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Lignin and Veratryl Alcohol Are Not Inducers of the Ligninolytic System of *Phanerochaete chrysosporium*

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Phanerochaete chrysosporium **is a white rot fungus which secretes a family of lignin-degrading enzymes under nutrient limitation. In this work, we investigated the roles of veratryl alcohol and lignin in the ligninolytic system of *P. chrysosporium* BKM-F-1767 cultures grown under nitrogen-limited conditions. Cultures supplemented with 0.4 to 2 mM veratryl alcohol showed increased lignin peroxidase activity. Addition of veratryl alcohol had no effect on Mn-dependent peroxidase activity and inhibited glyoxal oxidase activity. Azure-casein analysis of acidic proteases in the extracellular fluid showed that protease activity decreased during the early stages of secondary metabolism while lignin peroxidase activity was at its peak, suggesting that proteolysis was not involved in the regulation of lignin peroxidase activity during early secondary metabolism. In cultures supplemented with lignin or veratryl alcohol, no induction of mRNA coding for lignin peroxidase H2 or H8 was observed. Veratryl alcohol protected lignin peroxidase isozymes H2 and H8 from inactivation by H_2O_2. We conclude that veratryl alcohol acts as a stabilizer of lignin peroxidase activity and not as an inducer of lignin peroxidase synthesis.**

Lignin peroxidases and Mn peroxidases are known to catalyze the initial depolymerization of the lignin polymer (6, 7, 25). The best-characterized lignin degraders are white rot fungi, and among these, *Phanerochaete chrysosporium* has been the most extensively studied (9). Lignin peroxidase and Mn peroxidase are extracellular heme peroxidases which catalyze the oxidation of aromatic compounds (9). Glyoxal oxidase is involved in the generation of H_2O_2 for the extracellular peroxidases (8).

The ligninolytic system of *P. chrysosporium* is activated during secondary metabolism of fungal growth and is regulated by the availability of nutrients, oxygen, and trace metals and the pH (10; for review, see reference 17). Previous research also indicates that lignin peroxidase activity in the extracellular fluid of *P. chrysosporium* cultures increases in the presence of various substrates, such as lignin (23) and lignin model compounds (3). Several aromatic substrates and degradation products of lignin, such as chlorogenic acid, vanillic acid, veratric acid, veratraldehyde, and veratryl alcohol, have been assayed for their ability to increase ligninolytic activity (14). Of the aromatic compounds tested, veratryl alcohol is the most effective in stimulating lignin peroxidase activity and lignin degradation in *P. chrysosporium* cultures (3, 11, 13, 14, 23). The present study examines the mechanism by which veratryl alcohol increases lignin peroxidase activity. We conclude that veratryl alcohol acts by protecting the enzymes and does not act via an induction mechanism.

MATERIALS AND METHODS

Culture conditions. Wild-type *P. chrysosporium* BKM-F-1767 (ATCC 24725) was maintained on agar medium containing 1% malt, 0.2% Bacto-Peptone, 1% yeast extract, 0.1% asparagine, 0.2% KH_2PO_4, and 0.1% $MgSO_4 \cdot 7H_2O$. Experimental cultures of *P. chrysosporium* were grown in nitrogen-limited medium as described previously (21). The fungus was grown in shallow stationary cultures (10 ml in 125-ml flasks) containing 10^5 spores per ml. They were flushed on day 3 with water-saturated 100% O_2. Loblolly pine lignin, kindly provided by Champion Paper Co., was prepared as described by Faison et al. (4). The lignin suspension was added to the 10-ml fungal cultures on day 3 at a final concentration of 38 μg/ml. Veratryl alcohol was added on day 3 at various concentrations (0.4, 1.0, and 2.0 mM). Control cultures received an equivalent volume of water.

Enzyme assays. All enzyme assays were performed with the extracellular fluid, which was collected by filtration through cheesecloth. Data represent averages for three culture flasks. One unit of enzyme activity is equal to 1 μmol/min.

