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# Stabilization of Lignin Peroxidases in White Rot Fungi by Tryptophan

PATRICK J. COLLINS,[1] JIM A. FIELD,[2] PAULINE TEUNISSEN,[2] AND ALAN D. W. DOBSON[1]*

*Microbiology Department, University College, Cork, Ireland,[1] and Division of Industrial Microbiology, Department of Food Science, Wageningen Agricultural University, 6700 EV, Wageningen, The Netherlands[2]*

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**Supplementation of various cultures of white rot fungi with tryptophan was found to have a large stimulatory effect on lignin peroxidase activity levels. This enhancement was greater than that observed in the presence of the lignin peroxidase recycling agent veratryl alcohol. Using reverse transcription-PCR, we found that tryptophan does not act to induce lignin peroxidase expression at the level of gene transcription. Instead, the activity enhancement observed is likely to result from the protective effect of tryptophan against $H_2O_2$ inactivation. In experiments using a partially purified lignin peroxidase preparation, tryptophan and its derivative indole were determined to function in the same way as veratryl alcohol in converting compound II, an oxidized form of lignin peroxidase, to ferric enzyme, thereby completing the catalytic cycle. Furthermore, tryptophan was found to be a better substrate for lignin peroxidase than veratryl alcohol. Inclusion of either tryptophan or indole enhanced the oxidation of the azo dyes methyl orange and Eriochrome blue black. Stimulation of azo dye oxidations by veratryl alcohol has previously been shown to be due to its enzyme recycling function. Our data allow us to propose that tryptophan stabilizes lignin peroxidase by acting as a reductant for the enzyme.**

White rot fungi can degrade lignin (23, 31, 37) and a range of diverse environmental pollutants (2, 15, 17) by means of their extracellular ligninolytic systems. Among the best studied of these fungi are *Phanerochaete chrysosporium* and *Trametes versicolor*, both of which produce lignin peroxidase (LiP), an enzyme known to play an important role in this degradative ability (1, 18, 20). Purified forms of LiP have been found to directly oxidize recalcitrant xenobiotic compounds such as polycyclic aromatic hydrocarbons (19), chlorophenols (21), and azo dyes (38). LiP is a heme peroxidase which is secreted extracellularly at the onset of secondary metabolism, triggered by nitrogen limitation, in *P. chrysosporium* (43), but it can also be produced in nitrogen-sufficient conditions by other white rot fungi (9, 28). Along with LiP, the white rot fungal lignin-degrading system is composed of manganese-dependent peroxidases, laccases, $H_2O_2$-generating enzymes, veratryl alcohol (VA), and manganese (31, 37).

A number of aromatic LiP substrates and degradation products of lignin, such as vanillic acid, chlorogenic acid, veratric acid, and VA, have been tested for their abilities to enhance ligninolytic activity (33). The most effective stimulant was found to be VA, a secondary metabolite produced by ligninolytic cultures of white rot fungi (11, 34). VA plays an important role in LiP catalysis. LiP is oxidized by $H_2O_2$ to form a two-electron intermediate, compound I (42), which oxidizes substrates by one electron, forming the more reduced enzyme intermediate, compound II. Compound II can then oxidize substrates by one electron, returning the enzyme to the ferric state (35, 49). However, compound II has a very high reactivity with $H_2O_2$ (50, 51); therefore, in the presence of a poor substrate and excess $H_2O_2$, it is instead converted to an inactive form of the enzyme, compound III (35). VA, when present, is a more favorable substrate for compound II (40, 42) and functions to convert it to the resting enzyme, completing the catalytic cycle (32). It therefore recycles the enzyme and concomitantly prevents its inactivation by excess $H_2O_2$ (35, 49).

An additional role for VA, as a diffusible mediator in the LiP-catalyzed oxidation of lignin, had been proposed (8, 22). This suggestion has been questioned, however, on the basis that the VA cation radical ($VA^{\cdot +}$) is too short-lived to diffuse any distance from the catalytic center of the enzyme (27, 30), but it may act as an enzyme-bound species in the oxidation of LiP substrates (29). Considering this $VA^{\cdot +}$ instability, it is possible that white rot fungi produce alternative aromatic LiP substrates which can be oxidized to more stable cation radicals to function as diffusible oxidizing species.

In this work, we have identified L-tryptophan (TRP), an aromatic amino acid, as a substrate for LiP oxidation. We demonstrate that addition of TRP to the growth media of different white rot fungi results in large increases in LiP activity. Two possibilities for the stimulating effect of TRP on LiP activities were considered: direct induction of *lip* gene expression and protection of LiP against inactivation by $H_2O_2$. We have concluded that the presence of TRP, at micromolar concentrations, has a protective effect against inactivation of LiP by excesses of $H_2O_2$. Inclusion of TRP or its derivative indole prevents compound III formation and allows completion of the LiP catalytic cycle. In addition, we show that both TRP and indole enhance the oxidation by LiP of the azo dyes methyl orange and Eriochrome blue black in the same way as VA does.

