamy et al. 1985). Glucose oxidase-positive colonies produce a purplish or brownish violet discoloration (due to oxidation of orthoanisidine) of this medium in 5 to 8 days, whereas glucose oxidase-negative colonies show no discoloration.

Poly-R medium (Glenn and Gold, 1983) is the same as the Low N medium of Kirk et al. (1978) with the following amendments per 100ml: 4 g sorbose, 10 mg sodium deoxycholate and 2 g agar (Bacto-Difco). After autoclaving and cooling to about 60°C, 1 ml of poly-R 481 (Aldrich Chemical Co.; 2% filter sterilized solution) was added. Ligninolytic colonies produce a decolorized zone under and around the colony in 10-15 days whereas non-ligninolytic

colonies show no decolorization.

Mutagenisis and isolation of mutants. Conidia from cultures grown on malt extract agar for 7d were collected and mutagenized as previously described (Ramasamy et al. 1985). Mutagenized conidia were plated on GOX plates to screen for gox$^-$ mutants. This mutation procedure yielded approximately one mutant colony for every 30 colonies screened, whereas the spontaneous mutation rate was 3.4 x 10^{-4}. The glucose oxidase-negative mutants were also screened for ligninolytic activity by plating on poly-R medium.

To produce gox$^+$ revertants, conidia from the gox$^-$ mutant (GOX-10) were mutagenized as described above (Ramasamy et al. 1985). A large number of gox$^+$ revertants were isolated. The reversion frequency was approximately one to two revertants for every 100 colonies screened.

Preparation of cell extracts and assay of glucose oxidase activity. Cultures grown in low N medium (3 foam-plugged Erlenmeyer flasks containing 50ml each) for 6 days were used for the preparation of cell extracts as described previously (Ramasamy et al. 1985). Glucose oxidase in cell extracts was assayed spectrophotometrically by measuring the peroxidatic oxidation of orthodianisidine through a horseradish peroxidase coupled system (Reddy and Kelley, 1985).

Polyacrylamide gel electrophoresis of cell extracts, employed to demonstrate protein band(s) with glucose oxidase activity was performed as previously described

(Reddy and Kelley, 1985).

Other assays. Extracellular culture fluid from 6-day-old cultures grown in low N medium in 100% O_2 was concentrated 20-fold in an Amicon ultrafiltration unit equipped with a PM-10 filter (Amicon Co., Lexington, MA), and was used for determination of peroxidase and ligninase activity. Peroxidase and laccase activities were assayed spectrophotometrically as described by Harkin and Obst (1973). Ligninase activity was assayed spectrophotometrically by monitoring the conversion of veratryl alcohol to veratrylaldehyde (Tien and Kirk, 1984). Protein was assayed by the procedure of Lowry et al. (1951) using bovine serum albumin (IV, Sigma Chemical Co., St. Louis, MO, USA) as the standard.

Veratryl alcohol synthesis. Veratryl alcohol was extracted from 7-day-old cultures, grown in low N medium with 100% O_2, by the procedure of Shimada et al. (1981), and quantified by HPLC on a Micropac C18 reverse phase column (Varian Associates; 30 cm X 4mm; MCH-10) with water/methanol (1:1) as the eluting solvent at 2.0 ml/min. The chromatograph system comprised a Rheodyne model 7125 injector (Rheodyne, Inc., Berkeley, CA, USA), a Milton-Roy pump (Milton-Roy Corp., Riveria Beach, FL, USA) and a Laboratory Data Control UV detector with a 254 nm filter (model LDC III, Laboratory Data Control, Riveria Beach, FL, USA). Veratryl alcohol produced was estimated by monitoring the absorption at 254 nm. An authentic sample

of veratryl alcohol (Aldrich Chemical Co., Milwaukee, WI, USA) served as the standard.

Ligninolytic activity and hydroxyl radical production. Assay procedures for ligninolytic activity and hydroxyl radical production, as measured by ethylene produciton from KTBA, were performed as previously described (Forney et al. 1982; Kelley and Reddy 1982).

Enumeration of conidia. Conidial numbers were determined using 8-day-old cultures grown on malt extract plates. Conidia were suspended in 15 ml of sterile distilled water and their numbers determined using a hemocytometer.

