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Oxidation of Phenanthrene by a Fungal Laccase in the Presence of 1-Hydroxybenzotriazole and Unsaturated Lipids

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Phenanthrene, a polycyclic aromatic hydrocarbon, was efficiently oxidized by laccase in the presence of both 1-hydroxybenzotriazole and unsaturated lipids. 73 % of initially added phenanthrene was degraded within 182 hours to give phenanthrene-9,10-quinone and 2,2′-diphenic acid as the major products. The system was also able to peroxidize linoleic acid to its corresponding hydroperoxides suggesting the involvement of lipid peroxidation in laccase catalyzed phenanthrene oxidation. Lipid peroxidation by laccase required 1-hydroxybenzotriazole and did not depend on Mn^{2+} and H_2O_2 suggesting that the chemical reactions involved differ from those previously reported for manganese peroxidase.

White-rot fungi have evolved the nonspecific extracellular mechanisms required to degrade lignin, a major constituent of woody tissues (1). The nonspecific nature of these mechanisms is thought to enable white-rot fungi to also degrade a variety of toxic organopollutants, including PAHs (2, 3).

Lignin peroxidases, manganese peroxidases, and laccases are extracellular enzymes generally considered key components of the ligninolytic system of white-rot fungi (1). Lignin peroxidase catalyzes the oxidation of nonphenolic lignin structures to aryl cation radicals and has been shown to also oxidize several PAHs by the same mechanism (4). However, PAHs with ionization potentials above 7.55 eV such as phenanthrene are not lignin peroxidase substrates (4). Manganese peroxidase oxidizes Mn^{2+} to chelated Mn^{3+} which is a weak oxidant that oxidizes phenolic lignin structures to phenoxy radicals but is unable to degrade nonphenolic lignin structures (5). However, manganese peroxidase also promotes the peroxidation of unsaturated lipids, and under these conditions nonphenolic lignin and even phenanthrene are cooxidized (6, 7). The activity of laccase (EC 1.10.3.2), a blue copper oxidase, is also restricted to compounds with low ionization potentials such as phenols. However, it has been shown that the substrate range of laccase extends to nonphenolic lignin structures when so-called mediator compounds such as ABTS or HAA are present (8, 9). Recently, it has been demonstrated that such laccase/mediator couples also oxidize PAHs (10, 11), and that the ionization potential threshold value for the oxidation of PAHs appears to be similar to that of lignin peroxidase (10). However, the oxidation of PAHs with ionization potentials beyond the oxidative ability of lignin peroxidase such as phenanthrene by a laccase/mediator couple has not yet been reported.

Here we show that, in the presence of HBT, laccase oxidizes phenanthrene to 2,2′-diphenic acid via phenanthrene-9,10-quinone. The reaction is greatly enhanced by unsaturated lipids, most probably as a consequence of lipid peroxidation by the laccase/HBT couple.

MATERIALS AND METHODS

Organism and reagents. *Trametes hirsuta* D10 was isolated at our institute and was maintained on malt agar slants.

[9-^{14}C]phenanthrene (13.1 mCi mmol^{-1}) and [1-^{14}C]linoleic acid (58 mCi mmol^{-1}) were from Sigma and Amersham, respectively. 1-Monostearoyl-rac-glycerol (monostearin), 1-monolinoeoyl-rac-glycerol (monolinolein), Tween 80, *n*-dodecyl-*β*-D-maltoside, HBT, and ABTS were from Sigma. 2,2′-diphenic acid, phenanthrene-9,10-quinone, and linoleic acid were from Fluka. Tween 20 was from Promega. [S-(E,Z)]-9-hydroperoxy-10,12-octadecadieonic acid and [S-(E,Z)]-13-hydroperoxy-9,11-octadecadieonic acid were from Cayman Chemical, and stored at −70°C. All other chemicals were reagent grade and

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Abbreviations used: PAH, polycyclic aromatic hydrocarbon; HBT, 1-hydroxybenzotriazole; ABTS, 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); HAA, 3-hydroxyanthranilic acid.

