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p-Hydroxycinnamic Acids as Natural Mediators for Laccase Oxidation of Recalcitrant Compounds

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The capabilities of *p*-coumaric acid (PCA), ferulic acid (FA), and sinapic acid (SA) as laccase mediators are compared in oxidation of industrial dyes and polycyclic aromatic hydrocarbons (PAH). SA behaved as highly efficient mediator in decolorization of dyes, including the recalcitrant Reactive Black 5. This mediating capacity was related to the specificity constant of the enzyme oxidizing this *p*-hydroxycinnamic acid, which was 16 times higher than for the typical substrate 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The kinetics of ABTS oxidation by laccase in the presence of *p*-hydroxycinnamic acids suggested that the stable phenoxyl radical of a SA transformation product acts as laccase mediator. On the other hand, FA and, especially PCA, easily mediated benzo[a]pyrene oxidation, the latter also promoting the oxidation of the more recalcitrant phenanthrene. Phenanthrene transformation by laccase-PCA was enhanced by Tween 80. This fact, together with the detection of TBARS (thiobarbituric acid-reactive-substances) from unsaturated fatty acids, revealed that laccase can also initiate lipid peroxidation reactions in the presence of *p*-hydroxycinnamic acids enabling oxidation of the most recalcitrant PAH.

Introduction

Production of extracellular peroxidases and laccases confers white-rot fungi a unique capability to degrade the lignin polymer in wood, and a variety of recalcitrant contaminants (*1, 2*). Laccases are multicopper oxidases produced by most white-rot basidiomycetes with broad substrate specificity (*3*). Moreover, the presence of synthetic redox mediators, like ABTS (2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid)) or 1-hydroxybenzotriazole (HBT), enhances the oxidative capabilities of laccases allowing the oxidation of high-redox potential aromatic compounds (*4*). This fact together with the use of oxygen as electron acceptor, make laccases highly interesting for industrial and environmental applications. In fact, the so-called laccase-mediator system has been demonstrated to be efficient for degradation of aromatic contaminants (*5*), paper pulp bleaching (*6*), and pitch control (*7*) or dye decolorization (*8*). Nevertheless, the utilization of synthetic mediators in industrial processes is hindered by their high cost and the possible generation of toxic species. The availability of low cost and environmentally friendly natural mediators could facilitate the application of laccase-mediator systems in white-biotechnological processes.

Lignin biodegradation has been described as an "enzymatic combustion" (*9*) wherein enzymatic and radical reactions result in degradation of nonphenolic aromatic structures, even by white-rot fungi that produce laccase as the sole ligninolytic oxidoreductase (*10*). Contribution of free radicals from lignin-degradation products as laccase mediators might explain this phenomenon. Exploring the mediating capabilities of naturally occurring phenols is a good starting point for searching alternative mediators and might also help to understand the natural process of lignin degradation. The ability of some lignin-derived phenols, such as acetosyringone, syringaldehyde, or vanillin, to mediate laccase decolorization of dyes (*11*), oxidation of polycyclic aromatic hydrocarbons (PAH) (*12*), and removal of lignin (*13*) and recalcitrant lipids (*14*) from paper pulps has been proved.

p-Hydroxycinnamic acids are precursors of lignin in vascular plants being also present as extractives or forming lignin-carbohydrate bridges in grasses (*15*). *p*-Coumaric (4-hydroxycinnamic) acid (PCA) and ferulic (3-methoxy-4-hydroxycinnamic) acid (FA) are abundant in soil, where they incorporate to the humic substances (*16*). Moreover, *p*-hydroxycinnamic acids are ubiquitous in plant foods (seeds and fruits) where they act as antioxidants, e.g., the high concentration of sinapic (3,5-dimethoxy-4-hydroxycinnamic) acid (SA) in rapeseed oil has been related to its stability toward peroxidation (*17*). Treatment of *p*-hydroxycinnamic acids with laccase results in a variety of oxidation and coupling products depending on their aromatic ring substituents and the reaction conditions (*18, 19*).

PCA was included in a preliminary screening of natural phenols as laccase mediators (*11*) and yielded promising results in a recent study on transformation of PAH (*12*). Therefore, PCA and their analogues, FA and SA, are compared here regarding the kinetics of their oxidation by laccase or other oxidizers (antioxidant activity) and their capabilities to act as mediators in laccase oxidation of compounds of environmental interest (such as industrial dyes and PAH) including initiation of lipid peroxidation reactions for degradation of the most recalcitrant ones.

Materials and Methods

Chemicals. PCA, FA, SA, HBT, 2,6-dimethoxyphenol (DMP), Acid Blue 74, Azure B, benzo[a]pyrene, phenanthrene, linoleic acid, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were obtained from Sigma-Aldrich. ABTS was supplied by Roche, and Reactive Black 5 by Dye Star (Frankfurt, Germany). Syringaresinol (3,6-bis-(4-hydroxy-3,5-dimethoxyphenyl)-tetrahydrofuro[3,4-c]furan) was synthesized by oxidative β-β′ coupling of sinapyl alcohol in a "zulauf" dehydrogenation reaction catalyzed by horseradish peroxidase in the presence of hydrogen peroxide. Isolation of the dimeric product (with 25% yield) was performed using Biotage flash-chromatography equipment, and purity was checked by NMR.

Antioxidant Activity of *p*-Hydroxycinnamic Acids. The capacity of *p*-hydroxycinnamic acids for scavenging the ABTS cation radical ($ABTS^{\bullet+}$) was measured using the TEAC (Trolox

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equivalent antioxidant capacity) assay (*20*). Reactions were carried out in ethanol, phosphate buffer saline (PBS, pH 7.4) and sodium citrate (pH 5). $ABTS^{\bullet+}$ was generated by adding potassium persulfate to ABTS, diluted till 0.7 absorbance at 734 nm, 10 μL of each compound (1.25 mM) were added to 990 μL, and the decrease of absorbance (734 nm) after 6 min reaction was referred to that obtained with Trolox.

