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Influence of Veratryl Alcohol and Hydrogen Peroxide on Ligninase Activity and Ligninase Production by *Phanerochaete chrysosporium*

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Veratryl alcohol, added as a supplement to cultures of *Phanerochaete chrysosporium*, enhanced ligninase activity through protection of the ligninase against inactivation by hydrogen peroxide produced by this fungus in cultures. In the presence of veratryl alcohol, the loss of ligninase activity observed in non-protein-synthesizing cultures (cycloheximide-treated) equaled the extracellular protein turnover. When cultures were not supplemented with veratryl alcohol, inactivation of ligninase by hydrogen peroxide added to protein turnover, resulting in a more rapid loss of ligninase activity. Although all ligninase isoenzymes are sensitive to inactivation by hydrogen peroxide, only the isoenzyme of the highest specific activity (80.6 nkat · mg of protein^{-1}; M_r, 41,800; pI, 3.96) was found to be protected by veratryl alcohol. The concentration of veratryl alcohol necessary for full protection of ligninase activity varied according to the concentration of hydrogen peroxide present in the medium, which depended on the nature of the carbon source (glucose or glycerol). It is proposed that the nature of the carbon source influences the overall ligninase activity not only directly, by affecting the rate and the type of synthesized ligninase, but also by affecting the rate of hydrogen peroxide production, bringing about different rates of inactivation.

The white rot fungus *Phanerochaete chrysosporium* produces a variety of extracellular enzymes which are involved in the degradation of wood components (31). An enzyme, later identified as a peroxidase and designated ligninase or lignin peroxidase, has been discovered in this fungus (12, 31). The ligninolytic complex of this fungus, including the ligninase, is produced under nutrient-limited conditions (17). Multiple forms of the ligninase of *P. chrysosporium* have been isolated (18, 21, 23, 25, 28). This enzyme catalyzes the one-electron oxidation of various aromatic compounds in the presence of hydrogen peroxide, yielding radical cations which undergo further noncatalytic reactions (16). The mechanism involved in the degradation of model compounds related to lignin has been studied (31), and the reactions are identical to those previously shown in the degradation of polymeric lignin by *P. chrysosporium* (4, 5, 30).

Veratryl alcohol (3, 4-dimethoxybenzyl alcohol) is synthesized *de novo* from L-phenylalanine (26, 29) by *P. chrysosporium*, and its formation coincides with the onset of ligninolytic activity (10). This aromatic compound is a substrate for ligninase, which catalyzes its oxidation to veratraldehyde (20, 32). The possible role of veratryl alcohol in lignin degradation by *P. chrysosporium* is unclear. It has been proposed that veratryl alcohol could act as a mediator for certain lignin degradation reactions through the ligninase-catalyzed formation of a veratryl alcohol cation radical, which in turn would attack certain structures in lignin (14). This mechanism would allow degradation reactions to take place at a distance from the active site of the enzyme (14). Furthermore, veratryl alcohol has been shown to stimulate ligninolytic activity as well as ligninase production (8, 18, 25): supplementation at 0.4 mM in cultures resulted in a 2.1-fold enhancement of ligninase activity as well as a higher specific activity of ligninase and a different pattern of ligninase isoenzymes (9, 18). A 10-fold increase in ligninase activity was obtained by supplementation of cultures with 1.5 mM veratryl alcohol (25). Ulmer et al. previously reported the stimulation of lignin biodegradation by lignin addition in cultures (33). The mechanism responsible for this phenomenon could involve induction as proposed by Faison et al. (9). In addition, a protective effect of veratryl alcohol against inactivation of the ligninase by hydrogen peroxide has been reported by Haemmerli et al. (13). However, the concentration of hydrogen peroxide normally present in cultures is six to seven times lower than the concentration tested by these authors.

In the present study the effect of veratryl alcohol on ligninases is investigated.

MATERIALS AND METHODS

Organism and culture conditions. *P. chrysosporium* ME446 (ATCC 34541) was grown in nitrogen-limited cultures. The fungus was cultivated in 150-ml Erlenmeyer flasks containing 10 ml of growth medium (2) with no yeast extract and 2 ml of trace elements solution modified from that of Kirk et al. (19): 7.5 mM nitrilotriacetic acid, 12.17 mM $MgCl_2 \cdot 6H_2O$, 20.72 mM $MnCl_2 \cdot 4H_2O$, 17.11 mM NaCl, 0.36 mM $FeCl_2 \cdot 4H_2O$, 0.42 mM $CoCl_2$, 0.04 mM $CuCl_2$, 0.02 mM $AlCl_3$, 0.16 mM H_3BO_3, and 0.04 mM $Na_2MoO_4 \cdot 2H_2O$. Flasks were inoculated with conidia (3), flushed with 100% O_2, and incubated at 37°C in static conditions.