## MATERIALS AND METHODS

**Chemicals.** Methyl orange (acid orange 52) and $H_2O_2$ were purchased from BDH (Poole, United Kingdom). All other chemicals, including Eriochrome blue black (mordant black 17), were purchased from Sigma-Aldrich (Dorset, United Kingdom). $H_2O_2$ solutions were prepared daily.

**Organisms and media.** *T. versicolor* 290 (9), *P. chrysosporium* (ATCC 32629), and *Chrysosporium lignorum* PM1 (36) have previously been described. *T. versicolor* CU2 was isolated from decaying ash wood in County Cork, Ireland. Cultures were maintained at 4°C on glucose-malt extract slants (5 g of glucose, 3.5 g of malt extract, and 15 g of agar per liter). Experimental cultures of *T. versicolor* strains were grown in the nitrogen-rich (5 g of ammonium tartrate $liter^{-1}$) basal medium previously described (10), without the addition of 2,5-xylidine. A nitro-

* Corresponding author. Mailing address: Microbiology Department, University College, Cork, Ireland. Phone: 353-21-902743. Fax: 353-21-903101. E-mail: a.dobson@ucc.ie.

gen-limited (0.1 g of ammonium tartrate liter$^{-1}$) medium, modified from BIII medium (44) (Mn free, 10 g of glucose liter$^{-1}$, in 20 mM 2,2-dimethylsuccinate [pH 5.0] buffer), was used to culture *P. chrysosporium* and *C. lignorum*. Addition of TRP to cultures was to a final concentration of 2 mM except for the experiment which examined the effect on LiP activity in *T. versicolor* 290 cultures of TRP or VA additions at various concentrations (0.5, 1, 2, and 5 mM). TRP and VA were filter sterilized before addition to media.

**Culture conditions.** Aliquots (15 ml) of autoclaved media were transferred into presterilized 200-ml medical flat bottles (BDH) and were inoculated with two agar plugs (6 mm) from the outer circumference of a fungal colony growing (6 to 8 days for *T. versicolor* strains and 3 to 5 days for *P. chrysosporium* and *C. lignorum*) on a glucose-malt extract plate. Each bottle was loosely capped to allow passive aeration and incubated statically in darkness. The incubation temperatures were 26°C for *T. versicolor* strains and 30°C for *P. chrysosporium* and *C. lignorum.*

**LiP activity assays.** Ten-day-old cultures of the four fungi tested were assayed to determine the effect of TRP on LiP activity levels. LiP activity assays were performed on extracellular fluids which were collected by passive filtration through Miracloth (Calbiochem, Inc., La Jolla, Calif.). Low-molecular-weight supernatant components were removed by concentration in Fugisep 30 Centrifugal Concentrators (Intersep, Wokingham, United Kingdom) and washing three times with 100 mM sodium acetate buffer (pH 5.0). Enzyme samples were then resuspended in 100 μl of $H_2O$, and LiP activity was measured by monitoring the oxidation of VA to veratrylaldehyde at 310 nm ($\varepsilon_{310}$ = 9,300 $M^{-1}$ $cm^{-1}$) (44). Assay mixtures contained 50 mM sodium tartrate (pH 3.0), 2 mM VA, and 0.4 mM $H_2O_2$ in a total volume of 1 ml. One unit of LiP activity was defined as the amount of enzyme required to oxidize 1 μmol of VA per min. Datum points in all cases represent averages for triplicate cultures, with standard deviations represented by error bars.

**RNA preparation and reverse transcription (RT)-PCR amplification.** RNA was prepared from cultures of *T. versicolor* 290 grown in the presence of either 0, 0.5, 1, 2, or 5 mM TRP. Mycelium from triplicate cultures was combined, filtered through Miracloth, washed twice with distilled water, quick-frozen in liquid nitrogen, and ground to a powder by using a mortar and pestle. Total RNA was extracted by the method of Gromroff et al. (16). Poly(A)$^+$ mRNA was prepared from this material by using an Oligotex mRNA isolation kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's protocol and quantitated spectrophotometrically.

RT reaction mixtures contained 50 ng of poly(A)$^+$ mRNA, 40 ng of random hexamer primers (Boehringer-Mannheim, GmbH, Mannheim, Germany), 0.5 mM each deoxynucleoside triphosphate, 2 μg of bovine serum albumin, 1× RT buffer (Promega, Madison, Wis.), 200 U of Moloney murine leukemia virus reverse transcriptase (Promega), and 40 U of RNasin RNase inhibitor (Promega). Reaction volumes were adjusted to 20 μl with water. Reaction mixtures were incubated at 37°C for 1 h, and reactions were terminated by heating to 65°C for 10 min.

