Role of 1-hydroxybenzotriazole in oxidation by laccase from Trametes versicolor. Kinetic analysis of the laccase-1-hydroxybenzotriazole couple

FEMS Microbiology Letters

Role of 1-hydroxybenzotriazole in oxidation by laccase from *Trametes versicolor*.

Kinetic analysis of the laccase-1-hydroxybenzotriazole couple

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Abstract

In the current studies, we used Lineweaver-Burke analysis to examine the role of 1-hydroxybenzotriazole (HBT) in the oxidation of various compounds by laccase from *Trametes versicolor*. At low concentrations, HBT was a competitive inhibitor of the oxidation, but at high concentrations, it was a noncompetitive inhibitor. Analysis of the oxidation of ferrocytochrome c by the laccase-HBT couple showed that increasing the concentration of ferrocytochrome c did not affect the V_{max} but reduced the apparent K_m. In addition, in the manganese peroxidase-Mn(II) reaction, which is a typical oxidation system, the apparent K_m and V_{max} increased as the concentration of the substrate 2,6-dimethoxyphenol was increased. These results indicate that HBT is involved in the binding of laccase and substrates that laccase cannot oxidize alone.

Keywords: *Trametes versicolor*, laccase; 1-hydroxybenzotriazole; kinetic analysis; Lineweaver-Burke plot

1. Introduction

Laccase is a polyphenol oxidase and a member of the blue multicopper oxidase family [1]. This enzyme catalyzes the one-electron oxidation of four reducing-substrate molecules in conjunction with the four-electron reduction of molecular oxygen to water [1]. Laccase oxidizes a broad range of substrates, especially phenolic compounds and aromatic amines. Alone, laccase cannot oxidize nonphenolic compounds because it has a lower redox potential, but it can oxidize them in the presence of a suitable redox mediator [2].

The role of redox mediators in the laccase oxidation reaction is now well characterized. When a substrate is oxidized by a laccase, the redox mediator forms cation radicals (short-lived intermediates), that co-oxidize the substrate. These cation radicals can be formed by two mechanisms: (i) the redox mediator can perform either a one-electron oxidation of the substrate to a radical cation [3-5]; or (ii) the redox mediator can abstract a proton from the substrate, converting it into a radical [6, 7]. For example, 2,2′-azino-di-(3-ethyl-benzothiazolin-sulfonate) acts by the first mechanism [8, 9], whereas 1-hydroxybenzotriazole (HBT) acts by the second [10].

Kawai et al. reported that the laccase-HBT couple can catalyze the aromatic ring

cleavage of a nonphenolic β-*O*-4 lignin model dimer as well as cleavage of β-ethers and Cα-Cβ bonds and oxidation of Cα [11, 12]. Thus, the laccase-HBT couple carries out not only the oxidation of Cα in the lignin model compound but also the oxidation of the π-electron in the aromatic ring of the lignin model compound, which is similar to the reaction mediated by lignin peroxidases from white-rot fungi [13-15]. Because the nonphenolic β-*O*-4 lignin model dimer is chemically oxidized by the HBT radical, it appears that oxidation of the Cα in the lignin model compound occurs more easily than that of the π-electron in the aromatic ring of the lignin model compound. Therefore, HBT may have a function in addition to acting as a radical mediator in the laccase-HBT couple reaction. In the current studies, we carried out kinetic analysis of the laccase oxidation reaction to clarify the role of HBT.

2. Materials and methods

2.1 Fungi

Trametes versicolor IFO-6482 and *Phanerochaete chrysosporium* ME-446 were maintained on potato dextrose agar slants at 4°C.

2.2 Chemicals

Guaiacol (GU), horseradish peroxidase (HRP), and ferricytochrome c were purchased from Wako Pure Chemicals. All other chemicals were the highest purity available and used without further purification.

2.3 Oxidation of GU by laccase or HRP in the presence of HBT

Laccase from *T. versicolor* was purified as described in our previous report [16]. The oxidation of GU to the tetramer was monitored at 470 nm ($\varepsilon = 26.6\ mM^{-1}cm^{-1}$). GU reaction mixtures (1 ml) contained laccase (0.16 nkat), GU (75–300 μM), and HBT (0–5 mM) in 50 mM malonate buffer (pH 4.5). The reactions were initiated by the addition of laccase at 30°C. HRP reaction mixtures (1 ml) contained HRP (0.015 nkat), GU (0.7–6 mM), and HBT (0–5 mM) in 20 mM phosphate buffer (pH 7.0). The

reactions were initiated by the addition of 0.2 mM hydrogen peroxide at 30°C.

