

SUBSTRATE-INDUCED  $H_2O_2$  PRODUCTION IN MYCELIA FROM THE LIGNIN-  
DEGRADING FUNGUS PHANEROCHAETE CHRYSOSPORIUM

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Broken and subsequently washed mycelia from the lignin-degrading fungus Phanerochaete chrysosporium generated increased levels of  $H_2O_2$  when exposed to a lignocellulosic substrate (ground wheat straw) or substrate analogue (Poly B-411).  $H_2O_2$  production was observed only in cultures grown on limiting nitrogen, a condition known to induce the lignin-degrading activity of this organism.  $H_2O_2$  production was observed in the pH range of 4.5 to 8.4, but the highest levels of  $H_2O_2$  were generated around pH 6. The mycelia also exhibited catalase activity, which was 5- to 10-fold higher in nitrogen-limited cultures.

The biochemistry of lignin degradation by organisms such as the Basidiomycete (white rot) fungi is poorly understood. Studies with Phanerochaete chrysosporium Burds., the best characterized of these organisms, have indicated that lignin degradation is probably a secondary metabolic event, occurring after primary growth has slowed or stopped (1, 2). Commonly, limiting nitrogen is employed to stress the organism and induce lignolytic activity (1, 3). Initial lignin breakdown is an oxidative, extracellular process (4-7). It has been postulated that excreted oxidants such as  $H_2O_2$  or  $\cdot OH$  are involved in the initial depolymerization reactions (6, 7). Indeed, both  $H_2O_2$  and  $\cdot OH$  have been identified in the media and in preparations of P. chrysosporium cultures (6, 7), and the level of these oxidants appears to be correlated temporally with the amount of lignolytic activity. In our laboratory, the highest concentration

<sup>1</sup>The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

<sup>2</sup>Poly B-411 is a water-soluble, blue dye comprised of anthraquinone residues covalently linked to a polyvinyl backbone (16). Strong evidence has been provided which indicates that the lignin degrading system of P. chrysosporium also decolorizes Poly B-411 (17).

of  $\text{H}_2\text{O}_2$  found in the media of lignolytic P. chrysosporium cultures was low ( $<10^{-4}\text{M}$ ). By itself, this level of  $\text{H}_2\text{O}_2$  did not significantly react with lignin even in the presence of metal ions, which can catalyze a Fenton-type reaction to produce  $\cdot\text{OH}$  (unpublished data). The  $\cdot\text{OH}$  radical is an unlikely candidate for an excreted oxidant because of its extremely high reactivity with virtually all organic molecules (8). Furthermore, as a result of this high reactivity and low specificity, the diffusional lifetime of  $\cdot\text{OH}$  in complex solutions is extremely short (L. K. Patterson, personal communication). It is possible that relatively high local levels of  $\text{H}_2\text{O}_2$  (or  $\cdot\text{OH}$ ) could be produced enzymatically in close proximity to lignin, so that problems arising from short diffusional lifetimes and low bulk concentrations are effectively avoided. Such a mechanism requires a close physical association between the oxidant-producing enzyme system and the lignin substrate.

An attractive alternative has recently been suggested by Tien and Kirk (9). These authors purified an enzyme from the media of lignolytic P. chrysosporium cultures that, in the presence of  $\text{H}_2\text{O}_2$ , partially degraded lignin model compounds and solvent-extracted spruce and birch lignins. Based on this finding, Tien and Kirk concluded that at least one key reaction in lignin biodegradation is catalyzed by an extracellular  $\text{H}_2\text{O}_2$ -requiring enzyme.

In either model it would be advantageous for P. chrysosporium to possess a system capable of recognizing the presence of lignin in its environment. This would allow the organism to activate and/or excrete the lignin-oxidizing system only when substrate is present, thereby conserving metabolic energy. In this communication we report a necessary characteristic of a lignin recognition system in P. chrysosporium; namely, an increased production of extracellular  $\text{H}_2\text{O}_2$  in response to the presence of a lignocellulosic substrate or a lignin analogue.

