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NOTE

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Degradation of a nonphenolic *β-O*-4 lignin model dimer by horseradish peroxidase with 1-hydroxybenzotriazole

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Abstract Nonphenolic *β-O*-4 lignin substructure model dimer, 1,3-dihydroxy-2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane (**I**) was degraded by horseradish peroxidase (HRP) in the presence of hydrogen peroxide and 1-hydroxybenztriazole (HBT). 4-Ethoxy-3-methoxybenzoic acid (**II**), 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxypropanone (**III**), 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-1-formyloxypropane (**IV**), 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)propanone (**V**), 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane (**VI**), 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-2,3-cyclic carbonate (**VII**), and 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-1,2-cyclic carbonate (**VIII**) were identified as degradation products by gas chromatography-mass spectrometry. These degradation products were qualitatively the same as those of substrate **I** in the laccase/HBT system, but the yield of the products was apparently different. The products catalyzed by the HRP/H_2O_2/HBT system contained large amounts of the aromatic ring cleavage products **IV**, **VII**, and **VIII** compared with those catalyzed by the laccase/HBT system, while the amount of C*α*-C*β* cleavage product **II** is relatively low. These results suggest that the role of HBT is not in a simple one-electron transfer between the enzymes and substrates.

Key words Horseradish peroxidase · Laccase · Lignin model dimer · One-electron oxidation · Radical mediator

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Introduction

The laccase-mediator system is one of the most powerful lignin-degrading systems.[1–4] We previously reported that *Trametes versicolor* laccase catalyzed C*α*-C*β* cleavage, C*α*-oxidation, *β*-ether cleavage, and aromatic ring cleavage of a nonphenolic *β-O*-4 lignin model dimer, 1,3-dihydroxy-2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-propane (**I**) in the presence of 1-hydroxybenzotriazole (HBT).[5–7] Furthermore, we demonstrated that this system catalyzed the one-electron oxidation of substrate **I** to form both the *β*-aryl cation radical and the benzylic (C*α*) radical intermediates of substrate **I**, based on incorporation experiments from $H_2{}^{18}O$ and ${}^{18}O_2$.[7,8] However, it is poorly understood whether the mediator compounds truly act as radical mediators.

Horseradish peroxidase (HRP) is a commercially available enzyme and is used for the synthesis of dehydrogenatively polymerized lignin (DHP) and so on. The reaction mechanisms of laccase and HRP are very similar. They catalyze one-electron oxidation of phenolic substrates to form phenoxy radicals, which undergo polymerization and/or depolymerization, but they do not oxidize nonphenolic substrates such as compound **I**. The reduction of oxygen species, molecular oxygen for laccase and hydrogen peroxide for HRP, is accompanied by the one-electron oxidation. Thus, a HRP-mediator system might be a good model for providing the reaction mechanisms of the laccase-mediator system.

In the present study, we examined the oxidation of compound **I** by HRP in the presence of hydrogen peroxide and HBT under air or nitrogen atmosphere.

Materials and methods

Enzyme preparation

HRP (type X) was purchased from Sigma and used without further purification. Laccase from *Trametes versicolor* IFO

30340 was obtained and partially purified as described previously.[7–9] The enzyme activities were determined spectroscopically by measuring the absorption at 525 nm using syringaldazine.[10]

Substrate, authentic compounds, and reagents

Substrate **I**, 1,3-dihydroxy-2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)propane, was synthesized as previously described.[6]

The following compounds, namely, 4-ethoxy-3-methoxybenzoic acid (**II**),[5] the acetate of 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxypropanone (**III**-Ac),[11] the diacetate of 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-1-formyloxypropane (**IV**-Ac),[12] the diacetate of 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)propanone (**V**-Ac),[12] the triacetate of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane (**VI**-Ac),[12] the acetate of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-1,2-cyclic carbonate (**VII**-Ac),[7] and the acetate of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-2,3-cyclic carbonate (**VIII**-Ac),[7] were used from our previously synthesized stocks.

1-Hydroxybenzotriazole (HBT) was purchased from Nakalai Tesque. The substrate **I** and HBT were purified by preparative thin layer chromatography (TLC, Kieselgel 60 F_{254}, Merck) before use in the experiments. All other chemicals were reagent grade or of equivalent purity.

Enzymatic reactions

*The oxidation of substrate **I** by HRP/H_2O_2/HBT*

The oxidation of model compound with the HRP/H_2O_2/HBT system was assayed by a procedure similar to that used in the laccase/HBT system.[6–8] To the reaction vessel containing 350 μl of 0.2 M MacIlvaine buffer (pH 4.0), substrate **I** [530 nmol, 10 μl of *N*,*N*-dimethylformamide (DMF) solution] and HBT (74 nmol, 10 μl of DMF solution) were added. The reaction was started by addition of H_2O_2 (1.5 μmol) and HRP (400 nkat), and was carried out at 28°C for 2 h while being stirred. Control experiments lacked HBT in the reaction mixture. The vessel contents were then partitioned into ethyl acetate and water. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The residue was acetylated [acetic anhydride and pyridine (1:1, v/v)] and trimethylsilylated [TMSI-H (hexamethyldisilazane and trimethylchlorosilane in pyridine; 2:1:10, v/v) (GL Sciences)]. This fraction was analyzed by gas chromatography-mass spectrometry (GC-MS). The degradation products were identified by direct comparison of their mass spectra and retention times with those of corresponding authentic compounds.

