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Enhanced Expression of Laccase during the Degradation of Endocrine Disrupting Chemicals in *Trametes versicolor*

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A putative laccase cDNA from a white-rot basidiomycete, *Trametes versicolor*, that consisted of 1,769 nucleotides was cloned using the rapid amplification of cDNA ends (RACE)-PCR method. The deduced amino acid sequence had 4 putative copper binding regions, which are common to fungal laccases. In addition, the sequence was 57~97% homologous to sequences of other *T. versicolor* laccases. Additionally, the expression of laccase and manganese peroxidase in this fungus were both greatly increased under degrading conditions for bisphenol A, nonylphenol and two phthalic esters (benzylbutylphthalate and diethylphthalate), all of which are reportedly endocrine disrupting chemicals (EDCs). Furthermore, the estrogenic activities of the EDCs also decreased rapidly during incubation when examined in a two-hybrid yeast system. Finally, kojic acid inhibited the removal of estrogenic activities generated by bisphenol A and nonylphenol, which confirmed that laccase was involved in the degradation of EDCs in *T. versicolor*.

***Keywords*:** endocrine disrupting chemicals (EDCs), estrogenic activity, laccase, manganese peroxidase, *Trametes versicolor*

Many synthetic polymers have been used since the middle of 20th century; therefore, there is now considerable concern regarding their recalcitrant characteristics. Many chemicals used in the plastic and paint industries are known to affect the reproductive systems of various animals, including humans. Such chemicals, which are known as endocrine disrupting chemicals (EDCs), mimic our steroid sex hormones (Colborn *et al*., 1996). Bisphenol A [2,2-bis(4-hydroxyphenyl)propane], which is widely used for the production of epoxy and phenol resins, polycarbonates, polyester, and lacquer coatings on food containers, is released to the environment from factories that produce bisphenol A, as well as from plastic litter and landfill sites (Staples *et al*., 1998). In addition, nonylphenol and phthalate esters, which are largely produced for use as surfactants and/or plasticizers in a variety of plastics industries, are also known to have estrogenic activities (Tsutsumi *et al*., 2001; Dhooge *et al*., 2006). There are a wide variety of methods used to assay estrogenic activity, most of which use human cell lines such as transgenic HGPXR cells derived from HeLa cells (Mnif *et al*., 2007) or MELN cells from estrogen-sensitive human breast cancer cells (Berkmans *et al*., 2007). In addition, genetically transformed yeast cells are often used in assays for estrogenic activity (Suzuki *et al*., 2003; Fu *et al*., 2007).

EDCs can be degraded by ozone/UV treatment (Oh *et al*., 2006) or by bioremediation using various bacteria and fungi (Oshiman *et al*., 2007); however, lignin degrading white-rot basidiomycetes are the most promising candidates for the remediation of various EDCs. White-rot basidiomycetes have enzyme systems that enable the degradation of lignocellulose materials, and laccase, lignin peroxidase and manganese peroxidase are the key enzymes involved in these systems. These enzymes can utilize a wide variety of substrates; therefore, white-rot fungi play important roles in the degradation of chlorinated dioxins (Mori and Kondo, 2002), diethylphthalate (Lee *et al*., 2004) and 2,4,6-trinitortoluene (hereafter TNT) (Bumpus and Tatarko, 1994; Kim and Song, 2003). *Trametes versicolor* is a white-rot basidiomycete that has high degrading ability against a wide variety of materials, and we have isolated a *T. versicolor* strain that has degrading abilities towards many recalcitrant materials including aromatic hydrocarbons (Song, 1997), explosives (Cheong *et al*., 2006) and phenanthrene (Han *et al*., 2004). Therefore, we evaluated this strain of *T. versicolor* using a genetic transformation system (Kim *et al*., 2002), and cDNA of manganese-repressed peroxidase (Kim *et al*., 2005) and manganese-dependent peroxidase (MnP; Yeo *et al*., 2007) were cloned and evaluated. A laccase activity and its transcript level were increased during the degradation of TNT and its catabolic intermediates by *T. versicolor* (Cheong *et al*., 2006). In this study, we cloned full-length laccase cDNA and evaluated its expression during the degradation of several EDCs by *T. versicolor*. We also evaluated the expression of MnP under the same conditions. In addition, we used a yeast two-hybrid assay system (Tsutsumi *et al*., 2001) to analyze the estrogenic activity of each EDC during its degradation.

