

## 4-Hydroxybenzoic Acid Secreted from *Trametes versicolor* Catalyzes the Oxidation of a Nonphenolic $\beta$ -O-4 Lignin Model Dimer as Laccase Mediator

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### Abstract

To detect natural laccase mediator, the extracellular culture fluid of *Trametes versicolor* laccase production medium was fractionated using degradation of anthracene which can not be degraded with laccase alone. As a result, 4-hydroxybenzoic acid (HBA) was isolated from the culture fluid, and it was clear that HBA acted as a natural mediator and the degradation efficiency of anthracene by laccase greatly increased. The laccase-HBA system could also oxidized the nonphenolic  $\beta$ -O-4 lignin model dimer to produce the C $\alpha$ -oxidation,  $\beta$ -ether cleavage and aromatic ring cleavage products. The oxidation mechanisms of the laccase-HBA system are very similar to those for lignin peroxidase and laccase-1-hydroxybenzotriazol (HBT) system.

**Keywords:** Fungal metabolite; 4-Hydroxybenzoic acid; Natural laccase mediator, Nonphenolic  $\beta$ -O-4 lignin model compound

### INTRODUCTION

Laccase is an oxidase containing copper at its active center and it has been reported that it has oxidizing ability for the phenolic structure in lignin but cannot act on nonphenolic structure<sup>1)</sup>. However, Kawai et al.<sup>2)</sup> showed that oxidation of veratryl alcohol and 3,4,5-trimethoxybenzyl alcohol occurs by laccase in the presence of syringaldehyde, which is considered degradation product of lignin by white rot fungi. We propose the possibility that the nonphenolic structure in lignin that is not oxidized by laccase alone can be degraded by using such a low molecular weight compound as a radical mediator. In addition, Bourbonnais et al.<sup>3)</sup> showed that the degradation of nonphenolic  $\beta$ -1 lignin model compound by laccase with 2,2-azinobis(4-ethylbenzothiazoline-6-sulfonic acid ammonium salt) (ABTS), and these reactions did not proceed laccase only. Furthermore, various mediators including 1-hydroxybenzotriazole (HBT), which considered to be the most potent mediator, have been reported<sup>4-9)</sup>.

However, in natural lignin degradation by laccase derived from white rot fungi, synthetic compounds such as HBT are unlikely to become mediators. From this point of view, we have attempted to search for natural laccase mediators, suggesting that there are compounds that can act as mediators in extracellular fluid of white-rot fungi. In this study, therefore, the objective was to identify the chemical structure of the laccase mediator produced by *Trametes versicolor* and to clarify the effect on degradation of nonphenolic  $\beta$ -O-4 lignin model compound.

### EXPERIMENTAL

#### Organism and Laccase Preparation

*Trametes versicolor* IFO 30340 was incubated on potato dextrose agar (Wako Pure Chemical Industries) medium at 30°C for 7 days. The mycelial mats were homogenized and added to the Fåhræus and Reinhammar modified medium (150 ml in 500-ml Erlenmeyer flasks)<sup>10</sup>. The medium is as follows (per liter of distilled water): 30 g glucose, 2.5 g L-asparagine, 0.15 g DL-phenylalanine, 27.5 mg adenine, 2.0 mg thiamine-HCl, 1.0 g KH<sub>2</sub>PO<sub>4</sub>, 0.1 g Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 0.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 16 mg CuSO<sub>4</sub>·5H<sub>2</sub>O, 10 mg CaCl<sub>2</sub>, 10 mg FeSO<sub>4</sub>·7H<sub>2</sub>O, 1 mg MnSO<sub>4</sub>·H<sub>2</sub>O, 1 mg ZnSO<sub>4</sub>·7H<sub>2</sub>O. The culture medium was grown with agitation (150 rpm) at 30°C.

