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ORIGINAL PAPER

Removal of estrogenic activity of iso-butylparaben and *n*-butylparaben by laccase in the presence of 1-hydroxybenzotriazole

Hirohito Mizuno · Hirofumi Hirai · Shingo Kawai · Tomoaki Nishida

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Abstract In the presence of a redox mediator, 1-hydroxybenzotriazole (HBT), iso-butylparaben (iso-BP) and *n*-butylparaben (*n*-BP) were treated with laccase from white rot fungus *Trametes versicolor*. HPLC analysis demonstrated that iso-BP and *n*-BP almost completely disappeared from the reaction mixture after 4 h of treatment with the laccase-HBT system. Using the yeast two-hybrid assay system, it was also confirmed that the laccase-HBT system substantially removed the estrogenic activity of iso-BP and *n*-BP after 4 h of treatment. Furthermore, there was a linear relationship between the removal of estrogenic activity of both parabens and the decrease in their concentrations. These results demonstrate that the laccase-HBT system is effective in eliminating iso-BP and *n*-BP, and removing the estrogenic activity of both parabens.

Keywords iso-Butylparaben · *n*-Butylparaben · Estrogenic activity · Laccase · 1-Hydroxybenzotriazole · White rot fungus

Introduction

Parabens, alkyl esters of *p*-hydroxybenzoic acid, are used as preservatives in foodstuffs, cosmetics, toiletries, foods, and pharmaceuticals, because of their relatively low toxicity in humans and their effective antimicrobial activity (Elder 1984). Parabens have a similar *p*-hydroxy substituted phenol structure to alkylphenols, and this substitution on the aromatic ring has been recognized as an important requirement for the estrogenic activity of chemicals such as alkylphenols (Jordan and Lieberman 1984; Nishihara and Nishikawa 2001). Thus, the estrogenic activity of parabens has been studied by in vitro recombinant yeast assay, human estrogen receptor assay and E-screen (Lemini et al. 1997; Routledge et al. 1998; Blair et al. 2000; Hossaiani et al. 2000; Pedersen et al. 2000; Satoh et al. 2000; Okubo et al. 2001).

These in vitro studies have demonstrated that methylparaben, ethylparaben, propylparaben, and butylparaben are all weakly estrogenic, with butylparaben showing greater activity than methyl-, ethyl-, and propylparaben. In an in vitro yeast-based estrogen assay, butylparaben was three-fold more and 10,000-fold less potent than nonylphenol and natural steroidal hormone 17β-estradiol, respectively, (Routledge et al. 1998). Furthermore, in in vivo assays, propyl- and butylparaben have been shown to affect testosterone levels and sperm counts in rats and mice at oral doses fairly close to the ADI value (Oishi 2001, 2002a, b), while male offspring of exposed female rats show decreased gonad and accessory organ weights, as well as decreased sperm counts and motility (Kang et al. 2002). It has been also reported that propyl- and butylparaben are estrogenic in an in

H. Mizuno · H. Hirai · S. Kawai · T. Nishida (✉)
Faculty of Agriculture, Shizuoka University, Ohya 836, Surugaku, Shizuoka 422-8529, Japan
e-mail: aftnisi@agr.shizuoka.ac.jp

vivo fish assay of vitellogenin induction in rainbow trout, and that these parabens have estrogenic potencies comparable to bisphenol A in vivo (Pedersen et al. 2000).

White rot fungi are the best-known and most effective lignin-degrading microorganisms. There is currently great interest in these fungi and their ligninolytic enzymes due to their potential for degrading and detoxifying recalcitrant environmental pollutants, such as polychlorinated dioxins (Kamei et al. 2005), chlorophenols (Ehlers and Rose 2005), polycyclic aromatic hydrocarbons (Cambria et al. 2008), and dyes (Asgher et al. 2008). Furthermore, we recently demonstrated that ligninolytic enzymes such as manganese peroxidase (MnP) and laccase, which are produced extracellularly by white rot fungi, are effective in removing the estrogenic activities of nonylphenol (NP), bisphenol A (BPA), 4-*tert*-octylphenol, 17β-estradiol (E_2), ethinylestradiol, estrone, and genistein (Tsutsumi et al. 2001; Suzuki et al. 2003; Tamagawa et al. 2005, 2006, 2007), and 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 parabens using white rot fungi and ligninolytic enzymes, which are able to degrade various aromatic compounds.

