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Involvement of veratryl alcohol and active oxygen species in degradation of a quinone compound by lignin peroxidase

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Degradiation of 2-hydroxy-1,4-naphthoquinone (HNQ) by lignin peroxidase is discussed. Degradation rat was remarkably increased by an increase in veratryl alcohol concentration. Degradation is partly prevented by adding $OH^{\cdot}$ scavenger (mannitol or DMSO) to the reaction mixture. Addition of $O_2^{\cdot-}$ scavenger (Mn^{2+}) to the reaction mixture completely prevents the degradation. These results suggest that active oxygen species formed in the lignin peroxidase–H_2O_2–veratryl alcohol system play an important role in HNQ degradation.

Phanerochaete chrysosporium; Lignin peroxidase; Superoxide anion; Quinone; Decolorization; Veratryl alcohol

1. INTRODUCTION

When lignin model compounds are degraded by phenol oxidases such as lignin peroxidase (LiP), Mn-peroxidase (Mn-P), and laccase, quinone-type compounds are often produced as one of the main end products [1–3]. These quinone-type compounds have been thought to resist to the further oxidative degradation by these enzymes, and to cause polymerization of lignin samples during treatment with these enzymes [4,5]. On the other hand, the degradation of quinone-type structures was proved to be closely associated with the decolorization of the alkaline extraction stage effluent from pulp bleaching mills by the white rot fungus, *Phanerochaete chrysosporium* [6]. In previous reports, we showed that 2-hydroxy-1,4- naphthoquinone (HNQ) is degraded by LiP and H_2O_2 when veratryl alcohol is present in the reaction mixture and that the oxidation of veratryl alcohol proceeds only after HNQ degradation is completed [7,8]. In this report, we propose the roles of veratryl alcohol and active oxygen species on HNQ degradation by the LiP-H_2O_2 system.

2. MATERIALS AND METHODS

2.1. *Enzyme purification*

Extracellular fluid containing LiP was harvested from ligninolytic culture of *P. chrysosporium* Burds (ME-446) [8]. The concentrated crude enzymes were applied to a TOYO Parl DEAE-650M column and eluted by 5 mM acetate buffer (pH 5.5) with a 0–0.15 M NaCl gradient.

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2.2. *Degradation of HNQ in the presence of veratryl alcohol*

Lignin peroxidase activity was assayed at 30°C in 0.1 M Na-acetate buffer (pH 3.0) containing 100 μM veratryl alcohol and 60 μM H_2O_2.

HNQ was incubated at 30°C in 0.1 M Na-acetate buffer (pH 3.0) containing LiP (0.02 U), H_2O_2, HNQ, and veratryl alcohol. The reaction was initiated by adding H_2O_2 to the reaction mixture. UV-absorption spectra were recorded on a Shimadzu UV-240 spectrophotometer at 30°C.

2.3. *Effect of scavengers of active oxygen species*

The basic reaction mixture contained LiP (0.02 U), HNQ (50 μM), H_2O_2 (60 μM), veratryl alcohol (100 μM), and a scavenger at pH 3.0. UV spectra were recorded in the presence or absence of Mn^{2+} on a Shimadzu UV-160 spectrophotometer at 30 s intervals.

3. RESULTS

3.1. *Effect of veratryl alcohol*

The degradation of HNQ by LiP is shown in Fig. 1. HNQ was not degraded by H_2O_2 or LiP alone and was only slowly degraded by the LiP–H_2O_2 system. On the other hand, HNQ was rapidly degraded by adding veratryl alcohol to the LiP–H_2O_2 system (Fig. 1). Degradation was accelerated by increasing the amount of veratryl alcohol in the reaction mixture (Fig. 2).

HNQ was slowly degraded by the LiP–H_2O_2 system in the absence of veratryl alcohol. After HNQ was incubated with this sytem for 1–5 min, veratryl alcohol (final concentration was 0.5 mM) was added to the reaction mixture (Fig. 3). The degradation rate increased remarkably by this addition. The increase in degradation rates was almost independent of the time when veratryl alcohol was added.