After incubation for 14 days, cells were removed from the medium by using of glass fiber filters and membrane filters, successively. This filtrate was concentrated to about 50 ml using an ultrafiltration device (Advantec, Model-UHP-76 K) to obtain a crude enzyme solution. After dialysis, the crude enzyme solution was subjected to gel filtration chromatography (Sephadex G-50, 2.5 cm × 20 cm, 0.1 M phosphate buffer, pH 6.0). Each fraction was collected in 5.0 ml aliquots and the absorbance at 280 nm and laccase activity were measured. Fractions with laccase activity were collected used in the following experiments.

### Preparation of Mediator Candidate

The extracellular fluid (ca. 2.5L) produced by the above ultrafiltration was concentrated to 100 ml with a rotary evaporator. The concentrate was re-suspended into 100 ml of water, and the mixture was extracted with 500 ml of ethyl acetate (EtOAc) at room temperature for 48 h with stirring. The EtOAc layer was concentrated (181 mg) and used as a mediator candidate.

### Degradation of Anthracene in the Presence of Laccase Mediator Candidate

The reaction was performed in a total volume of 500 µl. To 50 mM malonate buffer (pH 4.5), 0.1 mM anthracene (50 µl DMSO solution), laccase 75 nkat, candidate mediator 153 µg (10 µl DMSO solution) were added and the reaction mixture was incubated at 37°C. After the reaction for a predetermined time (0, 3 and 6 h), 500 µl of acetonitrile and pyrene (internal standard) were added to the reaction mixture and the amount of anthracene decrease was quantified by HPLC (Shimadzu LC-10AT, column: Cadenza CD18 (Imtakt), 75 × 4.6 mm, eluent: acetonitrile: water = 6:4, flow rate: 1.0 ml/min). HBT 15.3 µg (0.1 µmol) was used as a positive control, and the amount of mediator candidate added was 10 times (153 µg) of HBT. As a negative control, the reaction was performed by adding only DMSO instead of mediator candidate.

### Fractionation of Mediator Candidate

The EtOAc soluble (151 mg) was fractionated by silica gel column chromatography (Kieselgel 60, 1.6 cm × 15 cm, solvent: EtOAc: Hexane = 1:8 (50 ml), 1:4 (50 ml), 1:1 (50 ml), 2:1 (50 ml), 1:0 (50 ml) and MeOH (100 ml)) and fractionated 50 ml each. Each fraction (Fr. 1 to Fr. 7) was concentrated under reduced pressure. These fractions were used as for anthracene oxidation above.

Furthermore, Fr. 7 (32 mg) was fractionated by preparative TLC (Kieselgel 60 F 254, solvent, 5% MeOH / chloroform, 3 times) to obtain three fractions, Fr. 7-1 (5.2 mg), Fr. 7-2 (8.2 mg) and Fr. 7-3 (6.1 mg). These fractions were subjected to anthracene degradation. For Fr. 7-1, experiments were also conducted with mediator concentrations at 5 times (76.5 µg) and 2.5 times (38.2 µg) of HBT.

### Gas Chromatograph Mass Spectrometry (GC-MS) Analysis

Fraction 7-1 was trimethylsilylated by TMSI-H (hexamethyldisilazane: trimethyl chloro-silane: pyridine = 2:1:10, GL Sciences) and analyzed by GC-MS (Shimadzu GCMS-QP 5050 gas chromatograph mass spectrometer, column: TC-1 (0.25 mm × 30 m, GL Science, column temperature: 100-270 °C, 5 °C/min).

### Effect of *p*-hydroxybenzoic Acid (HBA) Addition of Anthracene Degradation by Laccase

The reaction was performed in 500 µl. To 50 mM malonate buffer, 50 µl of 0.1 mM anthracene, laccase 50 nkat and HBA (Tokyo Chemical Industry, 76.5 µg, or 30.6 µg) were added and reacted at

37 °C for 24 h. The reaction was stopped by adding 500 µl of acetonitrile and pyrene, the amount of decrease in anthracene was determined by HPLC previously described.