Forward (5′-CGACGCIATCGCCATCTC-3′; I represents inosine) and reverse (5′-GAACGGCTCGGG[G/C]ACGAG-3′) PCR primers were based on conserved regions between previously described *lip* sequences from *T. versicolor* (3, 24–26). These primers should be isozyme nonspecific, allowing amplification of all LiP-encoding *T. versicolor* sequences. The predicted size for the cDNA amplification product was 304 bp. For PCR amplification, a 2-μl volume from each RT reaction was mixed with 75 ng of each primer, 5 μl of 10× KCl *Taq* buffer (Bioline, London, United Kingdom), 100 μM each deoxynucleoside triphosphate, and 1.25 U of *Taq* polymerase. Reaction volumes were adjusted to 50 μl with water. Amplification was performed in a DNA thermal cycler (Omnigene; Hybaid), using 30 cycles of denaturation (1 min at 94°C), annealing (1 min at 63°C), and extension (1 min at 72°C). A 10-μl aliquot from each reaction was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The *lip* PCR product was sequenced to confirm its identity.

**LiP preparations.** A crude LiP concentrate was prepared from the extracellular fluid of *T. versicolor* 290 cultures grown in the presence of 2 mM TRP. Concentration was by ultrafiltration through an Amicon YM10 membrane. The concentrate was washed five times, each time with 10× volumes of 50 mM potassium phosphate (pH 5.0). The partially purified LiP preparation from *P. chrysosporium* was obtained from Tienzyme, Inc. (State College, Pa.).

**LiP inactivation assays.** Assays were performed to determine the protective effect of TRP against LiP inactivation by high concentrations of $H_2O_2$. Assay mixtures were composed of the following: TRP (diluted to 0.025, 0.05, or 0.1 mM), LiP (initial activities of ca. 100 U liter$^{-1}$ for both crude and partially purified preparations), $H_2O_2$ (final concentrations of 0.1 and 0.02 mM in assay mixtures containing crude and partially purified LiP preparations, respectively), and 100 mM sodium acetate buffer (pH 5.0). Assay volumes were adjusted to 1 ml with distilled $H_2O$. After 0-, 15-, 30-, 45-, and 60-min periods of incubation at room temperature, 100-μl samples were removed from assay mixtures and their VA oxidizing activities were measured as described above. All data are expressed as percentages of the initial LiP activities remaining. Values represent the means of triplicate measurements, with standard deviations represented by error bars.

**Oxidation of VA in the presence of TRP.** The effect of TRP on the oxidation of VA by LiP was determined in reactions performed with 0.07 U of partially purified LiP, 1 mM VA, 0.4 mM $H_2O_2$, and 50 mM sodium tartrate (pH 3.0), with or without the addition of 1 mM TRP. The reaction volume was 1 ml. VA oxidation was determined by measuring the formation of veratrylaldehyde at 310 nm ($\varepsilon_{310}$ = 9,300), and results are presented as the micromolar concentration of veratrylaldehyde formed per minute.

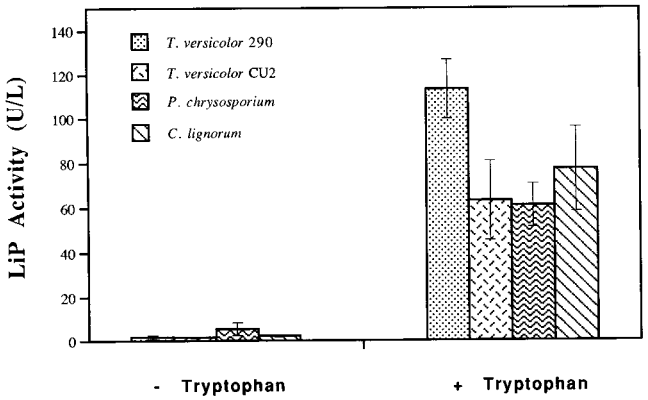


FIG. 1. Effects of supplementation with 2 mM TRP on LiP activity levels in cultures of four white rot fungi.

**Catalytic cycle analysis.** The catalytic cycle of LiP was monitored by using wavelength scans in the Soret region of the spectrum (46, 50, 51). Reaction mixtures contained 0.1 U of partially purified LiP, 0.1 mM $H_2O_2$, 100 mM sodium acetate buffer (pH 5.0), and either water, 2 mM VA, 2 mM TRP, or 2 mM indole. The final reaction volume was 1 ml. Scans were performed after 0, 1, 2, 4, and 8 min of incubation, using a Beckman (Fullerton, Calif.) DU600 spectrophotometer.

