Removal of estrogenic activity of 4-*tert*-octylphenol by ligninolytic enzymes from white rot fungi

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ABSTRACT: 4-*tert*-Octylphenol (4-*t*-OP) was treated with the white rot fungus *Phanerochaete sordida* YK-624 under ligninolytic condition with low-nitrogen and high-carbon culture medium. 4-*t*-OP completely disappeared after 5 days of treatment and the activities of ligninolytic enzymes, laccase and manganese peroxidase (MnP), were detected during this period, thus suggesting that the disappearance of 4-*t*-OP is related to these extracellular enzymes. Therefore, 4-*t*-OP was treated with laccase and MnP prepared from white rot fungi cultures. HPLC analysis demonstrated that 4-*t*-OP completely disappeared in the reaction mixture after 1 h of treatment with either laccase or MnP. Using the yeast two-hybrid assay system, it was also confirmed that laccase and MnP substantially removed the estrogenic activity of 4-*t*-OP after 1 and 2 h of treatment, respectively. These results strongly demonstrate that ligninolytic enzymes are effective in removing the estrogenic activity of 4-*t*-OP.

Keywords: 4-*tert*-Octylphenol; Estrogenic activity; Laccase; Manganese peroxidase;

White rot fungus

INTRODUCTION

Considerable concern has recently been expressed over the possibility that some man-made chemicals mimic the effects of hormones (particularly estrogens) and may adversely affect reproduction in wildlife and humans (Colborn et al., 1993; Stone, 5 1994). Like natural estrogens, these estrogenic chemicals can bind to the estrogen receptor and regulate the activity of estrogen-responsive genes. Such effects suggest that prolonged exposure to environmentally relevant concentrations of these chemicals may result in reproductive toxicity.

4-*tert*-Octylphenol (4-*t*-OP), which is used as a precursor in the manufacture of 10 nonionic surfactants such as octylphenol polyethoxylates (OPEOs), is a degradation product of the microbial breakdown of OPEOs in sewage treatment plants (STPs) (Ahel et al., 1994), and is then released into aquatic environments via STPs because of its recalcitrance. Surveys in STP effluents have quantified 4-*t*-OP at concentrations ranging from ng/L to µg/L in Canada, Germany, Japan and the USA (Lee and Peart, 15 1995; Rudel et al., 1998; Kuch and Ballschmitter, 2001; Isobe et al., 2001).

There is evidence that 4-*t*-OP can cause estrogenic effects in fish and other aquatic organisms. Effects noted in laboratory experiments with fish exposed to 4-*t*-OP include reduced male gonadosomatic index (Jobling et al, 1996), reduced male sexual behavior (Bayley et al., 1999; Gray et al., 1999), fertilization success, testis-ova, and 20 significant developmental abnormalities in offspring (Gray et al., 1999). Furthermore,

studies conducted *in vivo* have shown that administration of 4-*t*-OP to neonatal animals affects the male reproductive system in adults. Effects on the male reproductive system include small but significant decreases in testes weight and daily sperm production in rats (Sharpe et al., 1995), as well as in mice (vom Saal et al., 1998). Furthermore, a recent study has also found that 4 months of consuming water containing 4-*t*-OP at concentrations approximately 50-fold lower than levels measured in natural environments caused an increase in epididymal sperm with tail abnormalities that interfere with rat sperm motility (Blake et al., 2004).

As a consequence of its estrogenicity and recalcitrance, 4-*t*-OP was included on the List of Chemicals for Priority Action under the Oslo and Paris Commission (OSPAR) Hazardous Substances Strategy (OSPAR Commission, 2005). Moreover, the Japanese Ministry of the Environment (MOE) has recently released a report, “MOE’s Perspectives on Endocrine Disrupting Effects of Substances-ExTEND2005”, in which it was postulated that 4-*t*-OP has a strong endocrine disrupting effect on Japanese medaka (*Oryzias latipes*) at the concentrations believed to be present in aquatic environments of Japan (Ministry of the Environment, Japan, 2005).