### Degradation of Nonphenolic $\beta$ -O-4 Lignin Model Compound by Laccase in the Presence of HBA

The reaction was carried out in a total volume of 5 ml. To 50 mM malonate buffer (pH 4.5), 0.1 mM lignin model compound (I) (DMSO solution), laccase 125 nkat and 1.0 mM HBA (DMSO solution) were added and the reaction mixture was incubated at 30 °C. After 6 h and 24 h, 500 µl of the reaction solution was taken out and stopped by adding an equal amount of acetonitrile. Then, 3,5-dimethoxy-4-ethoxybenzaldehyde (internal standard) were added and subjected to HPLC analysis (column: Cadenza CD18, eluent: acetonitrile: water = 3:7, flow rate: 0.8 ml/min). As a control, the reaction was performed with laccase alone (DMSO was added instead of HBA).

After the reaction for 24 h, the reaction solution was partitioned between EtOAc and water, the EtOAc extract was washed with saturated brine, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The EtOAc extract was acetylated with pyridine (1.5 ml) and acetic anhydride (1.5 ml) for 48 h. The acetylated products were analyzed by GC-MS. A part of the product was trimethylsilylated with TMSI-H and analyzed by GC-MS.

### Substrate and Authentic Compounds

1-(4-Ethoxy-3-methoxyphenyl)-1,3-dihydroxy-2-(2,6-dimethoxyphenoxy) propane (I)<sup>6</sup> was used as a nonphenolic  $\beta$ -O-4 lignin model compound. The following compounds, 1-(4-ethoxy-3-methoxyphenyl)-3-dihydroxy-2-(2,6-dimethoxyphenoxy)propanone (II)<sup>6</sup>, 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxypropanone (III)<sup>11</sup>, 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxypropanone (IV)<sup>12</sup>, 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane (V)<sup>12</sup>, 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-3-formate ester (VI)<sup>12</sup>, 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-trihydroxypropane-2,3-cyclic carbonate ester (VII)<sup>8</sup>, 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-tri-hydroxypropane-1,2-cyclic carbonate ester (VIII)<sup>8</sup> were used from our previously synthesized stocks.

## RESULTS AND DISCUSSION

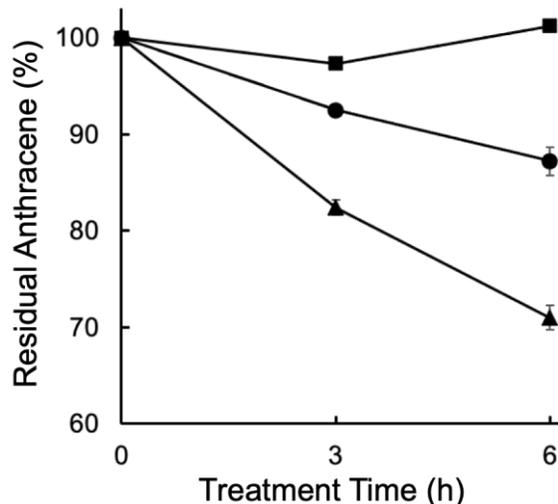
The extracellular fluid of *T. versicolor* laccase production medium was concentrated and it was extracted with EtOAc. The EtOAc soluble was used as a mediator candidate.

The degradation of anthracene by laccase was measured (Fig. 1). Laccase alone cannot degrade anthracene, but with the addition of EtOAc soluble, the degradation of anthracene improved to 8% in 3 h and 13% in 6 h. In the presence of HBT as a positive control, it was 18% in 3 h and 29% in 6 h. These results suggested that mediator compound(s) for the promoting anthracene degradation exists in the EtOAc soluble. Then, the EtOAc soluble was fractionated using silica gel column chromatography.

The degradation rate of anthracene for 24 h by laccase with the fractionated products is shown in Table 1. As a result, it was revealed that Fr. 5 to Fr. 7 had laccase mediator activity (Table 1) and that there was no activity from Fr. 1 to Fr. 4 (data not shown). In the case of Fr. 7, almost 70% of anthracene was degraded.