Lignin peroxidase activity was measured as described by Tien and Kirk (20) by monitoring the increase in the A_{310} associated with the oxidation of veratryl alcohol to veratraldehyde. Mn peroxidase activity was measured as described by Kuwahara et al. (12), with phenol red as the substrate. Glyoxal oxidase activity was determined as described by Kersten and Kirk (8), by using a modified horseradish peroxidase-coupled assay with phenol red as a substrate and monitoring the change in the A_{610}. Protease activity was assayed with Azocoll (Sigma Chemical Co.) as the substrate as described by Dosoretz et al. (2). Protein analysis was performed by the method of Lowry et al. (15) with bovine serum albumin as a standard.

RNA isolation and quantitation. Isolation of RNA from cultures of *P. chrysosporium* and Northern (RNA) blotting were done as described previously (19) except that 25 μg of total RNA was loaded per lane in the formaldehyde gel. Densitometric scanning (Quick Scan Jr.; Helena Laboratories) of the photographic negative (Polaroid 665 positive/negative instant-pack film) of this gel was used to quantitate the RNA levels. Hybridizations were carried out as described by Church and Gilbert (1).

Purification and characterization of lignin peroxidase. Lig-

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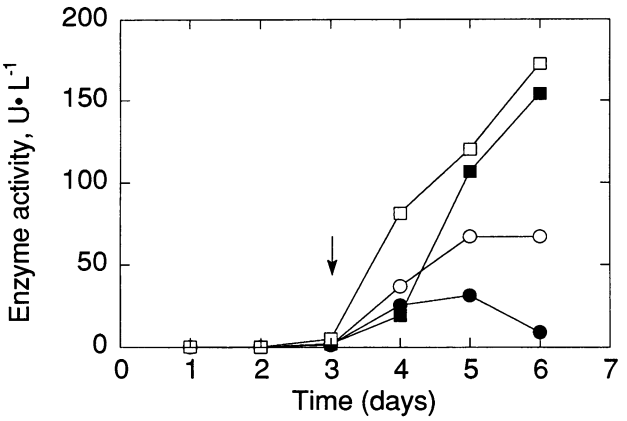

FIG. 1. Effect of veratryl alcohol addition on lignin peroxidase activity of *P. chrysosporium* stationary cultures. The veratryl alcohol concentrations used were (●) 0.0 mM, (○) 0.4 mM, (■) 1.0 mM, and (□) 2.0 mM. The arrow indicates the time of addition of veratryl alcohol.

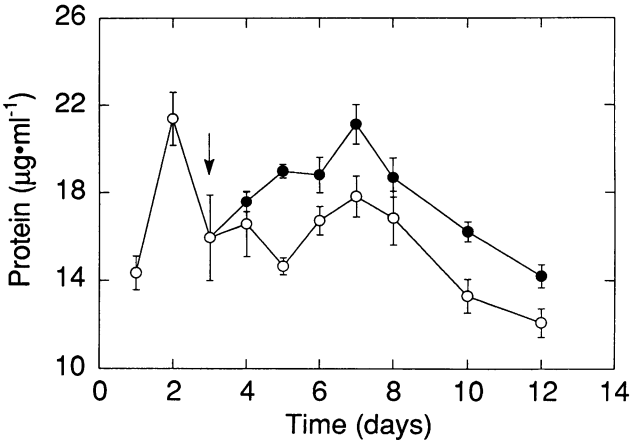

FIG. 2. Extracellular protein content as a function of culture age of *P. chrysosporium*. Results are given as the means of three datum points. Vertical bars represent the standard deviation of the mean. The arrow indicates the time of addition of veratryl alcohol.

nin peroxidase isozymes H2 and H8 were purified as described previously (21). Isozymes H2 and H8 (30 nM) were incubated separately for 20 min with 25 μM H_2O_2 in the presence or absence of 2 mM veratryl alcohol. The stability of the enzymes was assessed by measuring veratryl alcohol oxidation by the enzymes at various times during incubation.