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obtained from Merck. Unsaturated fatty acids and lipids were stored under argon at −20°C. Partially purified manganese peroxidase and lignin peroxidase were from Tienzyme.

Enzyme assays. Laccase and manganese peroxidase activities were determined by monitoring the oxidation of ABTS at 420 nm (ϵ = 43.2 cm^2 μmol^{-1}). The laccase assay contained 0.32 mM ABTS, and 50 mM sodium acetate (pH 5). The manganese peroxidase assay contained 0.32 mM ABTS, 0.26 mM $MnSO_4$, 0.1 mM H_2O_2, and 50 mM sodium malonate (pH 4.5). In crude fungal extracts, laccase interfered with the manganese peroxidase assay and oxidized ABTS in the absence of $MnSO_4$ and H_2O_2. To calculate manganese peroxidase activity, the rate obtained was subtracted from that measured in the presence of $MnSO_4$ and H_2O_2.

Lignin peroxidase activity was measured by monitoring the oxidation of veratryl alcohol at 310 nm (ϵ = 9.3 cm^2 μmol^{-1}). The assay contained 2 mM veratryl alcohol, 0.4 mM H_2O_2, and 50 mM sodium tartrate (pH 2.5).

1 U of activity was defined as the amount of enzyme that formed 1 μmol of product per min.

Laccase production and purification. Precultures of *Trametes hirsuta* were prepared by blending the mycelium from a pregrown malt agar plate in 20 ml of water and inoculating 3 ml of the suspension into 2 l Erlenmeyer flasks, containing 100 ml medium according to Roy-Arcand & Archibald (12). The flasks were incubated for 4 days at 130 rpm and 28°C. The precultures were then homogenized and 40 ml of the resulting suspension was inoculated into 2 l Erlenmeyer flasks containing 300 ml of fresh medium. The flasks were incubated as above, and laccase production was induced on day 3 by adding 200 μM 2,5-dimethylaniline. After 12 days of cultivation, the cultures were harvested and filtered through cotton wool. The filtrate was frozen, thawed, and precipitated polysaccharides were removed by centrifugation (10 min at 5000 × g). The supernatant was concentrated by ultrafiltration (10-kDa cutoff membrane, Filtron), dialyzed against 20 mM sodium acetate buffer (pH 5), and loaded on a gel permeation chromatography column (Bio-Gel P-100 Gel fine, Bio-Rad), equilibrated with 0.1 M NaCl in the same buffer. Eluted fractions containing laccase activity were pooled, concentrated by ultrafiltration, and subjected to preparative polyacrylamide gel electrophoresis (491 Prep Cell, Bio-Rad) according to Ornstein (13). Stacking and resolving gels were 8% polyacrylamide, and the electrophoresis was run at 12 W constant power with 20 mM sodium acetate (pH 5) as the elution buffer. Fractions containing laccase activity were pooled, concentrated by ultrafiltration, and dialyzed against 20 mM sodium acetate (pH 5).

Oxidation of phenanthrene. Reactions (0.6 ml) were carried out in capped 4 ml vials at room temperature, and were rotary shaken for 64 h or 182 h in the dark at 130 rpm. Water and buffer were autoclaved, and all other reagents were filtered through 0.22 μm pore size filters. Phenanthrene and fatty acid esters were added to the vials as solutions in organic solvents that were evaporated under a stream of nitrogen. The residue was then emulsified in the surfactant stock solution (10% wt/vol Tween, or 3% wt/vol *n*-dodecyl-β-D-maltoside) by Vortex mixing. Buffer and other reagents were added as aqueous solutions, and the reactions were started with the addition of enzyme. The concentrations of the ingredients were as follows. 3.6 μM phenanthrene (6×10^4 dpm), 2.5 mM Tween 80, 2.5 mM Tween 20, 6 mM *n*-dodecyl-β-D-maltoside, 4 mM monolinolein, 4 mM monostearin, 1 mM HBT, 0.4 mM $MnSO_4$, 50 mM sodium acetate (pH 5), and 1 U ml^{-1} enzyme (laccase, manganese peroxidase, or lignin peroxidase). In long-term reactions (182 h), additional 0.4 U ml^{-1} laccase, 0.3 mM HBT, and 1.25 mM Tween 80 were added after 30 h, 63 h, and 138 h incubation time.