Enzyme assays. Ligninase activity in cultures was measured by determining the rate of oxidation of veratryl alcohol to veratraldehyde at 37°C by the method of Tien and Kirk (32), as modified by Buswell et al. (3), without Tween 80. In certain cases (for very concentrated samples), 2 mM veratryl alcohol was used instead of 0.4 mM. Enzymic activity is expressed in nanokatals (nkat).

The ligninase activity was determined in 5- or 6-day-old cultures. Each flask received 0.5 ml of a fresh aqueous solution of cycloheximide (0.025 mM final concentration). Immediately, 0.5 ml of either water or an aromatic alcohol-water solution was added. Cultures were further incubated

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at 37°C. Ligninase activity was followed versus time for 8 h in each individual culture by means of 0.4-ml samples. Control experiments without cycloheximide but with veratryl alcohol or water were run in parallel.

Radiolabeling experiments. Rates of intracellular and extracellular protein turnover were estimated from the loss of radioactivity of protein labeled with 1.67 nmol of L-[U-^{14}C]leucine (18.5 kBq in each flask in 0.4 ml). Two different methods were used to stop the incorporation of label: either 0.5 ml of a fresh solution of cycloheximide (0.025 mM final concentration) or 0.5 ml of a solution of unlabeled L-leucine (0.25 mM final concentration) was added after 15 min. One milliliter of ice-cold L-leucine (0.5 mM final concentration) was added to each flask. The culture was then filtered on glass-fiber filters (GF/D, Whatman). Mycelium was washed with 3 ml of the ice-cold leucine solution, followed by three washes with 10 ml of ice-cold 10% (wt/vol) trichloroacetic acid and three more washes with 10 ml of ice-cold 95% ethanol. Each filtrate then received 1 ml of ice-cold bovine serum albumin (10 mg/ml), followed by 10 ml of ice-cold 50% (wt/vol) trichloroacetic acid, and was held on ice for at least 1 h. The acid-insoluble precipitates were collected by filtration on two GF/D filters and washed three times with 10 ml of ice-cold 10% (wt/vol) trichloroacetic acid, followed by three washes with 10 ml of ice-cold 95% ethanol. Filters were transferred to a 6-ml scintillation vial, and 4 ml of scintillation fluid (Pico-fluor 30, Packard) was added to each vial. The radioactivity was determined with a Kontron Betamatic II Scintillation Counter. In pulse-labeling experiments, L-[U-^{14}C]leucine was added to 5-day-old cultures for 15, 10, or 5 min. The filtrates were concentrated by ultrafiltration and tested by fast-protein liquid chromatography (FPLC) (see below). The peaks were collected, and the radioactivity of the fractions was evaluated.

Enzyme purification and analysis. The extracellular medium was recovered by filtration through a gauze filter and concentrated by ultrafiltration (32). For further elimination of salts, 100 ml of water was added to the 10-times-concentrated supernatant, and the concentration was then continued. Samples were concentrated 20- or 200-fold and stored at −18°C.

FPLC analysis was performed on a Pharmacia LCC 500 system using a Pharmacia FPLC Mono Q anion-exchange column. Operating conditions were as described by Kirk et al. (18), except that the gradient was applied over a 20-min period at a flow rate of 0.5 ml · min^{-1}. Effluent was monitored at 405 and 280 nm.

The apparent molecular weights for the three major ligninases were determined by native polyacrylamide gel electrophoresis using the PhastSystem system (Pharmacia) with an acrylamide gradient of 8 to 25% or 10 to 15% (Pharmacia). Molecular weights were evaluated by comparison of mobilities of reference proteins from a molecular-weight calibration kit (Pharmacia). Protein isoelectric points (pI's) were obtained by isoelectric focusing with the PhastSystem and Phastgel IEF 3-9 and 4-6.5 (Pharmacia pI Calibration Kit was used in determining protein pI's). The gels were stained either by the silver staining method or by the Coomassie blue method (methods adapted for the PhastSystem by Pharmacia).

Protein concentrations were determined by the Bradford method (1) using bovine serum albumin as a standard.

