Effective Removal of Endocrine-Disrupting Compounds by Lignin Peroxidase from the White-Rot Fungus Phanerochaete sordida YK-624

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journal or publication title	Current Microbiology
volume	64
number	3
page range	300-303
year	2011-12-28
出版者	Springer
権利	(C) Springer, Part of Springer Science+Business Media. The original publication is available at www.springerlink.com
URL	http://hdl.handle.net/10297/6730

doi: 10.1007/s00284-011-0067-2

Original Paper

Effective removal of endocrine disrupting compounds by lignin peroxidase from the white-rot fungus *Phanerochaete sordida* YK-624

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Running title: Removal of ECDs by LiP

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This work was partially supported by a Grant-in-Aid for Young Scientists (B) (No. 21780296) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Effective removal of endocrine disrupting compounds by lignin peroxidase from the white-rot fungus *Phanerochaete sordida* YK-624

The removal of endocrine disrupting compounds (EDCs) by lignin peroxidase from white-rot fungus *Phanerochaete sordida* YK-624 (YK-LiP1) was investigated. Five endocrine disruptors, *p*-t-octylphenol (OP), bisphenol A (BPA), estrone (E_1), 17β-estradiol (E_2) and ethinylestradiol (EE_2) were eliminated by YK-LiP1 more effectively than lignin peroxidase from *P. chrysosporium* (Pc-LiP), and OP and BPA were disappeared almost completely in the reaction mixture containing YK-LiP1 after a 24-h treatment. Particularly, the removal of estrogenic activities of E_2 and EE_2, which show much higher estrogenic activities than other EDCs such as BPA and OP, were removed following 24-h treatment with YK-LiP1. Moreover, 5,5’-bis(1,1,3,3-tetramethylbutyl)-[1,1’-biphenyl]-2,2’-diol and 5,5’-bis-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-biphenyl-2,2’-diol were identified as the main metabolite from OP or BPA, respectively. These results suggest that YK-LiP1 is highly effective in removing of EDCs by the oxidative polymerization of these compounds.

Introduction

The occurrence of endocrine disrupting compounds (EDC) in the aquatic environment has generated worldwide interest because these chemicals can cause feminization of fish as well as interfere with the reproduction and development of other aquatic organisms [5, 12, 17]. Various natural and synthetic chemical compounds have been identified as EDCs; including pharmaceuticals, pesticides, industrial chemicals, and heavy metals [4]. Typical EDCs of anthropogenic origin with estrogen-like action include *p*-*t*-octylphenol (OP) and bisphenol A (BPA). Natural estrogens, i.e., 17β-estradiol (E_2) and estrone (E_1), and synthetic estrogen, i.e., ethynylestradiol (EE_2), are excreted into wastewater by humans and mammals mainly through their urine. The effluent concentrations of estrogens typically range from a few ng/L to a few tens of ng/L [9, 11], but even these amounts are often high enough to cause endocrine-disrupting effects in some aquatic species such as trouts [24] and minnows [16]. Estrogenic activities of estrogens are two or three orders of magnitude higher than those of EDCs such as BPA [18, 23].

In recent years, white-rot fungi which can degrade lignin effectively are focused since white-rot fungi can degrade various environmental pollutions such as polychlorinated dioxin [10], lindane [3], heptachlor [27], trichlorobenzene [14] polycyclic aromatic hydrocarbons [1]. Moreover, ligninolytic enzymes such as manganese peroxidase (MnP) and laccase were shown to be effective in removing the estrogenic activities of bisphenol A, nonylphenol [25], 4-*tert*-octylphenol [21], and steroidal hormones [20, 22]. However, the detail mechanisms on the detoxification of these compounds are still unknown.

White-rot fungus *Phanerochaete sordida* YK-624, which has been isolated from rotted wood, showed much higher ligninolytic activity and selectivity than either *P. chrysosporium* or *Trametes versicolor* [6]. The major extracellular ligninolytic enzymes of this strain are MnP [6] and LiP [13]. Particularly, this strain produces 2 novel LiP (YK-LiP1 and YK-LiP2), and these enzymes degrade lignin model compounds more effectively than LiP from *P. chrysosporium* (Pc-LiP H8) [7, 19] . In the present study, YK-LiP1 was applied to the removal of EDCs, and the removal properties were compaed with Pc-LiP H8. Moreover, the structures of metabolites from BPA and OP were determined to clarify the removal mechanism of EDCs by YK-LiP1.

Materials and methods

Fungi

P. sordida YK-624 (ATCC 90872) and *P. chrysosporium* ME446 were used in this study. These fungi were maintained on potato dextrose agar slants at 4°C.