Laccase Production and Purification. Laccase was produced by Beldem (Belgium) from hyperproducer *P. cinnabarinus* ss3 (*21*) and purified according to Camarero et al. (*11*). Activity was estimated by 5 mM ABTS oxidation (ε_{436} 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate, pH 5, at 24 °C.

Laccase Oxidation of *p*-Hydroxycinnamic Acids: Kinetic Constants. *p*-Hydroxycinnamic acid oxidation was estimated spectrophotometrically (Shimadzu UV-160) using 100 mU of enzyme in 50 mM sodium citrate, pH 5, at 24 °C. Oxidation rates were calculated from the decrease of substrate absorbance. The molar absorbances of PCA (ϵ_{312} 11 100 M^{-1} cm^{-1}), FA (ϵ_{320} 12 500 M^{-1} cm^{-1}), and SA (ϵ_{312} 16 100 M^{-1} cm^{-1}) were estimated by substrate consumption under reaction conditions. ABTS and DMP (ε_{469} 27 500 M^{-1} cm^{-1}) were also used as substrates. Mean values and 95% confidence limits of K_m and k_{cat} values were obtained.

UV−visible spectra during enzymatic oxidation of 50 μM *p*-hydroxycinnamic acids by 50 mU/ml laccase in 0.1 M sodium citrate, pH 5, at 24 °C, were collected (for a total of 8 h) using a Hewlett-Packard 8453 diode-array spectrophotometer.

Dye Decolorization Assays. Decolorization of Acid Blue 74 (indigoid), Reactive Black 5 (diazo), and Azure B (heterocyclic) dyes (25 μM) were performed with 100 mU/mL laccase and 50 or 250 μM PCA, FA, SA, or syringaresinol. Reactions were carried out in 0.1 M sodium citrate, pH 5, for 2 h at 24 °C and 160 rev/min. Absorbance decrease was followed at the Acid Blue 74 (608 nm), Reactive Black 5 (598 nm), and Azure B (647 nm) maxima (deducting the absorbances of the oxidized mediators at these wavelengths).

ABTS Oxidation in the Presence of Laccase and *p*-Hydroxycinnamic Acids. First, 50 μM ABTS was oxidized with 50 mU/mL laccase in 0.1 M sodium citrate, pH 5 at 24 °C, for 5 min. Once oxidation to the $ABTS^{+\bullet}$ radical was completed, 50 μM PCA, FA, or SA was added, and changes in the UV−visible spectra were monitorized in a diode-array spectrophotometer until maximal reoxidation of ABTS was attained (240, 500, and 650 s for laccase-SA, laccase-FA, and laccase-PCA, respectively). Spectra of *p*-hydroxycinnamic acids oxidized by laccase alone were recorded for comparison.

Oxidation of PAH by Laccase in the Presence of *p*-Hydroxycinnamic Acids. Oxidation of benzo[a]pyrene (50 μM) was investigated with 2 U/mL of laccase and 500 μM PCA, FA, SA, HBT, or ABTS. Reactions were performed in 0.1 M sodium acetate, pH 5, with 0.1% Tween 20 (as surfactant) in 4 mL Teflon-sealed vials under continuous rolling (6 rev/min) in darkness at 30 °C, for 24 or 48 h. Oxidation of phenanthrene by laccase was investigated analogously, but in the presence of 1% Tween 80 (as source of unsaturated lipids) or 1% Tween 20 (as control), and incubated up to 9 days. Samples from benzo[a]pyrene and phenanthrene treatments were extracted with ethyl acetate, evaporated and dissolved in 100 μL of acetonitrile for HPLC analyses according to Cañas et al. (*12*), using a linear gradient of 20% acetonitrile/80% water (0.1% acetic acid).

Lipid Peroxidation: TBARS Production. Lipid peroxidation by laccase-*p*-hydroxycinnamic acids and laccase-HBT was assayed with 2 U/mL of enzyme in 0.1 M sodium acetate, pH 5, in 4 mL vials at 24 °C, under continuous rolling and darkness. Two concentrations of mediator (0.5 or 2 mM) and 0.7 mM linoleic acid or 2 mM Tween 80 were used. TBARS production was analyzed after different times in 0.5 mL samples, by adding 1.1 mL of 3.3 M sodium acetate, pH 3.5, 1.3 mL of 0.85% thiobarbituric acid and 40 μL of 0.8% butylated hydroxytoluene (in acetic acid) (*22*). Reactions were boiled for 30 min and, after adding 3 mL of butanol, clarified by centrifugation, and absorbance measured at 532 nm.

TABLE 1. Antioxidant Activities of *p*-Hydroxycinnamic Acids and HBT Measured by the Absorbance Decrease (734 nm) of Chemically-Generated $ABTS^{\bullet+}$ After 6-min Reaction (Referred to Trolox)

	ethanol	PBS (pH 7.4)	citrate (pH 5)
PCA	0.47	2.02	1.93
FA	1.08	2.20	1.88
SA	1.15	2.23	2.08
HBT	0.33	0.06	0.00
Trolox	1.00	1.00	1.00

TABLE 2. Kinetic Constants for Oxidation of the Three *p*-Hydroxycinnamic Acids by *P. cinnabarinus* Laccase Compared with Two Typical Substrates (ABTS and DMP), Acid Blue 74 Dye and Syringaresinol

	K_m (μM)	k_{cat} (s^{-1})	specificity constant (μM^{-1} s^{-1})
PCA	88.1	0.75	0.01
FA	7.4	13.0	1.77
SA	5.4	24.6	4.54
ABTS	18.7	5.5	0.29
DMP	3.6	8.7	2.43
Acid Blue 74	2.8	0.05	0.02
Syringaresinol	5.8	7.4	1.27

Results

Oxidation of *p*-Hydroxycinnamic Acids. The antioxidant activities of PCA, FA, SA, and HBT were evaluated by measuring the reduction of chemically generated $ABTS^{\bullet+}$ (Table 1). Activities were tested as usual in ethanol and PBS, but also in citrate, pH 5. SA exhibited the highest antioxidant activity in the three media assayed.

