A major laccase isozyme from *Grifola frondosa* (Lac 1) was found to be effective for decolorizing of synthetic dyes and degrading of bisphenol A. The oxidative capability of Lac 1 toward synthetic dyes and bisphenol A was enhanced in the presence of the redox mediator, 1-hydroxybenzotriazole. The major product from the degradation of bisphenol A by Lac 1 was determined to be 4-isopropenylphenol.

**Key words:** laccase; *Grifola frondosa*; dye decolorization; bisphenol A degradation; redox mediator

Laccase (EC 1.10.3.2) is well-known as one of the ligninolytic enzymes produced by most white-rot fungi. Fungal laccases have received considerable attention due to their high capacity for oxidizing lignin-related compounds, as well as highly recalcitrant environmental pollutants such as bisphenol A, poly-cyclic aromatic hydrocarbons and synthetic dyes. These abilities make laccases highly interesting enzymes for a wide variety of applications such as pulp bleaching, textile dye decolorization and bioremediation.

*Grifola frondosa* (Dicks.: Fr.) S.F. Gray (maitake in Japanese) is a very important edible mushroom on the food market, especially in Japan. This fungus produces and secretes laccases into the medium during growth. The laccase of *G. frondosa* has clear advantages in industrial applications, since the use of this enzyme is assumed to be safe even for food processing, and the spent substrate from the cultivation of *G. frondosa*, which is generated by mushroom factories in large amounts, is anticipated to be a good source of laccase. We have recently purified and characterized a major laccase isozyme from *G. frondosa* (Lac 1) and reported that Lac 1 had broad substrate specificity and high stability over a broad pH range and at high temperature, indicating its strong potential for biotechnological applications. We describe in this paper the biotechnological applications of Lac 1 from *G. frondosa* to decolorizing synthetic dyes and degrading of bisphenol A, an endocrine-disrupting chemical.

The decolorization of synthetic dyes was tested in this study with six chemically different samples: azo dye (Methyl Orange), diazo dyes (Congo Red and Reactive Black 5), triphenylmethane dye (Bromophenol Blue, BPB), antraquinone dye (Remazol Brilliant Blue R, RBBR) and heterocyclic dye (Acridine Orange). The effect of different dyes to varying degrees. This enzyme was able to decolorize RBBR and BPB with the highest effectiveness (more than 95% decolorization). Lesser effects on decolorization were observed for Methyl Orange and Congo Red (about 65% and 20%, respectively). However, very low decolorization was apparent for Reactive Black 5 and Acridine Orange. The effect of 1-hydroxybenzotriazole (HBT), a well-known redox mediator, was examined in order to facilitate the enzymatic decolorization. The addition of 2 mM HBT to the reaction mixture markedly enhanced the decolorization of Methyl Orange, Congo Red, Reactive Black 5 and Acridine Orange which all were insufficiently decolorized by Lac 1 alone. Acridine Orange is a widely used dye that is a serious pollutant in waste water. To our knowledge, there is only one report on the enzymatic decolorization of this dye, in which it was mentioned that the yellow laccase from *Pleurotus ostreatus* D1 decolorized this dye with very low efficiency (only 8.5% decolorization within 10 d). This present work is therefore the first reported instance of enzymatic decolorization of *Acridine Orange* with high effectiveness. The enhancement of dye decolorization in the presence of HBT has also been observed with other laccases. It has been suggested that phenolic and nonphenolic compounds with high redox potential can be oxidized by laccases through the mediation of small, redox-active substrates like HBT. It is noteworthy that Lac 1 effectively decolorized RBBR without any mediators, since it has been suggested that decolorization activity toward RBBR is correlated with the degradation capacity toward poly-
Decolorization of the synthetic dyes in the absence (black bars) or presence (gray bars) of HBT was determined spectrophotometrically by measuring the decrease in the absorbance at the wavelength for maximum absorption with each dye. Decolorization was evaluated as Decolorization (% = ([initial absorbance] – [final absorbance]) / [initial absorbance]) × 100.

We next examined the oxidative degradation of bisphenol A by Lac 1. Bisphenol A (0.015%) was incubated with 1.5 U mL⁻¹ of Lac 1 in 50 mM citrate buffer (pH 4.0) and 28 °C in the presence or absence of 2 mM HBT. The remaining amount of bisphenol A in the reaction mixture was quantitatively analyzed at intervals by reverse-phase HPLC, using a Develosil ODS-5 column (4.6 × 250 mm; Nomura Chemicals, Seto, Japan). Gas chromatographic separation was performed in an Inertcap 1MS column (30 m × 0.25 mm ID; GL Sciences, Tokyo, Japan). The oven temperature was programmed to rise from 50 °C to 300 °C at 10 °C min⁻¹, and MS was taken at 70 eV.