A portion (0.1 ml) of each reaction was subjected to high-performance liquid chromatography (HPLC) on a reversed-phase column (Vydac 201TP54, 4.6 × 250 mm). HPLC was done on a Hewlett-Packard model 1100 instrument equipped with a diode array detector. The column was eluted at 1 ml min^{-1} with acetonitrile-water-H_3PO_4 (100:900:1) for 1 min, followed by a linear gradient to acetonitrile-water-H_3PO_4 (900:100:1) between 1 and 35 min. The eluate was monitored at 254 nm, and 0.9 ml fractions were collected and assayed for ^{14}C on a Wallac 1409 liquid scintillation counter.

To identify the labeled products, HPLC fractions containing **II** were pooled, acidified with formic acid, extracted with ether, dried over Na_2SO_4, and evaporated to dryness. The residue was then redissolved in methanol-water (9:1), and esterified with diazomethane in ether at room temperature. Excess diazomethane and ether were evaporated, and the dry residue was redissolved in either methanol for reanalysis by HPLC as described above, or toluene for gas chromatography-mass spectrometry (GC-MS). GC-MS was performed on a Hewlett-Packard model 5971 instrument using a HP-5 capillary column (50 m × 0.2 mm i.d. × 0.33 μm film thickness). HPLC fractions containing **III** were pooled, extracted with ether, dried over Na_2SO_4, and evaporated to dryness. The residue was then redissolved in toluene for GC-MS, or reduced with $NaBH_4$ (1 mg ml^{-1} solution in 95% ethanol) for 1 h at room temperature. Excess $NaBH_4$ was decomposed by adding formic acid, after which the sample was evaporated to dryness and redissolved in methanol for reanalysis by HPLC as described above.

Oxidation of linoleic acid. Reactions (1 ml) were carried out in capped 7 ml vials at room temperature, and were rotary shaken for 1.5 h at 130 rpm. Reagents were added to the vials as described above for phenanthrene. The concentrations of the ingredients were as follows. 0.3 mM linoleic acid (4.4×10^5 dpm), 2.5 mM Tween 20, 1 mM HBT, 0.4 mM $MnSO_4$, 0.8 mM H_2O_2, 50 mM sodium acetate (pH 5), 0.006 U ml^{-1} laccase, and 0.5 U ml^{-1} manganese peroxidase.

The reactions were terminated by adding 0.5 ml acetonitrile, and portions (0.2 ml) were analyzed by HPLC. The column was eluted at 1 ml min^{-1} with acetonitrile-water-H_3PO_4 (490:510:1) for 25 min, followed by a linear gradient to acetonitrile-H_3PO_4 (1000:1) between 25 min and 46 min. The eluate was monitored at 234 nm, and 0.5 ml fractions were collected and assayed for ^{14}C by liquid scintillation counting.

To identify the labeled products, HPLC fractions containing **VII** were pooled, acidified with tartaric acid, extracted with CH_2Cl_2, dried over Na_2SO_4, evaporated to dryness, and reduced with $NaBH_4$ as described above. The reaction mixture was extracted with CH_2Cl_2, dried over Na_2SO_4, and evaporated to dryness. The residue was then esterified with diazomethane in ether and analyzed by HPLC and GC-MS as described above.