Chemicals

BPA, E_1, E_2 and EE_2 were purchased from Tokyo Chemical Industry, Tokyo, Japan. OP was obtained from Wako Pure Chemical Industries, Osaka, Japan. All other chemicals were extra-pure grade and were used without further purification.

LiP preparation and determination of LiP activity

YK-LiP1 from *P. sordida* YK-624 and Pc-LiP (isozyme H8) from *P. chrysosporium* were prepared and purified as described by Sugiura et al. [19] and Wariishi and Gold [26], respectively. LiP activity was measured by monitoring the oxidation of veratryl alcohol (VA) to veratraldehyde ($\varepsilon_{310} = 9.3$ mM^{-1}cm^{-1}). The reaction mixture (1 ml) contained VA (1 mM) and H_2O_2 (0.2 mM) in 20 mM succinate buffer (pH 3.0). One katal (kat) was defined as the amount of enzyme producing 1 mol of product per second.

LiP treatment of EDCs

LiP reactions were performed in 1 ml of reaction mixture containing 2 nkat each LiP, 100 μM EDCs, and 100 μM H_2O_2 in 20 mM succinate, pH 3.0. Reactions were performed in triplicate for 24 h at 30°C and mixing at 150 rpm. The amount of EDCs was determined by high-performance liquid chromatography (HPLC) under the following conditions: column, Wakosil-II 5C18HG (4.6 mm x 150 mm, Wako Pure Chemical Industries, Japan); mobile phase, 50% aqueous acetonitrile containing 0.1% acetic acid (E_1, E_2, EE_2, and BPA) or 80% aqueous methanol containing 0.1% acetic acid (OP); flow rate, 1.0 ml/min; detection wavelength, 275 nm (BPA) , 277 nm (OP), or 285 nm (E_1, E_2, and EE_2).

Estrogenic activity of E_1, E_2, or EE_2 treated with LiP

The estrogenic activities of E_1, E_2, or EE_2 before and after LiP 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. [15]. The concentrations of E_1, E_2, and EE_2 before enzymatic treatment were 1 μM in the assay system (2.5 μl of reaction mixture containing 100 μM E_1, E_2, or EE_2 added to 50 μl of yeast culture and 200 μl of SD medium). Relative estrogenic activity (%) was defined as the percentage of β-galactosidase activity of enzyme-treated E_1, E_2, or EE_2 compared to that of untreated E_1, E_2, or EE_2.

Metabolism experiments

OP or BPA (final concentration 100 μM) were incubated at 30°C for 24 h in a 100-mL reaction mixture containing 200 nkat YK-LiP1 and 200 μM H_2O_2 in 20 mM succinic acid buffer, pH 3.0. The reaction mixtures were extracted 3 times with equal volume of ethyl acetate (EtOAc). The EtOAc extract were dried over anhydrous sodium sulfate and then evaporated to dryness. The concentrates were analyzed by thin-layer chromatography (TLC), HPLC, HR-ESI-MS and NMR. Silica gel plates (Merck

F254, Merck, Darmstadt, Germany) was used for analytical TLC. The metabolite of OP or BPA was further separated by HPLC (column: Wakosil-II 5C18HG) by 80% aqueous methanol containing 0.1% acetic acid or 70% aqueous methanol containing 0.1% acetic acid, respectively. The purified metabolites were then analyzed by HR-ESI-MS and NMR including COSY, HMQC, and HMBC experiments. The HR-ESI-MS data were measured by a JMS-T100LC mass spectrometer. ^{1}H-NMR spectra were recorded by a Jeol lambda-500 spectrometer at 500 MHz, while ^{13}C-NMR spectra were recorded on the same instrument at 125 MHz.

Results and Discussion

We previously showed that YK-LiP1 from *P. sordida* YK-624 degrade dimeric lignin model compounds more effectively than Pc-LiP [7, 19]. Therefore, YK-LiP1 was applied to the removal of EDCs in the present study.

After a 24-h reaction using 2 nkat each LiP, the elimination of EDCs was determined (Fig. 1). Although YK-LiP1 belongs to LiP group, the substrate specificity of YK-LiP was different from that of Pc-LiP [7, 19]. Although same amounts (mole) of enzymes are popularly applied to these experiments, same activities of LiPs were used in this degradation experiment. Approximately 10% of LiP activities remained in each reaction mixture after 24-h. YK-LiP1 effectively removed OP, BPA, E_1, E_2, and EE_2. Particularly, OP, BPA, E_2, and EE_2 were disappeared almost completely in the reaction mixture containing YK-LiP1 whereas Pc-LiP removed OP, BPA, E_1, E_2, and EE_2 by 46.1%, 52.5%, 23.9%, 38.2%, and 45.0%, respectively. These results indicate that YK-LiP1 have a higher affinity than Pc-LiP for these phenolic compounds which were relatively high molecular weight (M.W. 206-296) since Huang et al. has reported that the activity of Pc-LiP toward various phenols is very low [8].