RESULTS

Activity of ligninolytic enzymes. Experiments were conducted to determine the effect of veratryl alcohol on the lignin peroxidase activity of *P. chrysosporium* cultures. In accord with previous results (4, 14, 22), cultures with added veratryl alcohol (0.4 mM) showed greater lignin peroxidase activity than control cultures which received no veratryl alcohol (Fig. 1; also see Fig. 3A). Increasing the concentration of veratryl alcohol above 0.4 mM resulted in a further increase in lignin peroxidase activity. The increase in activity is associated with an increase in extracellular protein levels (Fig. 2). This suggests that the increase in activity is not due solely to activation of the enzyme by veratryl alcohol.

Mn peroxidase activity was not altered by the addition of 2 mM veratryl alcohol to the medium (Fig. 3B). Mn peroxidase activity typically appears before lignin peroxidase activity; it was first detected on day 3. Maximal activity was detected on day 4 at 421 U/liter. After day 4, the activity decreased rapidly.

In contrast, glyoxal oxidase activity was significantly lowered by the addition of 2 mM veratryl alcohol. Whereas control cultures peaked at 4 U of glyoxal oxidase activity per liter on day 4, in agreement with previously published values (8), cultures amended with veratryl alcohol exhibited only 0.34 U of activity per liter (Fig. 3C).

Activity of proteases. To investigate the possibility that proteolytic cleavage is responsible for reduced lignin peroxidase activity in the absence of added veratryl alcohol, we assayed for acid protease activity with azure-casein. An initial increase in protease activity was correlated with the change from primary to secondary metabolism (Fig. 4). This was observed both in control cultures and in cultures with added veratryl alcohol. Protease activity decreased during the early stages of secondary metabolism in control and veratryl alcohol-supplemented cultures, while lignin peroxidase activity was at its peak.

RNA quantitation. To address the question of whether veratryl alcohol or lignin caused an induction of lignin peroxidase mRNA synthesis, total RNA was isolated from *P. chrysosporium* cultures grown with and without 2 mM veratryl alcohol or 38 μg of lignin per ml. The 16s rRNA subunit was used as a standard to quantitate the RNA levels (Table 1). The RNA was subjected to electrophoretic separation on a formaldehyde gel, transferred to nylon membranes, and probed with random-primed cDNAs encoding lignin peroxidase isozymes H2 and H8. The blots showed no increase in mRNA specific for either H2 (Fig. 5A) or H8 (Fig. 5B) upon addition of either veratryl alcohol or lignin. Surprisingly, less of these mRNAs was detected in cultures supplemented with veratryl alcohol than in the control cultures.

Protection from inactivation. In the absence of induction, it is possible, as previously proposed (22), that veratryl alcohol increases lignin peroxidase activity via enzyme stabilization. Previous reports had shown that lignin peroxidase is rapidly degraded in the presence of H_2O_2 and the absence of reducing substrate (22). To investigate this possibility, purified isozymes H2 and H8 were incubated with 25 μM H_2O_2, with and without veratryl alcohol, and their activity was assayed at various times during the incubation period. Figure 6 shows that veratryl alcohol clearly protects both isozymes from inactivation, especially after several minutes. Isozyme H8 was inactivated faster in the presence of H_2O_2 than isozyme H2 under these conditions.