Materials and Methods

Microorganisms and culture conditions

T. versicolor monokaryon 9522-1 (Cheong *et al*., 2006), which was used through out this experiment, was grown at 30°C

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in liquid YMG medium using previously described methods (Cheong *et al*., 2006). Fungal cells in the liquid culture were then ground in a Waring blender, after which the homogenate was transferred (10%, v/v) to fresh liquid medium (100 ml) that contained one of the following EDCs (Aldrich, USA) such as benzylbutylphthalate (BBP, 98%), bisphenol A (BPA, 99+%), diethylphthalate (DEP, 99.5%) or nonylphenol (NP, technical grade). The concentration of the EDCs added to the medium were as follows: 300 mg/L (BBP), 100 mg/L (BPA), 400 mg/L (DEP) or 20 mg/L (NP). The culture supernatants were then collected regularly to measure the laccase and estrogenic activities. Fungal cells

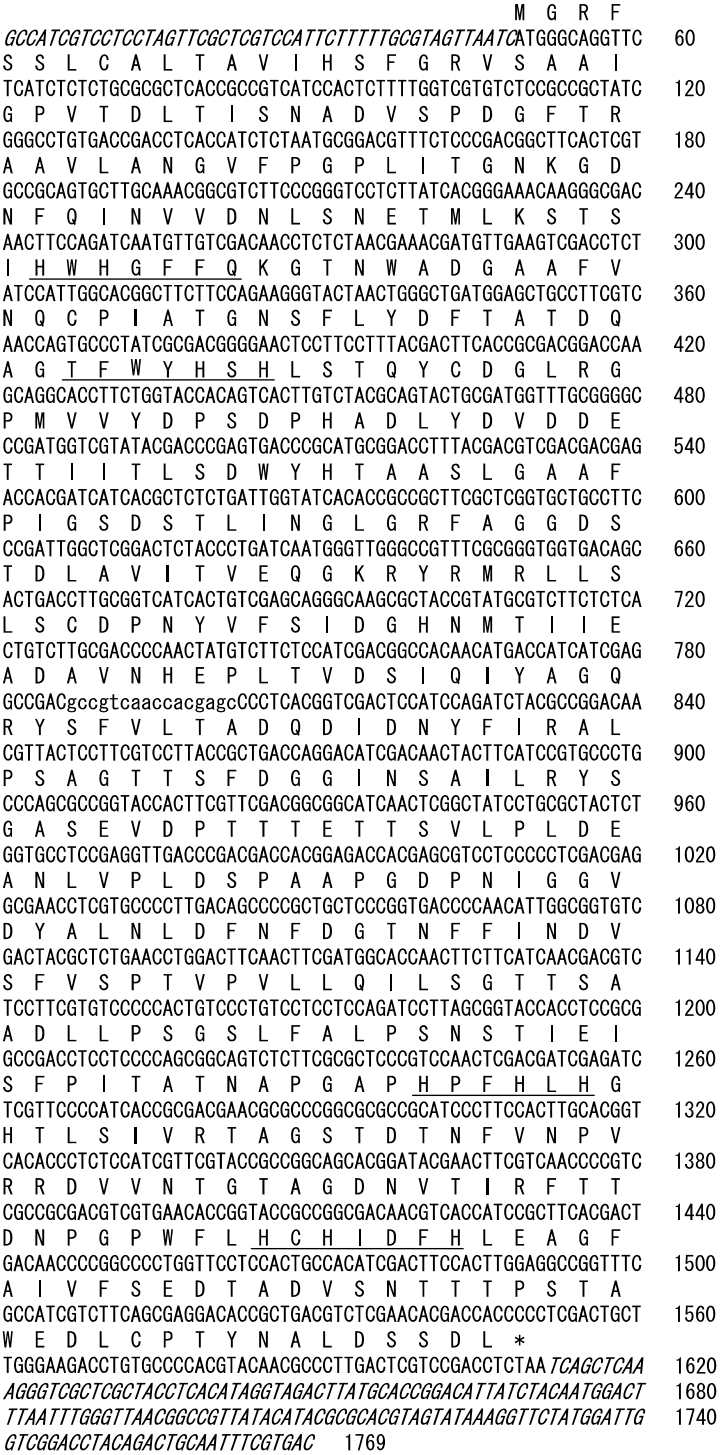

Fig. 1. The cloned cDNA (*klc1*) sequence and deduced amino acid sequence of a laccase isolated from *Trametes versicolor*. Untranslated 5'- and 3'-regions are shown in italics and the star represents the stop codon. The four putative copper binding domains are underlined.

were also harvested, after which the expressions of the laccase and manganese peroxidase gene were evaluated by real-time PCR using laccase or manganese peroxidase gene-specific primers.

