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Elimination of carbamazepine by repeated treatment with laccase in the presence of 1-hydroxybenzotriazole

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ABSTRACT

Carbamazepine (CBZP) is used as an antiepileptic drug and is highly persistent. In this study, CBZP was treated with laccase from white rot fungus *Trametes versicolor* in the presence of a redox mediator 1-hydroxybenzotriazole (HBT). A single treatment with laccase and HBT eliminated CBZP by about 22% after 24 h, and repeated treatments with laccase and HBT, which were added to the reaction mixture every 8 h, helped eliminate about 60% of CBZP after 48 h. This suggests that repeated treatment is effective in eliminating CBZP. Mass spectrometric analyses demonstrated that two degradation products of CBZP, 10,11-dihydro-10,11-epoxycarbamazepine and 9(10H)-acridone, were formed via repeated treatment with laccase and HBT.

Keywords:

Carbamazepine; Elimination; Ligninolytic enzymes; Laccase; 1-Hydroxybenzotriazole

1. Introduction

During the last decade, the occurrence and fate of residual pharmaceuticals in wastewater treatment and the environment has attracted increasing interest due to the potential adverse environmental and human health effects. The antiepileptic drug carbamazepine (CBZP) is highly persistent and its elimination efficiency by wastewater treatment plants (WWTPs) is below 10%. As a result, residual CBZP has been ubiquitously detected in WWTP effluents [1].

Recent studies have confirmed the capacity of lignin-degrading white rot fungi for degrading pharmaceuticals such as CBZP, ibuprofen, ketoprofen, clofibric acid, atenolol and propranolol [2-4]. However, no significant elimination of CBZP by ligninolytic enzymes, laccase, manganese peroxidase (MnP) and lignin peroxidase (LiP) from white rot fungi has been observed [2, 5]. On the other hand, we recently demonstrated that ligninolytic enzymes such as laccase and MnP are effective in degrading endocrine disruptors [6-10], pharmaceuticals [11] and personal care products [12]. In this study, we report that repeated treatment with laccase and 1-hydroxybenzotriazole (HBT) is effective in eliminating CBZP.

2. Materials and methods

2.1 Enzyme assay and preparation

Laccase and MnP activities were determined by monitoring the oxidation of

2,6-dimethoxyphenol (DMP), as reported previously [10]. Partially purified MnP was prepared from cultures of *Phanerochaete chrysosporium* ME-446 [6], and laccase was prepared from cultures of *Trametes versicolor* IFO-6482 [13], as described previously.

2.2 Treatment of CBZP with ligninolytic enzymes

For treatment with laccase, the reaction mixture consisted of 2×10^{-5} M CBZP (Wako, Osaka, Japan), partially purified laccase (10 nkat mL^{-1}) and 50 mM malonate buffer (pH 4.5). The laccase-HBT system was used in the same manner, except that HBT (0.2 mM) was added to the reaction mixture. Repeated treatments with laccase and HBT were carried out as follows: laccase (10 nkat mL^{-1}), or both laccase (10 nkat mL^{-1}) and HBT (0.2 mM), was repeatedly added to the reaction mixture every 8 h. For treatment with MnP, the reaction mixture consisted of 2×10^{-5} M CBZP, partially purified MnP (10 nkat mL^{-1}), 50 mM malonate buffer (pH 4.5), $MnSO_4$ (0.1 mM) and glucose (25 mM) plus glucose oxidase (3.33 nkat mL^{-1}; Wako) for H_2O_2 supply. The reaction was performed at 30ºC with stirring at 150 rpm. Each reaction mixture (0.5 mL), before and after enzymatic treatment, was mixed with methanol (0.5 mL) in order to stop the enzymatic reaction, and was kept at -20°C until high-performance liquid chromatography (HPLC) analyses.