3.2. *Effect of scavengers of active oxygen species*

The degradation of HNQ in the presence of hydroxy radical scavengers is shown in Fig. 4. Both mannitol

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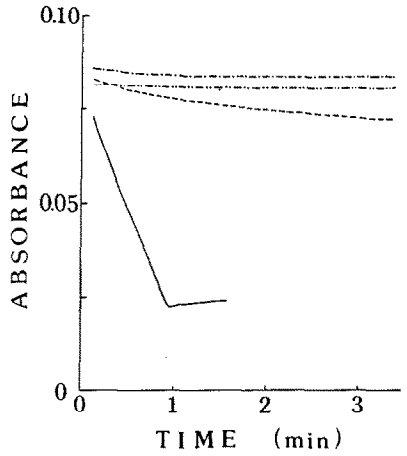

Fig. 1. Degradation of HNQ by LiP-H_2O_2-veratryl alcohol system. Concentrations of HNQ, veratryl alcohol, and H_2O_2 are 50, 100, and 60 μM, respectively. The reaction was started by adding H_2O_2 to the reaction mixture. Absorbance was monitored at 360 nm. (-·-·-·-·-) in the absence of LiP; (-----) in the absence of H_2O_2; (– – –) in the absence of veratryl alcohol; (——) in the presence of all components.

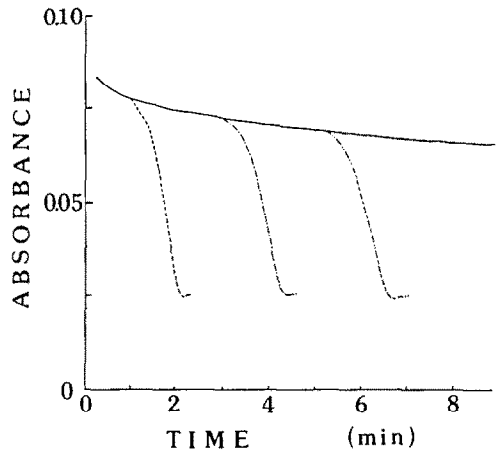

Fig. 3. Appearance of degradation activity by the veratryl alcohol addition. Veratryl alcohol (final concentration was 0.5 mM) was added into the reaction mixture after 1–5 min incubation of HNQ (50 μM) with LiP-H_2O_2 (60 μM) system. Veratryl alcohol was added after 1 (-----), 3 (-·-·-·) and 5 (-·-·-·-·-) minutes incubation, respectively.

(Fig. 4a) and DMSO (Fig. 4b) prevented the degradation of HNQ by the LiP-H_2O_2-veratryl alcohol system to some extent. However, the veratryl alcohol oxidation was not affected by mannitol or DMSO. Although thiourea, a powerful $OH^{\bullet}$ scavenger, inhibited HNQ degradation, it also inhibited veratryl alcohol oxidation when HNQ was absent.

As a scavenger of $HO_2^{\bullet}$, Mn^{2+} was examined. When HNQ was absent, the presence of Mn^{2+} did not inhibit veratryl alcohol oxidation. The presence of 1 mM Mn^{2+} inhibited the HNQ degradation perfectly (Fig. 5). Furthermore, it was found that when HNQ is present, the veratryl alcohol oxidation is also inhibited by the addition of Mn^{2+}. Figure 6a showed that neither the decrease in absorbance at 360 nm (corresponding to the HNQ degradation), nor the formation of an absorption maximum at 310 nm, characteristic for veratraldehyde, are observed. This in contrast with the spectral change of HNQ-veratryl alcohol mixture without Mn^{2+} (Fig. 6b), where subsequent occurrence of HNQ degradation and veratryl alcohol oxidation is clearly demonstrated [7].