**Oxidation of azo dyes.** In experiments to determine the effect of VA, TRP, or indole on the oxidation of the azo dyes methyl orange and Eriochrome blue black, assay mixtures contained 15 μg of methyl orange or 45 μg of Eriochrome blue black, 0.07 U of partially purified LiP, 0.4 mM $H_2O_2$, 50 mM sodium tartrate (pH 3.0), and the aromatic compounds at a concentration of 2 mM or 400 μM for the oxidation of methyl orange or Eriochrome blue black, respectively. The reaction volume was 1 ml. Oxidation of methyl orange and Eriochrome blue black was measured by monitoring the decrease at their absorption maxima, i.e., 500 and 515 nm, respectively. Data are expressed as the percentage of the initial amount of each dye oxidized after 1 min of incubation.

## RESULTS

**Stimulation of LiP activity in fungal cultures.** The effects of addition of 2 mM TRP to cultures of four white rot fungi are illustrated in Fig. 1. In the absence of TRP, LiP activity levels in supernatants of both *T. versicolor* 290 and *T. versicolor* CU2 were very low (1.2 and 0.6 U liter$^{-1}$, respectively). The inclusion of 2 mM TRP resulted in increases in enzyme activity levels of 63-fold in *T. versicolor* 290 cultures and 40-fold in *T. versicolor* CU2 cultures. Similarly, for cultures of *P. chrysosporium* and *C. lignorum*, the presence of 2 mM TRP resulted in LiP activity increases of 11- and 34-fold, respectively.

Of the four fungi tested, the highest level of LiP stimulation by TRP was observed for *T. versicolor* 290. This fungus was used in an experiment to determine the effect of culture supplementation with various concentrations of TRP or VA on LiP activity levels (Fig. 2). A direct relationship was found to exist between LiP activity and the concentration of either TRP or VA provided in the culture medium. Furthermore, TRP proved to have a greater stimulatory effect than VA did.

**RT-PCR amplification.** To determine if the stimulating effect of TRP on LiP activity was due to induction at the level of gene transcription, RT-PCR amplification was used to compare *lip* mRNA transcript levels in *T. versicolor* 290 cultures grown in the presence of various TRP concentrations (Fig. 3). TRP did not induce LiP expression at the level of gene transcription; in fact, increased TRP concentrations in the culture medium corresponded to decreases in the level of *lip* mRNA detected. In cultures containing 5 mM TRP, a low level of *lip*

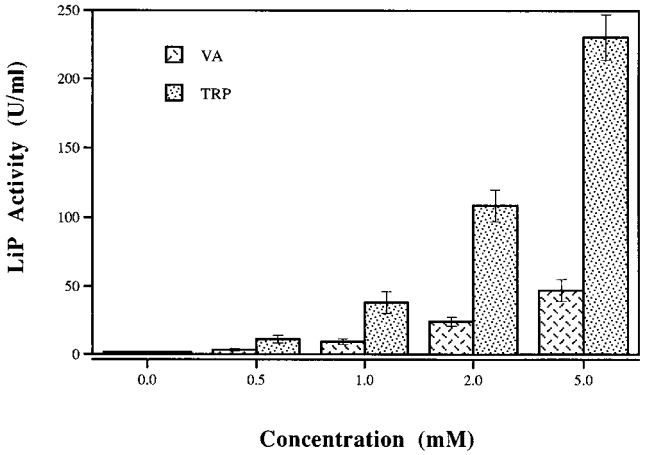


FIG. 2. Effects of supplementation with various concentrations of VA or TRP on LiP activities in *T. versicolor* 290 cultures.

transcript was present. This evidence indicates that the presence of TRP has a repressive effect on *lip* gene transcription.

**Protection against $H_2O_2$ inactivation of LiP.** TRP protects LiP from inactivation by high concentrations of $H_2O_2$ (Fig. 4). In reaction mixtures containing no TRP, LiP is rapidly inactivated, with no detectable activity remaining after 30 min of incubation. When TRP at concentrations as low as 25 μM is included, a protective effect on LiP activity can be observed, with some activity still remaining after the 60-min incubation period. As the concentration of TRP added to the reaction mixture is increased, its protective effect on LiP also increases. In the presence of 0.1 mM TRP, the highest concentration tested, no decrease in enzyme activity occurred during the assay period.