DISCUSSION

It has been well established that the addition of veratryl alcohol to cultures of *P. chrysosporium* enhances lignin peroxidase activity (3, 4, 13, 14, 16, 22). This study confirms these earlier results, showing that supplemental veratryl alcohol causes an increase in lignin peroxidase activity in the extracellular fluid under nitrogen limitation. The findings that the Mn peroxidase activity is unaffected and that glyoxal oxidase activity is actually inhibited with supplemental ver-

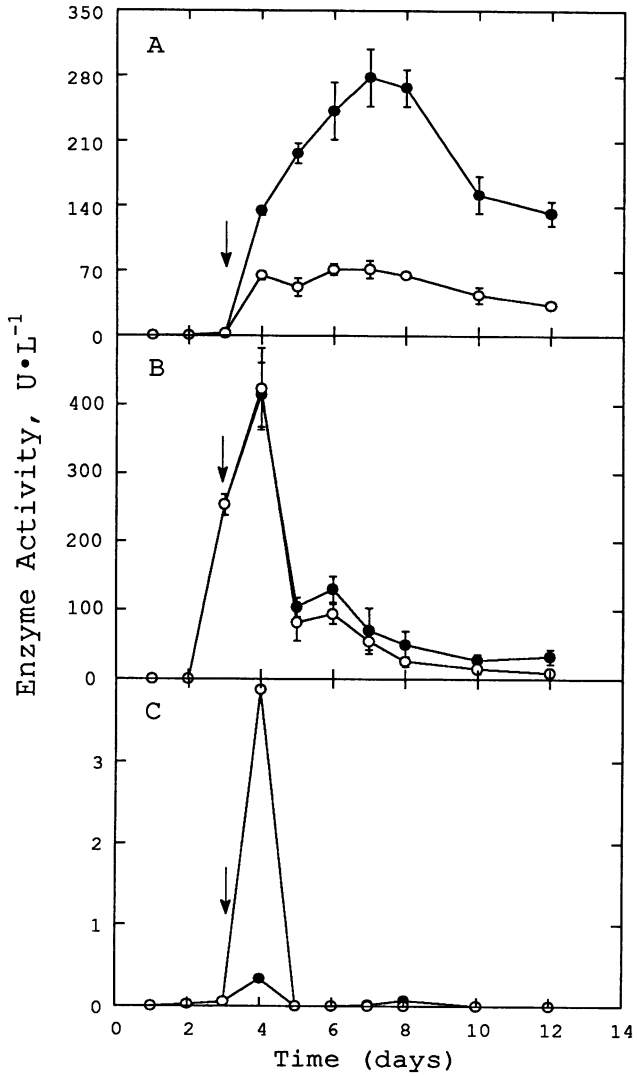

FIG. 3. Effect of veratryl alcohol on extracellular enzyme activity of *P. chrysosporium*. (A) Lignin peroxidase; (B) Mn-dependent peroxidase; (C) glyoxal oxidase. Results are given as the means of three datum points. Vertical bars represent the standard deviation of the mean. The arrow indicates the time of addition of veratryl alcohol. The veratryl alcohol concentrations used were 0.0 mM (○) and 2.0 mM (●).

atryl alcohol demonstrate that if the mode of action is induction, the entire ligninolytic system is not induced by this metabolite. The basis for the decrease in glyoxal oxidase activity in the presence of veratryl alcohol is not well understood but has been observed previously (8).

The increase in lignin peroxidase activity can be attributed, in part, to the ability of veratryl alcohol to protect lignin peroxidase from H_2O_2-dependent inactivation. In a study by Haemmerli et al. (5) on the oxidation of benzo[*a*]pyrene by crude and partially purified enzymes, veratryl alcohol stabilized lignin peroxidase in the presence of peroxide concentrations which would normally destroy the enzyme. Tonon and Odier (22) have clearly shown that in the absence of the reducing substrate veratryl alcohol, the decrease in lignin peroxidase activity in the presence of H_2O_2 is 21.3% · h^{-1}, whereas it is only 5.4% · h^{-1} in the