The kinetic constants for oxidation of PCA, FA, and SA by *P. cinnabarinus* laccase were estimated under steady-state conditions, and compared with those for the typical substrates ABTS and DMP (Table 2). SA was promptly oxidized by laccase, with an outstanding specificity constant (16-fold ABTS, twice DMP and more than 400-fold PCA).

UV−visible spectra in the course of SA oxidation by laccase are shown in Figure 1. The maximum around 300 nm decreased (10 s) and was replaced by maxima at 334 nm (30 s) and 512 nm (60 s). At an advanced stage (1 h) a maximum at 425 nm appeared, which is the only one in the visible region at the end of the reaction (8 h). These results suggest the existence of at least three chemical species during SA oxidation.

Decolorization of Industrial Dyes. Different dyes were treated with the enzyme in the absence or presence of each *p*-hydroxycinnamic acid. *Pycnoporus cinnabarinus* laccase was able to directly oxidize Acid Blue 74. The enzyme has high affinity for this dye as revealed by the K_m value that was the lowest among the different substrates and mediators investigated (Table 2). However, due to its low catalytic constant, 2 h incubation was required for high decolorization (Table 3). *p*-Hydroxycinnamic acids promoted laccase oxidation of Acid Blue 74 after short incubation times, attaining 90% decolorization after 5 min with SA. However, the mediating effect of the *p*-hydroxycinnamic acids was especially significant for the recalcitrant dyes Reactive Black 5 and Azure B (Table 3) that were not modified by the enzyme alone due to their high redox potential (both around 1.3 V)

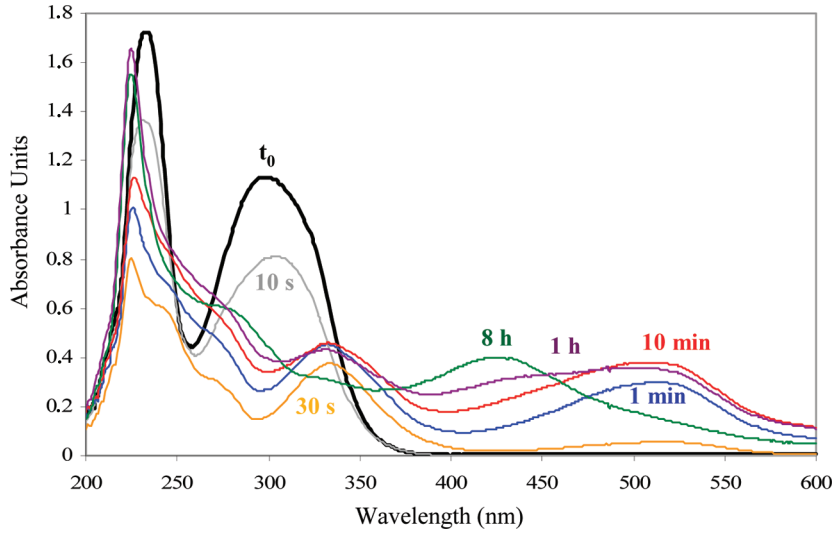

FIGURE 1. Changes in the UV−visible spectrum of SA (50 μM) during oxidation by laccase. The different scans correspond to 0 (black line), 10 s (gray line), 30 s (orange line), 60 s (blue line), 10 min (red line), 1 h (purple line), and 8 h (green line).

TABLE 3. Dye (25 μM) Decolorization (%) after 5 min and 2 h Treatment with Laccase Alone or in the Presence of *p*-Hydroxycinnamic Acids or Syringaresinol Using 2 or 10 Mediator/Dye Ratios (Mean Values with Standard Deviation Values below 5% Are Shown)

		Laccase alone	PCA		FA		SA		Syringaresinol	
			2	10	2	10	2	10	2	10
Acid Blue 74	5 min	4.1	44.1	52.0	23.8	4.0	90.5	85.5	70.9	5.1
	2 h	96.1	100	100	96.1	100	100	92.1	100	100
Reactive Black 5	5 min	1.8	6.9	7.8	6.5	5.2	12.4	11.7	3.0	1.5
	2 h	1.8	16.7	21.6	8.0	7.4	56.8	94.1	34.7	86.0
Azure B	5 min	0.0	1.3	11.5	10.3	3.2	1.6	3.9	0.0	4.5
	2 h	0.0	19.3	57.6	20.6	38.5	6.1	18.9	2.7	8.3

(*23*). SA produced almost complete decolorization of Reactive Black 5, and near 60% decolorization of Azure B was obtained in the presence of PCA after 2 h.

Syringaresinol, a phenolic dimer analogue to those formed by oxidative β-β' coupling of SA catalyzed by laccase, was also assayed as substrate being easily oxidized by the enzyme (Table 2). Moreover, its mediating capability was demonstrated by the increase of decolorization rates compared with the use of laccase alone, in a similar way to SA (Table 3).

Kinetics of ABTS Oxidation in the Presence of Laccase and *p*-Hydroxycinnamic Acids. When an equimolecular concentration of *p*-hydroxycinnamic acid was added to $ABTS^{\bullet+}$, previously obtained by 300 s incubation of ABTS with laccase (gray line in Figure 2), very rapid reduction of the cation radical was produced (red line), both the 425 nm peak and the broad maximum around 700 nm disappearing.