Then, the Fr. 7 was further fractionated by PTLC, and obtained three fractions, Fr. 7-1, Fr. 7-2 and Fr. 7-3, were used for the degradation of anthracene. After 24 h incubation, the anthracene was degraded by the addition of Fr. 7-1 (60%), Fr. 7-2 (33%), Fr. 7-3 (38%), respectively (Table 1). Since the degradation of anthracene depends on the addition of Fr. 7-1, it could be inferred that natural mediator(s) were included in this fraction.

Then, GC-MS analysis was performed using Fr. 7-1 as a TMS derivative, and the constituent components were investigated (Fig. 2a). From the mass spectra of the compound having a retention time of 15.6 min (Fig. 2b), it was expected to be *p*-hydroxybenzoic acid (HBA). The mass spectra and retention time were completely identical with those of the authentic compound (Fig. 2c). This result indicates that *T. versicolor* synthesized the mediator HBA by itself and secreted it outside the fungus.



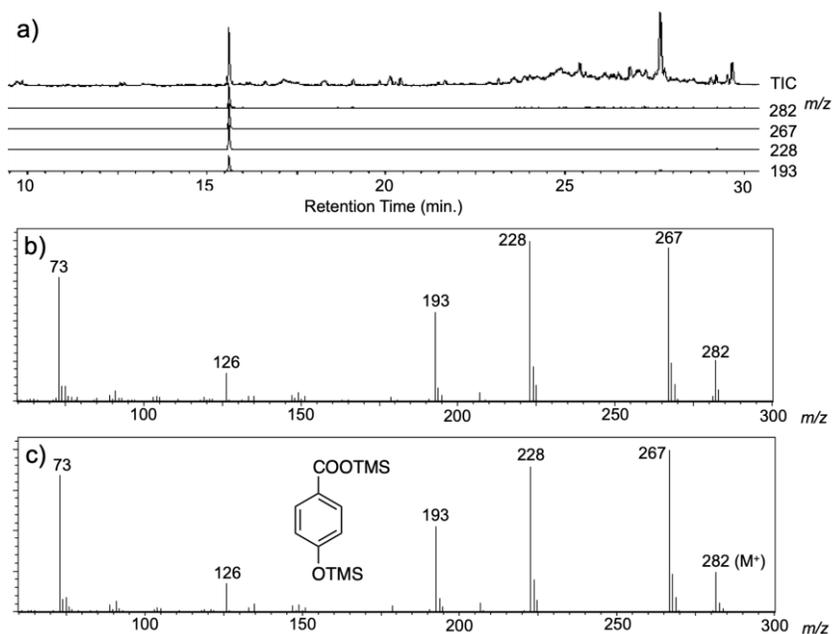
**Fig. 1.** Decrease in concentration of anthracene by enzymatic treatment with laccase, laccase-EtOAc soluble system or laccase-HBT system. Indicated for each point are the mean and standard deviation of three experiments. (■) laccase alone, (●) laccase plus EtOAc soluble, (▲) laccase-HBT system.

**Table 1.** Degradation rate of anthracene by laccase in the presence of various mediator candidate.

Sample	Amount		degradation rate (%)
	µg	µmole	
HBT	15.3	0.1	99.0
Fr.5	153	-	32.8
Fr.6	153	-	27.8
Fr.7	153	-	69.5
Fr.7-1	153	-	59.9
Fr.7-1	76.5	-	49.6
Fr.7-1	38.2	-	13.9
Fr.7-2	153	-	33.2
Fr.7-3	153	-	38.4
HBA	30.6	0.22	85.9
HBA	76.5	0.55	95.5