Hydrogen peroxide assay. Hydrogen peroxide concentration was determined by the peroxidase-dependent oxidation of extracellular *o*-dianisidine by the method of Faison and Kirk (7), modified as follows. To 1 ml of supernatant were added 0.1 ml of an ethanolic solution containing 3.1 μmol of *o*-dianisidine and 0.5 ml of an aqueous solution containing 45.6 U (Purpurogallin units) of horseradish peroxidase. After 5 min, A_{500} values were read against a water blank. The amount of hydrogen peroxide was determined from a standard curve prepared with permanganate-titrated H_2O_2 solutions processed in the same way.

Chemicals. Cycloheximide, *o*-dianisidine (3,3'-dimethoxybenzidine), bovine serum albumin, and horseradish peroxidase (EC 1.11.1.7) with a specific activity of 10,000 U (Purpurogallin units) per 137 mg of enzyme were purchased from Sigma Chemical Co. L-[U-^{14}C]leucine (11.1 GBq/mmol) was obtained from the Commissariat à l'Energie Atomique (Saclay, France); veratryl alcohol (Fluka) was distilled and checked for purity by ^{1}H-nuclear magnetic resonance before use. Benzyl alcohol was from Prolabo, and 3,4,5-trimethoxybenzyl alcohol was obtained from Fluka.

RESULTS

Ligninase activity in non-protein-synthesizing cultures of *P. chrysosporium*. Preliminary experiments verified that protein synthesis inhibition by cycloheximide was complete less than 10 min after addition to cultures (data not shown), precluding the induction of ligninase by veratryl alcohol.

Protein turnover in glycerol-grown cultures of *P. chrysosporium* ME-446, determined after labeling of protein with L-[U-^{14}C]leucine, was near 5% · h^{-1} for both intracellular and extracellular proteins. The two methods used for stopping incorporation of label into protein gave identical results (Table 1). Veratryl alcohol (1 mM) had no effect on protein turnover.

In cycloheximide-treated cultures, the decrease of ligninase activity was higher than protein turnover (11.5% · h^{-1}) except when 1 mM veratryl alcohol was added (Fig. 1A). At the concentration of 0.2 mM, veratryl alcohol had little effect on the rate of decrease in ligninase activity. This rate was higher in cultures with glucose (Fig. 1C) than in those with glycerol (both at 1% concentration), and 2 mM veratryl alcohol was necessary for maximum protection. However, the effect of veratryl alcohol was similar in both cases (Table

TABLE 1. Turnover of intracellular and extracellular proteins in *P. chrysosporium*[a]

Cellular compartment	Addition (concn [mM])	Turnover[b] (% · h^{-1})	*n*[c]	Significance level[d]
Intracellular	Cycloheximide (0.025)	4.8 ± 0.6	9	HS
	L-Leucine (0.025)	4.2 ± 0.3	3	S
	Cycloheximide (0.025) plus veratryl alcohol (1.000)	5.0 ± 0.8	8	S
Extracellular	Cycloheximide (0.025)	4.7 ± 0.8	7	HS
	L-Leucine (0.025)	4.8 ± 0.5	4	S
	Cycloheximide (0.025) plus veratryl alcohol (1.000)	4.6 ± 0.8	8	HS

[a] Cultures were grown in nitrogen-limited medium with glycerol (see Materials and Methods). Labeling was stopped by the addition of L-leucine or cycloheximide.

[b] Slope calculated by linear regression (percent of loss of radioactivity per hour ± standard error).

[c] Number of individual determinations (each in duplicate or triplicate).

[d] Significance level of the slope according to Student's test (*t*) for a bilateral distribution: HS, the slope is highly significant at a level of 1%; S, the slope is significant at a level of 5%.

