In vitro reduction of coplanar PCB congeners by ABTS oxidases from the culture of Trametes versicolor

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Introduction

In recent years, the environmental contamination by the harmful polluted chemicals becomes more serious. Among them, especially, the dioxins such as coplanar PCBs (Co-PCBs) and PCDDs are hard to be decomposed due to their stability and hydrophobic nature, leading to the world-wide contamination[1, 2]. To clean up the polluted environment, bioremediation using a microorganism is expected to solve the environmental pollution problem because of cost-effective alternative to the more established engineering method. There are some reports on the biodegradation of dioxins using various organisms, in which basidiomycetes, so-called white-rot fungi, have been extensively studied in the process of lignin degradation[3-6]. As a result unique extracellular oxidative lignin-degrading enzymes, such as lignin peroxidase, manganese-dependent peroxidase and laccase were supposed to be responsible for degrading dioxins. Overall many studies on biodegradation of dioxins or other chlorinated aromatic hydrocarbons have focused on using white-rot fungi; however, few reports referred to the metabolism of these environmental pollutants in the in vitro reaction by use of lignin-degrading enzymes produced by white-rot fungi[7, 8]. In this study, we reported the reduction of levels of Co-PCBs in the in vitro incubation with the fractions which had the oxidase activity toward 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonate) (ABTS) in the culture fluid of white-rot fungus, *Trametes versicolor*.

Materials and Methods

Chemicals

Co-PCB congeners of tetrachlorobiphenyl (TeCB), pentachlorobiphenyl (PeCB), hexachlorobiphenyl (HxCB) and heptachlorobiphenyl (HpCB) were purchased from Wellington Labs (Ontario, Canada). Each congener in toluene was mixed and diluted to a concentration of 50

ng/ml in dimethylsulfoxide. ABTS was obtained from SIGMA CHEMICAL CO. (St. Louis, USA). All solvents were pesticide free reagent grade. All other chemicals used were of analytical grade.

Organism and culture condition

The white-rot fungus *T. versicolor* (UAMH 8272) was obtained from University of Alberta Microfungus Collection & Herbariumecotype in Canada. The culture medium contained 10 g of polypeptone, 10 g of glucose, 1.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 2 mg of thiamin hydrochloride, and 16 mg of $CuSO_4 \cdot 5H_2O$ per liter. The final pH of the medium was adjusted to 5.6. The fungus was grown in 1 liter conical flask containing 300 ml of culture medium at 26 °C on a reciprocal shaker (50 rpm) for 7 days in darkness.

ABTS oxidase activity assay

ABTS oxidase activity was determined by using 0.5 mM ABTS as the substrate. Oxidation of ABTS was monitored spectroscopically by absorbance measurement at 420 nm. One unit of ABTS oxidase activity was defined as the amount of protein required to present 0.1 absorbance per min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to confirm the existence of enzyme protein(s) showing ABTS-oxidizing activity. The gel was stained to visualize ABTS-oxidizing activity by using ABTS.

Preparation of ABTS oxidase-containing fractions from the culture

The extracellular culture supernatant was filtered through gauze, concentrated 3-fold by a vacuum evaporation, and then ammonium sulfate was added to the concentrated supernatant to a final concentration of 65 % (w/v) to precipitate proteins. The precipitate was dissolved in 0.2 M succinate buffer, pH 5.6, and then dialyzed against 0.02 M succinate buffer, pH 5.6. The dialyzed solution was applied to an ion-exchange DEAE-Sepharose column (2.5 x 20 cm, Hiprep 16/10 QFF, Amersham Biosciences) equilibrated with 0.02 M succinate buffer, pH 5.6, and then proteins were eluted with a linear sodium chloride gradient which increased from 0 to 1.0 M. The ABTS oxidase-containing fractions were collected, dialyzed against 0.02 M succinate buffer, pH 5.6, concentrated, and finally stored at -20 °C.

In vitro incubation of Co-PCBs with ABTS oxidase-containing fractions and preparation of samples for GC-MS

In vitro incubation of Co-PCBs was carried out for 4 days at 37 °C with shaking in the glass-stoppered test tubes containing 250 μl of 0.2 M succinate buffer, pH 5.6, 50 μl of 11 Co-PCB congeners mixture (50 ng/ml each), and 10,000 or 50,000 units of each ABTS oxidase fraction in a total volume of 1.5 ml filled up with distilled water. As a control experiment, heated ABTS oxidase-containing fractions at 98 °C for 20 min were used instead of native fractions in the in vitro incubation mixture. As internal standards for the precise measuring of each Co-PCB congener, ^{13}C-

3,3',4,4'-TeCB for tetrachloro-congeners analysis and ^{13}C-3,3',4,4',5'-PeCB for penta, hexa, and heptachloro-congeners analysis, were added to the incubation mixture, and then the mixture was hydrolyzed with 1 M KOH overnight. Co-PCBs were extracted with hexane 3 times, and then acid hydrolysis of the hexane extract with 18 M sulfuric acid was repeated until the brown color disappeared in the aqueous layer. After rinsing, dehydration and concentration of the hexane extract, a column chromatography was performed on the two-layer column packed with a silica gel for upper layer and an alumina for lower layer. The elution of Co-PCBs was performed with 2 % (v/v) dichloromethane in hexane, and then ^{13}C-3,3',4,4',5,5'-HxCB was added to the concentrated solution of Co-PCBs as the syringe spike-standards for a correction of injection volume in GC-MS.