Great interest has been currently expressed in lignin-degrading white rot fungi and their ligninolytic enzymes due to the recognized potential for degrading and detoxifying recalcitrant environmental pollutants such as dioxins (Bumpus et al., 1985), chlorophenols (Joshi et al, 1993), polycyclic aromatic hydrocarbons (Bezalel et al.,

1996; Collins et al., 1996) and dyes (Ollikka et al., 1993). Manganese peroxidase (MnP), laccase, and lignin peroxidase (LiP) produced extracellularly by white rot fungi are involved in the degradation of lignin, and have been shown to degrade these pollutants.

5 MnP is a heme peroxidase that catalyzes the oxidation of Mn(II) to Mn(III) in the presence of hydrogen peroxide (H₂O₂). 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 (Glenn et al., 1986; Wariishi et
10 al., 1989). Fungal laccase is a multicopper oxidase that catalyzes the single-electron oxidation of phenolic compounds by reducing molecular oxygen to water (Reinhammer, 1984). We recently demonstrated that MnP and laccase are effective in removing the estrogenic activities of nonylphenol (NP), bisphenol A (BPA), 17 β -estradiol (E₂), and ethinylestradiol (Tsutsumi et al., 2001; Suzuki et al., 2003) and
15 in degrading methoxychlor (Hirai et al., 2004) and the antifouling compound Irgarol 1051 (Ogawa et al., 2004). These studies prompted investigation into removing the estrogenic activity of 4-*t*-OP using ligninolytic enzymes, which are able to degrade various aromatic compounds.

In this study, we examined the disappearance of 4-*t*-OP by the white rot fungus
20 *Phanerochaete sordida* YK-624 and investigated the enzymes related to this

disappearance. Furthermore, we applied the related enzymes, MnP and laccase, to the treatment of 4-*t*-OP and describe the removal of its estrogenic activity, as measured using a yeast two-hybrid system (Nishikawa et al., 1999).

5 MATERIALS AND METHODS

Treatment of 4-*t*-OP with white rot fungus

P. sordida YK624 (ATCC 90872) was used in this study. The fungus was maintained in potato dextrose agar (PDA; Difco Laboratories) slants. Fresh PDA plates were inoculated with the fungus, and were then precultured for 5 days at 30°C. Five disks
10 punched from the growing edge of the precultured mycelium were homogenized for 30 s with 50 mL of PMY medium (3.0% glucose, 1.0% peptone, 1.0% malt extract, 0.4% yeast extract), and this was added to a 500-mL Erlenmeyer flask containing 150 mL of PMY medium. This flask was then shaken at 150 rpm and 30°C to give a mycelium suspension culture. After 3 days, 2.2 mL of this culture was again homogenized for 30
15 s with 17.8 mL of Kirk medium (Tien and Kirk, 1988), and was added to a 100-mL Erlenmeyer flask containing 200 µL of 10⁻² M 4-*t*-OP (final concentration, 10⁻⁴ M 4-*t*-OP; Wako, Osaka, Japan). The flask was then shaken at 150 rpm and 30°C.

Enzyme assay and preparation

20 Laccase activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol

(DMP) at 470 nm and 30°C. 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. LiP activity was determined by monitoring the oxidation of veratryl alcohol (VA) at 310 nm and 30°C. The reaction mixture contained 1 mM VA, 20 mM succinate buffer (pH 3.0), and 0.2 mM H₂O₂. Enzyme activity (katal; kat) was calculated using extinction coefficients of 49.3 mM⁻¹cm⁻¹ (at 470 nm) or 9.3 mM⁻¹cm⁻¹ (at 310 nm) for DMP or VA, respectively (Lee and Edlin, 1985; Wariishi et al., 1992).

Partially purified MnP and laccase were prepared from cultures of *P. sordida* YK-624 and *Trametes versicolor* IFO-6482, respectively, as described previously (Tsutsumi et al., 2001). The partially purified MnP did not contain any laccase, and *vice versa*. LiP activity was not detected in both enzyme preparations.

Treatment of 4-t-OP with ligninolytic enzymes

For treatment with MnP, the reaction mixture consisted of 50 µM (5×10⁻⁵ M) 4-*t*-OP, partially purified MnP (10 nkat/mL), 50 mM malonate buffer (pH 4.5), MnSO₄ (0.1 mM) and glucose (25 mM) plus 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) was used instead of MnP, and MnSO₄, glucose, and glucose oxidase were omitted from the

reaction mixture.