RESULTS AND DISCUSSION

Oxidation of phenanthrene. As shown in Fig. 2A, laccase efficiently oxidized [^{14}C]phenanthrene in the presence of HBT and Tween 80. 73 % of initially added phenanthrene was degraded within 182 hours. Two major ^{14}C-labeled products, **II** and **III,** were formed that eluted identically with authentic standards of 2,2′-diphenic acid and phenanthrene-9,10-quinone, respectively, by reversed-phase HPLC. Furthermore, the UV spectra of the phenanthrene oxidation products **II** (absorption maxima at 224 and 285 nm) and **III** (absorption maxima at 264, 326 and 425 nm) were identical to those of the authentic standards. To confirm the identifications, product **II** was collected, methylated with diazomethane, and subjected to GC-MS analysis. The mass spectrum was the same as that of identically treated 2,2′-diphenic acid: *m/z* (relative intensity) 270 (M^+, 3), 239 (3), 211 (100), 196 (13), 180 (11), 168 (5),

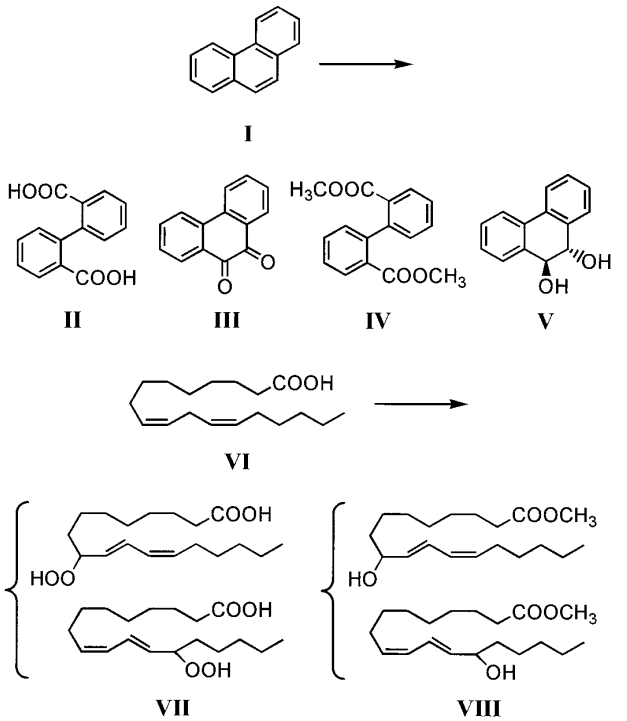

FIG. 1. Products obtained when phenanthrene and linoleic acid were oxidized by laccase/HBT, and chemical derivatization products as described in the text.

152 (10), 139 (7). Product **III** was collected and directly subjected to GC-MS. The mass spectrum was the same as that of authentic phenanthrene-9,10-quinone: *m/z* (relative intensity) 208 (M^+, 28), 180 (100), 152 (36), 126 (7), 76 (18).

Quantitative analysis was done by chemically converting the collected HPLC peaks and rechromatographing them by HPLC: **II** was methylated with diazomethane, and **III** was reduced with sodium borohydride. HPLC analysis showed that [^{14}C]**II** and [^{14}C]**III** were almost quantitatively converted to [^{14}C]**IV** and [^{14}C]**V** that coeluted with identically treated, authentic 2,2′-diphenic acid and phenanthrene-9,10-quinone, respectively (Figs 1, 2B, and 2C).

Moen and Hammel (7) proposed that phenanthrene is oxidized to 2,2′-diphenic acid via phenanthrene-9,10-quinone as an intermediate during lipid peroxidation by manganese peroxidase. However, phenanthrene-9,10-quinone was not identified by these authors and thus our results strongly support their hypothesis.

ABTS and HAA have been shown to mediate the oxidation of lignin and lignin model compounds in laccase catalyzed reactions (8, 9). However, in contrast to HBT, neither ABTS nor HAA stimulated phenanthrene oxidation under the experimental conditions employed (not shown). This finding was not further investigated but it seems likely that HBT forms more reactive intermediates than ABTS and HAA do when oxidized by laccase. In fact, the ABTS cation radical is very stable in aqueous solutions (14) whereas the nitroxy radical derived from HBT is not (15), and rapidly reacts with various aromatic compounds (16). The involvement of free radicals in phenanthrene oxidation by laccase/HBT was supported by the observation that equimolar amounts (1 mM) of the radical scavenger butylated hydroxytoluene (BHT) inhibited the reactions by 90% (not shown).