The GC-MS analysis of the reaction in the presence of HBT shows that the peak for 4-isopropenylphenol was only detected in the early period of the reaction without HBT, probably due to the formation of polymerized products as previously reported.3,14 To identify the reaction product in the aqueous solution, the reaction mixture was centrifuged at 14,000 g for 15 min to remove the water-insoluble precipitate, and then the reaction products in the supernatant were extracted with ethyl acetate. The ethyl acetate layer was evaporated to dryness, and the resulting residue was redissolved in 100 μL of ethyl acetate. An aliquot (5 μL) of the solution was injected into the HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) which was interfaced with a JMS-SX102A mass spectrometer (JEOL, Tokyo, Japan). Gas chromatographic separation was performed in an Inertcap 1MS column (30 m × 0.25 mm ID; GL Sciences, Tokyo, Japan). The oven temperature was programmed to rise from 50 °C to 300 °C at 10 °C min⁻¹, and MS was taken at 70 eV.

Figure 3 shows two major peaks (A and B) that were detected on the GC chromatogram. Peak A exhibited m/z [relative intensity (%)] shown in parentheses] of 134(M⁺, 100), 119(99), 105(20), 94(17), 91(72), 77(32), 65(44) and 51(7), corresponding to the reported fragmentation pattern of 4-isopropenylphenol.3 Peak B was determined to be the remaining bisphenol A exhibiting m/z of 228(M⁺, 19), 213(100), 119(36), 107(23), 91(37), 77(17), and 65(23). These data reveal that the major product from the oxidative degradation of bisphenol A by Lac 1 was 4-isopropenylphenol. A similar result has previously been reported with the laccase from Trametes villosa.5

The GC-MS analysis of the reaction in the presence of HBT shows that the peak for 4-isopropenylphenol could only be detected in the early period of the reaction. We next examined the oxidative degradation of bisphenol A by Lac 1. Bisphenol A (0.015%) was incubated with 1.5 U mL⁻¹ of Lac 1 in 50 mM citrate buffer (pH 4.0) and 28 °C in the presence or absence of 2 mM HBT. The remaining amount of bisphenol A in the reaction mixture was quantitatively analyzed at intervals by reverse-phase HPLC, using a Develosil ODS-5 column (4.6 × 250 mm; Nomura Chemicals, Seto, Japan). Gas chromatographic separation was performed in an Inertcap 1MS column (30 m × 0.25 mm ID; GL Sciences, Tokyo, Japan). The oven temperature was programmed to rise from 50 °C to 300 °C at 10 °C min⁻¹, and MS was taken at 70 eV.

Figure 3 shows two major peaks (A and B) that were detected on the GC chromatogram. Peak A exhibited m/z [relative intensity (%)] shown in parentheses] of 134(M⁺, 100), 119(99), 105(20), 94(17), 91(72), 77(32), 65(44) and 51(7), corresponding to the reported fragmentation pattern of 4-isopropenylphenol.3 Peak B was determined to be the remaining bisphenol A exhibiting m/z of 228(M⁺, 19), 213(100), 119(36), 107(23), 91(37), 77(17), and 65(23). These data reveal that the major product from the oxidative degradation of bisphenol A by Lac 1 was 4-isopropenylphenol. A similar result has previously been reported with the laccase from Trametes villosa.5
reaction (within 1 h). This peak for 4-isopropenylphenol had disappeared within 24 h of the reaction, whereas the relatively intensive peak of benzotriazole was detected in the GC-MS profile (data not shown). This result suggests that 4-isopropenylphenol, which was generated in the oxidative degradation of bisphenol A, had been completely polymerized to give a water-insoluble product by the action of the HBT radical formed by Lac 1. This is the first report on the fate of the oxidative degradation product from bisphenol A, 4-isopropenylphenol, in the laccase-mediated system. The HBT radical eventually decayed to benzotriazole. This is consistent with the previous report on the laccase/HBT redox system in which it was mentioned that the half-life of the HBT radical was short due to its high reactivity and that it readily decayed to benzotriazole.15)

We have shown here for the first time that Lac 1 from G. frondosa is an effective enzyme having high potential for environmental applications.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (22580376) from the Japan Society for the Promotion of Science (JSPS).

References