2.4 Oxidation of ferrocytochrome c (CYC) by the laccase-HBT couple

CYC was prepared as described by Wariishi et al. [17]. The oxidation of CYC to ferricytochrome c was monitored at 550 nm. Reaction mixtures (1 ml) contained laccase (0.06 nkat), HBT (1–4 mM), and CYC (2.47–9.86 μM) in 50 mM malonate buffer (pH 4.5). The reactions were initiated by the addition of laccase at 30°C.

2.5 Oxidation of 2,6-dimethoxyphenol (DMP) by the manganese peroxidase (MnP)-Mn(II) couple

Purified MnP was isolated from a culture of *P. chrysosporium* ME-446 as described by Wariishi et al. [18]. The oxidation of DMP to the coerulignone by the MnP-Mn(II) couple was monitored at 470 nm ($\varepsilon = 49.6\ mM^{-1}cm^{-1}$). The reaction (1 ml) contained MnP (0.05 nkat), $MnSO_4$ (5–20 μM), and DMP (0.5–5 mM) in 50 mM malonate buffer (pH 4.5). The reactions were initiated by the addition of 0.2 mM hydrogen peroxide at 30 °C.

3. Results

3.1 Kinetic analysis of GU oxidation by laccase or HRP in the presence of HBT

We analyzed the ability of HBT to inhibit the oxidation of GU by laccase and HRP. If HBT is a substrate of laccase and HRP, it should act as a competitive inhibitor vs. GU. Firstly, we analyzed the effect of HBT concentration on the inhibition of oxidation of GU by laccase and HRP. Under the presence of 1 mM HBT, the oxidation of GU by both enzymes was hardly inhibited. On the other hand, the oxidation of GU by both enzymes was drastically inhibited over the presence of 5 mM HBT, and did not obey in principle the Michaelis-Menten model. It is thought that both enzymes are inactivated by high concentration of HBT. Therefore, 1-5 mM HBT was used in the present study. Lineweaver-Burke plots (1/initial velocity vs. 1/concentration of HBT) gave parallel lines at various concentrations of HBT (Fig. 1), indicating uncompetitive inhibition vs. GU. Similarly, Lineweaver-Burke plots for the laccase-mediated oxidation of GU showed parallel lines when HBT was present (Fig. 2); however, the plot intersected on the Y-axis in the absence of HBT and on the X-axis in the presence of 1 or 5 mM HBT. These results indicate that the mechanism of laccase inhibition by HBT depends on its concentration; at low concentrations, HBT acts as a competitive inhibitor and at high concentrations, it acts as a noncompetitive inhibitor.

3.2 *Kinetic analysis of CYC oxidation by the laccase-HBT couple*

We next examined the kinetics of CYC oxidation, which is oxidized by the laccase-HBT couple but not by laccase alone. Lineweaver-Burke plots at various concentrations of HBT in the presence of 2.47–9.86 μM of CYC showed that increasing the concentration of CYC had no affect on the V_{max} but reduced the apparent K_m (Fig. 3 and Table 1).

For comparison, we also examined the kinetics of DMP by the MnP-Mn(II) couple, which is a typical oxidation system. DMP is oxidized by the MnP-Mn(II) couple by not by MnP alone. In this reaction, Mn(II) is a radical mediator, and Mn(III), which is produced by MnP, directly oxidizes DMP. Lineweaver-Burke plots of the initial rate of DMP oxidation showed parallel lines, and both the V_{max} value and apparent K_m values increased as the concentration of DMP was raised (Fig. 4 and Table 2).

4. Discussion

Laccase is a copper-containing enzymes that catalyzes the oxidation of electron-rich substrates such as phenols. Alone, laccase is of limited use for bioremediation due to its specificity for phenolic subunits in lignin. Redox-mediated catalysis by laccase, however, is used in a wide range of applications such as delignification of wood pulp, organic synthesis, and degradation of polycyclic aromatic hydrocarbons, pesticides, and insecticides [19]. HBT is widely used as redox mediator for these reactions, but oxidized HBT is very unstable and decays rapidly [3], bringing into question whether HBT is a radical mediator.