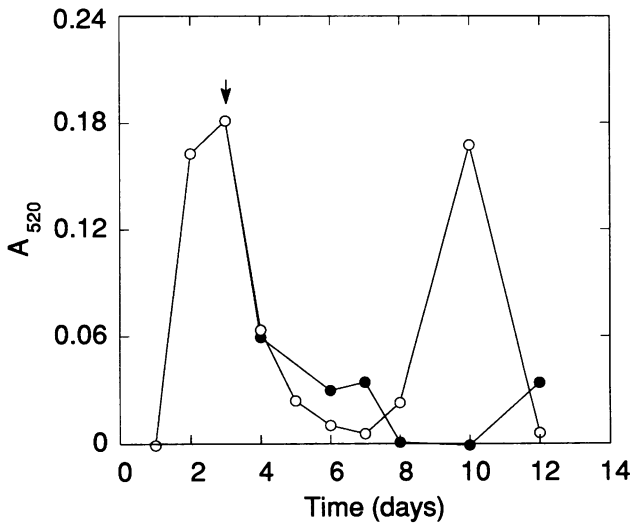

FIG. 4. Extracellular acid protease activity of *P. chrysosporium* cultures. The veratryl alcohol concentrations used were 0.0 mM (○) and 2.0 mM (●). The arrow indicates the time of addition of veratryl alcohol.

presence of veratryl alcohol. In cultures treated with cycloheximide to stop protein synthesis, extracellular protein turnover and inactivation caused a marked decrease in enzyme activity; addition of veratryl alcohol prevented this inactivation (22). Additionally, Tonon and Odier (22) found no evidence for an increase in synthesis of individual isozymes in the presence of veratryl alcohol in pulse-labeling experiments. This indicates that veratryl alcohol does not stimulate higher enzyme activity by inducing de novo protein synthesis. These authors conclude that veratryl alcohol protects the enzyme from inactivation by excess H_2O_2 produced by the fungus in culture. Valli et al. (24) also found that veratryl alcohol protects lignin peroxidase from inactivation by H_2O_2. We have confirmed these findings in vitro with purified isozymes H2 and H8.

Paszczynski and Crawford (18) demonstrated that veratryl alcohol can also protect lignin peroxidase by completing the catalytic cycle. For example, certain azo dyes can only be oxidized by compound I of lignin peroxidase, not by compound II. Consequently, incubation with these dyes in the absence of veratryl alcohol results in accumulation of compound II. The addition of veratryl alcohol resulted in conversion of the enzyme to the resting state and also in stimulation of dye oxidation.

Other researchers have suggested that veratryl alcohol may induce the synthesis of lignin peroxidase (3). However,

TABLE 1. Quantitation of 16S rRNA[a]

Addition to culture	Area (cm^2) on day:						
	1	2	3	4	5	6	7
Water	1.2	1.1	1.1	0.9	0.9	0.9	ND[b]
Veratryl alcohol (2 mM)	ND	ND	ND	1.0	0.9	1.0	0.9
Lignin (38 μg/ml)	ND	ND	ND	1.0	1.0	1.0	1.0

[a] Prior to blotting, the gels used for the Northern blot analysis (Fig. 5) were stained with ethidium bromide, and the 16S rRNA was quantitated. Values represent the area determined by densitometry on the day of culture that *P. chrysosporium* RNA was extracted.

[b] ND, not done.