Analyses of 4-*t*-OP by HPLC

Residual 4-*t*-OP concentrations in the fungal cultures or enzymatic reaction mixtures
5 were determined by high-performance liquid chromatography (HPLC). Each of the
whole fungal cultures containing mycelia, in which naphthalene was added as an
internal standard after incubation, was homogenized for 30 s and then extracted with
ethyl acetate (EtOAc) after acidification (pH 2-3) with 1 N-HCl. The EtOAc layer was
dried over anhydrous Na₂SO₄ and was evaporated under reduced pressure. The residue
10 was dissolved in methanol, which was analyzed by HPLC. Each of the enzymatic
reaction mixtures was subjected directly to HPLC. HPLC analyses were carried out
with a Wakosil-II 5C18HG (Wako) column using isocratic elution with 80% methanol
aqueous at a flow rate of 1 mL/min and detection at 277 nm.

15 Estrogenic activity of 4-*t*-OP treated with ligninolytic enzymes

Estrogenic activity of 4-*t*-OP before and after enzymatic treatment was evaluated by an
in vitro screening test for chemicals with hormonal activity that used the yeast
two-hybrid estrogenic assay system, as developed by Nishikawa et al. (1999). Yeast
Y190 transformed with the pGBT9-estrogen receptor ligand-binding domain
20 (pGBT9-ER LBD) and pGAD424-coactivator was provided by Dr. Nishihara (Osaka

University). In the yeast, the GAL4 DNA binding domain-ER LBD and the GAL4 activation domain-coactivator fusion proteins were expressed by these expression plasmids. Y190 harbors a GAL4 binding site upstream of the *lacZ* gene, and thus β -galactosidase activity corresponds to the strength of interaction between the estrogen receptor and coactivator. The protein-protein interaction between estrogen receptor and coactivator is strictly dependent on the presence of estrogen (Nishikawa et al., 1999).

In the assay system, 2.5 μ L of test sample was added to 50 μ L of yeast culture and 200 μ L of Sabouraud's dextrose medium (Tsutsumi et al., 2001). The test sample for the assay system was prepared as follows; each reaction mixture (400 μ L) was lyophilized and dissolved in dimethyl sulfoxide (20 μ L), thus ensuring that the concentration of 4-*t*-OP before enzymatic treatment corresponded to 10^{-5} M in the assay system. Relative estrogenic activity (%) was defined as the percentage of β -galactosidase activity of enzyme-treated 4-*t*-OP, as compared to that of untreated 4-*t*-OP. For the dose-response curves of estrogenic activity depicted in Fig. 1, 2.5 μ L of various concentrations of BPA, NP and E₂ dissolved in dimethyl sulfoxide were used instead of the reaction mixture.

RESULTS AND DISCUSSION

Estrogenic activity of 4-*t*-OP

The estrogenic activity of 4-*t*-OP, expressed in terms of β -galactosidase activity, was

compared with those of BPA, NP and E₂, which are known endocrine disrupters (Fig. 1). The activity of 4-*t*-OP (10⁻⁵ M) was comparable to that of E₂ (10⁻⁸ M). Furthermore, the activities of BPA (10⁻⁴ M) and NP (10⁻⁵ M) were similar to that of E₂ (~5×10⁻⁹ M). These findings are consistent with a previous report in which the relative estrogenicities of 4-*t*-OP, BPA and NP were found to be 8 × 10⁻⁴, 5 × 10⁻⁵, and 4 × 10⁻⁴, respectively, as compared with E₂ (Nakamuro et al., 2002).

Treatment of 4-*t*-OP with white rot fungus

Ligninolytic activity of white rot fungi appears as a secondary metabolic event, and nutritional nitrogen or carbon limitation (or both) allows extensive degradation of lignin (Keyser et al., 1978; Kirk et al., 1978). Thus, the treatment of 4-*t*-OP with *P. sordida* YK-624 was investigated under ligninolytic condition with low-nitrogen and high-carbon culture medium (0.02% ammonium tartrate, 1% glucose) (Tien and Kirk, 1988). Figure 2 shows the decrease in 4-*t*-OP observed during treatment with *P. sordida* YK-624. No decrease in 4-*t*-OP was observed during the first day of treatment, but marked decreases were noted after 2 days of treatment and 4-*t*-OP was undetectable after 5 days of treatment.