2.3 Analyses of CBZP by HPLC

Residual CBZP concentrations in the enzymatic reaction mixtures were determined by

HPLC. HPLC analytical conditions were as follows: Cadenza 5CD-C18 column (75 mm × 4.6 mm i.d.; 5 μm; Imtakt, Kyoto, Japan); mobile phase of 20 mM phosphate (X) and methanol (Y); elution with a linear gradient of 30-100% Y in 30 min after isocratic elution with 30% Y for 5 min; flow rate of 1.0 mL min^{-1}; and detection at 254 nm. In the experiment on repeated treatment with laccase and HBT, residual CBZP concentrations were corrected by the fluid volume of test sample for HPLC analyses and the laccase and HBT solutions that were added to the reaction mixture every 8 h.

2.4 Isolation and characterization of enzymatic intermediate of CBZP

After 48 h of repeated treatment with addition of both laccase and HBT every 8 h, the reaction mixture was extracted with EtOAc after acidification. EtOAc extracts were then repeatedly purified by preparative thin-layer chromatography (PTLC) (Merck Kieselgel 60 F_{254}, solvent: EtOAc/*n*-hexane, 2/1) to give two fractions containing the degradation products of CBZP. The degradation products were analyzed by direct inlet-mass spectrometry (DI-MS, electron impact). DI-MS analysis was performed with a Shimadzu GCMS-QP 5050 gas chromatograph mass spectrometer (70 eV). Authentic 10,11-dihydro-10,11-epoxycarbamazepine and 9(10H)-acridone were obtained from Sigma-Aldrich Co. (St. Louis, MO) and Wako, respectively.

3. Results and discussion

3.1 Elimination of CBZP by treatment with ligninolytic enzymes

In the present study, it was confirmed that no significant elimination of CBZP was obtained by treatment with laccase, which agrees with the results of Marco-Urrea et al. [2] (Fig. 1). However, the laccase-HBT system facilitated the elimination of CBZP when compared to laccase alone; CBZP concentration decreased by about 22% after 24 h of treatment. On the other hand, MnP treatment also eliminated CBZP by about 14% after 24 h (Fig. 1). These results indicate that CBZP elimination by MnP is possible, but the laccase-HBT system is most effective in eliminating CBZP among the three enzymatic treatments.

Figure 2 shows the time courses of residual laccase activity and CBZP elimination during treatment with the laccase-HBT system. The initial laccase activity (10 nkat mL^{-1}) decreased rapidly during the first 4 h of treatment and the residual laccase activity was below 10% after 8 h of treatment, while the profiles for the decrease in laccase activity and CBZP concentration were synchronized. These results indicate that laccase is inactivated by CBZP and/or HBT during treatment. Therefore, the effects of CBZP and HBT on laccase were examined (Fig. 3). In the absence of CBZP and HBT, about 90% and 80% of initial laccase activity remained until 8 and 24 h of incubation, respectively, and no differences were seen in the decrease of laccase activity between the presence and absence of CBZP. On the other hand, marked decreases in laccase activity were observed in the presence of HBT and the behavior

of laccase inactivation in the presence of HBT was the same as that in the presence of both HBT and CBZP. These results strongly suggest that HBT is involved in laccase inactivation, but CBZP is not. It has been proposed that when HBT is oxidized by laccase, the generated HBT radicals actively oxidize nonphenolic lignin model compounds, which are not oxidized by laccase alone [14]. Thus, it is likely that the HBT radicals generated in the laccase-HBT system can eliminate CBZP, and cause laccase to become inactive.

3.2 Elimination of CBZP by repeated treatments with laccase and HBT

As mentioned above, the laccase-HBT system showed potential for the elimination of CBZP, but residual laccase activity was below 10% after 8 h of treatment. We therefore attempted repeated treatment with laccase and HBT in order to enhance the efficiency of CBZP elimination. In this experiment, laccase (10 nkat mL^{-1}), or both laccase (10 nkat mL^{-1}) and HBT (0.2 mM), was added to the reaction mixture every 8 h. As shown in Fig. 4, repeated treatment with the addition of both laccase and HBT helped eliminate about 60% of CBZP after 48 h, during which 8-h treatments were repeated six times. On the other hand, treatment with laccase alone showed about 40% elimination of CBZP after 48 h.