In this study, we examined the disappearance of iso-butylparaben (iso-BP) and *n*-butylparaben (*n*-BP) by the white rot fungus *Trametes versicolor* and investigated the enzymes related to their disappearance. Furthermore, we applied the related enzyme laccase to the treatment of iso-BP and *n*-BP, and describe the removal of their estrogenic activity, as measured using a yeast two-hybrid system (Nishikawa et al. 1999).

Materials and methods

Fungal treatment of iso-BP

T. versicolor (IFO 6482) was used in this study. The fungus was maintained in potato dextrose agar (PDA; Difco Laboratories) slants. A new PDA plate was inoculated with the fungus, and was then precultured for 5 days at 30°C. Five disks 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 5 days, 2.2 ml of this culture was again homogenized for 30 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 iso-BP (final concentration, 10^{-4} M iso-BP; Wako, Osaka, Japan). The flask was then shaken at 150 rpm and 30°C.

Enzyme assay

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 $MnSO_4$ and 0.2 mM H_2O_2. Lignin peroxidase (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 H_2O_2. Enzyme activity (katal; kat) was calculated using extinction coefficients of 49.3 mM^{-1} cm^{-1} (at 470 nm) and 9.3 mM^{-1} cm^{-1} (at 310 nm) for DMP and VA, respectively.

Enzyme preparation

Laccase was partially purified from cultures of *T. versicolor* IFO 6482 by ammonium sulfate fractionation and gel filtration chromatography (Sephacryl S-200 HR; GE Healthcare life sciences), as described previously (Fujisawa et al. 2001). Partially purified laccase did not contain any MnP or LiP.

Enzymatic treatment of iso-BP and *n*-BP

For treatment with laccase, the reaction mixture (20 ml) consisted of partially purified laccase (10 nkat/ml), 10^{-4} M iso-BP or *n*-BP, and 50 mM malonate buffer (pH 4.5). The laccase-mediator system was used in the same manner, except that different concentrations of 1-hydroxybenzotriazole (HBT) were added to the reaction mixture. Reactions were performed at 30°C with stirring at 150 rpm.

Analyzes of iso-BP and *n*-BP by HPLC

Residual iso-BP or *n*-BP concentrations in the fungal cultures or enzymatic reaction mixtures were determined by high-performance liquid chromatography (HPLC). Each of the whole fungal cultures containing mycelia, in which 3′,4′-dimethoxyacetophenon 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 Na_2SO_4 and was evaporated under reduced pressure. The residue was dissolved in acetonitrile, which was analyzed by HPLC. Each of the enzymatic reaction mixtures was directly subjected to HPLC. HPLC analytical conditions were as follows: Wakosil-II 5C18HG (Wako) column; mobile phase of 1% acetic acid (X) and acetonitrile (Y); elution with a linear gradient of 5–100% Y in 50 min; flow rate of 1.0 ml/min; and detection at 254 nm.

Estrogenic activity of iso-BP and *n*-BP

Estrogenic activity of iso-BP before and after fungal treatments was evaluated by an in vitro screening test for chemicals with hormonal activity, based on a yeast two-hybrid estrogenic assay system (Nishikawa et al. 1999). In the assay system, 2.5 µl of test sample was added to 50 µl of yeast culture and 200 µl of Sabouraud's dextrose medium. The test sample for the assay system was prepared as follows; each of the whole fungal cultures (20 ml) containing mycelia was homogenized and then extracted EtOAc after acidification. EtOAc extracts were dissolved in dimethyl sulfoxide (DMSO) (2 ml), thus ensuring that the concentration of iso-BP before fungal treatment corresponded to 10^{-5} M in the assay system.

Estrogenic activity of iso-BP and *n*-BP before and after enzymatic treatments was also evaluated using the yeast two-hybrid estrogenic assay system. Each reaction mixture (25 µl) before and after enzymatic treatments was lyophilized and dissolved in DMSO (2.5 µl), which means that the concentrations of iso-BP and *n*-BP 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 fungal- or enzyme-treated iso-BP and *n*-BP, as compared to that of untreated iso-BP or *n*-BP. For the dose-response curves of estrogenic activity depicted in Fig. 1, 2.5 µl of various concentrations of BPA, NP, E_2, iso-BP, and *n*-BP dissolved in DMSO were used instead of the reaction mixture.