Table 1 shows the oxidation of phenanthrene by laccase under various reaction conditions and a reaction time of 64 h. The best results were obtained in the presence of HBT and Tween 80. Phenanthrene was not degraded in the absence of HBT or laccase, in the presence of boiled laccase (not shown), and was poorly degraded when Tween 80 was replaced by Tween 20. Tween 80 contains unsaturated fatty acid esters, whereas Tween 20 does not. To further investigate whether unsaturated lipids are essential for phenanthrene oxidation by the laccase/HBT couple, Tween 80 was replaced by monolinolein, emulsified in *n*-dodecyl-*β*-D-maltoside. Monolinolein was not as effective as Tween 80 but did support the oxidation of phenanthrene, whereas monostearin did not. Similar results were obtained when monolinolein and monostearin were emusified in Tween 20 (not shown). The results show that the laccase/HBT couple was able to oxidize phenanthrene to a limited extent and that this reaction was greatly enhanced by unsaturated lipids.

Control experiments were done to demonstrate that

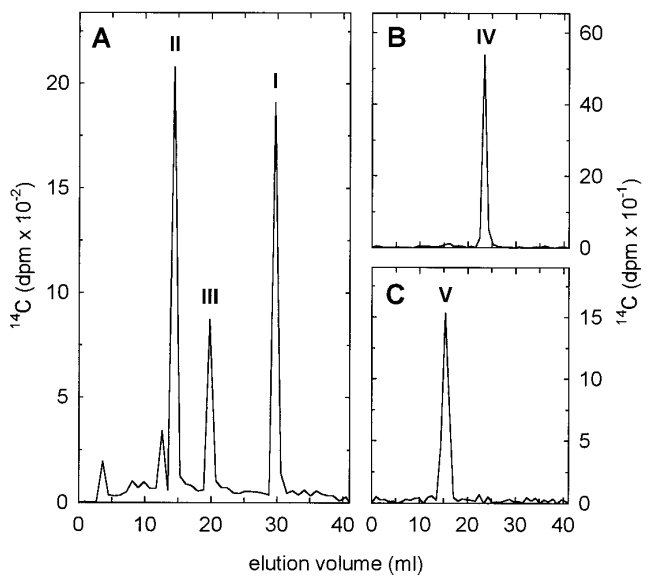

FIG. 2. HPLC analysis of ^{14}C-labeled products from the oxidation of phenanthrene (**I**) by laccase/HBT in the presence of Tween 80 (A), and from chemically derivatized **II** (B) and **III** (C). Roman numerals refer to chemical structures depicted in Fig. 1.

TABLE 1

Degradation of [^{14}C]Phenanthrene by Laccase, Manganese Peroxidase, and Lignin Peroxidase under Various Conditions

Enzyme[a]	HBT	Mn^{2+}	Lipid/surfactant	Product yield (%)[b]			Recovery (%)[c]
				Total	II	III	
Laccase	+	−	Tween 80	30.7	9.7	5.7	91
Laccase	−	−	Tween 80	0.7	0.0	0.0	95
Laccase	+	+	Tween 80	33.6	7.7	9.6	86
Laccase	−	+	Tween 80	1.2	0.0	0.0	84
Laccase	+	−	Tween 20	5.9	1.6	1.7	84
Laccase	−	−	Tween 20	0.6	0.0	0.0	98
Laccase	+	−	ML/DM	9.4	1.6	3.7	83
Laccase	+	−	MS/DM	5.1	1.1	2.0	83
Laccase	+	−	DM	5.1	1.2	2.2	92
Laccase	−	−	DM	0.7	0.0	0.0	81
MnP	+	−	Tween 80	2.1	0.3	0.4	85
MnP	−	−	Tween 80	1.0	0.0	0.0	81
MnP	+	+	Tween 80	24.4	6.5	4.0	80
MnP	−	+	Tween 80	39.9	13.9	5.0	81
LiP	+	−	Tween 80	0.4	0.0	0.0	85
None	+	−	Tween 80	0.2	0.0	0.0	91