It has been reported that in the laccase-HBT system, HBT is partly converted to benzotriazole (BT), which is inactive as a mediator [15, 16], but the underlying mechanisms of this reaction remain unclear. A possible explanation for the difference in CBZP elimination between these treatments is that the concentration of active HBT differs during repeated

treatment with addition of both laccase and HBT and with laccase alone.

During HPLC analyses of residual CBZP in the reaction mixtures after repeated treatment with the addition of both laccase and HBT, four new chromatographic peaks (three peaks with shortened retention when compared with CBZP, and one peak with delayed retention time) became apparent, and these peaks were thought to be CBZP degradation products. Thus, the isolation of CBZP degradation products was achieved by PTLC and the isolated degradation products were analyzed by DI-MS. The two degradation products with shortened retention when compared with CBZP were then identified as 10,11-dihydro-10,11-epoxycarbamazepine (CBZP-epoxide) and 9(10H)-acridone (Table 1). The mass spectra and retention times of HPLC analyses were identical to those of authentic compounds. Further studies including isolation and characterization of the other two degradation products are presently being conducted in our laboratory.

4. Conclusions

The present study revealed that CBZP may be effectively eliminated by repeated treatment with laccase and HBT. Very little is actually known about the enzymatic elimination of CBZP. Therefore, the present data are the first on the elimination of CBZP and its degradation products by the ligninolytic enzyme laccase in the presence of mediator. It has been reported that two identified degradation products, CBZP-epoxide and 9(10H)-acridone,

are present in the effluent from wastewater treatment plants [17], and that CBZP-epoxide is just as pharmaceutically active as the parent drug CBZP [1, 18]. Thus, evaluation of the ecotoxicity of CBZP degradation products formed by repeated treatment with laccase and HBT should be the subject of further studies.

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Figure Legends

Fig. 1. Decrease in CBZP concentration by enzymatic treatment with MnP, laccase or laccase-HBT system. Indicated for each point are the mean and standard deviation of five experiments. (▲), MnP; (■), laccase; (♦), laccase-HBT system.

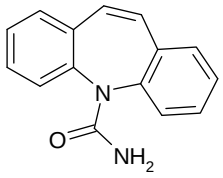
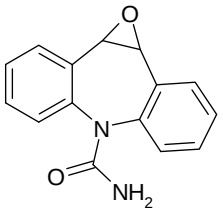
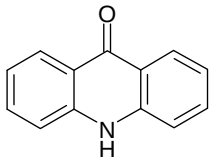
Fig. 2. Relationship between residual laccase activity and CBZP elimination during treatment with laccase-HBT system. Indicated for each point are the mean and standard deviation of five experiments. (■), residual laccase activity; (♦), CBZP elimination.

Fig. 3. Effects of HBT and CBZP on laccase activity. Indicated for each point are the mean and standard deviation of five experiments. (●), without CBZP and HBT; (▲), with CBZP, without HBT; (■), with HBT, without CBZP; (♦), with HBT and CBZP.

Fig. 4. Decrease in CBZP concentration by repeated treatment with addition of both laccase and HBT or laccase alone. Indicated for each point are the mean and standard deviation of five experiments. (♦), with laccase and HBT; (■), with laccase.

Table 1

Chemical structures and analytical data of the CBZP degradation products.