RESULTS AND DISCUSSION

Several hundred presumptive gox$^-$ mutants were isolated from the GOX plates. The lack of glucose oxidase in these mutants was confirmed by the spectrophotometric glucose oxidase assay and a small number of these were used for further study (Table 1). All the gox$^-$ mutants tested were deficient in ligninolytic activity. Ethylene production from KTBA (believed to be a measure of hydroxyl radical production and the ability to decolorize poly-R dyes have been shown to be associated with ligninolytic activity in Phanerochaete chrysosporium (Forney et al. 1982; Kelley and Reddy 1982; Glenn and Gold, 1983; Kutsuki and Gold, 1982). Consistent with this, the gox$^-$ mutants showed depressed levels of ethylene production and were unable to decolorize poly-R 481 dye on plates (Table 1). Furthermore, Y. H. Ko

and C. A. Reddy (unpublished data) quantified poly-R dye decolorization in liquid cultures by measuring the A_{513}/A_{362} absorption ratios as described by Glenn and Gold (1983), and showed that the mutants had less than 10% of the wild type's ability to decolorize poly-R 481. Cell yields (mg dry wt. of mycelium/ml culture) of the gox$^-$ mutants in malt extract broth or in low N medium were 83.6 to 100% of the wild type cell yield (results not shown) indicating that the deficiency in glucose oxidase and ligninolytic activities in the mutants is not due to their poor growth.

An extracellular, lignin-degrading enzyme ("ligninase") has been purified and characterized from ligninolytic cultures of P. chrysosporium (Gold et al. 1984; Tien and Kirk, 1984). Ligninase activity was absent in wild type cultures grown under non-lignin degrading conditions as well as in cultures of a non-ligninolytic mutant of this organism. The enzyme is a heme protein and has been shown to be an unique H_2O_2-requiring oxygenase that catalyzes the oxidation of a variety of lignin model compounds and the partial depolymerization of spruce and birch lignins (Tien and Kirk, 1984). Peroxidase activity was shown to be intrinsic to this enzyme. It was therefore of interest to determine whether the gox$^-$ mutants are deficient in ligninase/peroxidase activity or not. Our results showed that these mutants had little or no ligninase/peroxidase activity (Table 1). Thus, the lack of

ligninolytic activity in gox⁻ mutants can not be solely attributed to their deficiency in glucose oxidase activity, but to their loss of several other enzyme activities including ligninase, peroxidase and perhaps other enzyme(s) in the lignin degradation pathway. Attempts to restore lignin degradation to gox⁻ mutants by the addition of glucose oxidase purified from P. chrysosporium (R. L. Kelley and C. A. Reddy, unpublished data) and/or ligninase (a gift from T. K. Kirk, U.S. Forest Products Laboratory, WI, USA) to a gox⁻ mutant (GOX-10) did not enhance ligninolytic activity (Table 2). Similar results were obtained when glucose oxidase from P. chrysosporium was replaced in the above experiment by commercially available A. niger glucose oxidase.

Lignin degradation is known to be a secondary metabolic event (Kirk, 1984; Reddy, 1984). Evidence published to date shows that nutritional and physical factors which affect lignin degradation have a parallel effect on veratryl alcohol production. Gold et al. (1982) showed that a phenol oxidase-less mutant (phe⁻) of P. chrysosporium was defective not only in its ability to degrade lignin and various lignin model compounds, but also in its ability to produce fruiting bodies and synthesize veratryl alcohol, a typical secondary metabolite, and concluded that it is a pleiotropic mutant for a set of secondary metabolic characteristics. To determine the possibility that our gox⁻ strains are pleiotropic mutants defective in a set of secondary metabolic characteristics,

similar to the *phe*$^-$ mutants of Gold et al. (1982), we tested the ability of these strains to produce veratryl alcohol and to conidiate. The results (Table 1) showed that all the *gox*$^-$ mutants were able to produce substantial levels of veratryl alcohol, although at levels 2 to 3 times lower than those of the wild type. Conidiation was variable among the mutants; mutants GOX-4 and GOX-10 produced conidial numbers comparable to those of the wild type. The other three mutants also produced high numbers of conidia although less than those of the wild type. These results indicate that the *gox*$^-$ mutants studied lack several metabolic activities associated with lignin degradation, but unlike the pleiotropic mutants of the type described by Gold et al. (1982), our mutants retained some of the major secondary metabolic characteristics.

A number of *gox*$^+$ revertants were isolated by subjecting conidia from GOX-10 to UV mutagenesis. These revertants, regained not only their ability to produce glucose oxidase but also most of the characteristics of the wild type that were lost in the *gox*$^-$ mutants. These included: the ability to degrade lignin; decolorize poly-R 481; produce ligninase, peroxidase, veratryl alcohol and conidia; and to produce ethylene from KTBA (Table 1). The above results suggest the possibility that the structural genes for glucose oxidase, ligninase, peroxidase and perhaps other lignin degrading enzymes are regulated by a common regulatory gene which is inactivated in *gox*$^-$ mutants

and is reactivated in *gox*$^+$ revertants.