#### MATERIALS AND METHODS

Phanerochaete chrysosporium Burds. was grown as previously described (10) following the basic method of Kirk *et al* (3). Low-nitrogen medium contained 0.6 mM  $\text{NH}_4\text{NO}_3$  and 0.6 mM asparagine. High-nitrogen medium contained 6 mM  $\text{NH}_4\text{NO}_3$  and 6 mM asparagine. Cultures were grown in a volume of 500 ml in 3 liter Fernbach flasks without shaking. After 10 days at  $37^\circ\text{C}$ , a large mat

of entangled mycelia was present in the flask. The mycelial mat (about 15 g wet weight) was harvested by filtration and washed twice with 300 ml of 20 mM  $K_2HPO_4$  (pH 6.2). Size and physical characteristics made the mycelial mat unsuitable for the type of experiments conducted in this study. Therefore, it was broken into smaller pieces and slurried in a Waring Blendor (3 repetitions of 10 sec) containing 50 ml of 0.4 M KCl, 20 mM  $Na_2HPO_4$  (pH 6.2). The resulting suspension was centrifuged at 20,000 X g for 5 min. The pelleted pieces of mycelial mat were gently resuspended in 50 ml of fresh 0.4 M KCl, 20 mM  $Na_2HPO_4$  (pH 6.2) and centrifuged again. The washed pellet was resuspended in fresh medium (0.4 M KCl, 20 mM  $Na_2HPO_4$  at pH 6.2, unless otherwise stated) to a final concentration of ca. 2 mg protein·ml<sup>-1</sup>. The final mycelial preparation contained small pieces (<3 mm diameter) of the original mycelial mat, each composed of numerous intertwined segments of apparently intact mycelia.

Changes in  $O_2$  concentration in the mycelial preparation were monitored polarographically in a thermostatted (30°C), water-jacketed cell (Gilson Medical Electronics) using a Clark electrode connected to a strip chart recorder. Protein concentration of the mycelial preparation was determined by the method of Lowry *et al* (11), after disruption of the mycelia with a Branson model S125 sonifier. Wheat straw, obtained locally, was ground to pass a 2-mm screen and washed repeatedly with distilled water. Catalase and Poly B-411 were purchased from Sigma. All other chemicals were reagent grade.

## RESULTS

Catalase-mediated  $H_2O_2$  decomposition has been described for numerous biological systems (12-15). Because the presence of catalase activity associated with the washed *P. chrysosporium* mycelial preparation would reduce apparent  $H_2O_2$  production, the catalase activity of our preparation was assayed by adding exogeneous  $H_2O_2$  and monitoring subsequent  $O_2$  evolution. Considerable catalase activity was exhibited by mycelia from both high and low nitrogen cultures. In both cases the activity could be greatly inhibited (>90%) by 200  $\mu$ M  $NaN_3$  (Fig. 1), although mycelia grown on low nitrogen always exhibited 5-10 times higher catalase activity than mycelia grown on high nitrogen.

The level of  $H_2O_2$  in extracts from nitrogen-limited cultures of *P. chrysosporium* has been shown to increase dramatically at the same time the cultures develop the ability to convert  $^{14}C$ -lignin into  $^{14}CO_2$  (6). This correlation suggests that  $H_2O_2$  is involved in extracellular lignin degradation. The possibility of  $H_2O_2$  involvement is further supported by the data presented in Figure 2, which shows the rate of extracellular  $H_2O_2$  production by washed mycelia from low-nitrogen cultures in the presence of various substrates. With no substrate present, the mycelia produced  $H_2O_2$  slowly for a period of several hours. If ground wheat straw was present, the amount of  $H_2O_2$  produced was increased more than twofold. The water-soluble lignin analogue, Poly