However, the intensities of the *p*-hydroxycinnamic acid maxima around 300 nm were only slightly modified (a small displacement to higher wavelengths occurred). After that, new UV−visible spectra were collected revealing parallel decreases of the peak around 300 nm and increase of the 425 nm peak (arrows in Figure 2). After 240 s (blue lines) reoxidation was (i) complete in the presence of SA (Figure 2A) that recovered the previous intensities at 425 and 700 nm; (ii) intermediate in the presence of FA (Figure 2B) that required 500 s for spectrum stabilization; and (iii) nearly absent in the case of PCA (Figure 2C).

In the case of SA, a maximum around 512 nm appeared simultaneously to the decrease of the 300 nm peak that was displaced to 334 nm (Figure 2A and inset). Similar maxima had been detected during SA oxidation by laccase (orange and blue lines in Figure 1). In the case of FA, the initial displacement of the 300 nm peak till 325 nm was the only spectral change observed during ABTS reoxidation, in agreement with the absence of maxima of interest in the spectrum of oxidized FA (Figure 2B and inset). The spectral changes in the PCA reactions were very limited (Figure 2C), and ABTS reoxidation was extremely low (no changes at 425 nm during the first 240 s, although partial reoxidation was found later).

Transformation of Benzo[a]pyrene. The capabilities of the three *p*-hydroxycinnamic acids as laccase mediators were also tested in oxidation of PAH, and compared with ABTS and HBT (Figure 3A). Benzo[a]pyrene was poorly transformed by the laccase alone (only around 10% in 48 h). However, FA and PCA significantly promoted benzo[a]pyrene removal by the enzyme (no effect was caused by SA). The use of PCA resulted in high transformation rates (over 90%) similar to those obtained with HBT and higher than with ABTS.

Transformation of Phenanthrene and Lipid Peroxidation. No phenanthrene was transformed by *P. cinnabarinus* laccase alone and only 10% was transformed after 9 days treatment using PCA (or HBT) as mediator (Figure 3B). However, the presence of Tween 80 produced a significant enhancement of phenanthrene transformation by laccase-HBT and also by laccase-PCA, especially after long incubation times.

Production of TBARS from oxidation of linoleic acid by laccase in the presence of 2 mM PCA or SA was observed, whereas FA (or lower PCA or SA concentrations) did not promote TBARS formation (Figure 4A). The amount of TBARS obtained with PCA was significantly higher than obtained with SA. When compared with laccase-HBT, the TBARS levels from oxidation of linoleic acid with laccase-PCA were higher, although HBT seemed to act more rapidly promoting linoleic acid peroxidation (Figure 4B). Peroxidation of Tween 80 was