Cloning of laccase cDNA

Fungal laccases generally have four conserved copper-binding regions; therefore, we amplified a genomic DNA fragment by PCR using 2 primers specific for copper-binding region I and III (Cheong *et al*., 2006). Briefly, total RNA was isolated using an RNeasy Plant Mini Kit (QIAGENE), and the first strand of cDNA was then synthesized from 1 μg of total RNA using PowerScrip Reverse Transcriptase (Promega) following the manufacturer's instructions. The laccase cDNA fragment was then amplified by PCR using the aforementioned primers, which gave a 1,017 bp product. Next, the PCR product was cloned into T-vector for nucleotide sequencing. The following primers were designed for RACE-PCR: forward primer; 5'-CCCTCACGGTCGACTC CATCCAGA-3' and reverse primer; 5'-CTGATGGAGCTG CCTTCGTC-3'. These primers were used in conjunction with the RACE primers to amplify the 5'- and 3'-regions of the laccase cDNA.

Determination of laccase activity, laccase and MnP expression, residual concentrations of EDCs and estrogenic activity

Laccase activity was measured by spectrophotometry using *o*-tolidine as the enzyme substrate (Ko *et al*., 2001). Total RNA was isolated from fungal cells obtained at different time periods using Trizol extraction buffer (Invitrogen) and then 3 μg of RNA from each culture that contained EDC were used as the template in the synthesis of cDNA. Real-time PCR was performed using 5 μl out of 20 μl from the cDNA mix, 12.5 μl of iTaq SYBR Green Supermix (Bio-Rad), and 1 μl each of laccase-specific forward primer; 5'-C ATCCCTTCCACTTGCACG-3' and reverse primer; 5'-GAT GTGGCAATGGAGGAACC-3' in a 25 μl reaction mixture. Real-time PCR for MnP was conducted following the same protocol, except the following MnP-specific primers were used: forward primer; 5'-GCGCTCCTTCCCCGTCATCGA-3' and reverse primer; 5'-TCGGCGGTGGTAGGTTGTG-3'. The CT value of each sample was obtained using the ABI PRIAM 7000 SDS software, and the relative expression level of the laccase and MnP gene from each sample was then compared with the expression level of the actin gene.

To determine the residual concentrations of the EDCs, the fungal culture, including the cells and supernatant, was extracted with n-hexane and then ethylacetate in a 50 ml centrifuge tube, after which the mixture was separated by centrifugation at 24,900×g for 20 min at 4°C. The organic phase containing the residual EDCs was then analyzed by Waters HPLC (HP 1,525 series, Gemini 5 μm C6-phenyl 110A 150×4.6 mm column) using a flow rate of 1.0 ml/min and a mixture of acetonitrile and water (90:10) as the elution solvent. The retention time of each EDC was determined by monitoring the effluent, and the identification of each EDC was accomplished by comparing the retention times of the samples to those of analytical-grade EDCs.

The yeast strain used for the estrogenic activity assay of was kindly provided by Dr. T. Nishida of Shizuoka University, Japan, and the activity was evaluated using a yeast two-hybrid system that has been previously described by Tsutsumi *et al.* (2001).