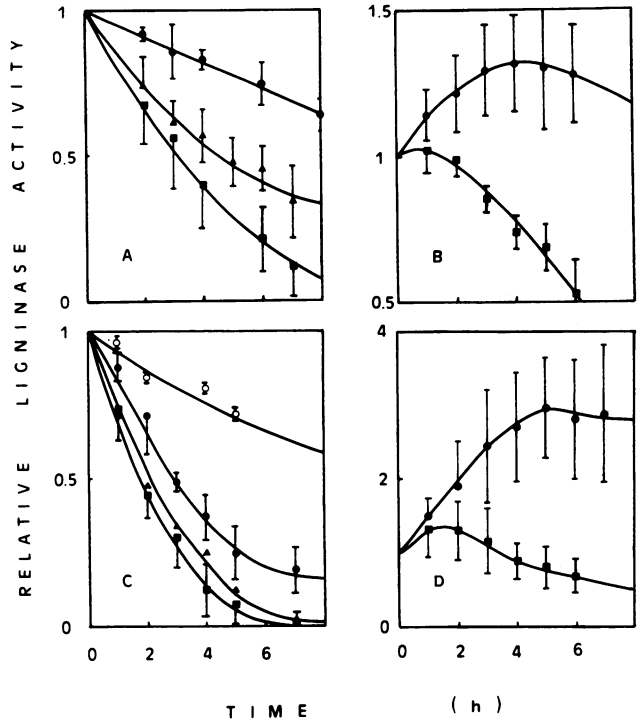

FIG. 1. Effect of veratryl alcohol supplementation on ligninase activity with glycerol (A and B) and glucose (C and D) as carbon sources. Additions to 5-day cultures (symbols): (A and C) cycloheximide plus distilled water (■), cycloheximide plus veratryl alcohol (0.2 mM final concentration) (▲), cycloheximide plus veratryl alcohol (1 mM final concentration) (●), and cycloheximide plus veratryl alcohol (2 mM final concentration) (○); (B and D) veratryl alcohol (1 mM final concentration) (●), and distilled water (■). All additions were made at time zero. The concentration in cycloheximide was 0.025 mM. Ligninase activity is expressed relative to the initial ligninase activity at time zero.

2). Experiments using supernatants of *P. chrysosporium* cultures instead of whole cultures showed an identical change of ligninase activity with and without veratryl alcohol compared with cultures in which protein synthesis was inhibited by cycloheximide (data not shown).

Aromatic compounds related to veratryl alcohol, such as benzyl alcohol (which is not a substrate for ligninase) and 3,4,5-trimethoxybenzyl alcohol, were compared with veratryl alcohol for their effect on the rate of decrease in ligninase activity in non-protein-synthesizing cultures. Such compounds were found to have no effect (the loss of ligninase activity was 12.3 ± 3.6 and 10.9 ± 0.2% · h^{-1}, respectively).

Effect of veratryl alcohol on ligninase synthesis. When veratryl alcohol was added at 1 mM to ligninase-synthesizing cultures grown on glycerol, ligninase activity increased at a higher rate than in control cultures supplemented with distilled water (Fig. 1B). In glucose-grown cultures, a significant difference was also apparent in the rate of increase with or without veratryl alcohol (Fig. 1D). Maximal ligninase activity levels when veratryl alcohol was added were 1.3 and 2.7 times more than the initial activity for glycerol and glucose media, respectively. In both cases, when water was added to cultures, the ligninase activity increased slightly and then decreased to less than the initial value.

The protective effect of veratryl alcohol might be limited in time because in both cases (glycerol or glucose medium) the ligninase activity became stable or slowly decreased 4 or 5 h after the addition.

Ligninase isoenzymes. Purification of ligninase isoenzymes from supernatants of cultures of *P. chrysosporium* on glycerol resulted in the separation of four peaks. Three of these peaks had measurable ligninase activity (peaks 1, 2, and 3). Peaks 2 and 3 corresponded to ligninases with molecular weights of 33,200 and 41,800 and pI's of 4.12 and 3.96, respectively. Peak 1 consisted of five proteins, each with a molecular weight of 39,200, and pI's of 4.43, 4.52, 4.59, 4.69, and 4.75. FPLC analysis of extracellular protein concentrates established that all the isoenzymes were sensitive to inactivation by hydrogen peroxide (data not shown). This was confirmed by an enzyme assay on concentrated dialyzed peaks (Fig. 2). This experiment showed that all peaks were sensitive to inactivation by hydrogen peroxide, but that peak 3 was protected by veratryl alcohol addition from inactivation by hydrogen peroxide.

The study of ligninase isoenzyme synthesis by pulse-labeling resulted in all three peaks being radiolabeled. The incorporation of label for each isoenzyme was essentially proportional to the relative abundance of each peak (Table 3). Supplementation with veratryl alcohol (1 mM final concentration) 30 min before pulse-labeling resulted in a reduced incorporation of L-[^{14}C]leucine (20% of control without added veratryl alcohol). In comparison with the control, a 2.1-fold increase of isoenzyme peak 3 area under the A_{405} curve was observed when veratryl alcohol was added, while the other areas, corresponding to isoenzyme peaks 1 and 2 and the hemoprotein of peak 4, were unchanged. This result was in agreement with the protective effect described.

Specific activities of the isoenzymes for the oxidation of veratryl alcohol were 56.9, 11.5, and 80.6 nkat · mg^{-1}, respectively, for peaks 1, 2, and 3. The absorption spectra of the peak 1 and 3 hemoproteins had Soret maxima at 408 nm with visible bands at 496 nm and 634 nm.