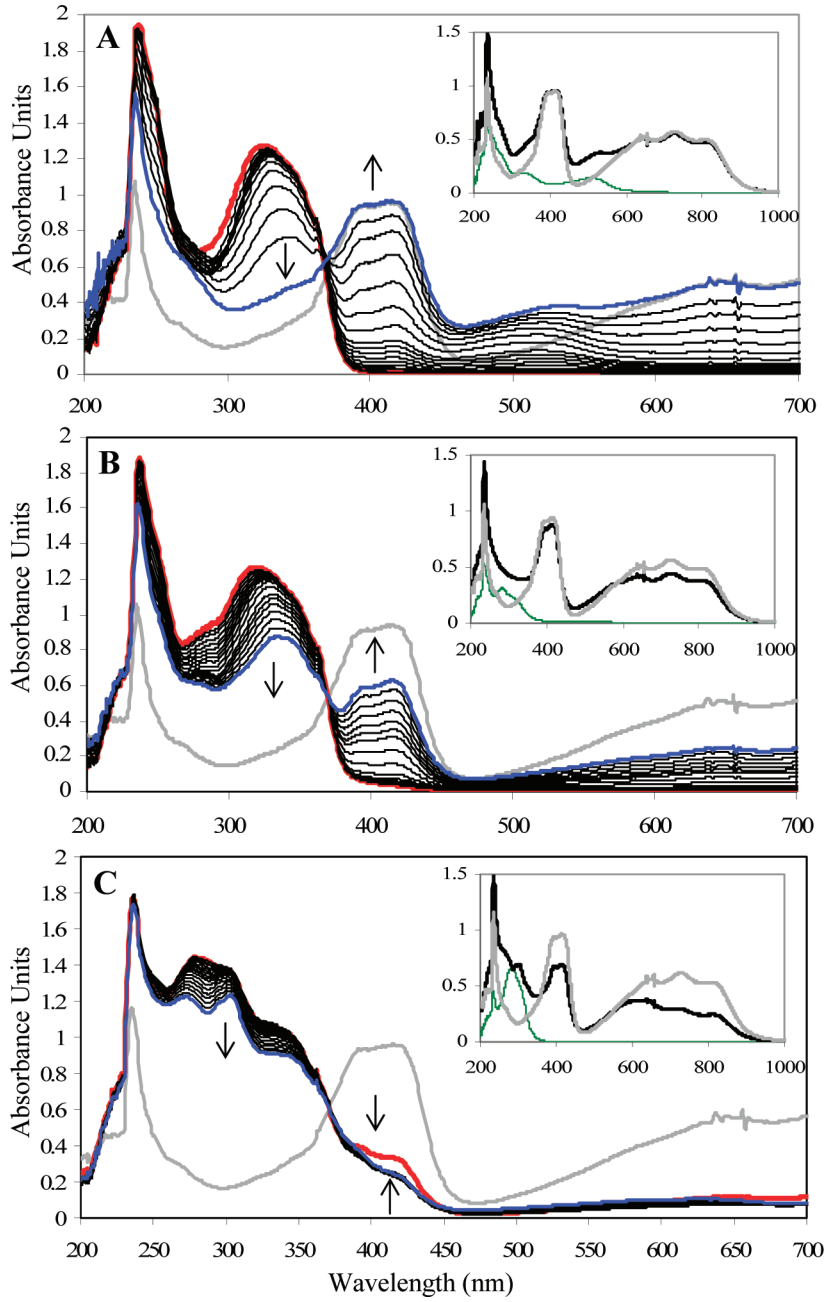

FIGURE 2. Changes in the UV−visible spectra during reoxidation of ABTS by laccase in the presence of SA (A), FA (B), or PCA (C). ABTS (50 μM) was oxidized by laccase during 300 s (gray spectra). Afterward SA, FA, or PCA (50 μM) were added and reduction of $ABTS^{+\bullet}$ was immediately observed (red spectra). Reoxidation by laccase-mediator was then followed during additional 240 s (scans each 10 s during first 100 s, and each 20 s until 240 s) (blue line, final spectra). Insets show the spectra of maximal ABTS oxidation attained by laccase-SA (240 s), laccase-FA (500 s), and laccase-PCA (650 s), compared with the spectrum of completely oxidized ABTS (gray line) and the spectra of SA, FA, and PCA oxidized by laccase (green line).

also detected with both laccase-mediator systems, but the TBARS levels attained were significantly lower than obtained from linoleic acid according to the lower unsaturation degree of Tween 80.

Discussion

Antioxidant Activities of *p*-Hydroxycinnamic Acids. *p*-Hydroxycinnamic acids appeared as strong antioxidants. Their antioxidant activities were significantly lower in ethanol than in aqueous solution. The electron-donating effect of the cinnamate group would be responsible for these differences since ethanol does not favor deprotonation of cinnamic acids (pK_a 4−5) whereas they are expected to be fully deprotonated at pH 7.4 (and partially deprotonated at pH 5). The presence of one or two electron-donor methoxy groups increased the antioxidant activity of FA and SA with respect to PCA, as seen by the TEAC values in ethanol, where no deprotonation interferences take place.

***p*-Hydroxycinnamic Acids As Laccase Substrates.** Laccase activity on the three *p*-hydroxycinnamic acids was in agreement with their antioxidant power (SA > FA > PCA). SA was the best substrate of laccase among those investigated here or reported in the literature (*11*, *24*). The higher laccase activity toward hydroxycinnamic than simple phenols is related to the more extended conjugated double-bond system facilitating electron abstraction.

Formation of successive chemical species during *p*-hydroxycinnamic acid oxidation by laccase was shown by the spectral changes observed with SA. The phenoxyl radical

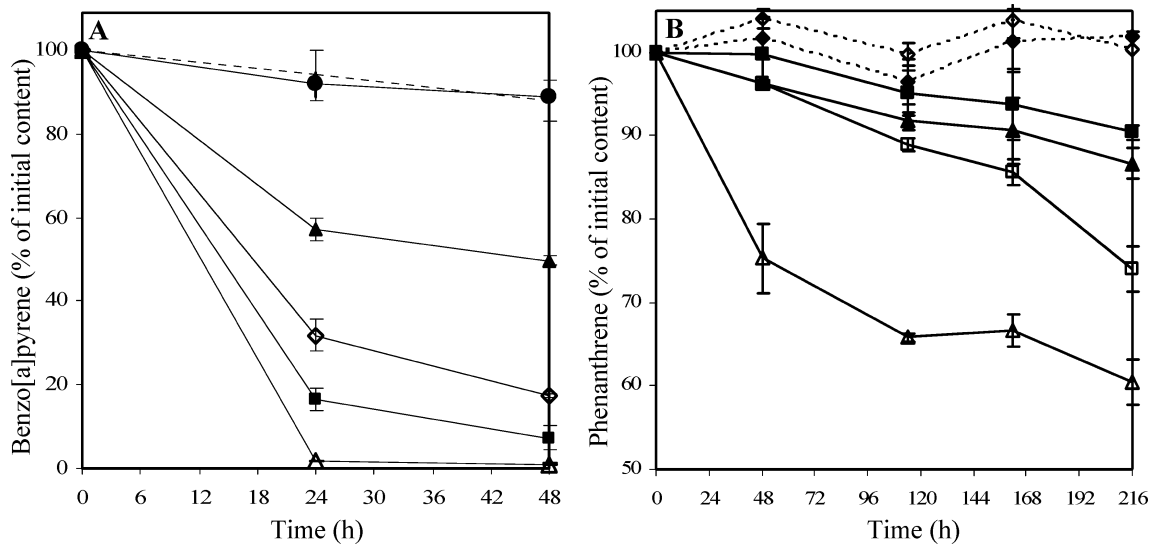

FIGURE 3. (A) Benzo[a]pyrene (50 μM) transformation (%) by *P. cinnabarinus* laccase alone (---) or using 500 μM PCA (■), FA (▲), SA (●), HBT (Δ) or ABTS (◇) as mediators (0.1% Tween 20). B) Phenanthrene (50 μM) transformation (%) by laccase alone (rhombuses) or using 500 μM HBT (triangles) or PCA (squares) in the presence of 1% Tween 20 (black symbols) or Tween 80 (open symbols). Mean values and 95% confidence limits are shown.