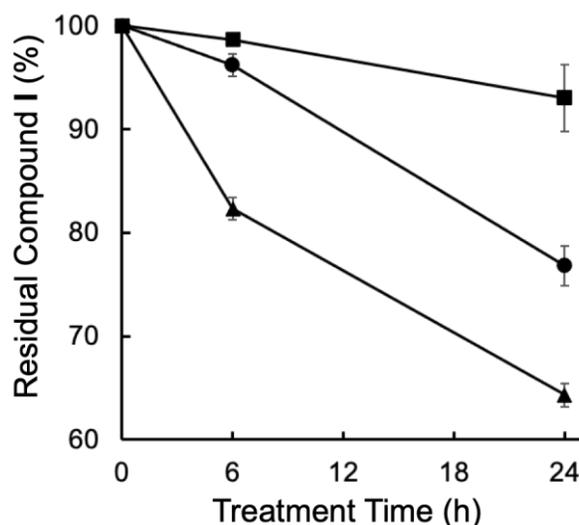
Next, the degradation of anthracene by laccase-HBA system was performed. The results indicated the anthracene degradation by this system (Table 1). When the amount of HBA added was 76.5 µg (0.55 µmol), almost 100% degradation of anthracene was observed as in the case of HBT (0.1 µmol). Furthermore, even when the amount of HBA added was twice the mole (0.22 µmol) of HBT, more than 80% of anthracene degradation was confirmed. These results suggested that HBA works effectively as a mediator of anthracene degradation by laccase. Johannes et al.<sup>13)</sup> have searched for phenolic mediators involved in the degradation of polycyclic aromatic compounds by laccase and listed HBA as one of the mediator compounds.

To investigate whether the laccase-HBA system catalyzes the degradation of lignin, a non-phenolic β-O-4 lignin model compound **I** was treated by this system. The results are shown in Fig. 3. The laccase-HBA system could catalyze the degradation of this compound **I**. It is revealed that the decrease of the compound **I** progresses with time and HBA dose dependent, and with HBA 2 mM addition, the degradation rate for compound **I** reached about 35 %. In the case of laccase alone without HBA, a slight decrease in compound **I** was observed. Since it has been reported that laccase alone cannot degrade non-phenolic compounds<sup>1,2,14)</sup>, we concluded that the degradation of compound **I** proceeded only with the addition of HBA in this experiment.



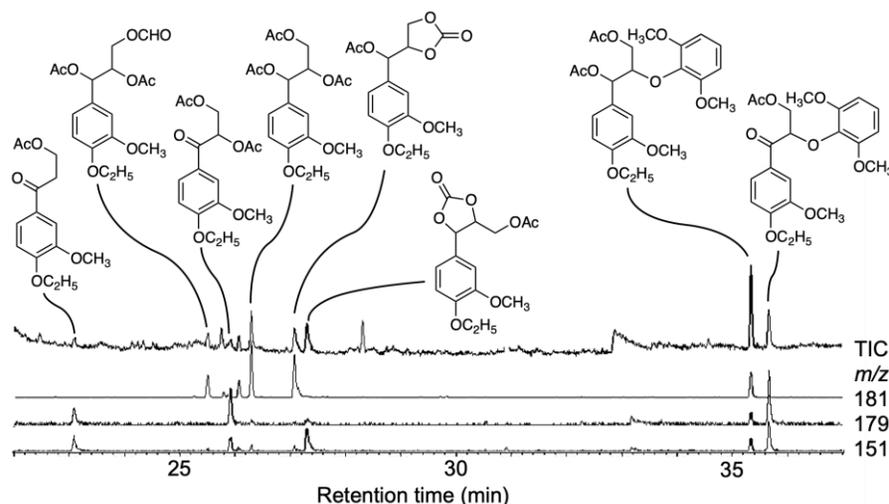
**Fig. 2.** GC-MS analyses of Fr. 7-1.

(a) total ion chromatogram (TIC) and mass chromatograms, (b) mass spectra of metabolite at 15.6 min, (c) mass spectra of TMS ester of 4-hydroxybenzoic acid.



**Fig. 3.** Effects of HBA on decrease in the nonphenolic  $\beta$ -O-4 lignin model compound **I** during treatment with laccase. Indicated for each point are the mean and standard deviation of three experiments. (■) laccase alone, (●) laccase and HBA (1 mM), (▲) laccase and HBA (2 mM).