Because removal of toxicity is essential for the biodegradation of environmental pollutants, we examined estrogenic activities of E_1, E_2, and EE_2 treated with each LiP since these EDCs shows much higher estrogenic activities that OP or BPA [20, 21, 25]. Although the estrogenic activity of E_1 treated with YK-LiP1 was hardly decreased, treatments of E_2 and EE_2 by YK-LiP1 reduced the estrogenic activities by 72.6% and 82.6%, respectively (Fig. 2). On the other hands, estrogenic activities of E_1, E_2, and EE_2 treated with Pc-LiP were not reduced.

The removal of EDCs' estrogenic activities by ligninolytic enzymes from white rot fungi (MnP and lassace) has been reported [20, 21, 25]. However, there have been no studies focusing on the metabolic product of these ECDs by ligninolytic enzymes. Therefore, we attempted to identify the metabolites of OP and BPA, relatively simple structures compared with E_1, E_2, and EE_2. The metabolites were detected in the analysis of TLC and HPLC (data not shown). The HR-ESI-MS data for the metabolite of OP, which yielded a molecular ion at *m/z* 409.3084 [M-H] $^-$ (calculated for $C_{28}H_{41}O_2$, 409.3061), indicated that the molecular formula of this compound was $C_{28}H_{42}O_2$. This formula suggested that the metabolite might be a dimer of OP. The structure of the purified metabolite was further characterized by NMR analyses. The ^{13}C-NMR and ^{1}H-NMR spectra indicated that the metabolite of OP had four carbon atoms, one methylene, two methyls and 1,2,4 substituted benzene (data not shown). The 1,1',3,3'-tetramethylbutyl moiety was indicated by HMBC correlations (Fig. 3a) (H-4,4', H-2,2'/C-3,3', H-1'-CH_3,H2,2'/C-1,1'). In addition, HMBC correlations (H-1'-CH_3, H-2,2', H3,3'/C-5,5', H-4,4',H-6,6'/C-1,1') confirmed the metabolite was 5,5'-bis(1,1',3,3'-tetramethylbutyl)-[1,1'-biphenyl]-2,2'-diol. The HR-ESI-MS data for the metabolite of BPA, which yielded a molecular ion at *m/z* 453.2062 [M-H] $^-$ (calculated for $C_{30}H_{30}O_4$, 453.2058), indicated that the molecular formula of this compound was $C_{30}H_{31}O_4$. This formula suggested that the metabolite might be a dimer of BPA. The structure of the purified metabolite was further characterized by NMR analyses. The ^{13}C-NMR and ^{1}H-NMR spectra indicated that the metabolite of BPA had six carbon atoms, two methyls, five methylenes, 1,2,4 substituted benzene and 1,4 substituted benzene (data not shown). HMBC correlations (Fig. 3b) (H-2',3'-methyl/C-1, C-2, C-5, H-3/C-1, C-2, C-5) confirmed the metabolite was 5,5'-bis-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-biphenyl-2,2'-diol. Our current results suggest that each dimer was generated as a metabolite from OP or BPA by YK-LiP1. We propose that the formation of phenoxy radical followed by phenolic hydroxyl of OP was one-electron oxidized, and the radical was transferred to the *o*-position, the radical polymerization thus a dimer of OP was generated. BPA occurs a similar reaction was demonstrated, we proposed that the removal of the ECDs' estrogenic activities might be due to polymerization brought about by enzymatic oxidation.

Acknowledgement

This work was partially supported by a Grant-in-Aid for Young Scientists (B) (No. 21780296) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Figure Legends

Fig. 1. Decrease of BPA，OP，E_1，E_2 and EE_2 by YK-LiP1 and Pc-LiP. Reactions contained 2 nkat each LiP, 100 μM EDCs, and 100 μM H_2O_2 in 20 mM succinate, pH 3.0. Reactions were performed for 24 h at 30 °C and mixing at 150 r.p.m. Values are means ± SD of triplicate samples.

Fig. 2. Removal of estrogenic activities of E_1，E_2 and EE_2 by YK-LiP1 and Pc-LiP. Values are means ± SD of triplicate samples.

