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Oxidation of Anthracene and Benzo[*a*]pyrene by Laccases from *Trametes versicolor*

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The in vitro oxidation of the two polycyclic aromatic hydrocarbons anthracene and benzo[*a*]pyrene, which have ionization potentials of ≤7.45 eV, is catalyzed by laccases from *Trametes versicolor*. Crude laccase preparations were able to oxidize both anthracene and the potent carcinogen benzo[*a*]pyrene. Oxidation of benzo[*a*]pyrene was enhanced by the addition of the cooxidant 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS), while an increased anthracene oxidizing ability was observed in the presence of the low-molecular-weight culture fluid ultrafiltrate. Two purified laccase isozymes from *T. versicolor* were found to have similar oxidative activities towards anthracene and benzo[*a*]pyrene. Oxidation of anthracene by the purified isozymes was enhanced in the presence of ABTS, while ABTS was essential for the oxidation of benzo[*a*]pyrene. In all cases anthraquinone was identified as the major end product of anthracene oxidation. These findings indicate that laccases may have a role in the oxidation of polycyclic aromatic hydrocarbons by white rot fungi.

Polycyclic aromatic hydrocarbons (PAHs) are highly toxic organic pollutants which are widely distributed in terrestrial and aquatic environments (23). They consist of benzene homologs which have two or more fused aromatic rings in a linear, angular, or clustered alignment and are mainly formed as products of the incomplete combustion of fossil fuels (15). Major sources include motor vehicle emissions, forest fires, and industrial processes (10, 22, 33). Due to their toxic effects PAHs pose a serious health risk to animals, including humans, and many, such as benzo[*a*]pyrene, are known to be highly mutagenic and carcinogenic (9, 24).

Although some low-molecular-weight PAHs, such as the tricyclic anthracene, are not carcinogenic, their oxidation mechanisms are of considerable interest as the same arrangements of fused aromatic rings are found in the more complex carcinogenic PAHs, such as benzo[*a*]pyrene and benz[*a*]anthracene. The abilities of various white rot fungi to degrade the PAH constituents of creosote and coal tar have been extensively studied (7, 26). The extracellular ligninolytic enzyme systems of these fungi have been directly linked to the degradation of these compounds. Purified forms of two major ligninolytic enzymes, lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP), have been demonstrated to be capable of directly oxidizing PAHs which have ionization potential (IP) values of ≤7.45 eV (14, 18).

Hammel et al. (17) showed that LiP, in whole cultures of *Phanerochaete chrysosporium*, catalyzes the production of anthraquinone from anthracene. The major product of the in vitro oxidation of anthracene by both LiP and MnP from *P. chrysosporium* was also anthraquinone (14). These peroxidase-mediated reactions had molar yields of anthraquinone formed per mole of anthracene eliminated of 0.35 and 0.44 for LiP and MnP, respectively (14). The requirement by these reactions of a miscible solvent concentration of at least 10% was observed. This solvent concentration is believed to have been necessary to increase anthracene solubility to levels where it was available for bioconversion. However, whole cultures and extracellular fluids of *Bjerkandera* sp. strain BOS55 have been found to oxidize anthracene in the presence of a solvent concentration of ≤1% (21).

The third major group of extracellular oxidative enzymes involved in the white rot fungal lignin degradative process are laccases. These are extracellular copper-containing polyphenol oxidases which catalyze the reduction of one dioxygen molecule to two molecules of water, simultaneously oxidizing aromatic substrates (30). Laccases are produced by the majority of lignin-degrading fungi, the best-studied being those of *Trametes versicolor*. However, the precise role of laccases in wood delignification is still not understood (30). The substrate nonspecificity of laccases has led to them being examined as agents for the biodegradation of xenobiotic compounds, and their ability to oxidize compounds such as chlorinated phenols and polyphenols as well as aromatic amines is well documented (13, 30). Recently it has been reported that, although the lignin-degrading ability of the white rot fungus *Pleurotus ostreatus* correlates closely with its laccase activity, no link between laccase activity and the PAH oxidizing ability of this fungus could be established (1). It has been demonstrated that the laccase substrate range may be extended to nonphenolic or substituted aromatics by the inclusion of a cooxidant, such as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) (2, 27, 28). ABTS, which acts as a single electron donor and activator of the enzyme (28), has been shown to mediate laccase-dependent oxidation of nonphenolic lignin subunits of kraft pulp (3).