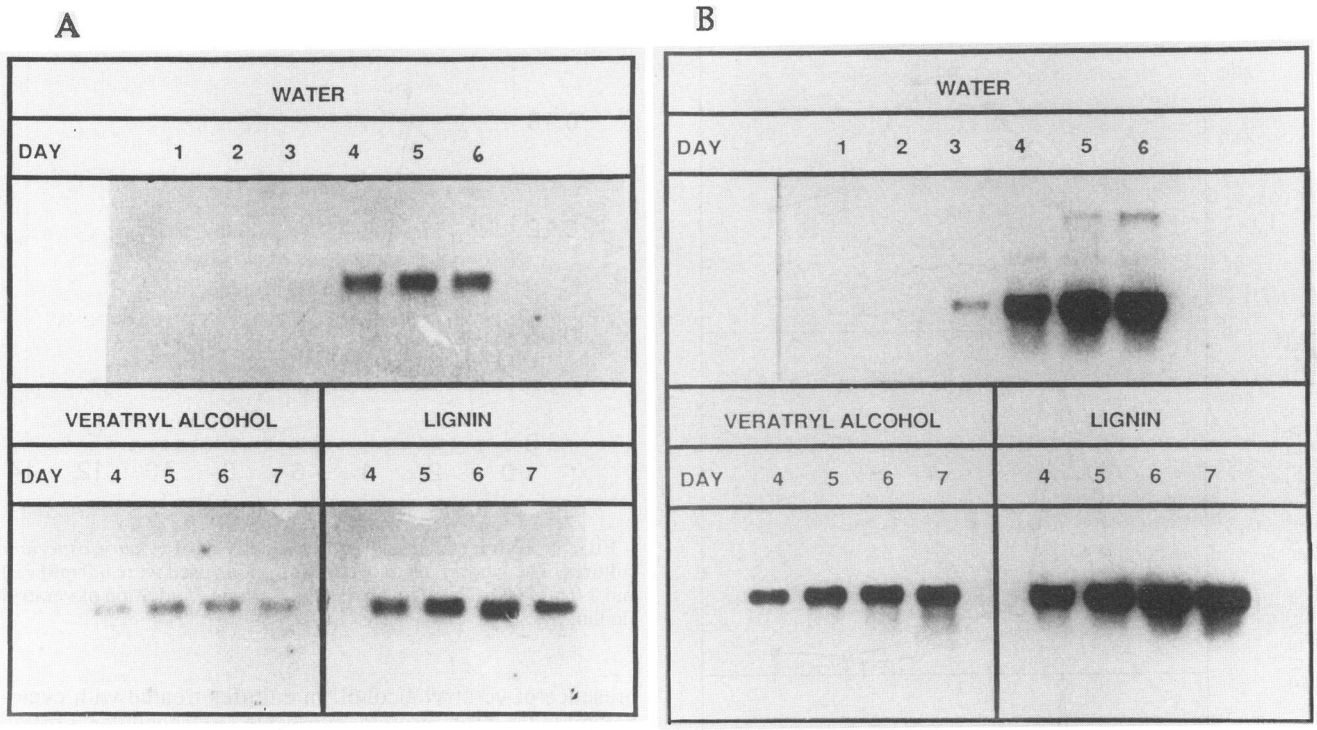

FIG. 5. Northern blot analysis of total RNA from days 4 to 7 for nitrogen-limited cultures with the cDNA for H2 (A) or H8 (B) used as a probe. Veratryl alcohol (2.0 mM final concentration), a lignin suspension (38 μg/ml final concentration), or water was added to the 10-ml fungal cultures on day 3. Total RNA (25 μg) was subjected to electrophoresis on a 1.5% agarose gel containing 0.66 M formaldehyde and transferred to nylon membranes. The blots were hybridized with the λML-6 (A) and λML-1 (B) cDNA *Eco*RI 1.3-kb insert.

the induction of the ligninolytic system has yet to be demonstrated for any aromatic or lignin-like compound. Ulmer et al. (23) were the first to suggest that an induction or activation mechanism is involved in lignin degradation by *P. chrysosporium*, because added lignin (1 mg/ml) enhanced lignin degradation in culture. Later work by this group showed that added veratryl alcohol activated the ligninolytic system, leading these authors to speculate that veratryl alcohol acts as an inducer of the system (14). Faison and Kirk (3) report that the activity of lignin peroxidase is markedly increased by the addition of natural or synthetic lignins (38 μg/ml) and by the addition of veratryl alcohol to ligninolytic cultures. Further work by these authors with Western immunoblot analysis showed that veratryl alcohol and lignin also caused an increase in total lignin peroxidase proteins (4). They suggested from these studies that an induction mechanism is involved in lignin degradation.