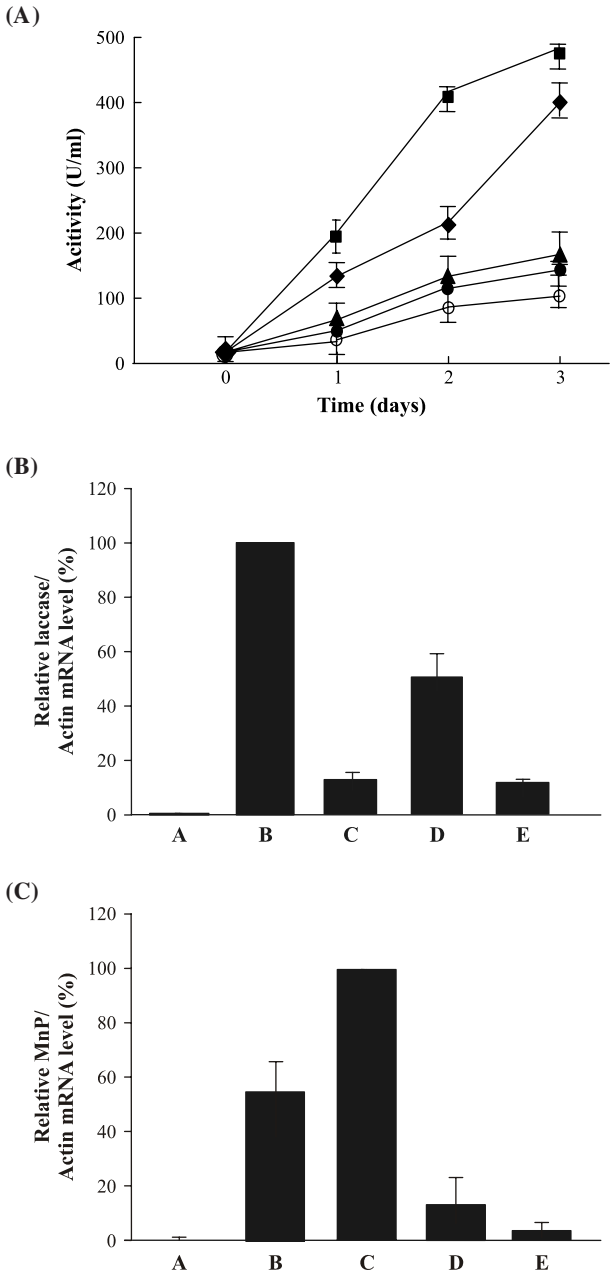

Fig. 2. (A) Assay of laccase activity during the degradation of EDCs. *o*-tolidine was used as the chromogenic substrate. (○), control; (■), benzylbutylphthalate (BBP); (▲), bisphenol A (BPA); (♦), diethylphthalate (DEP); (●), nonylphenol (NP). (B) Determination of laccase expression by real-time PCR using laccase-specific primers. Each value represents the relative amount of laccase expression to actin expression. The maximum level of laccase expression was set as 100%. A, control; B, BBP; C, BPA; D, DEP; E, NP. (C) Determination of MnP expression by real-time PCR was followed using the same procedure with MnP-specific primers. The legends are same as in (B).

To determine if laccase was involved in the degradation of EDCs, 3-day-old culture supernatants were used. Briefly, 400 μl of the culture supernatants were placed into tubes, after which BPA or NP was added (final concentration, 0.175 μM) with or without kojic acid. The samples were then incubated for 3 h, after which the estrogenic activity of each EDC was measured using the yeast two hybrid system.

Results and Discussion

T. versicolor has very good degrading ability towards various recalcitrant compounds; therefore, the ability of this fungus to degrade several EDCs was evaluated in this study. A putative laccase cDNA was successfully cloned by RACE-PCR using several primer sets, and its nucleotide and deduced amino acid sequences are presented in Fig. 1. The laccase cDNA isolated here has 4 putative copper binging regions, which is similar to that of other fungal laccases. In addition, the amino acid sequence of the laccase cDNA isolated here was found to be 57% (AB212731), 64% (AB212732), 96% (BAD98307), and 97% (Q12719) homologous with those of other *T. versicolor* laccases.

Different concentrations of EDCs were added to the cultures to obtain linear growth rates that were similar between all cultures evaluated here (ca. 70% growth of control culture). The laccase activity (more than 4 fold on day 3, Fig. 2A) and expression (Fig. 2B) increased rapidly in response to the addition of BBP or DEP to the culture broth. However, the addition of BPA or NP resulted in only a small increase in laccase activity (1.5 fold) and expression (Fig. 2). When the MnP expression was evaluated, BPA was found to induce an almost 100 fold increase in its expression, whereas BBP induced an increase in expression that was >50 fold. However, the remaining EDCs induced only a low level of MnP expression (Fig. 2C). Because BBP, BPA, DEP, and NP are widely used in diverse industries, it is important to develop methods to attenuate the estrogenic activities of these chemicals. Many studies have reported that white rot fungi are involved in the degradation of endocrine disrupting chemicals such as dioxin (Mori and Kondo, 2002; Sato *et al*., 2002) and phthalate (Lee *et al*., 2007). In addition, *T. versicolor* reportedly has degrading activity against trichloroethylene (Macro-Urrea *et al*., 2008), dye (Champagne and Ramsay, 2005; Gavril and Hodson, 2007), and explosives (Cheong *et al*., 2006). Laccase, which is one of the key enzymes involved in lignin degradation, is involved in the degradation of many recalcitrant compounds, and its enhanced activity has been reported during the degradation of recalcitrant chemicals such as 4-*n*-nonylphenol and aniline (Mougin *et al*., 2002), 2,5-xylidine (Kollmann *et al*., 2005), bisphenol A and nonylphenol (Cabana *et al*., 2007) and explosives (Cheong *et al*., 2006). In this study, we found that the activity and expression of laccase increased by the addition of phthalates (Fig. 2). In the case of bisphenol A and nonylphenol, however, the enzyme activity and expression level of laccase was only slightly higher than the enzyme activity and expression observed in the control culture. This finding indicates that several enzymes are involved in the degradation of EDCs by *T. versicolor* 9522-1.