In this work we present evidence which indicates, for the first time, that laccase has a role in PAH oxidation by white rot fungi. We demonstrate the ability of crude laccase preparations, as well as two purified isozymes, from *T. versicolor* to oxidize the nonphenolic PAHs anthracene and benzo[*a*]pyrene. The two laccase isozymes had similar reactivities with both PAHs. The oxidation of anthracene yielded, in all cases, anthraquinone as the major accumulation product.

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MATERIALS AND METHODS

Chemicals. The PAHs anthracene, benzo[*a*]pyrene, fluorene, and phenanthrene as well as anthraquinone were all obtained from Janssen Chimica (Til-

burg, The Netherlands). Glucose oxidase and ABTS were obtained from Boehringer Mannheim GmbH (Mannheim, Germany).

Laccase production. The organism used for laccase production was *T. versicolor* 290 (8). The culture medium was modified for laccase production from that of Jönsson et al. (19) to have the following composition: 10 g of glucose, 4 g of ammonium tartrate, 1.0 g of KH_2PO_4, 0.26 g of $NaH_2PO_4 \cdot H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.0066 g of $CaCl_2 \cdot H_2O$, 0.005 g of $FeSO_4 \cdot 7H_2O$, 0.0005 g of $ZnSO_4 \cdot 7H_2O$, and 0.00001 g of thiamine per liter of demineralized water. The medium was buffered at pH 5.0 by 2,2-dimethylsuccinic acid (20 mM). 2,5-Xylidine (0.3 mM) was added to the medium before inoculation to induce laccase production (11). Three plugs (6-mm diameter) from the growing edge of the mycelium on a malt extract agar plate were used to inoculate 100-ml volumes of this liquid culture medium in 5-liter Erlenmeyer flasks, which were then incubated statically in darkness at 27°C under a regime of passive aeration. After 12 days of growth, the laccase-containing extracellular culture fluid (CF) was separated from the mycelium by filtration through a layer of cotton wool; this was followed by centrifugation for 10 min at 5,000 × *g*. A crude enzyme concentrate (CE) was prepared by ultrafiltration of the CF by using an Amicon YM10 membrane and then dialyzing it against 10 mM sodium acetate, pH 5.0. The ultrafiltrate, in which all components were ≤10 kDa in size, was autoclaved at 121°C for 15 min and stored at 4°C.

Isoenzyme purification. The CE was applied to a 1-ml MonoQ column (Pharmacia, Tilburg, The Netherlands) which had been equilibrated with 10 mM potassium phosphate buffer, pH 6.0, and was eluted with a linear sodium chloride gradient which increased from 0 to 1.0 M over a 30-min period. Two major peaks of laccase activity were observed and pooled separately. Both were concentrated by ultrafiltration on an Amicon YM10 membrane and dialyzed against 10 mM sodium acetate, pH 5.0. Ammonium sulphate was added to each laccase preparation to a final concentration of 30%, and both were further purified by chromatography on a 1-ml phenyl Sepharose (Pharmacia) column. Elution was with a linear ammonium sulphate gradient which decreased from 75% to 5% over a 30-min period. The purified laccases, named laccase I and laccase II according to their order of elution from the MonoQ column, were finally dialyzed against 10 mM sodium acetate, pH 5.0, and stored at −20°C.

Enzyme assays. Laccase activities of the CF and the purified isozymes were determined by using 0.1 mM ABTS as the substrate (35). Oxidation of ABTS was monitored by determining the increase in A_{420} (ε_{420} = 36,000). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute. LiP activity was measured by the oxidation of veratryl alcohol at 310 nm (31), and MnP activity was measured by the oxidation of Mn(II) in a sodium malonate buffer at 270 nm (34).

The Bradford protein assay (5) was used to determine protein concentrations for calculating the specific enzyme activities of the purified isozymes by using bovine serum albumin as the standard.

