

**Removal of estrogenic activities of 17 β -estradiol and ethinylestradiol
by ligninolytic enzymes from white rot fungi**

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Abstract

We investigated whether manganese peroxidase (MnP) and the laccase-mediator system with 1-hydroxybenzotriazole (HBT) as mediator can remove the estrogenic activities of the steroidal hormones 17 β -estradiol (E₂) and ethinylestradiol (EE₂). Using the yeast two-hybrid assay system, we confirmed that the estrogenic activities of E₂ and EE₂ are much higher than those of bisphenol A and nonylphenol. Greater than 80% of the estrogenic activities of E₂ and EE₂ were removed following 1-h treatment with MnP or the laccase-HBT system; extending the treatment time to 8 h removed the remaining estrogenic activity of both steroidal hormones. HPLC analysis demonstrated that E₂ and EE₂ had disappeared almost completely in the reaction mixture after a 1-h treatment. These results strongly suggest that these ligninolytic enzymes are effective in removing the estrogenic activities of E₂ and EE₂.

Keywords: 17 β -Estradiol; Ethinylestradiol; Estrogenic activity; Manganese peroxidase;

Laccase-mediator

1. Introduction

Considerable concern has been expressed recently that steroidal hormones (estrogens) excreted by humans, domestic or farm animals, and wildlife into the environment, in part via sewage treatment plants (STPs), may be disruptive to the endocrine system. The hormones 17 β -estradiol (E₂) and ethinylestradiol (EE₂) have been detected ubiquitously in effluent from STPs and are thought to be responsible for most of the estrogenic effects found in such effluent [1-5].

It has been reported that E₂ is oxidized to estrone, which is further eliminated in aerobic batch experiments containing diluted slurries of activated sludge from an actual STP [6]. A gram-negative aerobic bacterium isolated from an STP, tentatively identified as a *Novosphingobium* sp., has also been demonstrated to degrade E₂ [7]. However, some reports have claimed that the elimination of E₂ is attributable primarily to adsorption of E₂ into the activated sludge or to other factors independent of microbial degradation [8, 9]. Furthermore, very little is known about the actual biodegradation of EE₂ [6, 7], and no attempts have been made, other than with bacteria, to degrade E₂ and EE₂ using microorganisms. Great interest is currently being expressed in lignin-degrading white rot fungi and their ligninolytic enzymes, due to a recognized potential for degrading and detoxifying recalcitrant environmental pollutants such as dioxins [10], chlorophenols [11], polycyclic aromatic hydrocarbons [12, 13], and dyes [14]. Manganese peroxidase (MnP), lignin peroxidase (LiP), and laccase produced

extracellularly by lignin-degrading fungi have been demonstrated to be involved in the degradation of lignin and these pollutants.

MnP is a heme peroxidase produced by white rot fungi and catalyzes the oxidation of Mn(II) to Mn(III) in the presence of hydrogen peroxide (H_2O_2). Malonate, oxalate, and α -hydroxy acids such as malate, lactate, and tartrate chelate the generated Mn(III) and release Mn(III) from the manganese-binding site of MnP. The released Mn(III)-organic acid complex in turn oxidizes various phenolic compounds, including lignin. Fungal laccase is a multicopper oxidase that catalyzes single-electron oxidation of phenolic compounds by reducing molecular oxygen to water. In the presence of a mediator such as 1-hydroxybenzotriazole (HBT) or 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate), laccase is capable of oxidizing nonphenolic compounds that it cannot oxidize alone. Recently, we demonstrated that MnP and the laccase-HBT system are effective in degrading polyethylene and nylon [15-19] and in removing the estrogenic activities of bisphenol A (BPA) and nonylphenol (NP) [20]. In this study, we report the application of MnP and the laccase-HBT system to the treatment of E_2 and EE_2 and describe the removal of their estrogenic activities as measured by the yeast two-hybrid system [21].

2. Materials and Methods

2.1 Chemicals and enzyme preparations

E₂ and EE₂ used in this study were extrapure grade (Tokyo Chemical Industry, Tokyo, Japan). All other chemicals, also of extrapure grade, were obtained from commercial sources and were used without further purification. Partially purified MnP and laccase were prepared from cultures of *Phanerochaete chrysosporium* ME-446 and *Trametes versicolor* IFO-6482, respectively, as described in our previous reports [20, 22]. The partially purified MnP contained no laccase activity, and vice versa; LiP activity was not detected in either enzyme preparation.

2.2 Enzyme assay

Laccase activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 470 nm. The reaction mixture contained 1 mM DMP and 50 mM malonate buffer (pH 4.5). MnP activity was determined in the same manner except that the reaction mixture also contained 0.1 mM MnSO₄ and 0.2 mM H₂O₂. One katal (kat) of enzyme activity is the amount of enzyme producing 1 mol of the quinone dimer from DMP per second [23].