The pattern of isoenzymes when glucose was the carbon source was different from that with glycerol: peak 1 was almost nonexistent, isoenzyme peak 2 was similar, and isoenzyme peak 3 was very developed but not as much as hemoprotein peak 4, the most important in this case (Fig. 3).

Evolution of ligninase activity in relation to hydrogen peroxide. Hydrogen peroxide in cultures of *P. chrysosporium* was produced on day 2 (with cessation of primary growth) in

TABLE 2. Decrease of ligninase activity according to carbon sources in non-protein-synthesizing cultures

Carbon source[a]	Veratryl alcohol concn (mM)	Decrease of activity[b] (% · h^{-1})	n[c]	Significance level[d]
Glycerol	0	11.5 ± 2.0	7	HS
	0.2	8.8 ± 2.1	8	HS
	1	4.6 ± 0.4	9	HS
Glucose	0	21.3 ± 2.7	8	S
	0.2	20.1 ± 2.3	8	S
	1	14.6 ± 1.2	8	HS
	2	5.4 ± 1.4	5	S

[a] In nitrogen-limited medium with glycerol or glucose (see Materials and Methods). The ligninase activities in glycerol- and glucose-grown cultures were 68.3 ± 50 and 153.3 ± 40 pkat · ml^{-1}, respectively.

[b] Slope of the loss of ligninase activity calculated by linear regression (± standard error).

[c] Number of individual determinations (each with repetitions).

[d] Significance level of the slope according to Student's test (t) for a bilateral distribution: HS, the slope is highly significant at a level of 1%; S, the slope is significant at a level of 5%.

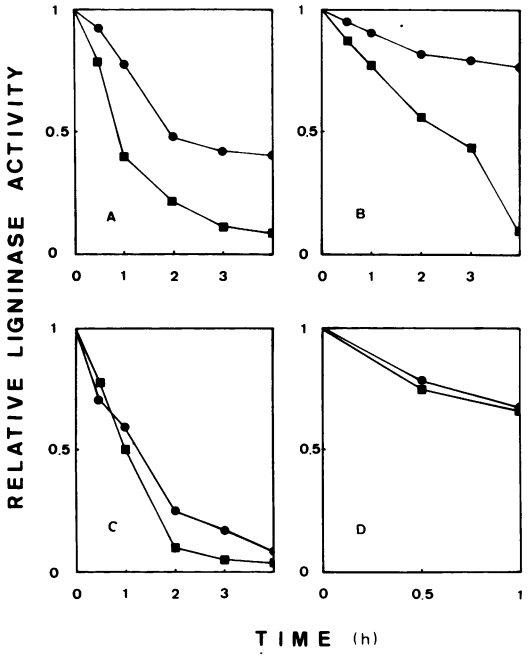

FIG. 2. Effect of veratryl alcohol supplementation on the inactivation of individual isoenzymes by hydrogen peroxide. Enzymes were purified by FPLC from 200-fold-concentrated, dialyzed supernatant. Additions to samples were (■) 0.05 mM hydrogen peroxide and (●) 0.05 mM hydrogen peroxide plus veratryl alcohol (1 mM final concentration). (A) Crude protein mixture; (B) peak 3; (C) peak 1; (D) peak 2.

glucose-supplemented cultures, or day 3 in glycerol-supplemented cultures. Hydrogen peroxide production peaked at day 5 for both carbon sources. At all incubation times, hydrogen peroxide concentration in the culture medium was two or three times higher in cultures with glucose than in cultures with glycerol (Fig. 4).

Veratryl alcohol at 1 mM did not affect the evolution of ligninase activity in concentrated-dialyzed supernatants from glycerol-grown cultures, whereas the same concentration of veratryl alcohol antagonized the rapid inactivation which occurred in mixtures to which 0.025 mM hydrogen peroxide was added (Fig. 5). When a higher concentration of hydrogen peroxide was added, the effect of 1 mM veratryl alcohol was only partial (Fig. 5).

P. chrysosporium INA-12, described by Buswell et al. (3), produced 33 nkat · ml^{-1} (M. Asther, personal communication) in optimum conditions for ligninase production. In these conditions, hydrogen peroxide concentration in cultures at day 5 (glycerol was the main carbon source) was 0.028 mM, comparable to what was observed with supernatant of strain ME446 from glucose cultures (0.024 mM).