The data presented in Fig. 5 show that in reaction mixtures containing 0.1 mM $H_2O_2$ and a crude LiP preparation from *T. versicolor*, TRP has a greater protective effect on LiP activity than VA. When 0.1 mM VA is present, a steady decline in the amount of LiP activity is observed. In contrast, when TRP is substituted for the VA, no enzyme inactivation occurs. This result suggests that TRP is a more effective reductant for LiP than VA. TRP also effectively protects a partially purified form of *P. chrysosporium* LiP from inactivation by $H_2O_2$ (Fig. 6). In the presence of 0.1 mM TRP, no LiP inactivation occurs, but when no TRP is included, a steady decline in LiP activity during the 60-min assay period is observed.

**Inhibition of VA oxidation.** TRP was found to inhibit the oxidation of VA by LiP. In reactions performed with no TRP, VA was oxidized to 142.7 ± 5.3 μM veratrylaldehyde $min^{-1}$. However, in reactions performed with equal concentrations of VA and TRP, only 8.94 ± 0.56 μM veratrylaldehyde $min^{-1}$ was formed. This result indicates that TRP is a more reactive substrate than VA for LiP and inhibits its oxidation.

**Completion of the LiP catalytic cycle.** TRP functions in the same way as VA to convert compound II to ferric enzyme and prevent formation of inactive compound III (Fig. 7). This was

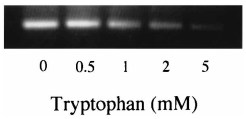


FIG. 3. RT-PCR detection of *lip* mRNA transcript levels in *T. versicolor* 290 cultures grown in the presence of various concentrations of TRP.

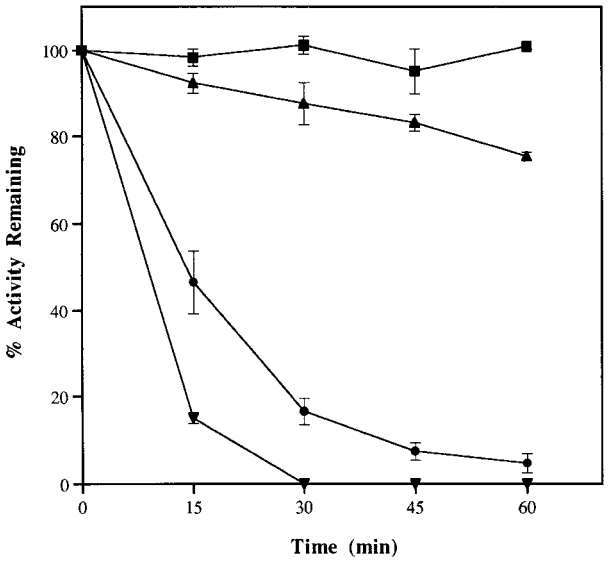


FIG. 4. Protective effects of 0 mM (▼), 0.025 mM (●), 0.05 mM (▲), and 0.1 mM (■) TRP concentrations against inactivation of a crude LiP preparation by 0.1 mM $H_2O_2$.

determined by examination of the heme moiety spectra in the Soret region when the enzyme was incubated with $H_2O_2$ alone or in the presence of VA or TRP. When neither VA nor TRP is included (Fig. 7A), ferric enzyme ($\lambda_{max}$ = 408 nm) is converted to compound III ($\lambda_{max}$ = 418 nm) after 1 min of incubation. $H_2O_2$ is in excess in the mixture, and no reductants are present; therefore, oxidation of compound III to compound III*, an irreversibly inactive form of compound III (50), would have occurred (49, 50). This would have been followed by bleaching of the heme, which is evident in Fig. 7A as the decreased absorbance at 418 nm observed after 4 and 8 min of incubation. In the reaction performed with VA, the heme peak is also shifted to 418 nm after 1 min (Fig. 7B). This peak represents compound II ($\lambda_{max}$ = 418 nm), which reacts with

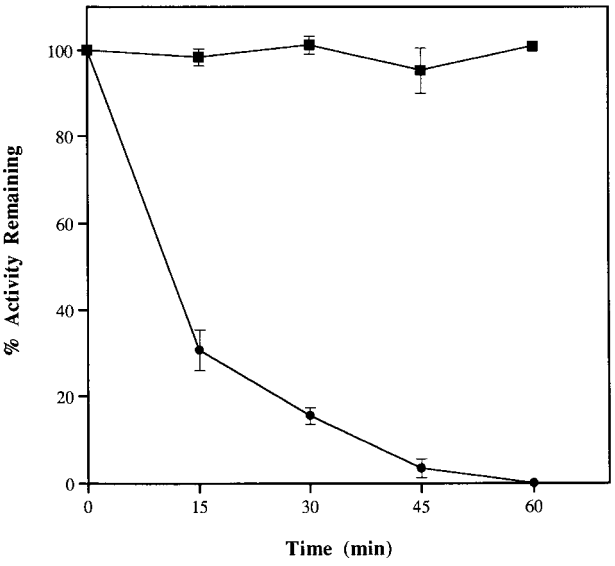


FIG. 5. Protective effects of 0.1 mM VA (●) and 0.1 mM TRP (■) against inactivation of a crude LiP preparation by 0.1 mM $H_2O_2$.