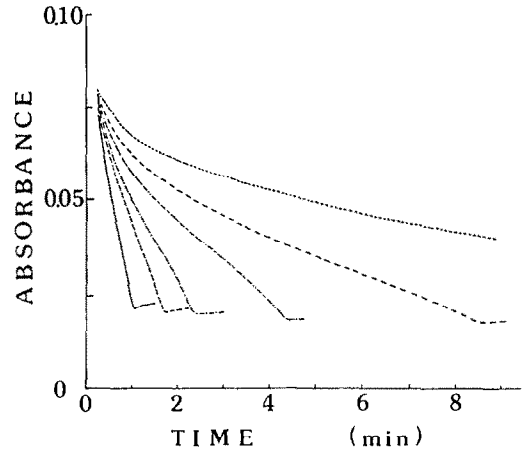

Fig. 2. Effect of veratryl alcohol concentration on HNQ degradation. Concentrations of HNQ and H_2O_2 are 50 and 60 μM, respectively. Veratryl alcohol concentrations are as follows: 10 μM (···); 20 μM (– – –); 30 μM (-·-·-·-·-·-); 40 μM (-·-·-); 60 μM (– – –); 100 μM (——). The reaction was started by adding H_2O_2 to the reaction mixture. Absorbance was monitored at 360 nm.

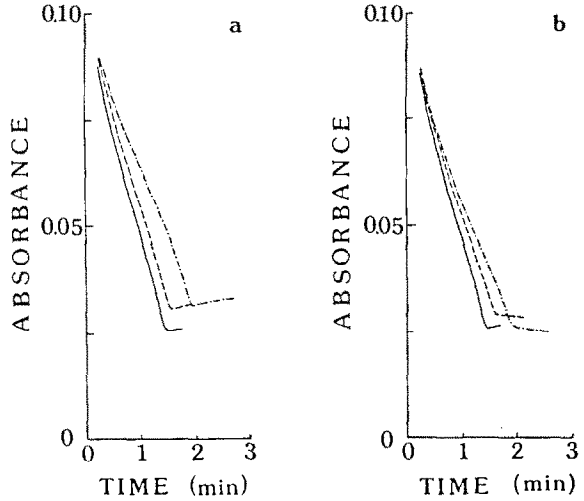

Fig. 4. Effect of hydroxy radical scavenger on HNQ degradation. HNQ (50 μM) was treated by the LiP-H_2O_2 (60 μM)-veratryl alcohol (100 μM) system in the presence of mannitol or DMSO. The reaction was started by adding H_2O_2 to the reaction mixture. Absorbance was monitored at 360 nm. (a) Effect of mannitol on HNQ degradation. Concentration of mannitol: 0 mM (——), 10 mM (– – –), 100 mM (-·-·-). (b) Effect of DMSO on HNQ degradation. Concentration of DMSO: 0 mM(——), 10 mM (– – –), 100 mM (-·-·-).

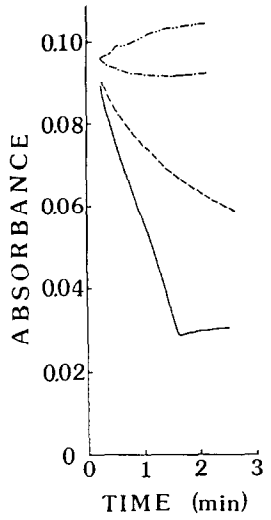

Fig. 5. Effect of superoxide anion radical scavenger on HNQ degradation. HNQ (50 μM) was degraded by LiP-H_2O_2 (60 μM)-veratryl alcohol (100 μM) system in the presence of Mn^{2+} in Na-lactate buffer (pH 3.0). The reaction was started by adding H_2O_2 to the reaction mixture. Absorbance was monitored at 360 nm. Concentration of Mn^{2+}: 0 mM (——), 0.1 mM (– – –), 1 mM (-·-·-), 10 mM (-·-·-·-·-).