GC-MS condition

Gas chromatograph (HP6890/HEWLETT PACKARD, USA) having SPB-5 capillary column (30m/SUPELCO, USA) equipped with high-resolution mass spectrometry detector (MStation JMS-700/JEOL, Japan) was used for the analysis of Co-PCB congeners. The mass value used for single ion monitoring was 291.9194, 325.8804, 359.8415 and 393.8025 for TeCBs, PeCBs, HxCBs and HpCB, respectively[9].

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Results and Discussion

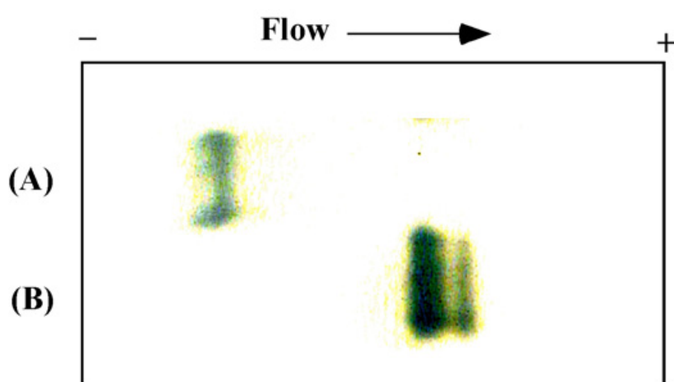

Figure 1 : SDS-PAGE of ABTS oxidases produced extracellularly by *T. versicolor*

PAGE was performed with 10% polyacrylamide gel at 150 V. Active bands of ABTS oxidases of the first fraction (A) and the second fraction (B) from a DEAE-Sepharose column were stained by use of ABTS.

Detection of ABTS oxidase activity in the culture fluid of T. versicolor

The first fraction containing ABTS oxidase activity was passed through the ion-exchange DEAE-Sepharose column during loading and washing with the equilibrating buffer. The second fraction was eluted with approximately 0.4 M sodium chloride in a linear sodium chloride gradient elution system from 0 to 1.0 M. SDS-PAGE showed the existence of three different forms of ABTS oxidases in the culture fluid of *T. versicolor* as shown in Figure 1. Form 1 which had the largest size appeared in the first fraction, and form 2 and 3 which were

smaller than form 1 existed in the second fraction. ABTS is widely used as a substrate for laccase activity assay, so SDS-PAGE experiment suggests that three different laccase isozymes were produced in the culture fluid by *T. versicolor*.

Reduction of Co-PCBs levels in the in vitro incubation with ABTS oxidases

As shown in Table 1, overall the first fraction containing ABTS oxidase has a higher activity to reduce each level of Co-PCB congeners compared to the second fraction. However, it is interesting that the reduction rate of HxCB congeners was larger in the second fraction containing 50,000 units of ABTS oxidase than that in the first fraction containing same units of ABTS oxidase. On the contrary, the first fraction containing 10,000 units of ABTS oxidase reduced the level of HxCB congeners more than the second fraction containing same units of enzyme. This result suggests that the substrate specificity against Co-PCB congeners is different between ABTS oxidase in the first fraction and that in the second fraction. In equally chlorinated congeners, the levels of non-ortho congeners were less reduced than ortho congeners, such as PCB 126 in PeCB and PCB 169 in HxCB, and also the reduction rate of non-ortho TeCBs, PCB 77 and 81 was the smallest in the Co-PCB congeners. It seems that this result is due to the difference of solubility in water between non-ortho and ortho congeners.

Table 1: Reduction of each level of Co-PCB congeners in the in vitro incubation by ABTS oxidases

Co-PCB congeners (IUPAC #)		Reduction rate of Co-PCB congeners (%)			
		First fraction		Second fraction	
		10,000 units	50,000 units	10,000 units	50,000 units
TeCB	77*	5.0	14.8	2.6	11.3
	81*	5.5	12.8	5.5	12.0
PeCB	105	12.0	31.7	9.2	25.7
	114	18.3	29.7	13.9	25.0
	118	12.8	31.8	11.6	22.3
	123	11.6	27.6	10.5	23.5
	126*	11.5	16.6	9.0	14.9
HxCB	156	17.8	24.1	13.4	24.5
	167	17.3	22.0	11.6	26.2
	169*	15.1	14.2	12.4	18.5
HpCB	189	11.8	12.2	9.6	12.6

* non-ortho congeners

ABTS is a well-known substrate for laccase activity assay[7], so ABTS oxidases are expected to be the same enzymes as laccases. Laccases are extracellular multicopper enzymes which catalyze the oxidation of a wide variety of phenolic compounds containing chlorinated phenols[10,11]. The substrate non-specificity of laccases leads us to examine them as candidates for the bioremediation of environmental pollutants. The enzyme preparation used in this study was crude which contained some other proteins besides ABTS oxidases, so purified ABTS oxidases have to be used to make sure of real agents for the reduction of Co-PCBs.

Acknowledgements

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