Degradation products	Chemical structure	HPLC retention time (min)	Mass spectrum *m/z* (relative intensity, %)
CBZP (starting compound)		18.3	237 (5.5), 236 (M^+, 30.5), 194 (15.6), 193 (100), 192 (37.5), 191 (21.1), 166 (5.7), 165 (18.1), 164 (5.7), 139 (3.3).
CBZP-epoxide		12.7	253 (7.9), 252 (M^+, 33.6), 224 (5.7), 223 (24.9), 209 (4.1), 208 (8.8), 207 (7.4), 196 (5.3), 195 (8.6), 181 (21.1), 180 (100), 179 (22.1), 153 (5.2), 152 (17.2), 127 (3.4).
9(10H)-acridone		16.5	196(14.5), 195 (M^+, 100), 168 (4.7), 167 (33.7), 166 (14.8), 148 (1.7), 140 (6.7), 139 (9.5), 98 (4.2), 83 (5.2).

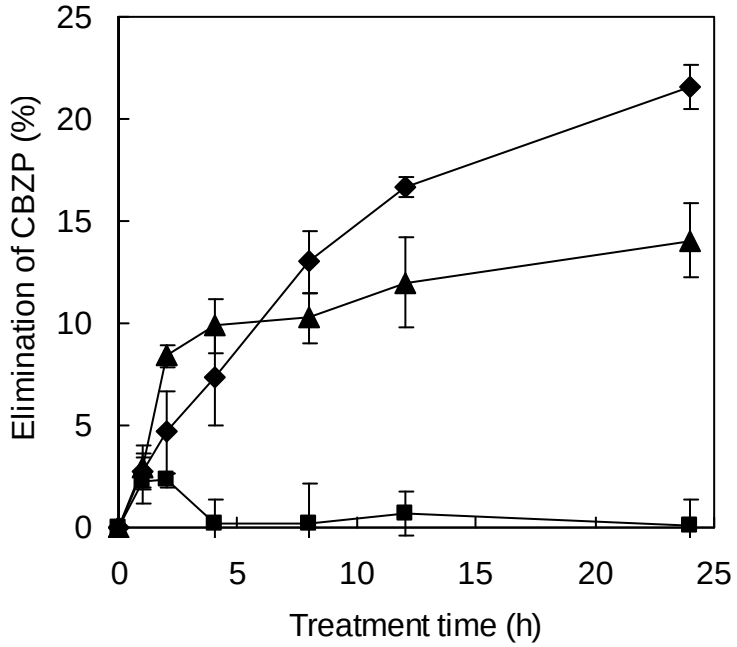
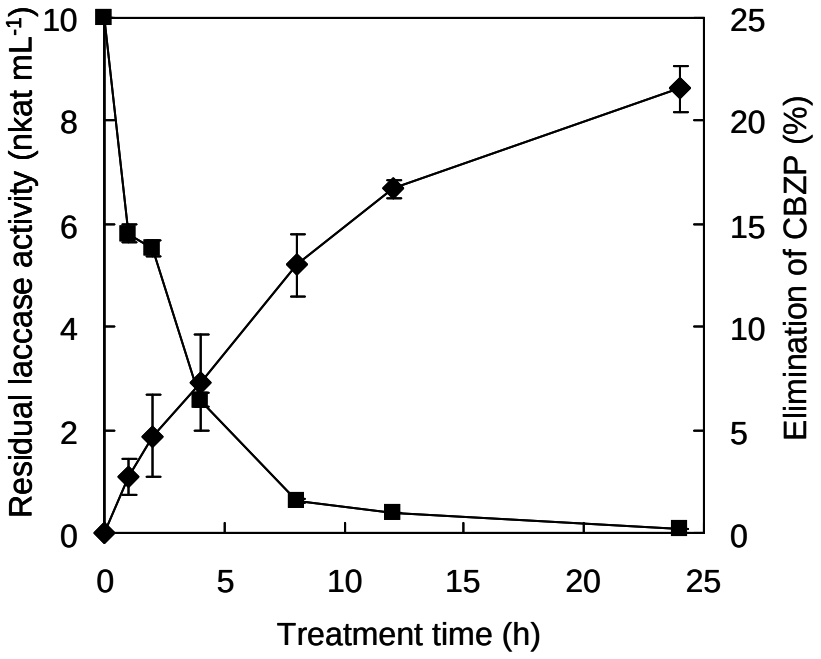


Fig. 1. Decrease in CBZP concentration by enzymatic treatment with MnP, laccase or laccase-HBT system. Indicated for each point are the mean and standard deviation of five experiments. (▲), MnP; (■), laccase; (♦), laccase-HBT system.