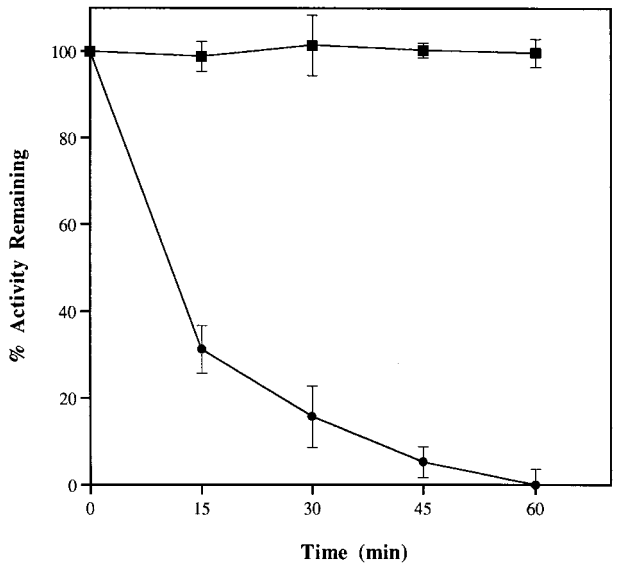


FIG. 6. Protective effects of 0 mM (●) and 0.1 mM (■) TRP against inactivation of a partially purified LiP preparation by 0.02 mM $H_2O_2$.

the VA and is converted back to the native enzyme within 4 min. After 8 min, the native heme peak has almost reverted to its original height. When TRP is included in place of VA in the reaction (Fig. 7C), a similar pattern can be seen. TRP causes reversion of the heme peak to ferric enzyme (408 nm) during the 8-min incubation period. Compounds II and III both maximally absorb at 418 nm in the Soret region, and so in order to distinguish between them, their visible spectra were examined. The compound II species obtained after 1 min of incubation with VA or TRP showed the expected visible peaks at 523 and 552 nm (49). By contrast, the compound III species obtained in the absence of an electron donor showed visible peaks at 543 and 577 nm.

To determine whether the indolic nucleus of TRP was responsible for reduction of compound II to ferric enzyme, indole was added in place of TRP to a reaction mixture. In the presence of indole, compound II also reverted to resting enzyme (data not shown), indicating that electron loss from TRP as a result of compound II reduction is from the aromatic component of the compound. Additional evidence that both TRP and indole are oxidized by LiP comes from the fact that as reactions in which they are present progress, increasing levels of background absorbance can be observed, which raise the levels of the complete spectra (as seen in Fig. 7C when TRP is present). These background absorbances correspond to the development in these reactions of a yellow color which is probably indicative of dimeric oxidation product accumulation.

**Enhancement of LiP-catalyzed oxidation of azo dyes.** Inclusion of either TRP or indole into reaction mixtures containing LiP enhances the oxidation of the azo dyes methyl orange and Eriochrome blue black (Table 1). It has previously been reported that VA is necessary for the efficient oxidation of azo dyes (38) by LiP, due to its function in completing the LiP catalytic cycle. The fact that TRP and indole also enhance oxidation of these dyes confirms that they too can have a function in the recycling of LiP.

## DISCUSSION

LiP is generally regarded as an important enzyme involved in the oxidative depolymerization of lignin by white rot fungi. In addition, LiP is capable of oxidizing a variety of xenobiotic compounds, including polycyclic aromatic hydrocarbons, polychlorinated phenols, nitroaromatics, and azo dyes (17). In the absence of a reducing equivalent, such as the secondary metabolite VA, compound II, the one-electron oxidized form of the enzyme, reacts with $H_2O_2$, forming the inactive compound III (35, 51). When excess $H_2O_2$ is present, compound III reacts further with it, leading to the formation of an irreversibly inactive species, compound III* (49). VA is a more favorable substrate for compound II than $H_2O_2$ (40, 42), and in its presence, their reaction functions to convert the enzyme back to its native state (32, 35, 49). This means that VA can provide LiP with protection from inactivation by excess $H_2O_2$. In this report, we provide evidence which indicates that the aromatic amino acid TRP can function similarly to VA, in allowing the completion of the LiP catalytic cycle while, as a secondary function, preventing $H_2O_2$ inactivation of the enzyme.

