PURIFICATION OF HIGHLY CHLORINATED DIOXNS DEGRADING ENZYME

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Introduction

Soil contamination caused by dioxins in and around sites of incinerators for municipal solid waste (MSW) is a concern in Japan. For example, scattering wastewater from a wet gas scrubber at an MSW incinerator facility in Nose, Osaka caused soil and surface water contamination. The concentration of dioxins in the soil was about 8,000 pg-TEQ/g. Other contamination sites include soils on which fly ash has been placed directly or improperly stored and landfill sites that have received bottom and fly ash over a long period. Some countermeasures are required immediately at these dioxins-contaminated sites.

We have previously developed bioreactor systems for dioxin-contaminated water and soil[1, 2]. We have shown that a fungus, *Pseudallescheria boydii (P. boydii)*, isolated from activated sludge treating wastewater that contained dioxins, has the ability to degrade highly chlorinated dioxins. A reaction product of octachlorinated dibenzo-p-dioxin (OCDD) was identified as heptachlorinated dibenzo-p-dioxin[1]. Therefore, one of the pathways for degradation of OCDD by this fungus was predicted to be as follows: OCDD is transformed by dechlorination and then one of the remaining aromatic rings is oxidized.

To apply *P. boydii* to on-site technologies (e.g., bioreactor systems), as well as *in situ* technologies, enzyme treatment using a dioxin-degrading enzyme from *P. boydii* needs to be developed because *P. boydii* is a weak pathogenic fungus, known to cause opportunistic infection[3]. As a result, we have studied enzyme purification of nonchlorinated dioxin, namely, dibenzo-p-dioxin (DD)[4]. However, we did not try to identify enzymes capable of degrading highly chlorinated dioxins.

This study has elucidated a method of enzyme assay for measuring OCDD-degrading activity, and has attempted to purify OCDD-degrading enzymes from *P. boydii* using enzyme assay. In addition, as first step toward purifying 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), 2,3,7,8-TCDD degradation tests were carried out using living cells, external cell enzymes, and membrane enzyme of *P. boydii*.

Materials and Methods

Fungus: The fungus was isolated from activated sludge in a leachate treatment facility associated with MSW landfill sites in Japan[2]. This leachate contained dioxins. The fungus was found to be *Pseudallescheria boydii* (*P. boydii*) by 18S rDNA and morphological analysis[4].

Cultivation conditions: The medium contained glucose, 1.0g; $(NH_4)_2SO_4$, 0.2 g; NaCl, 0.2 g; K_2HPO_4, 0.1 g; $MgSO_4 \bullet 7H_2O$, 0.1 g; $CaCO_3$, 0.2 g and 0.1 mL of a trace element solution ($FeSO_4 \bullet 7H_2O$, 0.01 g; $MnCl_2 \bullet 4H_2O$, 0.01 g and $ZnSO_4 \bullet 7H_2O$, 0.01 g per 10 mL of distilled water) per 100 mL of distilled water. After pre-cultivation was carried out in a 500 mL Erlenmeyer flask for two days at 30 °C, cultivation continued at 30 °C in an 8 L fermentor.

Preparation of external cell enzyme and membrane enzyme from *P. boydii*: We attempted to confirm which membrane enzyme or external cell enzyme was able to degrade OCDD or 2,3,7,8-TCDD. The membrane enzyme mixture was prepared using the method described by Ishii[4]. The external cell enzyme mixture was prepared by filtering the culture using a 0.2 μm of membrane filter.

Dioxins degradation tests: After a designated quantity of OCDD or 2,3,7,8-TCDD was injected into a 5 mL test tube, 1 mL of the external cell enzyme mixture, or 0.1 mL of the prepared membrane enzyme mixture and 0.9 mL of citric acid–trisodium citrate buffer (pH 3.5), or phosphoric acid–trisodium phosphate buffer (pH 6.9) containing 1.2 mM NAD^+, NADH or NADPH and 1 mM Mn, were added to the test tube. The test tube was then shaken slowly at 30 °C for 48 hours. After labeled OCDD or 2,3,7,8-TCDD and NaOH or H_2NO_3 and H_2SO_4 were added to the tube, OCDD or 2,3,7,8-TCDD was extracted by shaking three times with 0.3 mL of toluene. These alkalis and acid were used to extract dioxins absorbed in the organic matter in the samples as far as possible.

Enzyme purification: The membrane enzyme was desalted and then concentrated to approximately 5 mL by ultrafiltration with a 10,000 MW cut-off membrane filter (UK-10, Advantec). The concentrate sample was then subjected to anion-exchange chromatography (AEC). The electrical conductivity of the sample was adjusted to that of 50 mM tris-HCl (pH 8.0) (buffer B), which was used as the eluting medium during AEC.