Results and discussion

Estrogenic activity of iso-BP and *n*-BP

The estrogenic activity of iso-BP (nonlinear alkyl paraben) and *n*-BP (linear alkyl paraben), expressed in terms of *β*-galactosidase activity, was compared with that of BPA, NP, and E_2, which are known endocrine disrupters (Fig. 1). iso-BP showed a slightly higher estrogenic activity than *n*-BP at concentrations ranging from 5×10^{-6} to 5×10^{-5} M, and the highest estrogenic activity for iso-BP and *n*-BP was obtained at 10^{-5} and 5×10^{-5} M, respectively. The activity of iso-BP (10^{-5} M) and *n*-BP (5×10^{-5} M) was almost the same as that of BPA (10^{-4} M), NP (10^{-5} M), and E_2 ($\sim 5 \times 10^{-9}$ M). These findings are consistent with a previous report in which the relative estrogenicity of iso-BP, *n*-BP, and BPA was found to be 1.9×10^{-4}, 5.9×10^{-5}, and 4.2×10^{-5}, respectively, as compared with E_2 in vitro (Morohoshi et al. 2005).

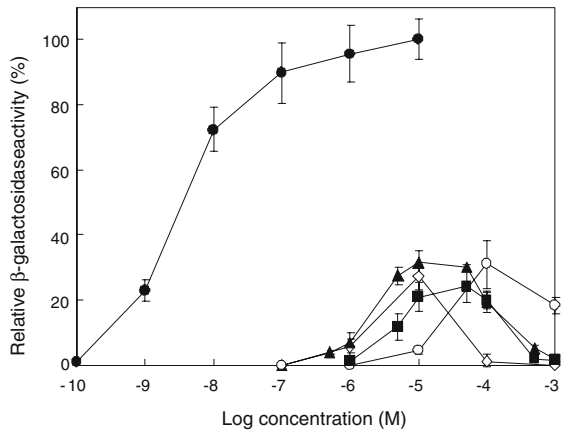

Fig. 1 Dose-response curves for estrogenic activity, as measured by yeast two-hybrid assay. E_2 at 10^{-5} M has a relative activity of 100%. Indicated for each point are the mean and standard deviation of five experiments for iso-BP, *n*-BP, E_2, NP and BPA. (▲), iso-BP; (■), *n*-BP; (●), E_2; (◇), NP; (○), BPA

Fungal treatment of iso-BP

One of the important characteristics of some white rot fungi is that the ligninolytic activity appears as a secondary metabolic event and nutritional nitrogen limitation permits extensive lignin degradation (Kirk et al. 1978). Thus, treatment of iso-BP with *T. versicolor* was investigated under ligninolytic condition with low-nitrogen and high-carbon culture medium (i.e., Kirk medium). Figure 2 shows the decrease in iso-BP observed during treatment with *T. versicolor*. The concentration of iso-BP decreased by 99% after 2 days of treatment and the profiles of the decrease in iso-BP concentration and the removal of its estrogenic activity were very similar.

It has been suggested that the MnP, LiP, and laccase produced extracellularly by white rot fungi are involved in the oxidative breakdown of lignin (Martínez et al. 2005). Therefore, these enzyme activities were determined during the treatment of iso-BP with *T. versicolor*. Laccase activity was detected after the first day of treatment (Fig. 2), but MnP and LiP activities were not detected throughout the treatment period (data not shown). Recent studies demonstrated that the expression of laccase gene from *T. versicolor* is increased under degrading conditions for 2,4,6-trinitrotoluene, BPA, NP, and two phthalic esters (Cheong et al. 2006; Kim et al. 2008). In the present study, the laccase gene expression during the degradation of iso-BP was not examined, but the data on enzyme production shown in Fig. 2 suggested that laccase may be involved in the decrease in iso-BP concentration and the removal of its estrogenic activity by *T. versicolor*.