Fig. 3. HMBC correlations of the identified OP (a) and BPA (b) metabolites.

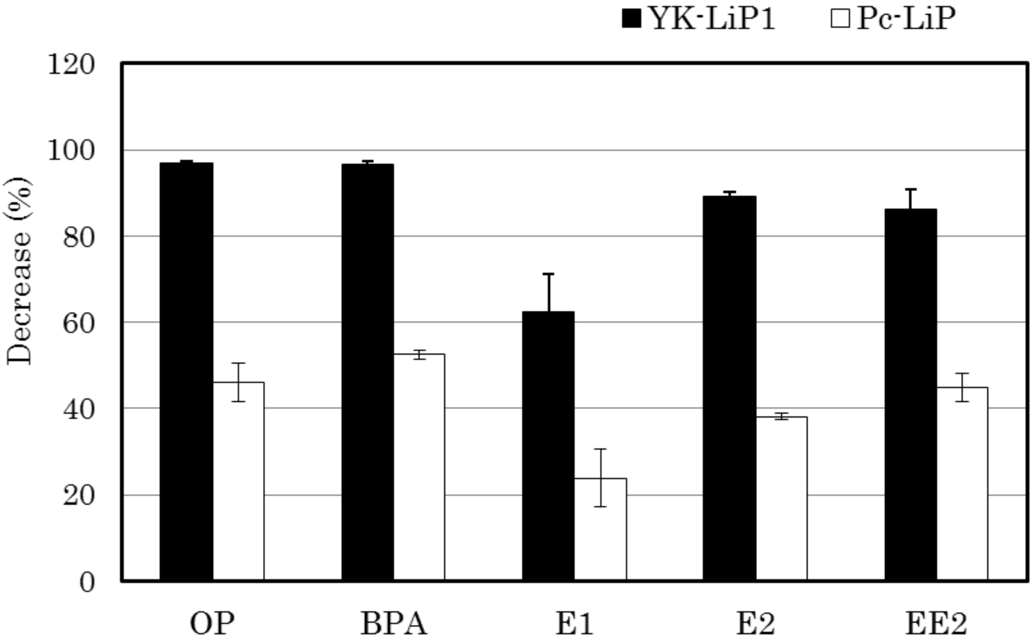

Fig. 1

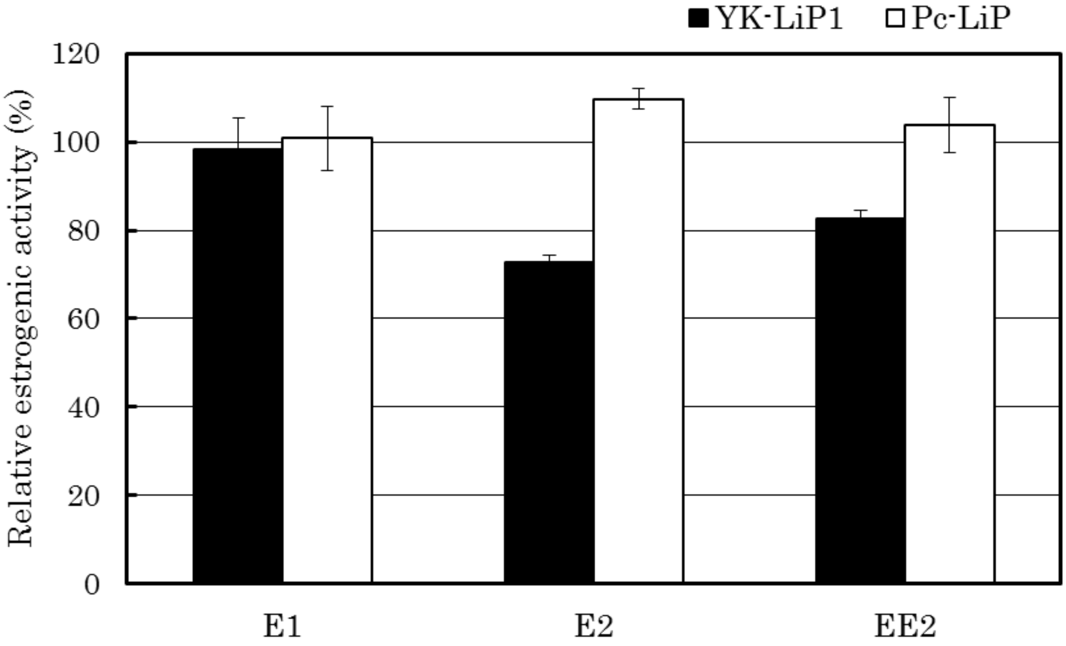
■ YK-LiP1
□ Pc-LiP
Relative estrogenic activity (%)
0
20
40
60
80
100
120
E1
E2
EE2