TABLE 3. Effect of veratryl alcohol on synthesis of ligninase isoenzymes and hemoprotein peak 4 by *P. chrysosporium*

Peak	Control cultures[a]		Veratryl alcohol[b]	
	Radioactivity incorporated[c] (Bq)	Area under A_{405} peak[d]	Radioactivity incorporated[c] (Bq)	Area under A_{405} peak[d]
1	23.5 (37.2)	11.5 (21.6)	5.5 (40.1)	12.9 (13.4)
2	5.2 (8.2)	3.4 (6.4)	1.0 (7.0)	3.4 (3.6)
3	33.0 (52.3)	36.6 (68.8)	6.6 (48.6)	77.5 (80.9)
4	1.4 (2.3)	1.7 (3.1)	0.6 (4.3)	2.0 (2.1)

[a] No veratryl alcohol added.
[b] Veratryl alcohol added at 1 mM final concentration 30 min before the addition of label to 5-day-old cultures.
[c] Five-day-old cultures each received 1.67 nmol of L-[U-^{14}C]leucine (18.5 kBq in each flask); incubation proceeded for 10 min, and then incorporation of label was stopped by addition of L-leucine (0.25 mM final concentration). Fourteen labeled cultures were collected before concentration of supernatants and protein analysis (see Fig. 2).
[d] After separation by FPLC and integration; relative units.

DISCUSSION

In non-protein-synthesizing cultures of *P. chrysosporium* a sharp decrease of ligninase activity was observed. Results showed that this decrease was related to extracellular protein turnover as well as to inactivation of the enzyme. This inactivation did not take place in cultures supplemented with 1 or 2 mM veratryl alcohol, respectively, for glycerol- and glucose-grown cultures. The effect of veratryl alcohol on the ligninase activity does not involve de novo protein synthesis. Indeed, the effect of veratryl alcohol on ligninase activity

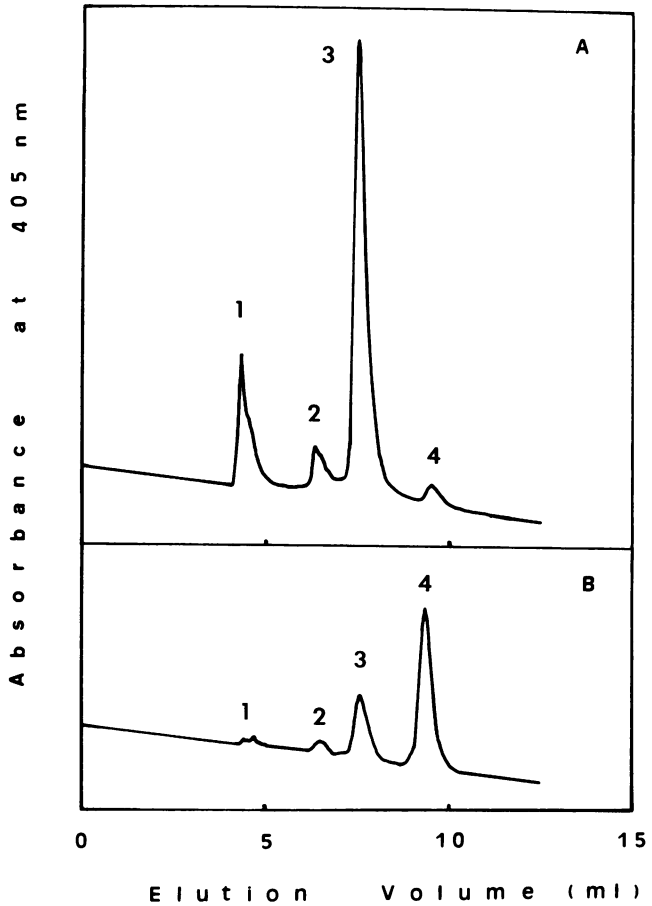

FIG. 3. FPLC profiles of extracellular fluids from 5-day cultures of *P. chrysosporium* ME446. Nitrogen-limited cultures were prepared and incubated as described in the text with glycerol (A) or glucose (B) as the carbon source. The 20-fold-concentrated enzymes were eluted at pH 6.0 with a linear sodium acetate gradient (from 10 mM to 1 M) over a 20-min period at a flow rate of 0.5 ml · min^{-1}, and A_{405} was measured.

remained the same when mycelium was removed. The 0.2 mM veratryl alcohol used by Faison et al. (9) only partially prevented the loss of ligninase activity in cultures. The carbon source used in the culture does not seem to influence intracellular protein turnover; results in this study are consistent with a previous report by Fenn and Kirk (10) in which glucose was the carbon source.