We have recently isolated another type of mutant (Lig-5) which had 74% of the wild type's ability to produce glucose oxidase, but only 8% of wild type's ability to produce ligninase indicating that ligninase and glucose oxidase activities can be uncoupled. This mutant showed very little ability to degrade (^{14}C) synthetic lignin to $^{14}CO_2$ until day 12, but after that the strain was somewhat erratic in its ability to degrade lignin (Fig. 1). Since we observed high reversion rates with this mutant, the variability in ligninolytic activity observed after the 12th day with the Lig-5 strain may be due to the emergence of wild type revertants.

In conclusion, we have characterized a number of *gox*$^-$ mutants which can be isolated and reverted with relative ease. These mutants are deficient not only in glucose oxidase activity but also in their ability to degrade ^{14}C-synthetic lignin to $^{14}CO_2$, and produce ligninase and peroxidase activities. The mutants appear to be pleiotropic for a set of secondary metabolic characteristics, but have retained others such as the ability to synthesize veratryl alcohol and to form conidiospores. The evidence suggests that the primary lesion in these mutants affects the regulation of the onset of a variety of secondary metabolic characteristics.

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Table 1. CHARACTERISTICS OF THE WILDTYPE, GLUCOSE OXIDASE-NEGATIVE MUTANTS AND REVERTANTS OF PHANEROCHAETE CHYRSOSPORIUM[a]

Strains	% 14 CO_2 Evolved From 2'-(^{14}C) Lignin[b]	Poly R	Ethylene Production[c] (nmol/hr/flask)	Glucose Oxidase[d] (nmol/min/mg)	Peroxidase[e] (nmol/min/mg)	Ligninase[e] (µmol/min/mg)	Veratryl[f] Alcohol Synthesis (nmol/ml)	Total[g] Conidiation (X 10^8)
A. Wild Type (ME-446)	12%	+	141.3	19.0	312.0	29.1	285	3.0
B. Mutants								
gox-1	2.8	-	11.7	0.0	0.0	0.7	n.d.	n.d.
gox-4	2.5	-	11.8	0.0	0.0	0.2	86	1.8
gox-6	1.0	-	0.9	0.0	0.0	n.d.	71	3.2
gox-10	1.0	-	1.3	0.0	14.0	n.d.	86	0.05
gox-K7	2.2	-	n.d.	0.0	0.0	0.0	71	4.2
gox-K10	1.2	-	n.d.	0.0	0.0	0.1	97	0.20
C. Revertants								
R-1	13.6	+	155.7	18.0	350.1	8.7	418	4.4
R-2	14.1	+	160.5	27.6	301.9	17.3	275	5.3
R-3	14.7	+	120.2	35.1	345.1	14.2	365	n.d.
KR-6	13.2	+	n.d.	23.2	391.5	16.4	220	n.d.
KR-8	12.8	+	n.d.	13.0	116.4	15.5	410	n.d.

[a] The strains were grown at 37°C from a plug inculum in low N medium as described in text.

[b] Initial concentration was 3.0 X 10^4 dpm or 2'-(^{14}C) synthetic lignin/flask.

[c] Ethylene production was determined from 6-day-old cultures (Kelley, et al., 1982).

[d] Glucose oxidase activity was determined from cell extracts of 6-day-old cultures as described (Kelley et al., 1986).

[e] Ligninase and peroxidase activity was determined using concentrated (20x) culture fluid as previously describe (Forney, et al., 1982) and (Harkin, et al., 1973), respectively.

[f] Veratryl alcohol from 7-day-old cultures was extracted as described and quantitated on HPLC.

[g] Conidia were enumerated from 8-day-old malt extract plates as describe in test.

n.d. = not determined.

values = average of three replicates.

S.D. were ± 10% of the values shown.

Table 2. The effects of exogenous addition of ligninase and glucose oxidase on lignin degradation by the wild type and a glucose oxidase-negative mutant of P. chrysosporium

	Ligninolytic Activity (dpm/day/flask)	
Treatment	Wild type	GOX-10
None	1274 ± 134	122 ± 10.1
Ligninase	1441 ± 127	115 ± 8.1
Glucose oxidase	1335 ± 123	94 ± 5.2
ligninase + glucose oxidase	1241 ± 173	106 ± 6.7

Ligninolytic activity was deterimined in low N medium on the 8th day as previously described (Forney et al. 1982). On the 6th day of growth, 2'-^{14}C-synthetic lignin (71,000dpm) and 2.0 U (50 ul) of ligninase and/or 0.5 U purified glucose oxidase were added per flask. One unit (U) of activity represents 1 umole product/min/mg.