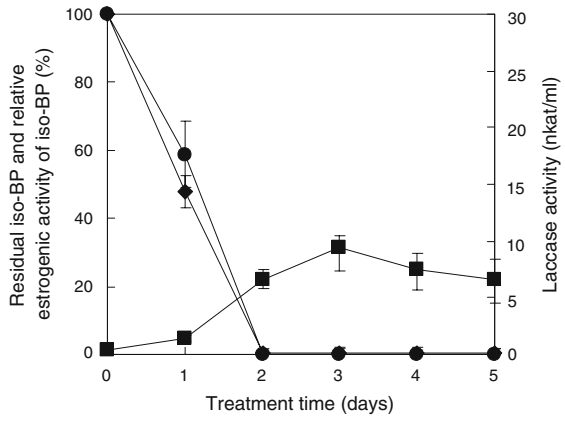

Fig. 2 Treatment of iso-BP with *T. versicolor*. Indicated for each point are the mean and standard deviation of five experiments. (◆), Residual iso-BP; (●), Relative estrogenic activity; (■), Laccase activity

Laccase treatment of iso-BP and *n*-BP

As shown in Fig. 2, *T. versicolor* produced about 2 and 7 nkat/ml laccase on the first and second days of treatment, respectively, and iso-BP almost completely disappeared after second day treatment with this fungus. Thus, 10 nkat/ml laccase was used for the treatment of iso-BP and *n*-BP.

The HPLC data demonstrated that the concentrations of iso-BP and *n*-BP in the reaction mixture decreased by about 15 and 5% after 2 h of treatment with laccase alone, respectively, and these levels were largely maintained thereafter (Fig. 3). These results suggest that degradation of parabens by laccase itself is possible, but is not sufficiently effective for application to the bioremediation of parabens, although several studies on laccase treatment of endocrine-disrupting (estrogenic) chemicals have demonstrated its efficiency (Tamagawa et al. 2005, 2006, 2007; Kim et al. 2008; Sei et al. 2008).

It has been demonstrated that the substrate range of fungal laccase is extended in the presence of redox mediators such as 1-hydroxybenzotriazole (HBT). For example, the laccase-HBT system can oxidize nonphenolic compounds that are not oxidized by laccase alone (Kawai et al. 2002). The proposed mechanism of the laccase-HBT system is as follows; when HBT is oxidized by laccase, the free radicals that are generated actively oxidize nonphenolic lignin model compounds (Fabbrini et al. 2002). This system has been applied to the removal of natural and synthetic hormones in municipal wastewater (Auriol et al. 2007) and the degradation of polycyclic aromatic hydrocarbons (Cambria et al. 2008). Therefore, we applied the laccase-HBT system in order to degrade iso-BP and *n*-BP.

The concentrations of parabens decreased markedly when HBT was added to a reaction mixture containing laccase (10 nkat/ml). Adding 2 mM HBT to the reaction mixture had the greatest effect; both iso-BP and *n*-BP decreased by 95% after 2 h of treatment and almost completely disappeared after 4 h of treatment (Fig. 3). These results indicate that the laccase-HBT system effectively decreases iso-BP and *n*-BP. However, the greatest focus regarding the biodegradation of

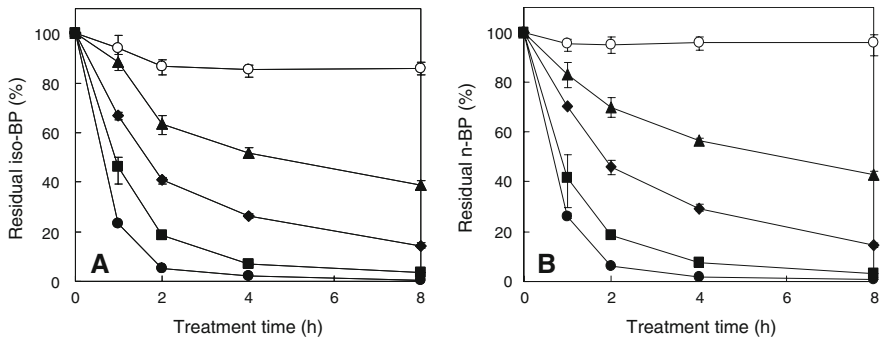

Fig. 3 Effects of HBT concentration on decreases in iso-BP (A) and *n*-BP (B) during treatment with laccase. Indicated for each point are the mean and standard deviation of triplicate experiments. (○), 0 mM; (▲), 0.2 mM; (◆), 0.4 mM; (■), 1.0 mM; (●), 2.0 mM

estrogenic chemicals should be on the removal of estrogenic activity. We therefore attempted to assay the estrogenic activity of the reaction mixtures of iso-BP and *n*-BP during treatment using the yeast two-hybrid estrogenic assay system.