In the present study, we performed kinetic analysis to clarify the role of HBT in oxidation by the laccase-HBT couple. For HRP, HBT was an uncompetitive inhibitor vs. GU, indicating that that HBT binds to the enzyme-substrate complex (HRP-GU), forming a ternary complex (HRP-GU-HBT). For laccase, on the other hand, the mechanism of inhibition of HBT depended on its concentration; at low concentrations (around 1 mM), HBT is a competitive inhibitor vs. GU, whereas at high concentrations (5 mM ≥ HBT ≥ 1 mM), it was a noncompetitive inhibitor. Moreover, these results show that the role of HBT is different in the laccase and HRP reactions.

We also found that increasing the concentration of DMP in the MnP-Mn(II)

couple increased the V_{max} and apparent K_m. This indicates that increasing the concentration of DMP raised the turnover rate but reduced the binding of Mn(II) to MnP. If HBT is a radical mediator, the kinetic pattern for the oxidation of CYC by the laccase-HBT couple would be the same as for oxidation of DMP by the MnP-Mn(II) couple. However, the V_{max} value did not change and the K_m value decreased as the concentration of CYC was raised. These results suggest that HBT is involved in the binding of compounds that cannot normally be bound and oxidized by laccase.

Piontek et al. reported that the trinuclear copper center of laccase from *T. versicolor* lies between domains 1 and 3 and is buried approximately 12 Å inside the enzyme, whereas the mononuclear copper of the T1 center is embedded in domain 3, approximately 6.5 Å below the enzyme surface [1]. The copper occupies a depression in the enzyme surface, delimited by a β-turn in domain 1 and two β-turns in domain 3 that are involved in substrate binding. This explains why large molecules cannot access the substrate binding site of laccase. Penetration of HBT into the depression of the enzyme surface, however, could provide access to the substrate binding site of laccase via an indirect electron transfer pathway.

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Table 1. Apparent kinetic parameters for laccase in the oxidation of CYC by the laccase-HBT couple.

CYC (μM)	K_m (mM)	V_{max} (μmol/min)
2.47	3.38	1.30
4.93	2.88	1.31
9.86	2.62	1.32

Table 2. Apparent kinetic parameters for MnP in the oxidation of DMP by the MnP-Mn(II) couple.

DMP (mM)	K_m (μM)	V_{max} (μmol/min)
0.5	5.68	1.02
1.0	6.82	1.25
1.5	10.1	1.83

Figure legends

Fig. 1. Lineweaver-Burke plots of GU oxidation by HRP. The reactions contained 0 (diamond), 3 (square), or 5 mM (triangle) HBT.

Fig. 2. Lineweaver-Burke plots of GU oxidation by laccase. The reactions contained 0 (diamond), 1 (square), 2 (triangle), or 5 mM (circle) HBT.

Fig. 3. Lineweaver-Burke plots of CYC oxidation by the laccase-HBT couple. The reactions contained 2.47 (diamond), 4.93 (square), or 9.86 μM (triangle) CYC.

Fig. 4. Lineweaver-Burke plots of DMP oxidation by the MnP-Mn(II) couple. The reactions contained 0.5 (diamond), 1 (triangle), or 5 mM (square) DMP.