Laccase treatment of PAHs. All oxidation treatments were performed in 2-ml reaction volumes in 30-ml serum bottles (12) containing laccase in the form of CF, CE, or purified laccase isozymes. Laccase titers in each treatment were adjusted to approximately 4 U ml^{-1} by dilution with the reaction buffer, 10 mM sodium acetate (pH 5.0). A 16-μl aliquot of acetone containing 2.5 g $liter^{-1}$ of either anthracene, benzo[*a*]pyrene, fluorene, or phenanthrene was introduced, providing final concentrations of acetone and PAH of 8 ml $liter^{-1}$ and 20 mg $liter^{-1}$, respectively. Tween 80 was added to all treatments to a final concentration of 2.5 g $liter^{-1}$ to increase PAH bioavailability. The influence of the artificial oxidation mediator, ABTS, on PAH oxidation was determined by adding it to some treatments to a final concentration of 1.0 mM. The possibility of the fungus secreting a natural oxidation mediator of low molecular weight was investigated by using either autoclaved ultrafiltrate, instead of reaction buffer, as the CE or purified isozyme diluent in some treatments. The bottles were flushed with 100% oxygen for 5 min, incubated statically in darkness at 27°C, and analyzed after 24 h of incubation. Boiled controls contained CF, CE, or purified isozymes that had been boiled vigorously for 10 min.

Extraction and analysis of PAHs. After incubation, each treatment was completely utilized for extraction. After addition of a 10-ml volume of acetonitrile, each bottle was shaken by 300 2-cm-long strokes min^{-1} for 1 h on a shaking table (Janke & Kundel, Staufen, Germany) in complete darkness. A 1-ml sample of each extract was centrifuged at 5,000 × *g* for 10 min, and the supernatant was analyzed by using high-performance liquid chromatography as previously described (12). For analysis, isocratic conditions were used with 15% water and 85% acetonitrile. The UV and visible light absorbances were monitored at 2 nm wavelength intervals from 210 to 600 nm. The wavelengths used to integrate peak areas were 252, 252, 265, 263, and 250 nm for determination of anthracene, anthraquinone, benzo[*a*]pyrene, fluorene, and phenanthrene, respectively.

Statistical methods. All PAH oxidation data presented here are the result of triplicate treatments. Datum points are represented by the mean, with standard deviation indicated by an error bar. Particular datum points were subjected to a Student *t* test to determine the significance ($P > 0.05$) of the apparent differences between them.

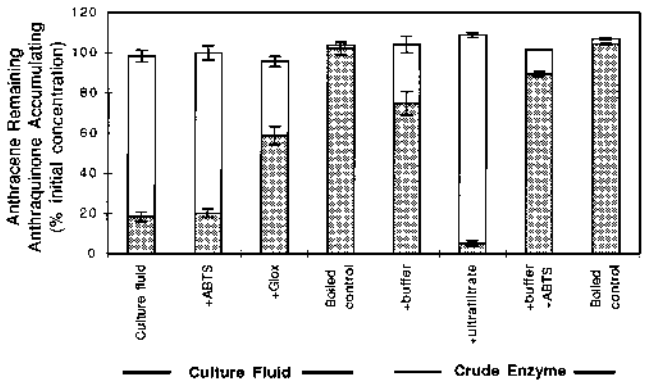

FIG. 1. Effect of various treatment additions on anthracene (shaded bars) elimination and anthraquinone (open bars) accumulation when either CF or CE was used as the laccase source. Glox, glucose and glucose oxidase addition.

RESULTS

Oxidation of anthracene and benzo[*a*]pyrene by crude laccase preparations. The crude laccase preparations (either CF or CE) for use in PAH treatments contained high laccase titers and extremely low levels of peroxidase activity (LiP and MnP activities were 1.97×10^{-3} U ml^{-1} and 5.1×10^{-4} U ml^{-1}, respectively). These crude laccase preparations catalyzed the oxidation of the PAHs anthracene (three ring) and benzo[*a*]-pyrene (five ring). The oxidation of these compounds, which both have IP values of ≤7.45 eV (29), was determined by their decreased absorbances after the 24-h incubation period. Phenanthrene and fluorene, both of which have IP values of >8.0 eV (32), were not oxidized by either CF or CE in the presence or absence of ultrafiltrate or ABTS.