Fig. 2

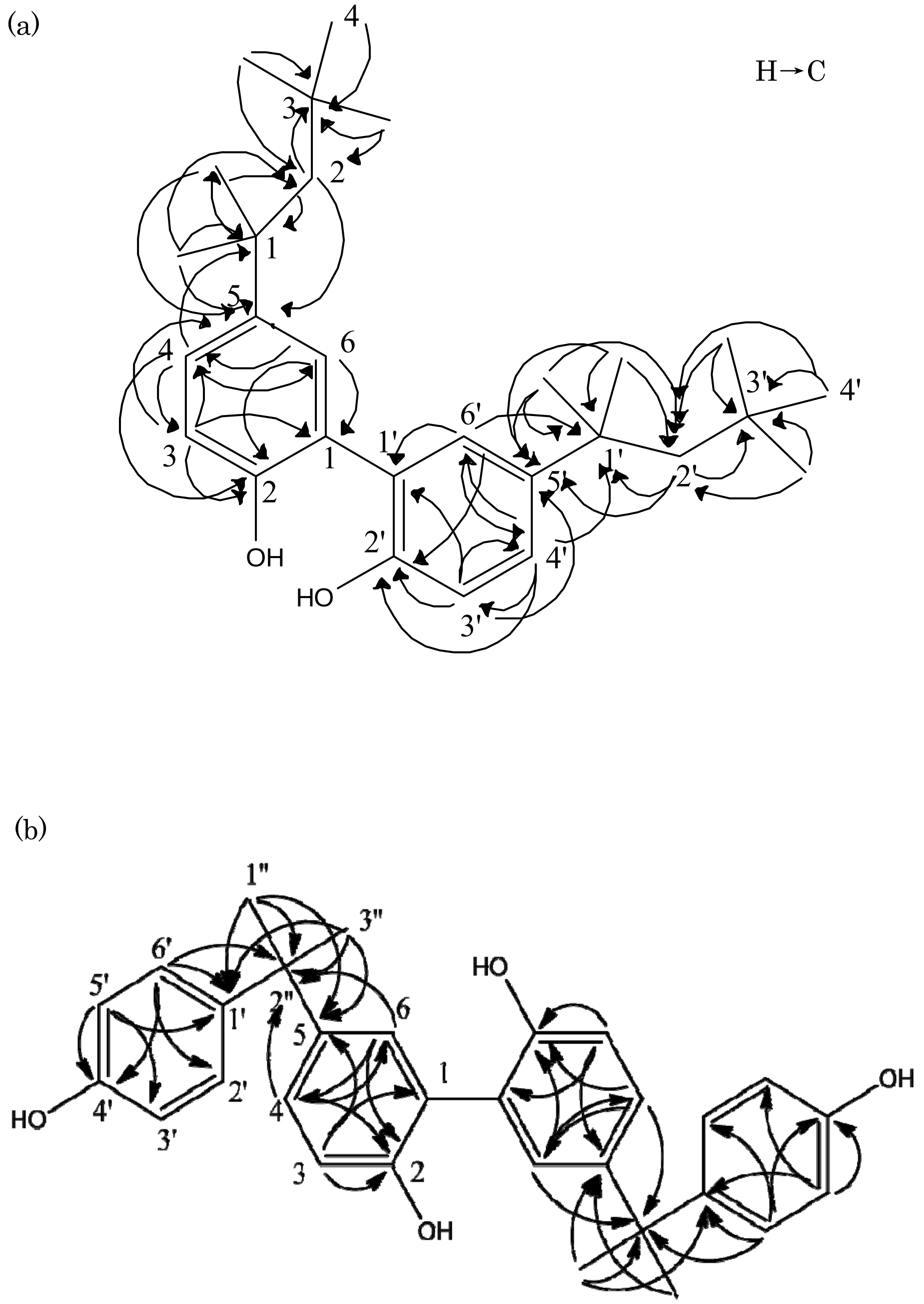
(a)
H→C
4
3
2
1
5
6
4
3
2
1
OH
6'
1'
2'
HO
3'
4'
5'
1'
2'
3'
4'
(b)
1"
3"
2"
6'
5'
1'
HO
4'
2'
3'
6
5
1
4
3
2
OH
HO
OH

Fig. 3