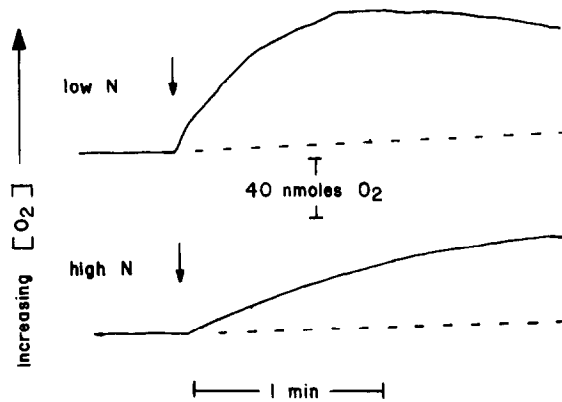


Figure 1. Endogenous catalase activity in *P. chrysosporium* mycelia. Mycelial preparations were from low-nitrogen (upper trace) and high-nitrogen (lower trace) cultures. Catalase activity was assayed in 1.3 ml of the respective preparations by monitoring  $\text{O}_2$  evolution after addition of  $\text{H}_2\text{O}_2$  to a final concentration of  $1.9 \times 10^{-4} \text{ M}$  (designated by the arrows). The dashed lines indicate the response observed in the presence of  $2 \times 10^{-4} \text{ M}$   $\text{NaN}_3$ . Protein concentrations for the low-nitrogen and high-nitrogen mycelial preparations were  $1.6 \text{ mg} \cdot \text{ml}^{-1}$  and  $2.1 \text{ mg} \cdot \text{ml}^{-1}$ , respectively. Standardization of changes in  $\text{O}_2$  concentration was accomplished by addition of  $300 \mu\text{g}$  of exogenous catalase and known quantities of  $\text{H}_2\text{O}_2$ .

B-411<sup>2</sup>, caused an even larger increase in the amount of extracellular  $\text{H}_2\text{O}_2$ . The production of extracellular  $\text{H}_2\text{O}_2$  was observed only in mycelia from cultures of *P. chrysosporium* grown with limiting nitrogen. Mycelia from cultures grown

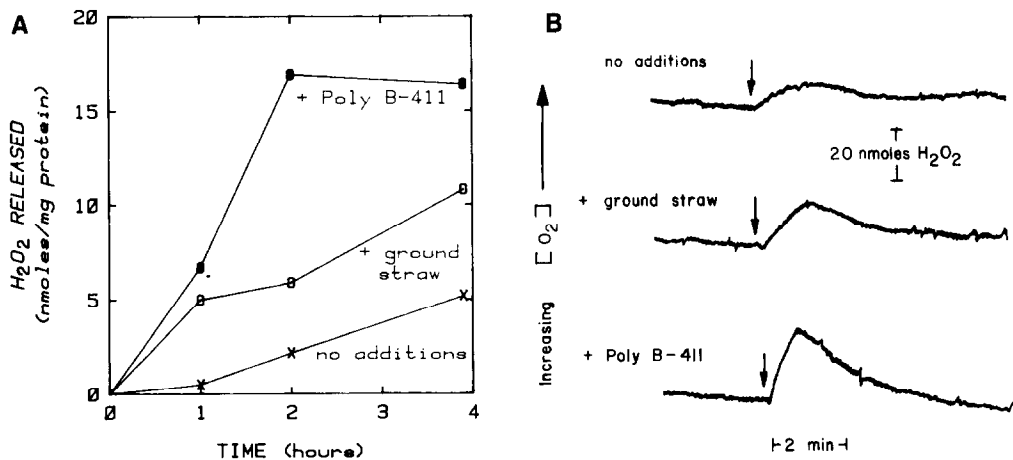


Figure 2. Extracellular  $\text{H}_2\text{O}_2$  production stimulated by ground straw or Poly B-411. A mycelial preparation ( $1.6 \text{ mg protein} \cdot \text{ml}^{-1}$ ) was prepared from a low-nitrogen culture and divided into three reaction vessels, each containing  $2 \times 10^{-4} \text{ M}$   $\text{NaN}_3$ . Aliquots ( $1.3 \text{ ml}$ ) were taken at the designated time intervals and  $\text{H}_2\text{O}_2$  content was assayed by overwhelming the system with exogenous catalase ( $300 \mu\text{g}$ ) and monitoring subsequent  $\text{O}_2$  evolution. Addition of a known quantity of  $\text{H}_2\text{O}_2$  was used for standardization. Panel (A): Additions to the reaction vessels were none (X),  $5 \text{ mg ground straw} \cdot \text{ml}^{-1}$  (O), or  $0.2 \text{ mg Poly B-411} \cdot \text{ml}^{-1}$  (●). Panel (B): Raw data after 4 hours incubation. The arrows designate the addition of catalase.

in high-nitrogen medium did not produce measurable amounts of  $H_2O_2$  after 4 hr in either the presence or absence of ground straw or Poly B-411 (not shown).

The effect of medium pH on  $H_2O_2$  production by the mycelial preparation in the presence and absence of Poly B-411 is shown in Figure 3.  $H_2O_2$  production was observed in the pH range of 4.5 to 8.4, and Poly B-411 stimulated the production throughout this range. Interestingly, maximal  $H_2O_2$  production occurred between pH 5.5 and pH 6.5. This was a somewhat unexpected result, because it was reported earlier that the optimum pH for lignin degradation is 4.5 (3). Throughout the pH range examined in Figure 3, endogenous catalase activity was found to be inhibited >90% by 200  $\mu$ M azide (not shown). Therefore, it is unlikely that this pH profile was skewed by residual catalase activity.