No appreciable removal of the estrogenic activity of iso-BP and *n*-BP was observed after treatment with laccase alone, but marked decreases were obtained by the laccase-HBT system (Fig. 4). Removal of the estrogenic activity of iso-BP and *n*-BP was plotted against decreases in concentrations of both parabens during treatment with the laccase-HBT system (Fig. 5), and a linear relationship was seen under all tested enzymatic reaction conditions. The correlation coefficient for the removal of estrogenic activity of iso-BP and the decrease in iso-BP was 0.945, whereas that for the removal of estrogenic activity of *n*-BP and the decrease in *n*-BP was 0.970. These results strongly indicate that the decreases in parabens observed throughout the treatment with the laccase-HBT system are associated with the loss of structural characteristics responsible for estrogens activity.

Conclusions

In this study, we demonstrated that the laccase-HBT system effectively eliminates iso-BP and *n*-BP and

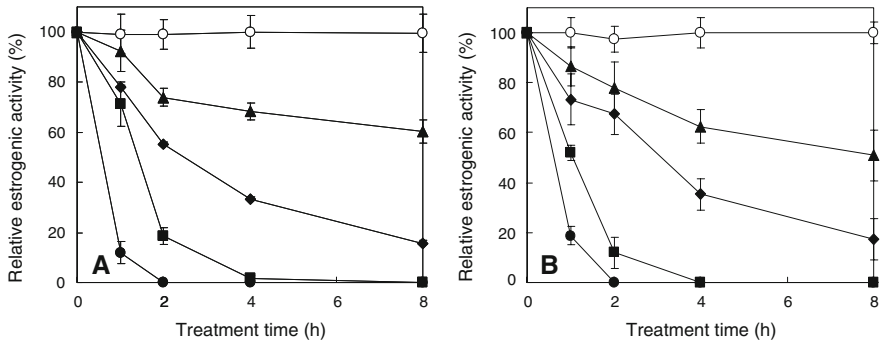

Fig. 4 Effects of HBT concentration on removal of estrogenic activity of iso-BP (A) and *n*-BP (B) during treatment with laccase. Indicated for each point are the mean and standard deviation of triplicate experiments. (○), 0 mM; (▲), 0.2 mM; (◆), 0.4 mM; (■), 1.0 mM; (●), 2.0 mM

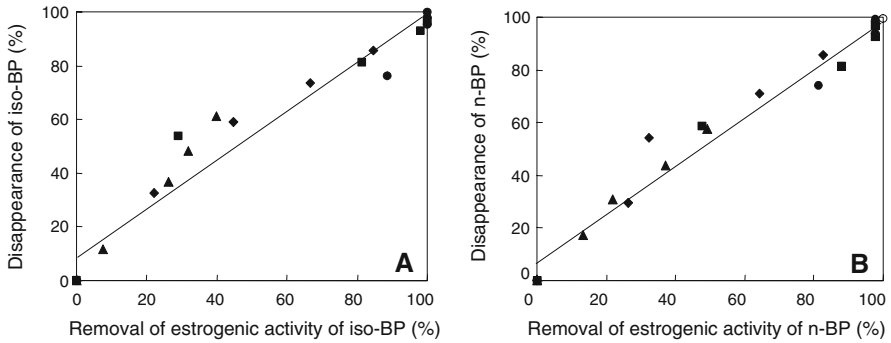

Fig. 5 Relationship between disappearance of iso-BP (A) and *n*-BP (B) and removal of estrogenic activity of iso-BP (A) and *n*-BP (B) during treatment with the laccase-HBT system. See Figs. 3 and 4 for explanation of symbols. For *panel A*, the correlation coefficient is 0.945. For *panel B*, the correlation coefficient is 0.970

removes estrogenic activity. To our knowledge, this is the first report dealing with the enzymatic removal of the estrogenic activity of iso-BP and *n*-BP. However, *T. versicolor* is not capable of producing the artificial mediator HBT during treatment of iso-BP, although the fungus can produce laccase and almost completely eliminate the estrogenic activity of iso-BP after 2 days of treatment (Fig. 2). Further investigation is needed to verify whether other fungal metabolites derived from the culture medium containing iso-BP may act as a mediator, or whether some other enzyme is involved in degradation of parabens by this fungus.

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