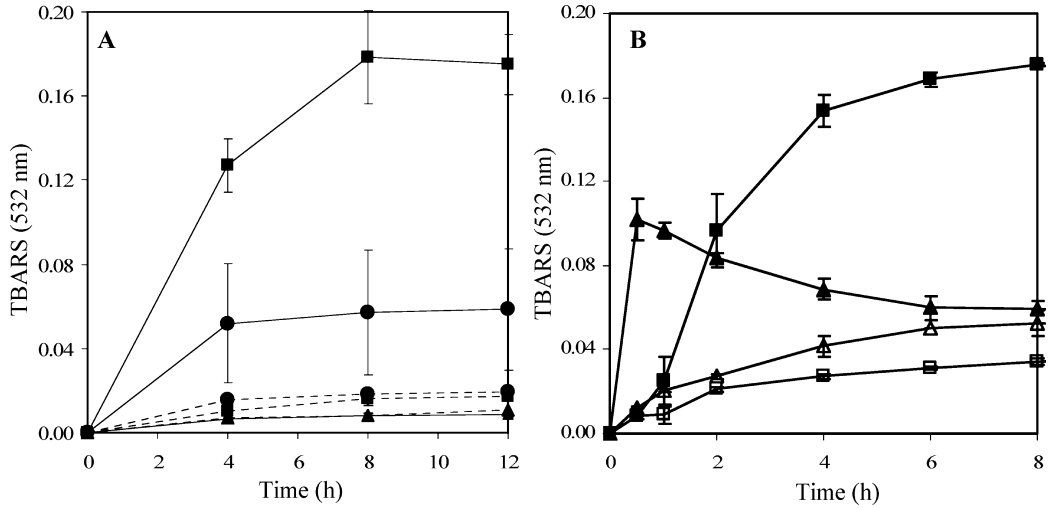

FIGURE 4. Lipid peroxidation. (A) Production of TBARS by laccase in the presence of 0.5 (---) or 2 mM (—) PCA (■), FA (▲) or SA (●) using 0.7 mM linoleic acid as substrate. (B) Comparison of TBARS production from 0.7 mM linoleic acid (black symbols) and 2 mM Tween 80 (open symbols) by laccase and 2 mM PCA (squares) or HBT (triangles) as mediators. Mean values and 95% confidence limits are shown.

would be the first species, followed by β-β' coupling of its quinone methide resonant form yielding a dihydrosinapic acid dimer. Side-chain lactonization will restore the phenolic groups forming the dehydrodisinapic acid dilactone (3,6-bis-(4-hydroxy-3,5-dimethoxy-phenyl)-tetrahydro-furo[3,4-c]furan-1,4-dione) (*19, 25, 26*). This dilactone is also substrate of laccase and yields one (or various) products absorbing at 512 nm (*19*). The high molar antioxidant capability of *p*-hydroxycinnamic acids is related to this fact, i.e. each cinnamic acid molecule reduced more than one $ABTS^{\bullet+}$ radical. The 512 nm peak shift to 425 nm, initiated after 1 h of reaction, was in agreement with nonenzymatic breakdown of the dimeric product yielding *p*-benzoquinone as an end-product from SA oxidation by laccase (*19*).

A wider range of condensation products is expected during oxidation of FA and PCA due to formation of 5−5′, β−5′, and 4-O-5′ linkages that are not being formed during SA oxidation due to the presence of the two methoxy groups (*27*).

Efficient Decolorization of Dyes by Laccase and Natural Mediators. The best substrate of *P. cinnabarinus*, laccase, SA, was the most rapid mediator in dye decolorization. Its strong mediating capacity might be explained by its fast oxidation by the enzyme producing a high concentration of free radicals.

Other dimethoxy-substituted phenols, acetosyringone and syringaldehyde, had been demonstrated to be good laccase substrates and efficient mediators for dye decolorization (*11*). The two methoxy substituents increase the lifetime of their phenoxyl free radicals by preventing 5−5′ coupling reactions, among others. In the case of the SA phenoxyl radicals, their high tendency for β-β' coupling (*19*) immediately rendered the above-mentioned lactones that would be oxidized by laccase yielding stable phenoxyl radicals since additional coupling reactions are not possible. Easy oxidation of these lactones is in agreement with their strong antioxidant activity (*26*). Thus, these phenolic dimeric products would act as laccase mediators as demonstrated here by the significant increase of dye decolorization by laccase in the presence of the homologous phenolic dimer syringaresinol.

Kinetics of *p*-Hydroxycinnamic Acid-Mediated ABTS Oxidation. ABTS was also used to investigate the behavior of *p*-hydroxycinnamic acid radicals as laccase mediators.

Reduced ABTS was obtained by adding *p*-hydroxycinnamic acid to enzymatically generated $ABTS^{\bullet+}$. After its immediate reduction, ABTS reoxidation started and the spectral changes produced were analyzed.

Despite the fact that SA was totally oxidized due to the equimolecular amount added, ABTS reoxidation proceeded slowly suggesting that additional reactions were occurring, probably involving formation of the phenolic lactones mentioned above (512 nm absorbance maximum appeared) acting as laccase mediators. Finally, both ABTS and SA were completely oxidized.

ABTS reoxidation was produced through the laccase-mediator systems and not by laccase alone since different oxidation rates were observed in the presence of each *p*-hydroxycinnamic acid. Preferred oxidation of SA and FA by the *P. cinnabarinus* laccase was in agreement with the lower K_m and higher k_{cat} values for these compounds compared with ABTS, and the affinity for their phenolic dimers should be in the same order, as found for syringaresinol.

Differences in the nature and yield of the coupling products from oxidation of *p*-hydroxycinnamic acids will affect the final yield and speed of the laccase-mediator reaction. Fast oxidation of SA results in high concentration of phenoxyl radicals and fast formation of phenolic dilactones acting as mediators. PCA is much more slowly oxidized by laccase, and its phenoxyl radicals could form a wider variety of dimers and oligomers with a lower phenolic content due to formation of 4-O-5′ and phenylcoumaran-type structures (*19, 25, 28*).