Our data clearly show that veratryl alcohol does not cause a net increase in the mRNAs encoding lignin peroxidase isozymes H2 and H8. The same conclusion can be drawn for lignin; lignin peroxidase mRNA levels appear to be equivalent in control cultures and those which received supplemental lignin. Surprisingly, RNA levels in cultures with added veratryl alcohol were lower than in the controls for both isozymes H2 and H8. This evidence does not support the role of induction for veratryl alcohol or lignin.

We have no evidence to suggest that veratryl alcohol has any effect on proteases. We found no difference in protease activity over a 12-day period between control and veratryl alcohol-supplemented cultures. Our findings are in agreement with those of Dosoretz et al. (2) and indicate that the decrease in lignin peroxidase activity after day 6 in *P.*

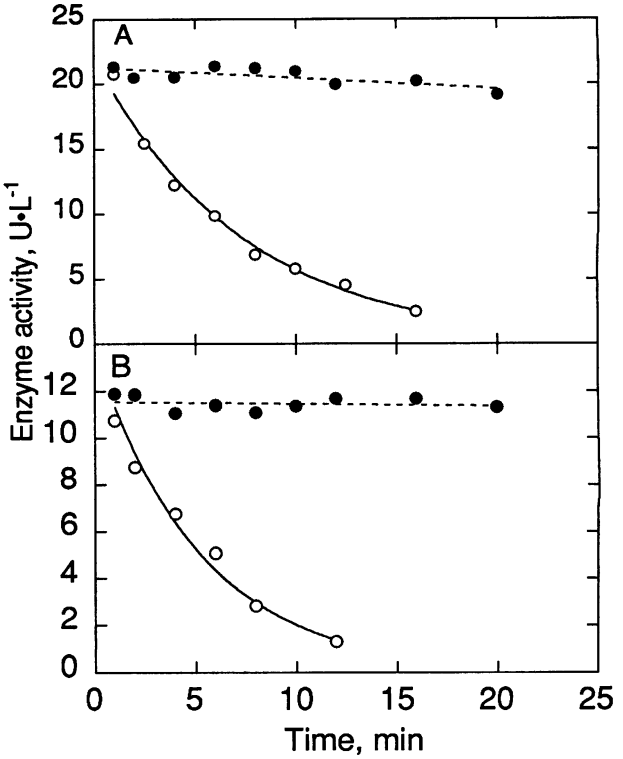

FIG. 6. Effect of veratryl alcohol on activity of purified lignin peroxidase isozymes H2 (A) and H8 (B) after addition of 25 μM H_2O_2. The veratryl alcohol concentrations used were 0.0 mM (○) and 2.0 mM (●).

chrysosporium cultures is correlated with the appearance of extracellular protease activity.

It is noteworthy that although greater lignin peroxidase activity is found in cultures supplemented with veratryl alcohol, less transcript is evident than in control cultures. From the results presented in this article, it would be difficult to comment on the significance of this decrease. There are a number of possible mechanisms involving feedback inhibition to explain this phenomenon. At this point, the nature of this mechanism is unknown and warrants further study. Nevertheless, our data provide no evidence for induction of lignin peroxidase mRNA by veratryl alcohol.