2.3 Enzymatic treatment of E₂ and EE₂

For treatment with MnP, the reaction mixture consisted of 10⁻⁵ M E₂ or EE₂,

partially purified MnP (10 nkat/ml), 50 mM malonate buffer (pH 4.5), MnSO₄ (0.1 mM), Tween 80 (0.1%), and glucose (25 mM) and glucose oxidase (3.33 nkat/ml; Wako, Osaka, Japan) to supply H₂O₂. The reaction was performed at 30°C while stirring at 150 rpm. Laccase treatment was conducted in the same manner except that laccase (10 nkat/ml) and 0.2 mM HBT were used and MnSO₄, Tween 80, glucose, and glucose oxidase were omitted from the reaction mixture.

2.4 Estrogenic activities of E₂ and EE₂ treated with ligninolytic enzymes

The estrogenic activities of E₂ or EE₂ before and after enzymatic treatment were evaluated by an *in vitro* screening test for chemicals with hormonal activities that used the yeast two-hybrid estrogenic assay system, developed by Nishikawa *et al.* [21]. The concentrations of E₂ and EE₂ before enzymatic treatment were 10⁻⁷ M in the assay system (2.5 μl of reaction mixture containing 10⁻⁵ M E₂ or EE₂ added to 50 μl of yeast culture and 200 μl of SD medium [20]). Relative estrogenic activity (%) was defined as the percentage of β-galactosidase activity of enzyme-treated E₂ or EE₂ compared to that of untreated E₂ and EE₂. For the experiments described in Fig. 1, 2.5 μl of various concentrations of E₂, EE₂, BPA, and NP dissolved in dimethyl sulfoxide were used instead of reaction mixtures to evaluate their estrogenic activities.

2.5 Analyses of E₂ and EE₂ treated with ligninolytic enzymes

Residual E₂ or EE₂ concentrations in the enzymatic reaction mixtures were determined by high-performance liquid chromatography (HPLC) analysis. HPLC analyses were carried out with a Wakosil-II 5C18HG (Wako) column using an isocratic elution with 50% acetonitrile aqueous containing 1% acetic acid, a flow rate of 1 ml/min, and detection at 285 nm.

3. Results and Discussion

Although the biodegradation of E₂ and EE₂ has been studied previously, those studies dealt with the consumption or disappearance of the target chemicals. The greatest focus concerning the biodegradation of an endocrine-disrupting (estrogenic) chemical should be on the removal of this activity. We therefore have attempted to assay the estrogenic activities of the reaction mixtures of E₂ and EE₂ during enzymatic treatment using the yeast two-hybrid estrogenic assay system. This system is newly developed and is based on the ligand-dependent interaction between the nuclear hormone receptor and its coactivator. The method is rapid and has been confirmed to be reliable for measuring estrogenic activity [21].

The estrogenic activities of E₂ and EE₂, expressed as β -galactosidase activities, are compared with those of BPA and NP (Fig. 1). The activities of E₂ (10^{-9} M) and EE₂ ($\sim 5 \times 10^{-9}$ M) were almost the same as those of BPA (10^{-4} M) and NP (10^{-5} M),

indicating that E₂ and EE₂ have much higher estrogenic activities at much lower concentrations than do BPA and NP, which are widely used in a variety of industrial and residential applications. This finding is consistent with that of the previous report in which the relative potencies of the estrogenicities of EE₂, BPA, and NP were 0.5, 5×10^{-5} , and 4×10^{-4} , respectively, compared with E₂ [24].

Figure 2 demonstrates that MnP and the laccase-HBT system reduced the estrogenic activities of E₂ and EE₂ by over 80% after a 1-h treatment and completely removed those activities after 8 h of treatment. On the other hand, HPLC detection with an ODS column (285 nm) of the residual E₂ and EE₂ during enzymatic treatment revealed that both E₂ and EE₂ almost completely disappeared in the reaction mixture after a 1-h treatment with either the MnP or the laccase-HBT system; the residual concentrations of E₂ and EE₂ were below HPLC detection limits (data not shown). Figure 1 shows that E₂ and EE₂ exhibit estrogenic activities at very low concentrations. Thus, the fact that 10-20% of the estrogenic activities of E₂ and EE₂ remained after a 1-h treatment (Fig. 2) may be due to residual traces of E₂ and EE₂. In HPLC analyses, we found no metabolites in the reaction mixtures after a 1-h treatment. These results suggest that the removal of estrogenic activities of E₂ and EE₂ may be due to cleavage of the aromatic ring of these compounds. This hypothesis should be a focus of forthcoming studies.