Semiquantitative experiments were performed as follows. The diacetate of 3-(hydroxy-3-methoxyphenyl)-1-propanol (5 μg) was added as an internal standard before extraction, and the extracts were analyzed as described above. The quantities of degradation products were determined by comparing the peak area of the internal standard on a total ion chromatogram with those of degradation products.

*The oxidation of substrate **I** by laccase/HBT*

The reaction mixture (350 μl in a 3-ml micro vial) contained substrate **I** (530 nmol), HBT (74 nmol), and laccase (400 nkat) in 0.2 M MacIlvaine buffer (pH 4.0). The reaction was carried out at 28°C for 2 h while being stirred. The reaction mixture was extracted and analyzed as described above.

*The oxidation of substrate **I** by HRP/H_2O_2/HBT under nitrogen atmosphere*

The vessel containing the reaction mixture (330 μl in a 3-ml micro vial) with H_2O_2 (1.5 μmol) and HRP (400 nkat) in MacIlvaine buffer (pH 4.0) was evacuated and then filled with nitrogen gas, and this procedure was repeated ten times. Finally, substrate **I** (530 nmol) and HBT (74 nmol) were added to the reaction mixture and it was incubated at 28°C for 2 h while being stirred. The reaction mixture was extracted and analyzed as described above.

Instruments

Enzyme activity was measured using a Shimadzu Multispec-1500 spectrometer. GC-MS was performed with a Shimadzu GCMS-QP 5000 gas chromatograph mass spectrometer (EI, 70 eV). GC-MS analyses were carried out on a capillary column (DB-1, 30 m × 0.25 mm i.d., film, 1 μm, J&W Scientific) at a rate of 5°C/min from 150° to 280°C using He as the carrier gas.

Results and discussion

The degradation products of substrate **I** treated with HRP in the presence of H_2O_2 and HBT were analyzed by GC-MS after acetylation and trimethylsilylation. The total ion chromatogram (TIC) of the degradation products is shown in Fig. 1A. The following seven compounds were identified: 4-ethoxy-3-methoxybenzoic acid (**II**), 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxypropanone (**III**), 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-1-formyloxypropane (**IV**), 2,3-dihydroxy-1-(4-ethoxy-3-methoxyphenyl)-propanone (**V**), 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane (**VI**), 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-1,2-cyclic carbonate (**VII**), and 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-2,3-cyclic carbonate (**VIII**). Their mass spectra and retention times were identical to those of the authentic compounds. The peak between **IV**-Ac and **V**-Ac [retention time (Rt) 24 min] in Fig. 1A was expected to be a regioisomer of

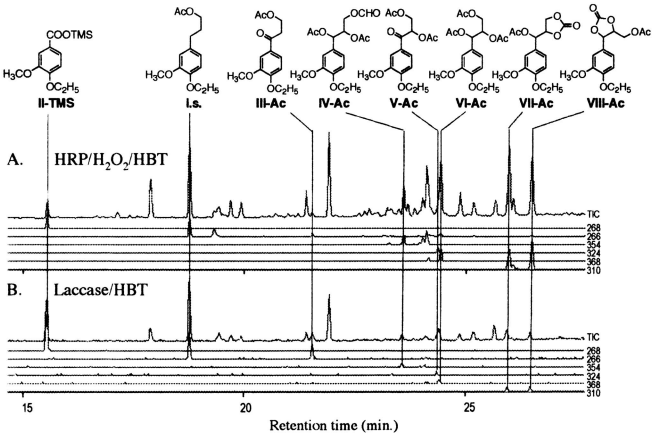

Fig. 1. Total ion chromatograms (*TIC*) and mass chromatgrams of the reaction residue of substrate **I** treated with horseradish peroxidase (*HRP*) (**A**) and laccase (**B**) in the presence of 1-hydroxybenzotriazole (*HBT*). *TMS*, trimethylsilyl ether; *Ac*, acetyl ester, *i.s.*, internal standard