Kirk et al. (18) have shown that the optimum concentration of veratryl alcohol for increase of activity is 0.4 mM for cultures on glucose medium, whereas 2 mM was better according to our results. However, in the former study, veratryl alcohol was added at the time of inoculation and could have interfered with fungal metabolism (24), whereas in the present study the additive was added at the time of the experiment. Leisola et al. (25) reported maximal enhancement of ligninase activity for veratryl alcohol between 1 and 2 mM concentration.

Benzyl alcohol and 3,4,5-trimethoxybenzyl alcohol, which are not good substrates for the ligninase, were inefficient in preventing decrease of ligninase activity. However, the former was reported to increase ligninase activity in the presence of veratryl alcohol by Kirk et al. (20).

The evolution of ligninase activity of dialyzed concentrated supernatants in the presence of various concentrations of hydrogen peroxide establishes, in agreement with previous reports (13), that hydrogen peroxide at a physiological level causes inactivation of the ligninase. These results show that veratryl alcohol acts as an enzyme protector against inactivation by hydrogen peroxide. Hydroxyl radical $HO^{\cdot}$ and superoxide anion radical $O_2^{\cdot-}$ are known to cause protein degradation (34); both radicals can be formed from hydrogen peroxide. Also, horseradish peroxidase can be converted to an inactive form similar to Compound III (or IV?) in the presence of excess peroxide (35), and a cholesterol oxidase from *Streptomyces* sp. is rapidly inactivated in presence of produced or added hydrogen peroxide (22). The mechanism of enzyme inactivation by hydrogen peroxide remains unknown.

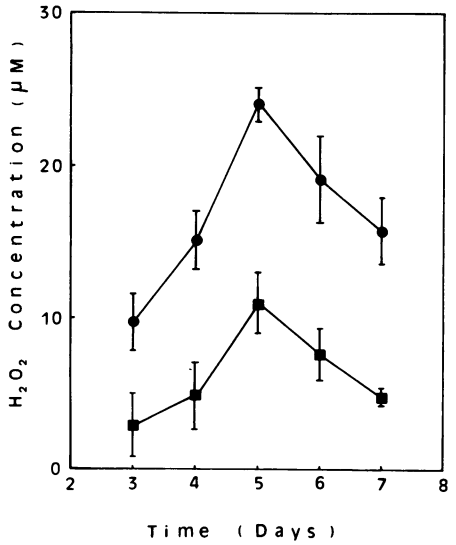

FIG. 4. Hydrogen peroxide production by *P. chrysosporium*. Kinetics of hydrogen peroxide production by whole cultures growing on nitrogen-limited medium with glycerol (■) or with glucose (●) as a carbon source were measured via the oxidation of *O*-dianisidine.

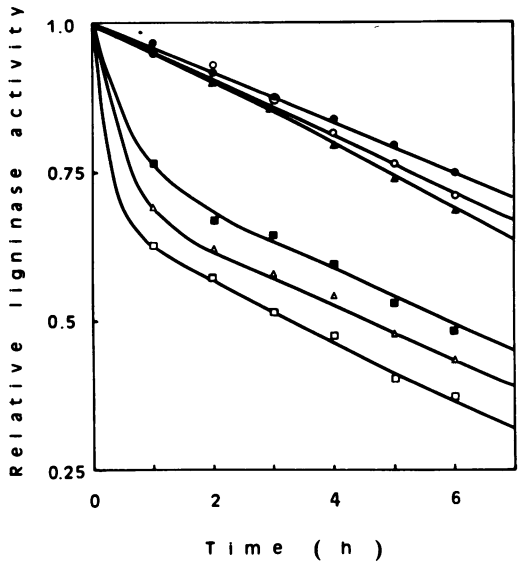

FIG. 5. Effect of hydrogen peroxide addition on concentrated supernatant. A 20-fold-concentrated supernatant from 5-day cultures grown with glycerol as the carbon source was prepared. Additions to the concentrated sample (symbols): no addition (control) (○), 1 mM veratryl alcohol (●), 0.025 mM H_2O_2 (△), 0.025 mM H_2O_2 plus 1 mM veratryl alcohol (▲), 0.05 mM H_2O_2 (□), and 0.05 mM H_2O_2 plus 1 mM veratryl alcohol (■).