## DISCUSSION

Evidence is accumulating which indicates that  $H_2O_2$  may play an important role in the mechanism of fungal lignin degradation (5-7, 9, 18). The observation that a lignocellulosic substrate or a lignin analogue accelerates the production of  $H_2O_2$  by *P. chrysosporium* mycelia (Figs. 2 and 3) further supports this hypothesis. The higher levels of catalase activity associated with lignin-

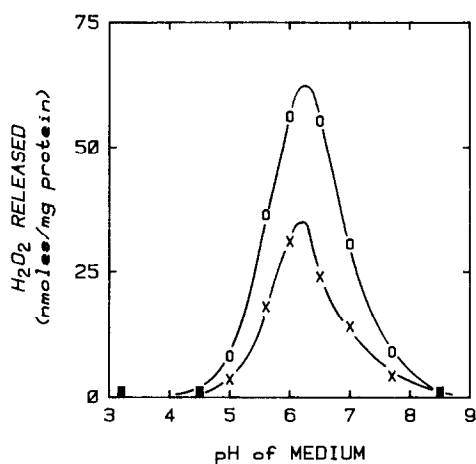


Figure 3. Effect of medium pH on  $H_2O_2$  production. A mycelial preparation ( $1.4 \text{ mg protein} \cdot \text{ml}^{-1}$ ) was suspended in  $0.4 \text{ M KCl}$ ,  $10 \text{ mM Na}_2\text{HPO}_4$ ,  $10 \text{ mM}$  2,2-dimethylsuccinate,  $200 \mu\text{M NaN}_3$  and was divided into reaction vessels. The pH of each suspension was then adjusted with NaOH or HCl. The symbols designate no additions (X) or the addition of  $0.2 \text{ mg Poly B-411} \cdot \text{ml}^{-1}$  (O) to the reaction vessels.  $H_2O_2$  content was assayed as in Figure 2 after 4 hours of incubation.

degrading cultures (Fig. 1) also supports this conclusion, since increased catalase activity would probably be required to protect intracellular components from the higher levels of  $H_2O_2$  in the lignin-degrading cultures. Furthermore, Tien and Kirk (9) have purified an extracellular lignin-degrading enzyme that requires  $H_2O_2$  for activity. It is possible that all or part of the catalase activity observed in our mycelial preparations (Fig. 1) may be attributable to the same enzyme, especially since many peroxidases are known to exhibit substantial catalase activity at higher  $H_2O_2$  concentrations (19). If this is indeed the case, we would predict the enzyme isolated by Tien and Kirk to be azide sensitive.

The observed pH optimum for  $H_2O_2$  production ( $\sim$ pH 6, Fig. 3) does not correlate well with the previously reported pH optimum for P. chrysosporium lignin degradation ( $\sim$ pH 4.5, Ref. 3). However, the pH optimum for lignin degradation was determined by measuring the production of  $^{14}CO_2$  derived from  $^{14}C$ -lignin. Numerous metabolic steps are involved in the conversion of lignin into  $CO_2$ . At least some of these steps probably occur extracellularly. The pH optimum of 4.5 as determined from measurements of  $^{14}CO_2$  evolution must therefore represent an average of the effects of pH on the many constituent reactions, or be the effect of pH on a rate-limiting metabolic step in the overall reaction.

Demonstration of lignocellulosic-induced  $H_2O_2$  production by P. chrysosporium mycelia suggests that this organism possesses a mechanism that responds to lignin in its environment. Two possible mechanisms for lignin recognition can be postulated. The first mechanism would utilize a receptor protein located on the surface of the mycelia. Because of the complex, three-dimensional structure and almost random arrangement of the substituted phenylpropane units in lignin (20), it seems likely that a lignin receptor would be rather nonspecific, perhaps recognizing a more general, substituted aromatic moiety. This relatively low specificity may explain why Poly B-411 is apparently degraded by the P. chrysosporium lignin degradation system (17). The second possible mechanism involves the recognition of a lignin breakdown product generated by an "idle

level" of excreted  $H_2O_2$ . Binding of this product, either extracellularly or intracellularly, could then induce accelerated  $H_2O_2$  production. Additional studies are underway to further explore the role of the mycelial cell surface in the mechanism of lignin biodegradation, and to elucidate the metabolic source of extracellular  $H_2O_2$ .

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