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REFERENCES

1. **Church, G. M., and N. Gilbert.** 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:**1991–1995.
2. **Dosoretz, C. G., H.-H. Chen, and H. E. Grethlein.** 1990. Effect of environmental conditions on extracellular protease activity in ligninolytic cultures of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **56:**395–400.
3. **Faison, B. D., and T. K. Kirk.** 1985. Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **49:**299–304.
4. **Faison, B. D., T. K. Kirk, and R. L. Farrell.** 1986. Role of veratryl alcohol in regulating ligninase activity in *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **52:**251–254.
5. **Haemmerli, S. D., M. S. A. Leisola, D. Sanglard, and A. Fiechter.** 1986. Oxidation of benzo(a)pyrene by extracellular ligninases of *Phanerochaete chrysosporium*. J. Biol. Chem. **261:**6900–6903.
6. **Hammel, K. E., and M. A. Moen.** 1991. Depolymerization of a synthetic lignin in vitro by lignin peroxidase. Enzyme Microb. Technol. **13:**15–18.
7. **Hammel, K. E., M. Tien, B. Kalyanaraman, and T. K. Kirk.** 1985. Mechanism of oxidative C_α—C_β cleavage of a lignin model dimer by *Phanerochaete chrysosporium* ligninase. J. Biol. Chem. **260:**8348–8353.
8. **Kersten, P. J., and T. K. Kirk.** 1987. Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*: synthesized in the absence of lignin in response to nitrogen starvation. J. Bacteriol. **135:**790–797.
9. **Kirk, T. K., and R. L. Farrell.** 1987. Enzymatic "combustion": the microbial degradation of lignin. Annu. Rev. Microbiol. **41:**465–505.
10. **Kirk, T. K., E. Schultz, W. J. Connors, R. D. Bleam, W. F. Hackett, and J. G. Zeikus.** 1978. Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. **117:**227–285.
11. **Kirk, T. K., M. Tien, P. J. Kersten, M. D. Mozuch, and B. Kalyanaraman.** 1986. Ligninase of *Phanerochaete chrysosporium*: mechanism of its degradation of the nonphenolic arylglycerol β-aryl ether substructure of lignin. Biochem. J. **236:**279–287.
12. **Kuwahara, M., J. K. Glenn, M. A. Morgan, and M. H. Gold.** 1984. Separation and characterization of two extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium*. FEBS Lett. **169:**247–250.
13. **Leisola, M. S. A., U. Thanei-Wyss, and A. Fietcher.** 1985. Strategies for production of high ligninase activities by *Phanerochaete chrysosporium*. J. Biotechnol. **3:**97–107.
14. **Leisola, M. S. A., D. C. Ulmer, R. Waldner, and A. Fiechter.** 1984. Role of veratryl alcohol in lignin degradation by *Phanerochaete chrysosporium*. J. Biotechnol. **1:**331–339.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
16. **Orth, A. B., M. Denny, and M. Tien.** 1991. Overproduction of lignin-degrading enzymes by an isolate of *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **57:**2591–2596.
17. **Orth, A. B., E. A. Pease, and M. Tien.** Properties of lignin peroxidases and their use in bioremediation. *In* G. R. Chaudhry (ed.), Biological degradation and bioremediation technologies. Dioscorides Press, Portland, Oreg., in press.
18. **Paszczynski, A., and R. L. Crawford.** 1991. Degradation of azo compounds by ligninase from *Phanerochaete chrysosporium*: involvement of veratryl alcohol. Biochem. Biophys. Res. Commun. **178:**1056–1063.
19. **Pease, E. A., A. Andrawis, and M. Tien.** 1989. Manganese-dependent peroxidase from *Phanerochaete chrysosporium*: primary structure deduced from cDNA sequence. J. Biol. Chem. **264:**13531–13535.
20. **Tien, M., and T. K. Kirk.** 1983. Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. Science **221:**661–663.
21. **Tien, M., and T. K. Kirk.** 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. Methods Enzymol. **161:**238–249.
22. **Tonon, F., and E. Odier.** 1988. Influence of veratryl alcohol and hydrogen peroxide on ligninase activity and ligninase production by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **56:**466–472.
23. **Ulmer, D. C., M. S. A. Leisola, and A. Fiechter.** 1984. Possible induction of the ligninolytic system of *Phanerochaete chrysosporium*. J. Biotechnol. **1:**13–24.
24. **Valli, K., H. Wariishi, and M. H. Gold.** 1990. Oxidation of monomethoxylated aromatic compounds by lignin peroxidase: role of veratryl alcohol in lignin biodegradation. Biochemistry **29:**8535–8539.
25. **Wariishi, H., K. Valli, and M. H. Gold.** 1991. In vitro depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem. Biophys. Res. Commun. **176:**269–275.