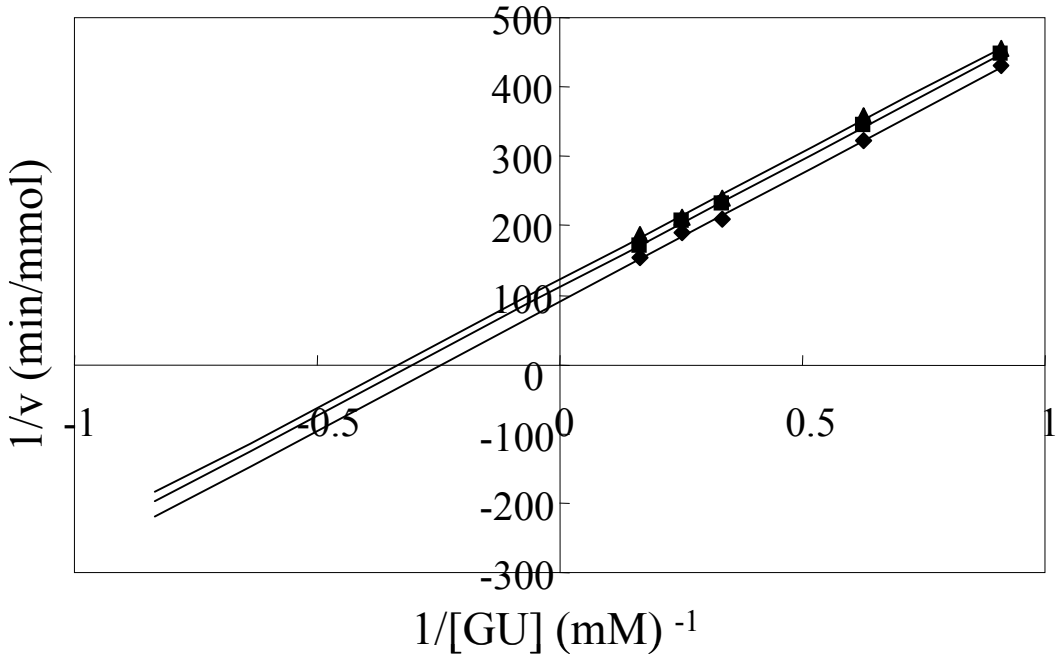
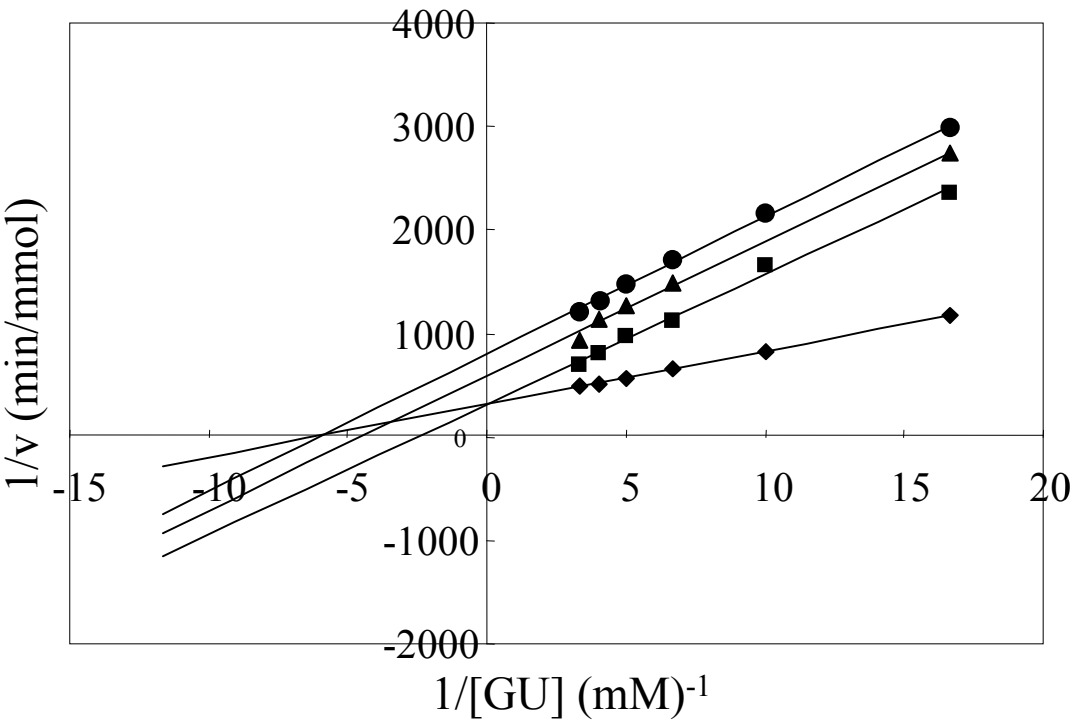