Environmentally Friendly Degradation of Recalcitrant Xenobiotics. In contrast with that observed for decolorization of dyes, SA did not mediate PAH oxidation, whereas FA and especially PCA significantly promoted anthracene and benzo[a]pyrene removal by laccase (*12*). SA is easily oxidized by the enzyme and their phenoxyl radicals are more reactive than PCA radicals, but the redox potential of the latter is significantly higher (0.8 V of PCA, compared with 0.7 and 0.6 V of FA and SA, respectively) (*29*). Therefore, besides the concentration and the reactivity/stability equilibrium of the mediator radicals, their oxidation power toward the target substrate will control the yield of the laccase-mediator reactions.

The higher redox potential of phenanthrene (1.50 V) compared with anthracene (1.09 V) or benzo[a]pyrene (1.27 V) (*30*) explains the inability of laccase to oxidize this PAH. The use of mediators allows laccase removal of anthracene and benzo[a]pyrene, but not of phenanthrene. However, laccase-PCA efficiently transformed this compound in the presence of Tween 80 due to lipid peroxidation reactions forming peroxyl radicals that act as strong oxidizers. The involvement of these lipid radicals was confirmed here by the TBARS assay.

The analysis of the different reaction products obtained by laccase-PCA and laccase-HBT vs laccase-ABTS during oxidation of PAH (*12*) suggested that *p*-hydroxycinnamic acid phenoxyl radicals follow an hydrogen atom transfer (HAT) oxidation mechanism, similar to that described for HBT nitroxyl radicals, and different from the electron transfer (ET) mechanism followed by $ABTS^{\bullet+}$ radicals (*31*). Peroxidation of lipids by laccase-PCA, or other phenols (*32*), supports this HAT oxidation mechanism that generates substrate free radicals. Moreover, the absence of TBARS with laccase-ABTS corroborates the ET mechanism and explains why no phenanthrene oxidation could be attained in the presence of Tween 80.

It had been reported that the addition of unsaturated lipids enabled phenanthrene transformation by laccase using HBT as mediator (*33*) but the present paper showed that more-environmentally friendly degradation of phenanthrene (or other recalcitrant PAH) *via* lipid peroxidation reactions can be attained using some laccase natural mediators, such as PCA. It its noteworthy that in this case both the level of phenanthrene transformation and the release of TBARS by laccase-PCA were closely similar than those obtained with HBT, one of the strongest laccase mediators described up to date.

Acknowledgments

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Supporting Information Available

Chemical structures of mediators, dyes and PAH. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Schoemaker, H. E.; Tuor, U.; Muheim, A.; Schmidt, H. W. H.; Leisola, M. S. A. In Biodegradation: Natural and Synthetic Materials; Betts, W. B., Ed.; Springer-Verlag: London, 1991; pp 157−174.

(2) Martínez, A. T.; Speranza, M.; Ruiz-Dueñas, F. J.; Ferreira, P.; Camarero, S.; Guillén, F.; Martínez, M. J.; Gutiérrez, A.; del Río, J. C. Biodegradation of lignocellulosics: Microbiological, chemical and enzymatic aspects of fungal attack to lignin. *Intern. Microbiol.* **2005**, *8*, 195–204.

(3) Thurston, C. F. The structure and function of fungal laccases. *Microbiology* **1994**, *140*, 19–26.

(4) Bourbonnais, R.; Paice, M. G.; Freiermuth, B.; Bodie, E.; Borneman, S. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. *Appl. Environ. Microbiol.* **1997**, *63*, 4627–4632.

(5) Collins, P. J.; Kotterman, M. J. J.; Field, J. A.; Dobson, A. D. W. Oxidation of anthracene and benzo[*a*]pyrene by laccases from *Trametes versicolor*. *Appl. Environ. Microbiol.* **1996**, *62*, 4563–4567.

(6) Camarero, S.; García, O.; Vidal, T.; Colom, J.; del Río, J. C.; Gutiérrez, A.; Gras, J. M.; Monje, R.; Martínez, M. J.; Martínez, A. T. Efficient bleaching of non-wood high-quality paper pulp using laccase-mediator system. *Enzyme Microbiol. Technol.* **2004**, *35*, 113–120.

(7) Gutiérrez, A.; del Río, J. C.; Ibarra, D.; Rencoret, J.; Romero, J.; Speranza, M.; Camarero, S.; Martínez, M. J.; Martínez, A. T. Enzymatic removal of free and conjugated sterols forming pitch deposits in environmentally sound bleaching of eucalypt paper pulp. *Environ. Sci. Technol.* **2006**, *40*, 3416–3422.

(8) Zille, A.; Munteanu, F. D.; Gubitz, G. M.; Cavaco-Paulo, A. Laccase kinetics of degradation and coupling reactions. *J. Mol. Catal. B: Enzym.* **2005**, *33*, 23–28.

(9) Kirk, T. K.; Farrell, R. L. Enzymatic "combustion": The microbial degradation of lignin. *Annu. Rev. Microbiol.* **1987**, *41*, 465–505.

(10) Geng, X.; Li, K. Degradation of non-phenolic lignin by the white-rot fungus *Pycnoporus cinnabarinus*. *Appl. Microbiol. Biotechnol.* **2002**, *60*, 342–346.

(11) Camarero, S.; Ibarra, D.; Martínez, M. J.; Martínez, A. T. Lignin-derived compounds as efficient laccase mediators for decolorization of different types of recalcitrant dyes. *Appl. Environ. Microbiol.* **2005**, *71*, 1775–1784.

(12) Cañas, A.; Alcalde, M.; Plou, F. J.; Martínez, M. J.; Martínez, A. T.; Camarero, S. Transformation of polycyclic aromatic hydrocarbons by laccase is strongly enhanced by phenolic compounds present in soil. *Environ. Sci. Technol.* **2007**, *41*, 2964–2971.