Ternes et al. have reported that the diluted slurry of activated sludge from an

actual STP degrades the natural hormone E_2 but that the contraceptive EE_2 persists [6]. A gram-negative E_2 -degrading bacterium isolated from an STP, which has tentatively been identified as a *Novosphingobium* sp., has also been demonstrated not to degrade EE_2 [7]. To our knowledge very little is known about the EE_2 degradation process, and no data have existed on the microbial removal of the estrogenic activities of E_2 and EE_2 . Therefore, this is the first report that the ligninolytic enzymes MnP and laccase from white rot fungi can effectively degrade E_2 and EE_2 and remove their estrogenic activities.

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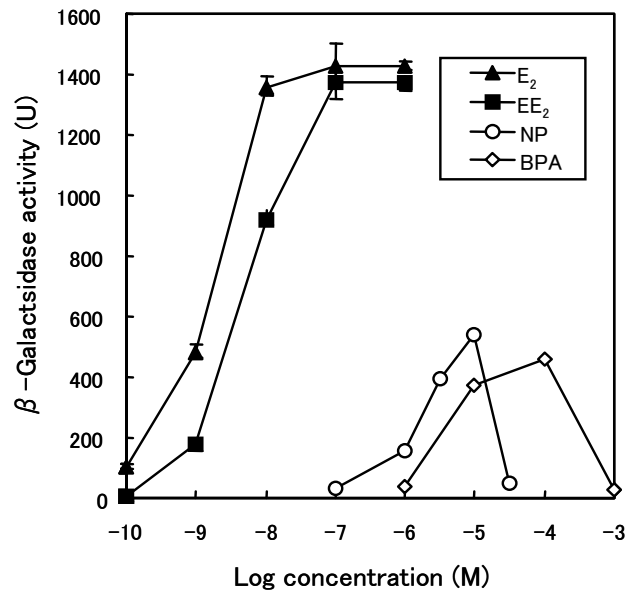


Fig. 1. Dose response curve for estrogenic activity as measured by yeast two-hybrid assay. Indicated for each point are the mean and standard deviation of five experiments for 17 β -estradiol (E₂), ethinylestradiol (EE₂), bisphenol A (BPA), and nonylphenol (NP).

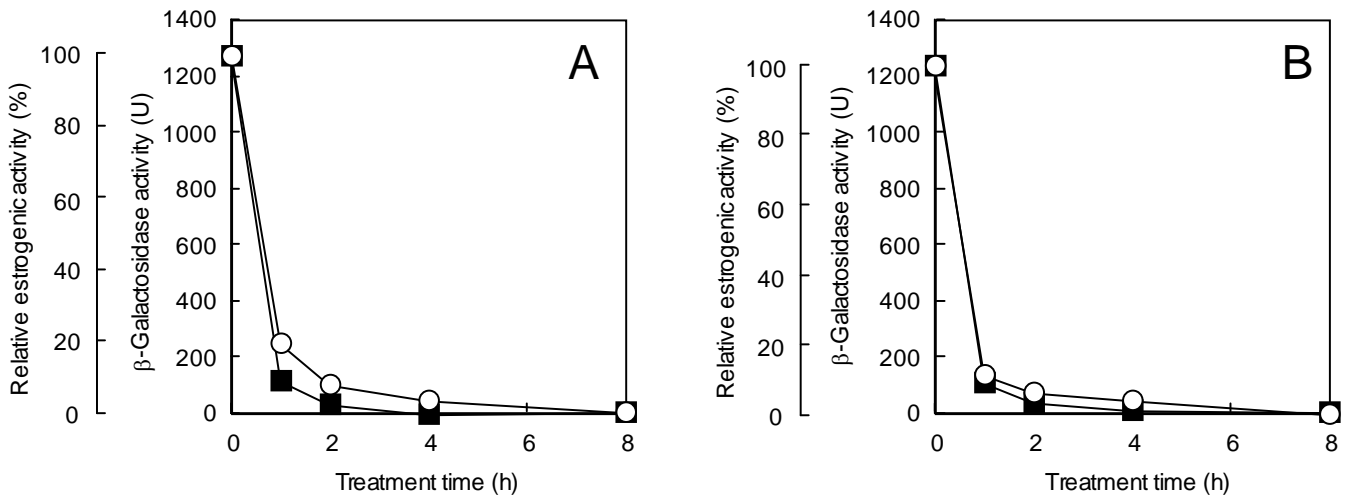


Fig. 2. Removal of estrogenic activities of 17 β -estradiol, E₂, (A) and ethinylestradiol, EE₂, (B) by enzymatic treatment with MnP (■) or the laccase-HBT system (○).