Note. + and −, Presence and absence of ingredient; ML, monolinolein; MS, monostearin; DM, *n*-dodecyl-β-D-maltoside.
[a] MnP, manganese peroxidase; LiP, lignon peroxidase.
[b] 100% = total ^{14}C in the HPLC chromatogram.
[c] 100% = ^{14}C initially added as [^{14}C]phenanthrene.

the laccase preparation used in our experiments was not contaminated with another fungal enzyme, MnP, which also oxidizes phenanthrene when unsaturated lipids are present (7). As shown in Table 1, phenanthrene oxidation by laccase required HBT and did not depend on Mn^{2+} whereas phenanthrene oxidation by MnP was partially inhibited by HBT and required Mn^{2+}. It is noteworthy that phenanthrene oxidation by MnP did not require a chelating buffer such as malonate which normally plays an important role in MnP catalysis (17).

Our laccase preparation did not show any detectable LiP activity, and a control experiment with purified LiP showed that this enzyme was unable to oxidize phenanthrene in the presence of HBT and Tween 80 (Table 1).

Oxidation of linoleic acid. Moen and Hammel (7) concluded from their results that MnP supported phenanthrene oxidation was a consequence of lipid peroxidation. To test the hypothesis that lipid peroxidation was also involved in phenanthrene oxidation by the laccase/HBT couple, reactions were done in the presence of [^{14}C]linoleic acid. Linoleic acid **(VI)** was converted to several labeled products (Fig. 3A) that absorbed at 234 nm (Fig. 3B) which is typical for conjugated double bonds in fatty acids. The HPLC peak eluting at 22.1 min **(VII)** had an absorption maximum at 234 nm and eluted identically with authentic standards of [S-(E,Z)]-9-hydroperoxy-10,12-octadecadieonic acid and [S-(E,Z)]-13-hydroperoxy-9,11-octadecadieonic acid which were not separated from each other in the chromatographic system used. These compounds are diagnostic for lipid peroxidation of linoleic acid. To confirm the identification, the HPLC peak fractions containing [^{14}C]**VII** were extracted, reduced with sodium borohydride, methylated with diazomethane, and rechromatographed by HPLC. The labeled peak then

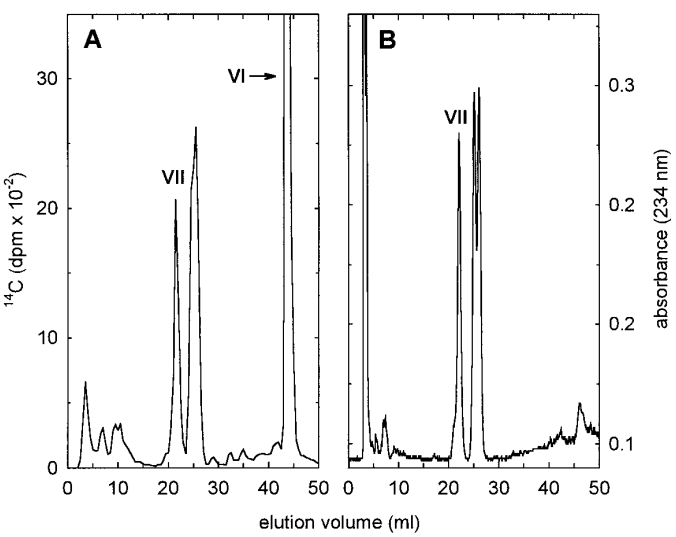

FIG. 3. HPLC analysis of products from the oxidation of linoleic acid (**VI**) by laccase/HBT. Products were detected by liquid scintillation counting (A) and UV-spectroscopy (B). Roman numerals refer to chemical structures depicted in Fig. 1.