TRP is a common aromatic amino acid which is produced de novo by basidiomycetes. In addition to being involved in protein synthesis, it functions as a precursor in the synthesis of a large number of N-substituted aromatic secondary metabolites of fungi (47). Eggert et al. (13) have recently identified 3-hydroxy-2-aminobenzoate, a TRP-derived metabolite, as a mediator in laccase-catalyzed oxidation reactions in the white rot fungus *Pycnoporus cinnabarinus*. Laccases have also been found to be reactive with the TRP derivative 4-hydroxyindole (5, 14). Furthermore, direct oxidation of N-substituted aromatic compounds by the white rot fungal enzyme manganese-dependent peroxidase (48) and by horseradish peroxidase (7) has been demonstrated. It is perhaps not surprising, therefore, that TRP can act as a substrate for another peroxidase, LiP.

The addition of TRP to cultures of four white rot fungi resulted in increased LiP activity levels (Fig. 1). This increase was significant in each case, ranging between 10- and 100-fold in activity enhancement. Thus, like VA, TRP acts across a range of white rot fungi as a stimulant of LiP activity. This stimulation was found not to be due to induction of *lip* gene expression at the level of gene transcription (Fig. 3). Indeed, the presence of TRP in cultures of *T. versicolor* 290 appears to have a repressive effect on *lip* mRNA levels. A similar effect has previously been observed for VA (6), whereby its presence in cultures of *P. chrysosporium* resulted in a decreased level of *lip* transcripts.

The LiP activity enhancement observed in *T. versicolor* 290 cultures containing TRP is likely to be due to protection by TRP of the enzyme from inactivation by $H_2O_2$. VA has previously been observed to have the same protective effect on LiP activity (6, 45). The correlation between the TRP concentration present in reaction mixtures and the degree of protection observed against $H_2O_2$ inactivation of a crude LiP preparation (Fig. 4) clearly illustrates the protective effect provided by TRP in this in vitro system. Evidence that the mechanism by which TRP protects LiP from $H_2O_2$ inactivation is similar to the mechanism involving VA comes from examination of the oxidation states of the enzyme (Fig. 7). When TRP is present, it causes reversion of compound II to the native enzyme (Fig. 7C). This is the same effect as seen when VA is present, as shown in this study (Fig. 7B) and as previously reported (35, 49). The presence of either TRP or VA as a reductant prevents compound III* formation and bleaching of the heme as observed in reactions when no reductant was present (Fig. 7A). Further proof that the mechanisms by which TRP and VA react with LiP are similar comes from the observation that TRP enhances the oxidation of azo dyes by LiP, in the same way that VA does (Table 1). Oxidation enhancement of these dyes by VA has previously been suggested to be due to its enzyme recycling role (38). All of this evidence indicates that