When the residual concentrations of the original compounds were evaluated by HPLC in 2-day-old cultures, 97± 0.16% of the bisphenol A was found to have been removed, while the other three EDCs were removed completely. In addition, assay using the yeast two-hybrid system revealed that the estrogenic activities of the EDCs were almost completely attenuated at day 2 (Fig. 3A). Additionally, even though nonylphenol showed the highest estrogenic activity, it was completely removed after 3 days of incubation (Fig. 3A). Kojic acid is reportedly a specific inhibitor of fungal laccase and tyrosinase (Murao *et al*., 1992); therefore, it was used in this experiment to determine if the degradation of EDCs was dependent on laccase. To accomplish this, kojic acid was added at a concentration of 0.175 μM to inhibit the laccase activity in the culture supernatants. As shown in Fig. 3B, removal of the estrogenic activities of bisphenol A and nonylphenol was inhibited by the addition of kojic acid. This indicates that, even though laccase was not the only enzyme involved in the degradation and removal of the EDCs and their estrogenic activities, laccase played a critical role in the degradation of EDCs by *T. versicolor* 9522-1. Laccase, lignin peroxidase and manganese peroxidase are major enzymes involved in the degradation of lignin. Lignin peroxidase was found to be important in the decoloration

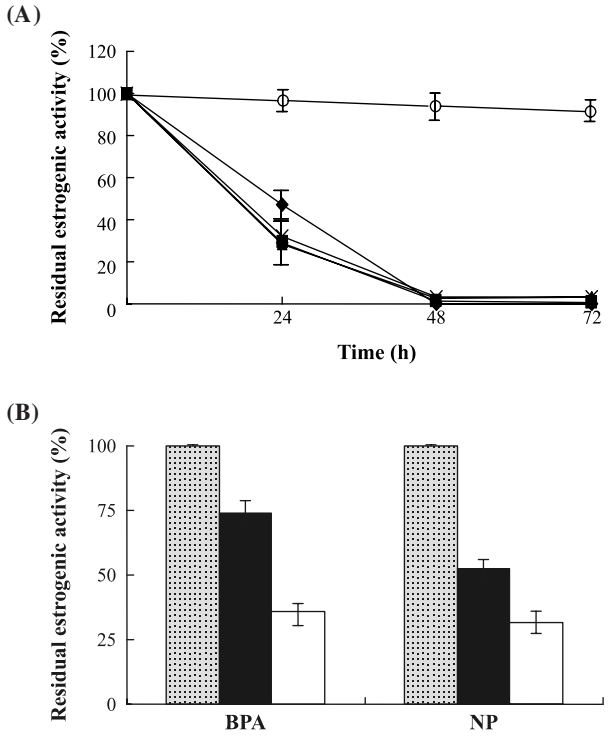

Fig. 3. (A) Removal of estrogenic activity from the culture supernatants of *T. versicolor*. (■), benzylbutylphthalate (BBP); (▲), bisphenol A (BPA); (♦), diethylphthalate (DEP); (×), nonylphenol (NP); (○), BPA without any treatment. (B) Confirmation of the involvement of laccase in the degradation of BPA and NP. Kojic acid was added to the culture supernatant (final concentration, 0.175 μM), and the estrogenic activity was determined after 3 h incubation. Hatched bar, negative control; black bar, inhibited by kojic acid; open bar, positive control (culture supernatant with laccase activity).

of Amaranth by *T. versicolor*, whereas laccase was not (Gavril and Hodson, 2007). The degradation of bisphenol A was confirmed in an *in vitro* experiment using manganese peroxidase (Hirano *et al*., 2000). In addition, manganese peroxidase was found to degrade bisphenol A and nonylphenol more effectively than laccase in an *in vitro* experiment; however, laccase used in conjunction with a mediator more effectively attenuated the estrogenic activities of bisphenol A and nonylphenol (Tsutsumi *et al*., 2001). Biocatalytic elimination of bisphenol A, nonylphenol and triclosan has also been shown to increase in response to the addition of a laccase mediator (Cabana *et al*., 2007). Finally, the kojic acid experiment conducted in the present study demonstrated that laccase played a critical role in the degradation of the 4 EDCs evaluated here.

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