It has been suggested that the MnP, LiP and laccase produced extracellularly by white rot fungi are involved in the oxidative breakdown of lignin (Tien and Kirk, 1983; Gold et al., 1984; Dodson et al., 1987). Therefore, these enzyme activities were

determined during the treatment of 4-*t*-OP with *P. sordida* YK-624 (Fig. 2). MnP and laccase activities were detected after 2 days of treatment, but LiP activity was not detected throughout the treatment period. The profiles for MnP and laccase production and 4-*t*-OP decrease during treatment were very similar. These results strongly suggest that MnP and laccase, which are produced by *P. sordida* YK-642 under ligninolytic condition, are involved in the disappearance of 4-*t*-OP.

Treatment of 4-*t*-OP with ligninolytic enzymes

We used two enzymatic treatments, MnP and laccase, in order to remove the estrogenic activity of 4-*t*-OP. As shown in Fig. 3, HPLC detection of residual 4-*t*-OP on an ODS column (277 nm) during these two enzymatic treatments revealed that 4-*t*-OP had completely disappeared from the reaction mixture after 1 h of treatment. This indicates that the two enzymatic treatments effectively decrease 4-*t*-OP. However, the greatest focus concerning the biodegradation of endocrine-disrupting (estrogenic) chemicals should be on the removal of estrogenic activity. We therefore attempted to assay the estrogenic activity of the 4-*t*-OP reaction mixtures during enzymatic treatment using the yeast two-hybrid estrogenic assay system. This recently developed system 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 (Nishikawa et al., 1999).

The data depicted in Fig. 4 demonstrate that laccase almost completely removed the estrogenic activity of 4-*t*-OP after 1 h of treatment, which is consistent with the HPLC data shown in Fig. 3. On the other hand, MnP substantially removed the estrogenic activity of 4-*t*-OP after 2 h of treatment but the activity remained at 40% in the reaction mixture after 1 h of treatment (Fig. 4), although no detectable level of residual 4-*t*-OP remained in the reaction mixture after a 1-h treatment (Fig. 3). These results strongly indicate that the estrogenic assay is necessary in studies of the bioremediation of endocrine-disrupting chemicals. In a previous report, we demonstrated that treatment of NP and BPA with MnP and laccase resulted in oligomeric reaction products through the formation of phenoxy radicals followed by radical coupling, and the reaction products must lose structural characteristics of acting as estrogens (Tsutsumi et al., 2001). Thus, the removal of estrogenic activity of 4-*t*-OP may be due to polymerization brought about by enzymatic oxidation, as 4-*t*-OP has a similar *para*-substituted phenol structure to NP and BPA. Furthermore, the fact that 40% of the estrogenic activity of 4-*t*-OP remained after 1 h of treatment with MnP may be due to the reaction products of 4-*t*-OP sharing a key structural feature for acting as estrogens.

It is well known that white rot fungi universally exist in ecosystems like forests or fields (Nishida et al., 1988). In this study, we showed that the ligninolytic enzymes laccase and MnP from white rot fungi could effectively eliminate 4-*t*-OP and remove

its estrogenic activity. To our knowledge, this is the first report dealing with enzymatic removal of the estrogenic activity of 4-*t*-OP.

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Fig. 1. Dose-response curves for estrogenic activity, as measured by yeast two-hybrid assay. Activity of E₂ at 10⁻⁵ M has a relative activity of 100%. Indicated for each point are the mean and standard deviation of five experiments for 4-*t*-OP, E₂, NP and BPA. (●), 4-*t*-OP; (◇), E₂; (□), NP; (△), BPA.

Fig. 2. Treatment of 4-*t*-OP with *P. sordida* YK-624. Indicated for each point are the mean and standard deviation of triplicate cultures. (◆), Residual 4-*t*-OP; (□), MnP activity; (○), laccase activity.

Fig. 3. Decrease in 4-*t*-OP concentration by enzymatic treatment with MnP or laccase. (■), MnP; (○), laccase.

Fig. 4. Removal of estrogenic activity of 4-*t*-OP by enzymatic treatment with MnP or laccase. (■), MnP; (○), laccase.