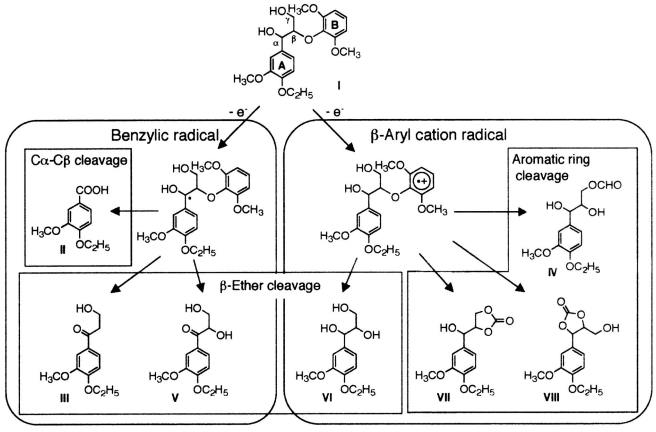

Fig. 2. Possible mechanisms for the formation of the degradation products from benzylic or aryl cation radicals of substrate **I** by one-electron oxidation

Table 1. The quantities of the degradation products of substrate **I** after catalytic action of the HRP/H_2O_2/HBT or laccase/HBT system

	Degradation products					
	II	**III**	**IV**	**V + VI**[a]	**VII**	**VIII**
HRP/H_2O_2/HBT/air atmosphere						
mmol[b]	8	7	8	17	24	16
%[c]	2.4	2.1	2.4	5.2	7.3	5.1
Laccase/HBT/air atmosphere						
mmol[b]	31	10	4	15	15	12
%[c]	10.0	3.5	1.3	4.8	4.8	3.8
HRP/H_2O_2/HBT/nitrogen atmosphere						
mmol[b]	3	7	nd	13	8	1
%[c]	0.8	1.9	–	3.6	2.2	0.3

HRP, Horseradish peroxidase; HBT, 1-hydroxybenzotriazole; nd, not detected.
[a] Products **V** and **VI** could not be completely separated under the gas chromatography-mass spectrometry (GC-MS) conditions.
[b] The quantities were calculated by comparing the peak area of the internal standard with those of degradation products in GC-MS analyses
[c] Percentage = 100 × (mmol of the formed products)/(mmol of degraded substrate **I**)

IV-Ac from the mass spectra, although it was not compared with the authentic compound. The other peaks (especially Rt 18 and 22 min) were detected in the TIC of the control experiment without HBT and H_2O_2, indicating that they were not the degradation products of the lignin model compound.

To compare the degradation products for the HRP/H_2O_2/HBT and laccase/HBT systems, the oxidation of substrate **I** by the laccase/HBT system was also conducted. A TIC of the degradation products for the laccase/HBT system by GC-MS analyses is shown in Fig. 1B. The degradation products in both systems were identical. We previously demonstrated that the laccase/HBT system catalyzed the one-electron oxidation of substrate **I** to form both the β-aryl cation radical and the benzylic (Cα) radical intermediates of substrate **I**, based on incorporation experiments from $H_2{}^{18}O$ and $^{18}O_2$.[7,8] Therefore, the possible reaction pathway for the HRP/H_2O_2/HBT system is quite similar to that for the laccase/HBT system as shown in Fig. 2. The β-aryl cation radical is converted to the products via an aromatic ring cleavage reaction, and the benzylic radical is cleaved between the Cα and Cβ carbons via a kind of Baeyer-Villiger reaction. The β-ether cleavage products were derived from both radical intermediates.

Although the degradation products in both systems were identical, the TIC profiles were different from each other. Then, semiquantitative analyses with the diacetate of 3-(4-hydroxy-3-methoxyphenyl)propanol as an internal standard were performed. The results indicated that 62% (330 mmol) and 60% (315 mmol) of substrate **I** were oxidized by the HRP/H_2O_2/HBT and laccase/HBT systems, respectively. The amounts of the degradation products were calculated as shown in Table 1, and the relative proportions of the degradation products based on the amount of the oxidized substrate **I** were also estimated. The yield of benzoic acid **II**, the Cα-Cβ cleavage product, formed by the

laccase/HBT system was four times higher than that formed by the HRP/H_2O_2/HBT system, whereas aromatic ring cleavage producing **IV**, **VII**, and **VIII** was the predominant reaction for the HRP/H_2O_2/HBT system. These differences in the yield of degradation products between the two systems imply that the role of HBT could not be a simple one-electron transfer catalyst between the enzymes and substrates. Shibata et al.[13] have demonstrated that HBT exhibits kinetically different inhibition in the guaiacol oxidation for laccase and HRP, respectively.

To elucidate the involvement of molecular oxygen in the oxidation of substrate **I** by HRP/H_2O_2/HBT, some experiments were performed under nitrogen atmosphere. GC-MS analysis showed that 69% (365 mmol) of substrate **I** was oxidized, but the yields of the degradation products **II**, **III**, **V**, **VI**, **VII**, and **VIII** were lower than those obtained when performed under air (Table 1) and novel unknown peaks appeared in the TIC (data not shown). This result indicated that molecular oxygen is not needed for the radical formation of substrate **I** and is only involved in the radical reactions causing Cα-Cβ cleavage, aromatic ring cleavage, and so on.

The present results could not elucidate the role of HBT in the laccase-mediator system, but it is clear that the HRP/H_2O_2/HBT system could oxidize the nonphenolic substrate.

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