The percentages of the initial anthracene concentration remaining in crude laccase treatments after the 24-h incubation period are presented in Fig. 1. Significant levels of oxidation ($P > 0.05$) occurred in treatments containing only CF and anthracene. No increase in the level of oxidation was observed following addition of the cooxidant ABTS. In the boiled control no significant degradation of anthracene occurred. When CE, diluted by addition of the reaction buffer (10 mM sodium acetate, pH 5.0), was used as the laccase source, the degree of anthracene oxidation observed was much less than that seen with CF. Addition of ABTS to the CE reaction mixture resulted in no enhancement of oxidation levels. However, when the low-molecular-weight extracellular fluid ultrafiltrate was added as the CE diluent instead of reaction buffer, a significant level of oxidation ($P > 0.05$), similar to that seen with CF, was observed. This result indicates that some factor involved in the mediation of anthracene oxidation is present in the ultrafiltrate. In all treatments the major product of laccase-mediated anthracene oxidation was identified as anthraquinone. Fig. 1 shows data for the accumulation of anthraquinone as a percentage of the initial anthracene concentration (20 mg $liter^{-1}$) supplied in each treatment. A direct relationship was observed in each case with one mole of anthracene eliminated yielding one mole of anthraquinone accumulated; i.e., the molar yield was 1.0.

It was necessary to determine whether the anthracene oxidation observed in these treatments could be attributed to the extremely low levels of peroxidase activity found in the crude laccase preparations. An H_2O_2-generating system comprising glucose oxidase (19 U $liter^{-1}$) and glucose (5 g $liter^{-1}$) was therefore added to one set of CF-anthracene treatments. This H_2O_2-generating system has previously been shown to stimu-

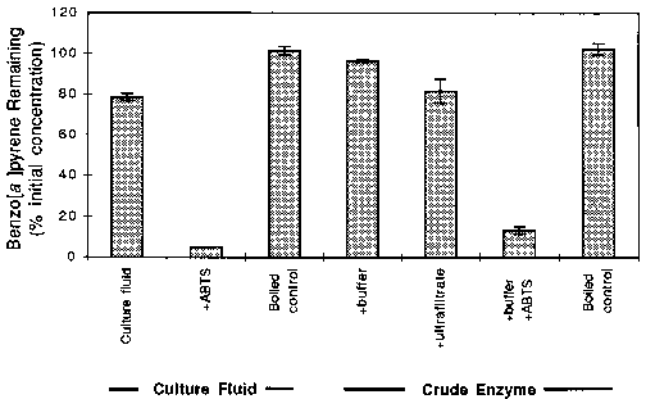

FIG. 2. Effect of various treatment additions on benzo[*a*]pyrene elimination when either CF or CE was used as the laccase source.

late anthracene oxidation by peroxidase-containing extracellular fluids of *Bjerkandera* sp. strain BOS55 (21). Instead of enhancing anthracene oxidation levels, however, addition of exogenous H_2O_2 caused a significant decrease ($P > 0.05$) in the amount of anthracene oxidized by the CF (Fig. 1). This reduction was probably due to laccase inactivation by the H_2O_2 produced, since after 24 h of incubation with this system, laccase activity was found to be decreased by 69.5% (data not shown).

The ability of the crude laccase preparations to oxidize benzo[*a*]pyrene was determined (Fig. 2). The percentage of the initial benzo[*a*]pyrene which was eliminated in treatments containing only CF and benzo[*a*]pyrene was low. A significant increase ($P > 0.05$) occurred when ABTS was added to the reaction mixture, resulting in levels of only 4.78% of the initial benzo[*a*]pyrene being detected after the 24-h incubation period. Benzo[*a*]pyrene oxidation levels in treatments containing CE, diluted to the working titer by addition of reaction buffer, were negligible. A small increase was observed when the reaction buffer was replaced with the low-molecular-weight ultrafiltrate. However, in an effect similar to that seen with CF, addition of the cooxidant ABTS significantly increased ($P > 0.05$) the level of benzo[*a*]pyrene oxidation. A number of compounds were observed to accumulate in reaction mixtures where benzo[*a*]pyrene oxidation occurred. The identity of these products, however, remains to be determined.

Purification of laccase isozymes. Two laccase isozymes were purified from a 2,5-xylidine-induced culture of *T. versicolor* 290 (Table 1) and were designated laccase I and laccase II on the basis of their elution order from ion-exchange chromatography on a MonoQ column. The specific activities of the purified laccases I and II were determined to be 225 U mg^{-1} and 214 U mg^{-1}, respectively. The molecular weights of laccases I and II were both approximately 63,000 as determined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3).