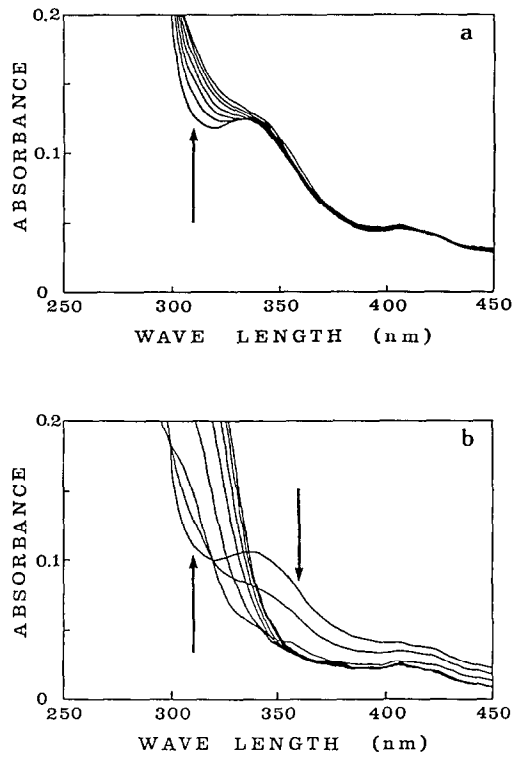

Fig. 6. Change in UV spectra during HNQ degradation by the LiP-H_2O_2-veratryl alcohol system in the presence or the absence of Mn^{2+}. HNQ (50 μM) was degraded by LiP-H_2O_2 (60 μM)-veratryl alcohol (100 μM) system; (a) in the presence of Mn^{2+} (1 mM), or (b) the absence of Mn^{2+}.

4. DISCUSSION

Kuwahara et al. proposed that veratryl alcohol plays a role as a mediator of radical cation in the lignin biodegradation [9]. Haemmerli et al. proposed that veratryl alcohol prevents the inactivation of LiP [10]. Wariishi et al. recently proposed that veratryl alcohol converts an inactivated enzyme (lignin peroxidase compound III*) to a resting enzyme [11] ('rescue' mechanism).

Results of the present study expressed in Figs. 1, 2 and 3 seem to be explained well by any of these proposed roles of veratryl alcohol. Because the increase in veratryl alcohol concentration might be linked to the increase of radical cation formation and/or to the increase of the chance of 'rescue', both of which result in the increase in apparent HNQ degradation activity of LiP. In Fig. 3 the increase in HNQ degradation activity by veratryl alcohol addition was almost constant at each addition. This would probably suggest that final inactivation of LiP, namely further reaction of LiP compound III* with H_2O_2 [11], does not occur to a significant extent in the presence of HNQ.

Contrary to the above description, the results of scavenger experiments (Figs. 4, 5 and 6) strongly suggest that active oxygen species are involved in the HNQ degradation. Among 3 scavengers, Mn^{2+} was most effective, suggesting that HOO$^{\bullet}$ is involved in the degradation of HNQ. Since mannitol and DMSO also had some effect on the HNQ degradation, the active oxygen species which react directly with HNQ might be OH$^{\bullet}$, which is formed via HOO$^{\bullet}$. As such a formation mechanism, Haber-Weiss reaction is known (HOO$^{\bullet}$ + $H_2O_2 \rightarrow$ OH$^{\bullet}$ + OH^- + O_2) [12].

Once a peroxidase (ferric form) is oxidized to compound I with a H_2O_2, an additional H_2O_2 could serve as an electron donor to reduce a peroxidase compound I back to a ferric form via compound II and compound III [11,13]. In such a cycle, no substrate other than H_2O_2 is oxidized and the oxidation product of H_2O_2 is HOO$^{\bullet}$ under an acidic condition. In addition to this, Wariishi and Gold revealed that LiP compound III* is formed from compound III under an excess amount of H_2O_2 and veratryl alcohol can 'rescue' it from the final inactivation by releasing $O_2^{\cdot-}$ [11]. The role of veratryl alcohol in our experiments is probably to stimulate the formation of active oxygen species [11].

When HNQ as well as veratryl alcohol are present in the LiP-H_2O_2 system, it seems as if a redox cycle of LiP only functions for the production of HOO$^{\bullet}$. Veratryl alcohol also functions to release HOO$^{\bullet}$ from LiP compound III* until HNQ is completely degraded by thus formed HOO$^{\bullet}$.

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