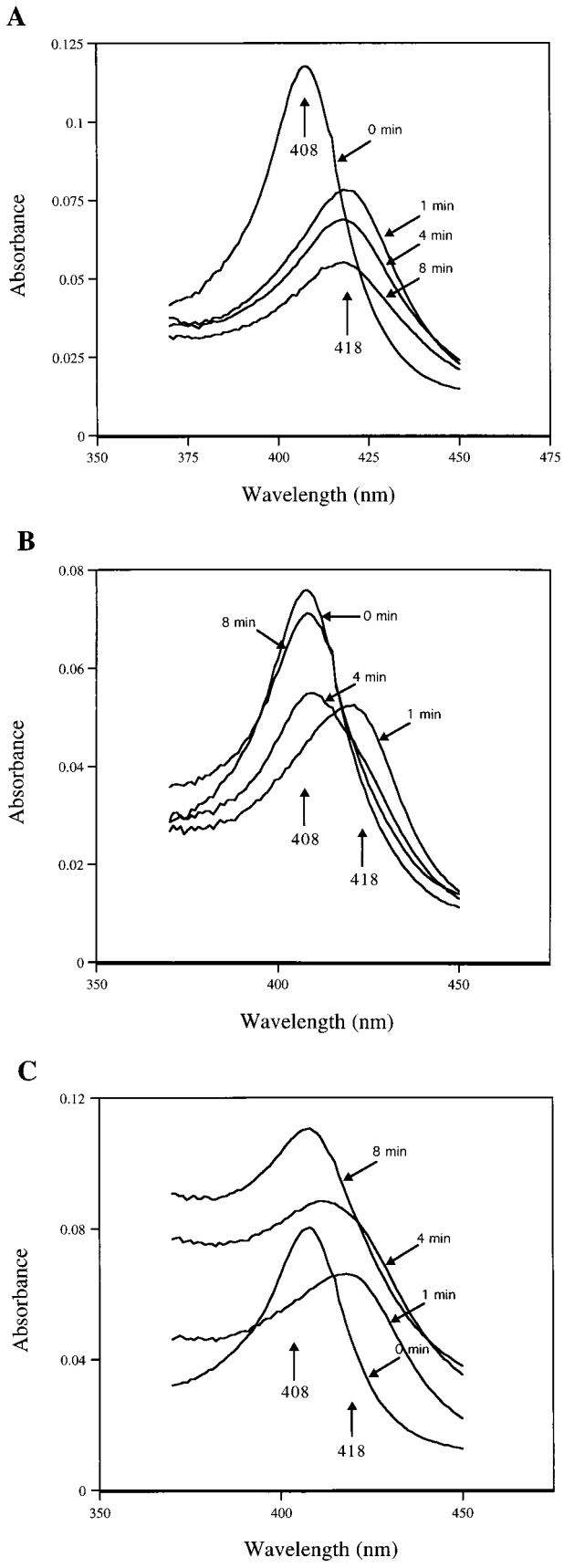


FIG. 7. Absorption spectra in the Soret region of a partially purified lignin peroxidase preparation when the enzyme was incubated with 0.1 mM $H_2O_2$ in the presence of water (A), 2 mM VA (B), or 2 mM TRP (C).

TABLE 1. Oxidation of azo dyes by LiP

| Addition | % of initial concn oxidized after 1 min (mean ± SEM [$n$ = 3]) | |
|---|---|---|
| | Methyl orange | Eriochrome blue black |
| Water | 4.55 ± 0.36 | 3.56 ± 0.61 |
| VA | 34.66 ± 1.67 | 25.47 ± 1.20 |
| TRP | 45.03 ± 1.89 | 38.24 ± 0.95 |
| Indole | 43.27 ± 3.36 | 40.20 ± 1.89 |

TRP functions in the same way as VA in providing a substrate for the conversion of compound II to resting enzyme.

To determine whether the indolic nucleus of TRP was responsible for its ability to act as a LiP substrate, we tested indole for its ability to react with compound II. The inclusion of indole resulted in reversion of compound II to the native enzyme in the same way as TRP did. Furthermore, the presence of indole enhanced the oxidation of azo dyes by LiP (Table 1). The degree of oxidation enhancement in the presence of indole was similar to that observed when TRP was present. This evidence indicates that the oxidation of TRP by LiP takes place on the indole moiety. Previous findings suggest that the point of electron loss could be the indole nitrogen (4).

The data presented in this study indicate that TRP not only acts as an alternative to VA as a compound II substrate but may be more reactive with compound II than VA is. First, supplementation of *T. versicolor* 290 cultures with TRP resulted in higher LiP activity levels than supplementation with an equal concentration of VA (Fig. 2). Second, TRP provided a much greater protective effect against $H_2O_2$ inactivation of the crude LiP preparation (Fig. 5). Third, inclusion of TRP gave rise to a greater degree of stimulation in the oxidation of azo dyes than VA did (Table 1). Finally, the oxidation of VA by LiP was inhibited by TRP. Although the kinetics of the reaction between compound II and TRP have not been studied, TRP would be a more thermodynamically favorable substrate than VA for the enzyme. The redox potential of the VA-$VA^{\cdot +}$ couple has been determined to be 1.28 V (30), whereas the corresponding potentials of indolic compounds are lower (4, 7); i.e., electron removal from indolic compounds would require less energy. TRP therefore exhibits a higher reactivity with LiP, perhaps providing an explanation for its greater ability to protect the enzyme from inactivation and enhance the oxidation of azo dyes.

Harvey et al. (22) were the first to propose an additional role for VA—that of a diffusible oxidation mediator—as a mechanism to explain the oxidation of lignin by LiP. As a result of the large size of the LiP molecule (8, 41) and the positioning of the heme moiety within it (12, 39), direct interaction between the enzyme and its insoluble polymeric lignin substrate is improbable. However, it has more recently been shown that although compound I oxidizes VA to $VA^{\cdot +}$, this radical is too short-lived to act as a diffusible oxidant (27, 30). We can speculate that white rot fungi may produce alternative LiP substrates which can be oxidized to stable cation radicals to function as oxidants in lignin degradation. TRP and its indolic derivatives may be one such set of compounds. These compounds have lower redox potential values than VA (4, 7), which would

indicate a greater kinetic stability for their cation radicals relative to $VA^{\cdot +}$.

In conclusion, this work demonstrates that TRP can function as a reductant for compound II in the same way as VA. As a secondary function, the process of enzyme recycling by TRP protects the enzyme from inactivation by excess $H_2O_2$, thus providing a possible explanation for the higher LiP activity levels observed in fungal cultures. Work is now under way to determine a possible role for TRP in the LiP-catalyzed oxidation of lignin and a range of xenobiotic environmental pollutants such as chlorinated phenols and polycyclic aromatic hydrocarbons.

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