TABLE 1. Summary of laccase isozyme purification

Purification step	Total act (μmol of ABTS min^{-1})	Sp act (U mg^{-1})	Recovery (%)
Initial enzyme (CF)	4,116	343	100.0
CE	3,503	313	85.1
Mono Q column			
Laccase I	953	307	23.2
Laccase II	1,067	324	25.9
Phenyl Sepharose column			
Laccase I	510	225	12.4
Laccase II	436	214	10.6

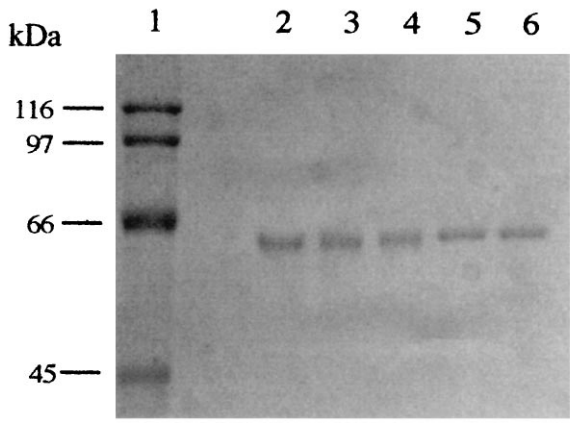

FIG. 3. SDS-PAGE of laccase samples (~2 μg per lane). Lanes: 1, molecular mass markers; 2, CE; 3, partially purified laccase I after MonoQ column; 4, purified laccase I after phenyl Sepharose column; 5, partially purified laccase II after MonoQ column; 6, purified laccase II after phenyl Sepharose column. The electrophoresis was performed on a 4 to 10% gel (Atto), and the staining was done with Coomassie blue.

Oxidation of anthracene and benzo[*a*]pyrene by laccase isozymes. The abilities of laccase I and laccase II to oxidize anthracene were determined in reaction buffer alone and in the presence of ultrafiltrate or ABTS (Fig. 4). A similar pattern of results was seen with both isozymes. A limited amount of oxidation occurred when enzyme and anthracene alone were present in the reaction. No significant increase in this level of oxidation was observed when ultrafiltrate was used instead of reaction buffer as the enzyme diluent, contrasting greatly with the effect seen when CE was used as the laccase source (Fig. 1). However the addition of ABTS to reactions containing purified isozymes had a significant stimulatory effect ($P > 0.05$) on the oxidation of anthracene. The major oxidation product of anthracene oxidation detected in these reactions was anthraquinone, as it was when CF or CE was used as the laccase source. A direct molar relationship between anthracene eliminated and anthraquinone accumulated, i.e., a molar yield of 1.0, also existed for anthracene oxidation by the purified laccase enzymes.

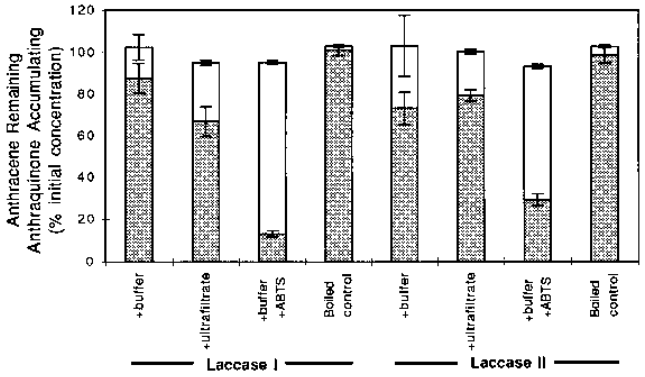

FIG. 4. Effect of various treatment additions on anthracene (shaded bars) elimination and anthraquinone (open bars) accumulation in the presence of either laccase I or laccase II.