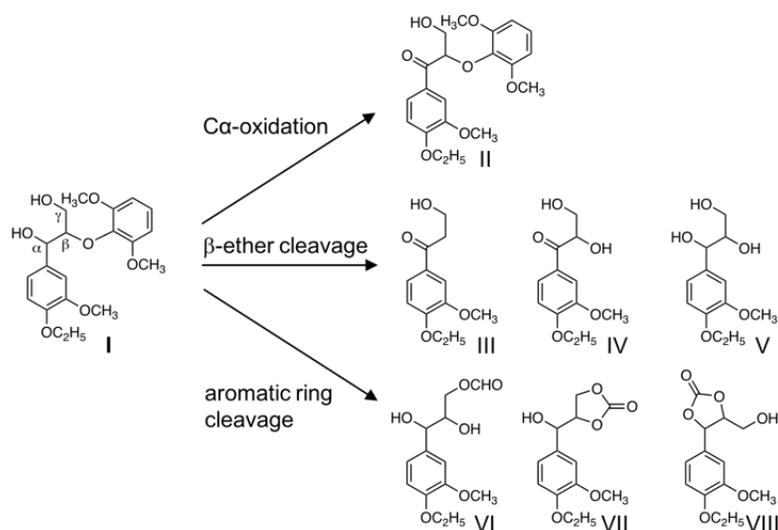
Then, the degradation products of compound **I** by the laccase-HBA system were analyzed by GC-MS. Fig. 4 shows the total ion chromatogram of the acetylated degradation products. As degradation products, a compound **II** which is a  $\alpha$ -position oxidation product, three kinds of  $\beta$ -ether cleavage products, compounds **III-V**, and aromatic ring cleavage products, compounds **VI-VIII** were confirmed as an acetyl derivative. Mass spectrum and retention time of these compounds were completely consistent with those authentic compounds.



**Fig. 4.** Total ion- and mass-chromatograms of the reaction residue of the nonphenolic  $\beta$ -O-4 lignin model compound **I** treated with laccase in the presence of HBA. The products were acetylated.

As the major route of lignin degradation by white-rot fungi, oxidation at the  $C\alpha$  position, cleavage of  $\beta$ -ether bond, cleavage of aromatic ring, and cleavage of  $C\alpha$ - $C\beta$  bond are known<sup>15,16</sup>. The present study indicated that all degradation pathways were confirmed except  $C\alpha$ - $C\beta$  cleavage. Furthermore, the degradation pathways of the compound **I** by lignin peroxidase<sup>11,17</sup>, laccase-HBT system<sup>6-8,18</sup> and horseradish peroxidase-HBT system<sup>19</sup> were quite similar in this study (Fig. 5). Therefore, we conclude that HBA is synthesized *de novo* by white-rot fungi and may act as a natural mediator in lignin degradation of white-rot fungi. The biosynthetic pathway of HBA and its relationship to lignin-degrading activity should be investigated in the future.

Currently, white-rotted wood by *T. versicolor* is being prepared, and we are trying to detect low molecular weight phenols from it. In addition, whether phenolic compounds (vanillin, syringaldehyde, etc.) expected as lignin degradation products function as laccase mediators is under consideration by using of lignin model compounds.



**Fig. 5.** Possible oxidation pathways for the nonphenolic  $\beta$ -O-4 lignin model compound **I** treated with laccase in the presence of HBA.

## CONCLUSIONS

1. Anthracene, which cannot be degraded by laccase alone, was degraded by coexisting the ethyl acetate extract of the extracellular culture fluid of *Trametes versicolor* laccase production medium with laccase.
2. 4-Hydroxybenzoic acid (HBA) was isolated from the culture fluid. It was revealed that this compound is synthesized *de novo* by *T. versicolor* and acts as a natural mediator of the degradation of anthracene by laccase.
3. The laccase-HBA system could also oxidize nonphenolic  $\beta$ -O-4 lignin model dimer. The oxidative mechanisms are very similar to those of the lignin peroxidase and laccase-1-hydroxybenzotriazole (HBT) systems.

## ACKNOWLEDGEMENT

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