TABLE 2

Peroxidation of [^{14}C]Linoleic Acid by Laccase and Manganese Peroxidase under Various Conditions

Enzyme[a]	HBT	Mn^{2+}	H_2O_2	Product yield (%)[b] Total	Product yield (%)[b] VII	Recovery (%)[c]
Laccase	+	−	−	48	9.3	87
Laccase	+	+	−	56	10.5	92
Laccase	+	−	+	43	8.9	89
Laccase	+	+	+	2	0.2	92
Laccase	−	+	+	0	0.0	95
Laccase	−	−	−	0	0.0	103
MnP	+	−	−	2	0.2	95
MnP	−	+	−	7	0.9	89
MnP	−	−	+	0	0.0	91
MnP	+	+	+	28	4.0	89
MnP	−	+	+	50	7.8	86
MnP	−	−	−	0	0.0	95

Note. + and −, Presence and absence of ingredient.
[a] MnP, manganese peroxidase.
[b] 100% = total ^{14}C in the HPLC chromatogram.
[c] 100% = ^{14}C initially added as [^{14}C]linoleic acid.

eluted at 18.9 min, the elution time for the identically treated authentic standards (results not shown). The two isomers (structures **VIII** in Fig. 1) were resolved by GC and exhibited distinct mass spectra. Mass spectrum of 9-isomer: *m/z* (relative intensity) 310 (M^+, 8), 292 (26), 279 (4), 185 (100), 155 (74), 153 (74). Mass spectrum of 13-isomer: *m/z* (relative intensity) 310 (M^+, 11), 292 (15), 279 (3), 236 (6), 207 (13), 99 (100). Both isomers were found in approximately equal amounts.

Table 2 shows the peroxidation of linoleic acid by laccase and MnP under various reaction conditions. Peroxidation of linoleic acid by laccase required HBT and did not depend on Mn^{2+} and H_2O_2. When both Mn^{2+} and H_2O_2 were present, the reaction was almost completely inhibited. In contrast, peroxidation of linoleic acid by MnP was partially inhibited by HBT, required Mn^{2+}, and was greatly enhanced by H_2O_2. Thus, the chemistry involved in laccase supported lipid peroxidation appears to be different from that previously reported for manganese peroxidase (7). The results are in good agreement with those obtained in the oxidation of phenanthrene as discussed above. The requirement for the presence of both HBT and active laccase (boiled laccase did not react) in the reactions rule out the possibility that free copper ions were responsible for lipid peroxidation by decomposing the lipid hydroperoxides that might have already been present in the linoleic acid preparation (18). Moreover, neither Cu^+ and Cu^{2+} (50 μM) nor mixtures of both stimulated lipid peroxidation under the conditions employed (results not shown). Our results strongly suggest that the laccase/HBT couple catalyzed the peroxidation of unsaturated fatty acids.

Conclusions. Laccase oxidized phenanthrene and also peroxidized linoleic acid when HBT was present. The nitroxy radical formed when HBT is oxidized by laccase is known as an potent electrophile that easily abstracts allylic hydrogens (16). Therefore, it seems likely that the laccase generated nitroxy radical oxidizes both phenanthrene and linoleic acid via hydrogen or electron abstraction. Oxy radicals generated during the peroxidation of fatty acids in turn are known to cooxidize PAHs (7, 19) which would explain the enhancement of phenanthrene oxidation by laccase/HBT in the presence of unsaturated lipids. However, more work is needed to determine the radical species that actually oxidize phenanthrene.

Recent work has provided evidence that manganese peroxidase supported lipid peroxidation may account for phenanthrene metabolism in white-rot fungi (7, 20). Here we show, for the first time, that laccase is equally capable of oxidizing phenanthrene by a similar mechanism. The results provide further evidence for the involvement of lipid peroxidation in fungal metabolism of recalcitrant aromatics. However, naturally occurring compounds that mediate phenanthrene oxidation by laccase have not yet been identified. Furture work will reveal whether laccase significantly participates in phenanthrene degradation by white-rot fungi.

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