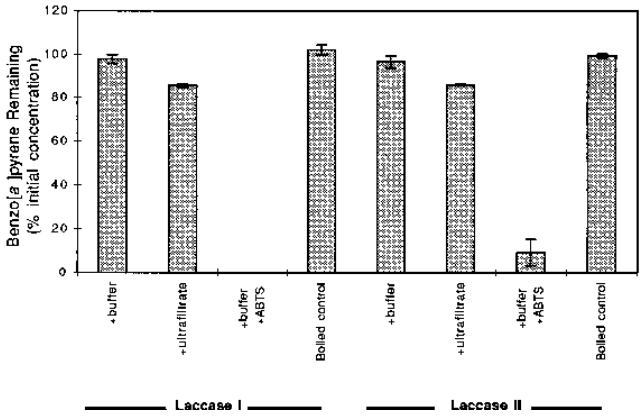

FIG. 5. Effect of various treatment additions on benzo[*a*]pyrene elimination in the presence of either laccase I or laccase II.

When benzo[*a*]pyrene was incubated with either laccase I or laccase II, in the absence of either ultrafiltrate or ABTS, no significant elimination compared with the boiled controls was observed (Fig. 5). Replacement of the reaction buffer with ultrafiltrate resulted in a slight increase in oxidation levels for both isozymes. However, addition of ABTS to reactions containing either laccase I or laccase II in reaction buffer caused a significant increase ($P > 0.05$) in the level of benzo[*a*]pyrene oxidation. No remaining benzo[*a*]pyrene could be detected in reactions containing laccase I, and only 9.2% of the initial benzo[*a*]pyrene was detected in reactions containing laccase II. Again, products of benzo[*a*]pyrene oxidation were observed in treatments where benzo[*a*]pyrene oxidation had occurred, but these products remain to be identified.

DISCUSSION

In this study we have demonstrated that laccases from *T. versicolor* can catalyze the initial oxidation step in the biotransformation of anthracene and benzo[*a*]pyrene. This catalysis appears to involve either a direct laccase oxidation mechanism or an indirect mechanism involving the participation of an oxidation mediator, such as ABTS, or a putative mediator present in the ultrafiltrate fraction. Although the oxidation of nonphenolic compounds by laccase in the presence of ABTS (2, 27, 28) or syringaldehyde (20) has been observed, this is the first report providing evidence for laccase oxidation of PAHs with direct incorporation of oxygen into the aromatic ring.

It is important to note that the PAH oxidation was shown not to be due to the action of the extremely low level of peroxidases ($\sim 2.5 \times 10^{-3}$ U ml^{-1}) present in the crude laccase preparations. This was achieved by demonstrating that the addition of an H_2O_2-generating system to the reactions containing CF did not result in increased levels of anthracene oxidation (Fig. 1). The H_2O_2-generating system used has been demonstrated to stimulate anthracene oxidation by peroxidase-containing extracellular fluids of *Bjerkandera* sp. strain BOS55 (21). Introduction of this exogenous H_2O_2 had the effect of causing a simultaneous reduction in laccase activity and in the level of anthracene oxidation, providing further evidence for the involvement of laccase in the PAH oxidation reactions.

Direct oxidation of anthracene by the two purified laccase isozymes was observed, but a marked increase in the levels of oxidation occurred when ABTS was present. In contrast, no significant direct oxidation of benzo[*a*]pyrene by the purified laccases occurred, and the presence of ABTS in the reaction mixture was essential for high levels of benzo[*a*]pyrene oxidation to occur. The mechanism by which ABTS plays a role in laccase-mediated oxidation reactions is still not completely clear. Although it has been suggested to act as a diffusible electron carrier (4), Potthast et al. (28) provide evidence that its role is that of a cooxidant, which transfers an electron to the enzyme, initiating the ability of the enzyme to accomplish electron transfer. Purified extracellular laccases from *P. ostreatus* have previously been reported to catalyse single electron transfer reactions when the lignin-related compound 3,5-dimethoxy-5-hydroxyacetophenone was used as substrate (36). This type of one electron mechanism would be similar to that previously seen to be catalyzed by LiP from *P. chrysosporium* (17) in the oxidation of anthracene to anthraquinone and of benzo[*a*]pyrene to a mixture of 1,6-, 3,6-, and 6,12-benzo[*a*]pyrene diones (16). The laccase-ABTS-mediated in vitro oxidation of anthracene observed in this study may proceed in a similar manner given that anthraquinone accumulates in the reaction medium. Thus, anthracene could be oxidized by a nucleophilic attack at either position 9 or 10, due to the high charge densities at these positions, resulting in the formation of a C-centered radical which would undergo further spontaneous nonenzymatic rearrangements to form 9,10-anthraquinone. Benzo[*a*]pyrene may be degraded by an analogous mechanism yielding quinones as reaction end products, but these have yet to be identified as products of this reaction.