(13) Camarero, S.; Ibarra, D.; Martínez, A. T.; Romero, J.; Gutiérrez, A.; del Río, J. C. Paper pulp delignification using laccase and natural mediators. *Enzyme Microb. Technol.* **2007**, *40*, 1264–1271.

(14) Gutiérrez, A.; Rencoret, J.; Ibarra, D.; Molina, S.; Camarero, S.; Romero, J.; del Río, J. C.; Martínez, A. T. Removal of lipophilic extractives from paper pulp by laccase and lignin-derived phenols as natural mediators. *Environ. Sci. Technol.* **2007**, *41*, 4124–4129.

(15) Lam, T. B. T.; Iiyama, K.; Stone, B. A. Determination of etherified hydroxycinnamic acids in cell walls of grasses. *Phytochemistry* **1994**, *36*, 773–775.

(16) Suominen, K.; Kitunen, V.; Smolander, A. Characteristics of dissolved organic matter and phenolic compounds in forest soils under silver birch (*Betula pendula*), Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*). *Eur. J. Soil Sci.* **2003**, *54*, 287–293.

(17) Koski, A.; Pekkarinen, S.; Hopia, A.; Wahala, K.; Heinonen, M. Processing of rapeseed oil: effects on sinapic acid derivative content and oxidative stability. *Eur. Food Res. Technol.* **2003**, *217*, 110–114.

(18) Carunchio, F.; Crescenzi, C.; Girelli, A. M.; Messina, A.; Tarola, A. M. Oxidation of ferulic acid by laccase: identification of the products and inhibitory effects of some dipeptides. *Talanta* **2001**, *55*, 189–200.

(19) Lacki, K.; Duvnjak, Z. Transformation of 3,5-dimethoxy,4-hydroxy cinnamic acid by polyphenol oxidase from the fungus *Trametes versicolor*: Product elucidation studies. *Biotechnol. Bioeng.* **1998**, *57*, 694–703.

(20) Arts, M. J. T. J.; Dallinga, J. S.; Voss, H. P.; Haenen, G. R. M. M.; Bast, A. A new approach to assess the total antioxidant capacity using the TEAC assay. *Food Chem.* **2004**, *88*, 567–570.

(21) Herpoël, I.; Moukha, S.; Lesage-Meessen, L.; Sigoillot, J. C.; Asther, M. Selection of *Pycnoporus cinnabarinus* strains for laccase production. *FEMS Microbiol. Lett.* **2000**, *9190*, 1–6.

(22) Moen, M. A.; Hammel, K. E. Lipid peroxidation by the manganese peroxidase of *Phanerochaete chrysosporium* is the basis for phenanthrene oxidation by the intact fungus. *Appl. Environ. Microbiol.* **1994**, *60*, 1956–1961.

(23) Zille, A.; Ramalho, P.; Tzanov, T.; Millward, R.; Aires, V.; Cardoso, M. H.; Ramalho, M. T.; Gübitz, G. M.; Cavaco-Paulo, A. Predicting dye biodegradation from redox potentials. *Biotechnol. Prog.* **2004**, *20*, 1588–1592.

(24) Shleev, S. V.; Morozova, O.; Nikitina, O.; Gorshina, E. S.; Rusinova, T.; Serezhenkov, V. A.; Burbaev, D. S.; Gazaryan, I. G.; Yaropolov, A. I. Comparison of physico-chemical characteristics of four laccases from different basidiomycetes. *Biochimie* **2004**, *86*, 693–703.

(25) Tranchimand, S.; Tron, T.; Gaudin, C.; Iacazio, G. Synthesis of bis-lactone lignans through laccase catalysis. *J. Mol. Catal. B: Enzym.* **2006**, *42*, 27–31.

(26) Liu, H. L.; Wan, X.; Huang, X. F.; Kong, L. Y. Biotransformation of sinapic acid catalyzed by *Momordica charantia* peroxidase. *J. Agric. Food Chem.* **2007**, *55*, 1003–1008.

(27) Arrieta-Baez, D.; Stark, R. E. Modeling suberization with peroxidase-catalyzed polymerization of hydroxycinnamic acids: Cross-coupling and dimerization reactions. *Phytochemistry* **2006**, *67*, 743–753.

(28) Daquino, C.; Foti, M. C. Coupling and fast decarboxylation of aryloxyl radicals of 4-hydroxycinnamic acids with formation of stable p-quinomethanes. *Tetrahedron* **2006**, *62*, 1536–1547.

(29) Lin, W. Z.; Navaratnam, S.; Yao, S. D.; Lin, N. Y. Antioxidative properties of hydroxycinnamic acid derivatives and a phenylpropanoid glycoside. A pulse radiolysis study. *Radiat. Phys. Chem.* **1998**, *53*, 425–430.

(30) Pysh, E. S.; Yang, N. C. Polarographic oxidation potentials of aromatic compounds. *J. Am. Chem. Soc.* **1963**, *85*, 2124–&.

(31) Cantarella, G.; Galli, C.; Gentili, P. Free radical versus electron-transfer routes of oxidation of hydrocarbons by laccase-mediator systems. Catalytic and stoichiometric procedures. *J. Mol. Catal. B: Enzym.* **2003**, *22*, 135–144.

(32) Srebotnik, E.; Boisson, J. N. Peroxidation of linoleic acid during the oxidation of phenols by fungal laccase. *Enzyme Microb. Technol.* **2005**, *36*, 785–789.

(33) Böhmer, S.; Messner, K.; Srebotnik, E. Oxidation of phenanthrene by a fungal laccase in the presence of 1-hydroxybenzotriazole and unsaturated lipids. *Biochem. Biophys. Res. Commun.* **1998**, *244*, 233–238.

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