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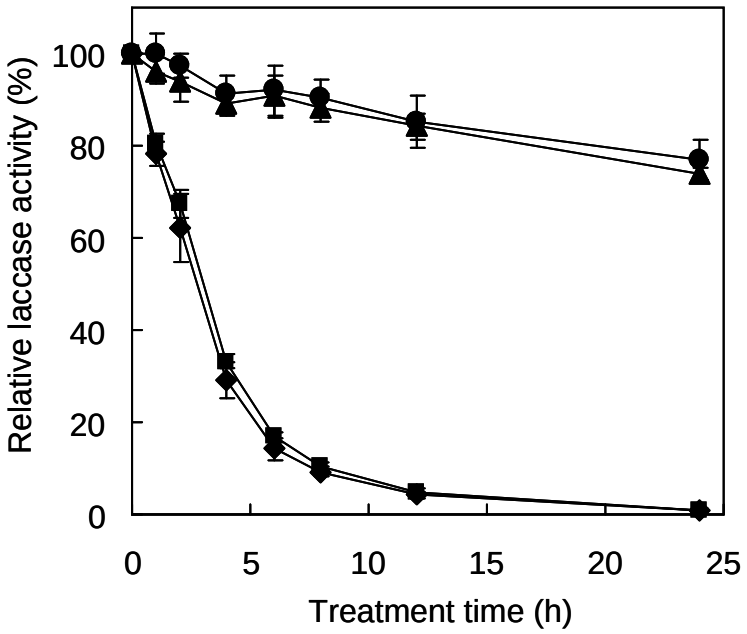

Fig. 3. Effects of HBT and CBZP on laccase activity. Indicated for each point are the mean and standard deviation of five experiments. (●), without CBZP and HBT; (▲), with CBZP, without HBT; (■), with HBT, without CBZP; (♦), with HBT and CBZP.

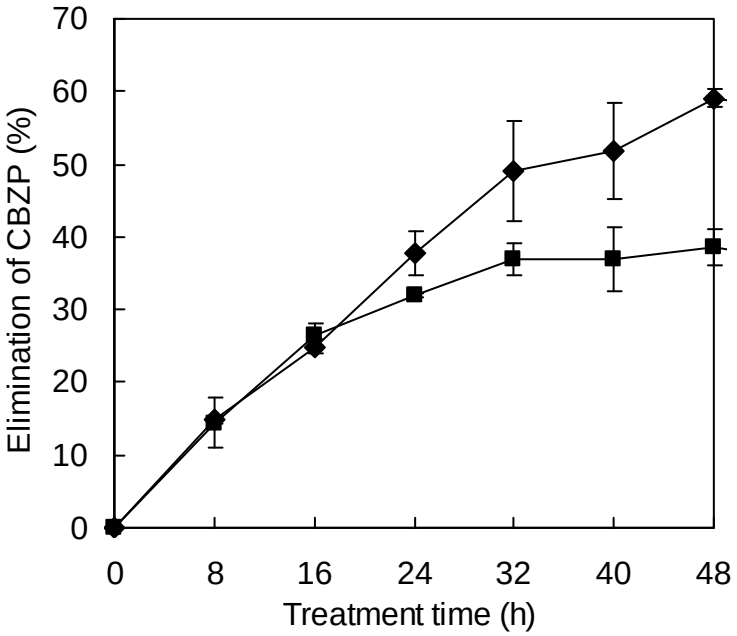

Fig. 4. Decrease in CBZP concentration by repeated treatment with addition of both laccase and HBT or laccase alone. Indicated for each point are the mean and standard deviation of five experiments. (♦), with laccase and HBT; (■), with laccase.