Although LiP, MnP, and laccase are likely to oxidize anthracene to anthraquinone by similar mechanisms involving single electron transfer reactions, the laccase-mediated oxidation observed in this study differs in two notable aspects. Firstly, LiP and MnP from *P. chrysosporium* oxidize anthracene with the molar yields of anthraquinone formed per mole of anthracene eliminated being 0.35 and 0.44, respectively (14). This implies that a large portion of the anthracene was converted to an alternative product, either with or without anthraquinone as an intermediate. In contrast to these peroxidase-catalyzed reactions, each mole of anthracene eliminated by laccase resulted in the accumulation of a mole of anthraquinone; i.e., the molar yield was 1.0. Secondly, for the in vitro oxidation of PAHs, a concentration of at least 10% miscible solvents is required by LiP (14, 18, 32) and MnP (14). However, the laccase-catalyzed degradation of anthracene observed here occurred in a solvent concentration of 0.8%, suggesting that the level of solubility required for PAH oxidation by laccase is lower than that required by LiP and MnP.

It is apparent from the data in Fig. 1 that the low-molecular-weight ultrafiltrate fraction contains some factor(s) which plays a role in the laccase-mediated oxidation of anthracene. This was demonstrated by the fact that when ultrafiltrate was added to CE, a significant increase in the level of anthracene oxidation was observed. This potential mediator, which may act as a laccase cooxidant, is $\leq$10 kDa in size and is heat stable. Furthermore, some high-molecular-weight component of the crude laccase preparations may play a role in the activity of this putative mediator, as evidenced by the fact that the stimulatory effect of the ultrafiltrate fraction on anthracene oxidation was less marked when added to purified laccase isozyme treatments than when present in CF or CE treatments. To our knowledge, this is the first report in which a fungus has been shown to produce a natural laccase mediator.

The IP value of the PAH appears to affect its susceptibility to oxidation by laccase. In this study, anthracene and benzo[*a*]pyrene, both of which have IP values of $<$7.45 eV, could be oxidized by laccase, while fluorene and phenanthrene, which have IP values of $>$8.0 eV, remained unoxidized. Similarly, LiP has been shown to be unable to oxidize PAHs with IP values

greater than a threshold value of ~7.55 eV (18). The oxidation of PAHs with IP values of ≤7.8 eV has been demonstrated with Mn(III) (6) leading to speculation that this may be the upper limit for direct PAH oxidation by MnP. However, oxidation of phenanthrene, which has an IP value of 8.18 eV, can be accomplished by MnP via an indirect method involving a lipid peroxidation mechanism (25). The IP threshold value for laccase-mediated oxidation of PAHs therefore appears to lie within the same range as that of LiP and MnP, i.e., 7.45 to 8.0 eV.

Previous attempts to determine a possible role for laccase in the oxidation of phenanthrene and pyrene in whole cultures of *P. ostreatus* failed to find a link between laccase activity and PAH degradation and suggested that the enzyme may not have a role in PAH oxidation (1). This work demonstrates that both crude laccase enzyme preparations and purified laccase isozymes from *T. versicolor* can oxidize PAHs. The substrate range for laccase activity was previously thought not to extend beyond substituted aromatics, and while the oxidation of nonphenolic compounds by laccase in the presence of catalytic amounts of ABTS (2, 27, 28) or syringaldehyde (20) has been observed, this is the first report of laccase oxidation of the nonsubstituted aromatic structures present in PAHs. Further investigations are now under way to determine the precise mechanism of PAH oxidation by laccases and to identify the factor present in the extracellular ultrafiltrate which appears to help mediate this oxidation. White rot fungi other than *P. chrysosporium*, including those such as *P. ostreatus* and *T. versicolor* which produce high laccase titers, may now warrant further study as potentially useful strains in the biotransformation of PAHs.

ADDENDUM IN PROOF

After this work was submitted for publication, Eggert et al. (FEBS Lett. **391:**144–148, 1996) presented work which identified a laccase mediator produced by the fungus *Pycnoporus cinnabarinus.*

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