Fig. 1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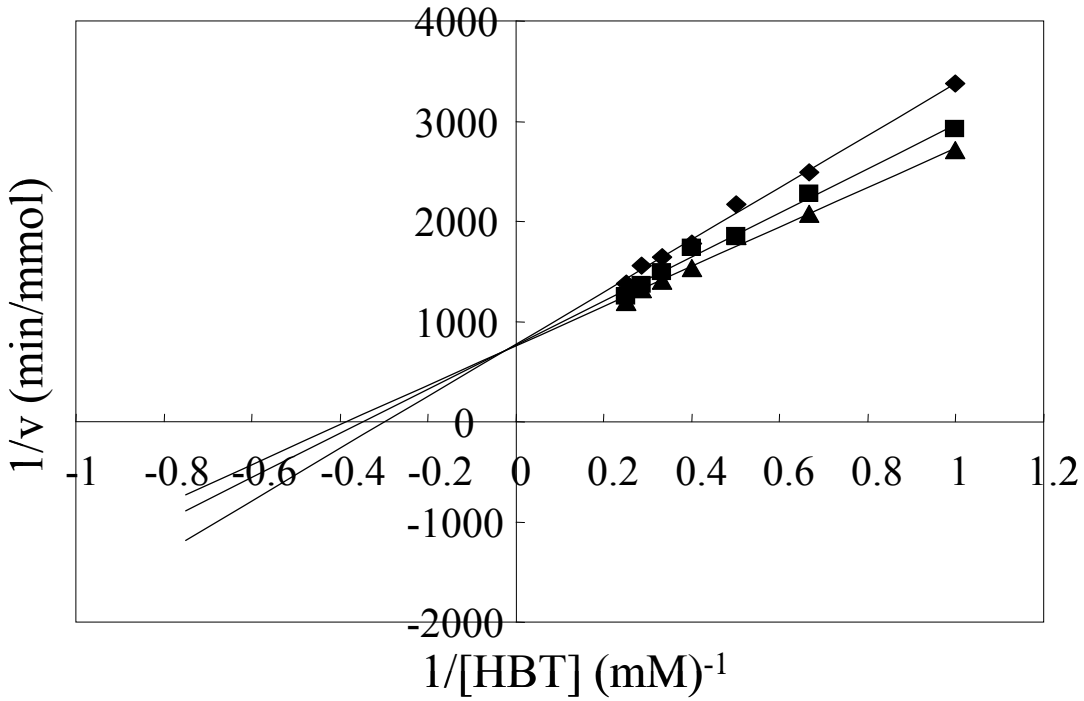
1/v (min/mmol)
1/[GU] (mM)-1

Fig. 2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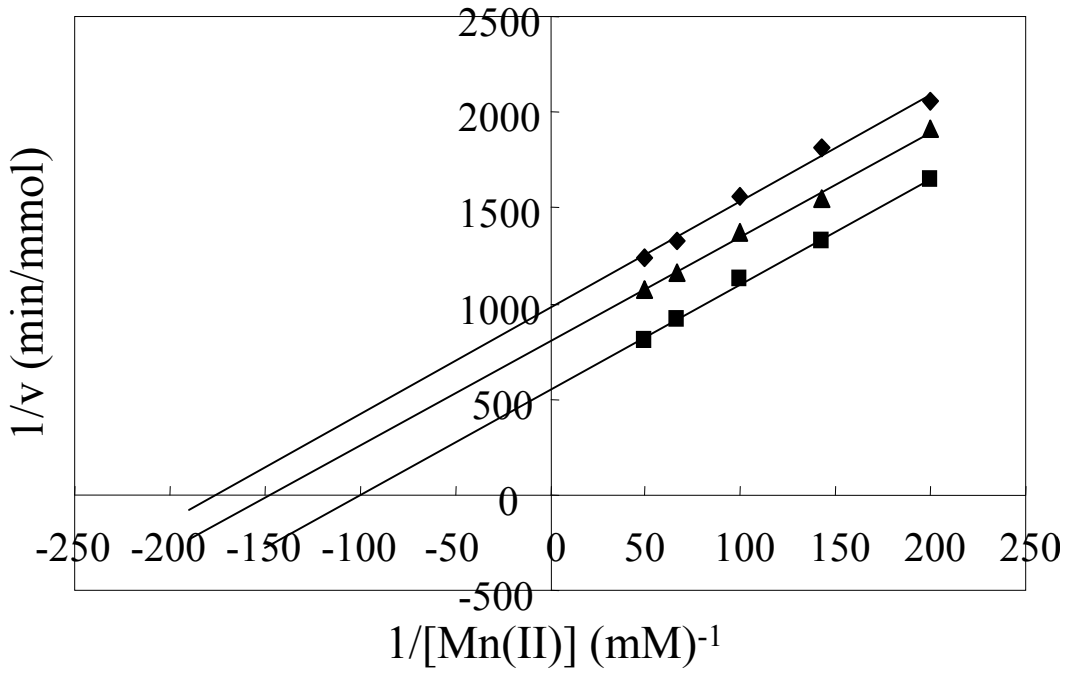
1/[HBT] (mM)-1

Fig. 3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1/v (min/mmol)
1/[Mn(II)] (mM)-1

Fig. 4