Figure 1. Release of $^{14}CO_2$ from 2-^{14}C-synthetic lignin by the wild type (○), *gox*$^-$ mutant (GOX-10; △) and LIG-5 mutant (●). Ligninolytic activity was determined as described in the legend for Table 2. Each value represents the average of three replicates ± S.D.

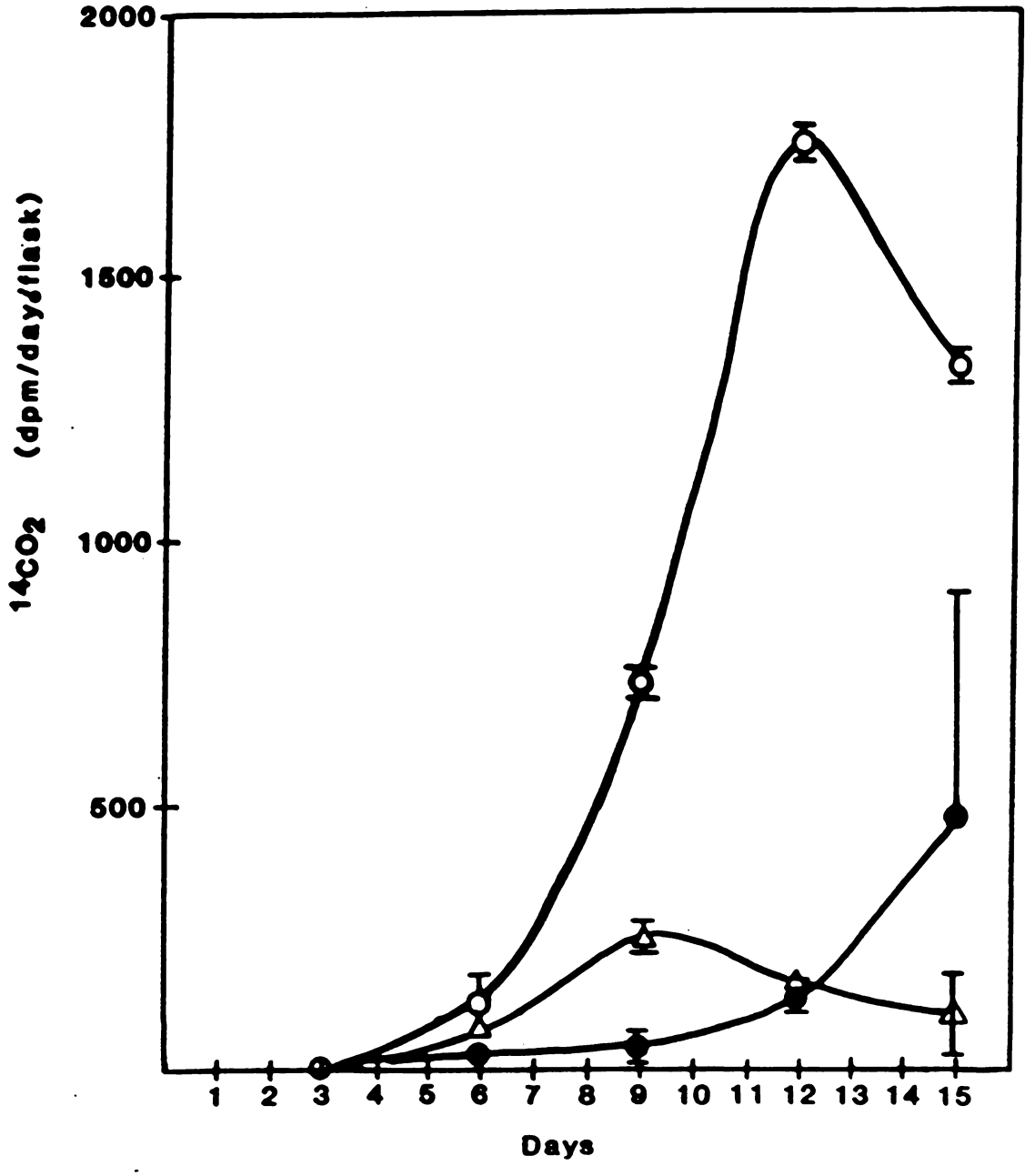
2000
1500
1000
500
$^{14}CO_2$ (dpm/day/flask)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days

Figure 1.