The carbon source used in cultures strongly influenced the rate of hydrogen peroxide production as well as the loss of ligninase activity in non-protein-synthesizing cultures. A higher concentration of veratryl alcohol was necessary to maintain the loss of ligninase activity at the level of protein turnover when glucose was the carbon source. Thus, these differences in ligninase activity for the two carbon sources may be explained by natural hydrogen peroxide production. The mechanism responsible for the higher hydrogen peroxide production rate when glucose was the carbon substrate is unknown. Perhaps the oxidation of D-glucose by glucose 2-oxidase (6) or by glucose-1-oxidase could be responsible for extracellular hydrogen peroxide production. The importance of glucose oxidase as a major source of hydrogen peroxide production has been suggested by Forney et al. (11) and Reddy et al. (27). This production would be localized in "periplasmic microbodies" (11), and these enzymes would be more efficient when glucose is the carbon substrate. An extracellular enzyme, glyoxal oxidase, has been isolated in ligninolytic cultures of *P. chrysosporium* (15). This enzyme is able to catalyze the oxidation of simple aldehyde, α-hydroxycarbonyl, or α-dicarbonyl compounds by molecular oxygen with concomitant formation of hydrogen peroxide.

FPLC analysis showed that all three ligninase isoenzymes, as well as the hemoprotein peak 4, are degraded in the same way by hydrogen peroxide in the absence of veratryl alcohol. The evolution profile of ligninase activity of individual purified isoenzymes in the presence of hydrogen peroxide with or without veratryl alcohol establishes that only the isoenzyme of peak 3 was protected by veratryl alcohol. The increase of ligninase activity observed when protein-synthesizing cultures were supplemented with veratryl alcohol is explained by the lower degradation of isoenzyme peak 3 relative to the other isoenzymes.

In this study, results of pulse-labeling experiments indicate that the ligninase isoenzymes are synthesized simulta-

neously in cultures. For an unknown reason, incorporation of label into protein was much lower with added veratryl alcohol then that of the control. The 30-min incubation used in the experiment is insufficient for any induction to be observed. In fact, no effect of veratryl alcohol on the synthesis of individual ligninase isoenzymes was apparent. Veratryl alcohol exerted a marked effect on the amount of isoenzyme peak 3 as estimated by A_{405}. The higher amount of isoenzyme peak 3 as estimated by A_{405} is consistent with a lower degradation of the protein, in agreement with experiments with purified isoenzymes. We propose that veratryl alcohol protects only certain ligninase isoenzymes (peak 3) against the inactivation reaction by H_2O_2, whereas no protection is possible with other isoenzymes (peaks 1 and 2). This capacity of veratryl alcohol to exert a protective effect against inactivation by hydrogen peroxide is related to an unknown structural feature of this isoenzyme. The isoenzyme of peak 3 constitutes the main source of ligninase activity in cultures, which explains why a crude ligninase mixture reacted almost the same way as did peak 3 alone to the addition of hydrogen peroxide with or without veratryl alcohol.

Results of pulse-chase labeling experiments did not suggest interconversion of one isoenzyme into another of different specific activity, interconversion which could account for variations of total ligninase activity.

The addition of veratryl alcohol to protein-synthesizing cultures grown on glycerol or glucose produced in both cases an increase of ligninase activity, as noted in glucose-grown cultures by Faison et al. (9).

Differences in specific activity depending on the composition of the growth medium have been reported previously (25, 28). However, there has been no evidence until now that certain ligninase isoenzymes are synthesized preferentially in certain cultivation conditions. The differences observed in ligninase isoenzymes could instead correspond to differences in hydrogen peroxide production in cultures, resulting in different ligninase degradation rates.

It has been suggested (25) that the higher ligninase activities obtained in carbon-limited conditions are the result of a more intense synthesis of isoenzymes with higher specific activities. Preliminary results suggest that the enhanced ligninase activity obtained in cultures of *P. chrysosporium* INA-12 supplemented with certain additives (Asther, unpublished data) corresponds to the presence of enzymes of higher specific activity.

P. chrysosporium INA-12 is able to produce significantly higher amounts of ligninase with a high overall specific activity (twice or three times higher than values previously reported [25, 28]). Hydrogen peroxide production by INA-12, however, is not enhanced proportionally. Indeed, hydrogen peroxide was shown to be the limiting factor for lignin biodegradation by this strain (3). The high specific activity of ligninases produced by INA-12 is likely to be related to the lower H_2O_2 production in cultures with glycerol in proportion to the ligninase synthesis.

In conclusion, hydrogen peroxide seems to be the main factor, apart from protein turnover, determining decrease in ligninase activity through inactivation of the enzyme in cultures. Veratryl alcohol can protect ligninase, particularly the isoenzyme of peak 3, against this action, and the protective effect is dependent on the balance of hydrogen peroxide versus veratryl alcohol versus enzyme in the medium. Our results provide no evidence of induction in the fungus when veratryl alcohol is added, but more specific experiments need to be done for a valid conclusion.

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