AEC was performed on an open column (30 x 150 mm) filled with Toyopearl DEAE-650M (Toso, Japan), which had been equilibrated with buffer B. After the sample was injected, the column was eluted with 100 mL of buffer B (approximately 1 mL/min), in which the NaCl concentration was progressively increased from zero to 400 mM in 50 mM increments. Each eluted fraction was concentrated to approximately 5 mL by the ultrafiltration.

Gel electrophoresis: Some fractions selected from the AEC fractions on the basis of enzyme assays were submitted to SDS-PAGE to confirm the purity of the enzymes. SDS-PAGE was performed by the method of Laemmli[7] on a 12.0% acrylamide gel containing 0.1% sodium dodecyl sulfate. The gel staining was performed with CBB (Wako, Japan). The molecular masses of the enzymes were estimated with the standard protein Perfect Protein Markers, 10-225 kDa (Takara).

Analysis of dioxins: After confirmation that there was no color of toluene, the toluene phase was applied to a GC-MS/MS apparatus (ThermoQuest GCQ plus ion trap mass spectrometer and TRACE GC 2000 gas chromatograph) in accordance with the method of Kemmochi and Arikawa[5].

Chemicals: All dioxins used in this study were made by CIL, Inc. The OCDD and 2,3,7,8-TCDD were ED-980 and ED-899, respectively, and the isotopically-labeled OCDD and 2,3,7,8-TCDD were ED-981 and ED-900, respectively. The other chemicals were all laboratory grade.

Results and Discussion

OCDD degradation tests using the membrane and external cell enzymes

The membrane enzyme had the activity of OCDD degradation. In particular, approximately 70%

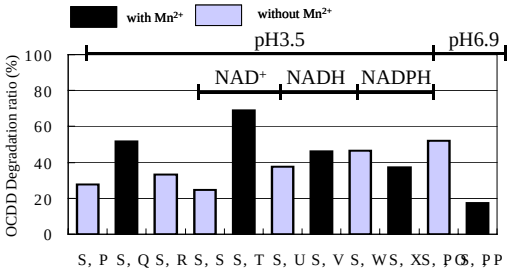

Figure 1 OCDD-degrading activity of the membrane

of OCDD was degraded when pH 3.5 and NAD^+ and Mn^{2+} were added, as shown in Figure 1. This indicated that NAD^+ and Mn^{2+} tended to promote the OCDD degradation. On the other hand, the external cell enzyme did not degrade the OCDD significantly (not shown in figures). Therefore, we decided to try to purify the OCDD-degrading enzyme from the membrane enzyme, and to add 1.2 mM NAD^+ and 1.0 mM Mn^{2+} to the enzyme at enzyme assay during purification of the OCDD-degrading enzyme.

Purification of OCDD-degrading enzyme

Figure 2 shows the result of AEC, suggesting that the OCDD degrading activity was the highest with 200 mM of chloride ion concentration. However, the activity was seen with the chloride ion concentration from 0 mM to 100 mM, because part of the OCDD-degrading enzyme, which could not be adsorbed with anion exchange resin, may elute from the column.

The fraction of 200 mM of chloride ion concentration was submitted to SDS-PAGE. The results revealed two bands (not shown in figures), whose molecular weights were estimated as 45 kDa and 27 kDa, respectively.

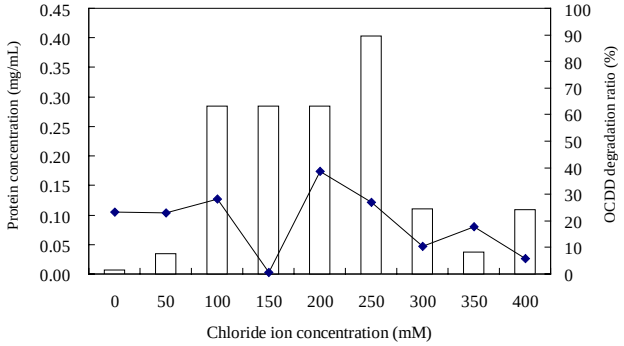

Figure 2 Result of anion-exchange

2,3,7,8-TCDD degradation tests

Living cells: First, 2,3,7,8-TCDD degradation tests using the living cells of *P. boydii* were conducted. Figure 3 suggests that 50 ng/mL of 2,3,7,8-TCDD was degraded by the living cells. The concentration of 2,3,7,8-TCDD was higher than in our previous experiment (approximately <2 ng/mL) [1]. This demonstrates that *P. boydii* has the ability to degrade very large quantities of 2,3,7,8- TCDD.

External cell enzyme: Figure 4 shows the remaining 2,3,7,8-TCDD in the test tube after the degradation tests using the external cell enzyme. It seems that the external cell enzyme did not have as great as 2,3,7,8-TCDD-degrading activity compared with blank data, where the external cell enzyme was heated to deactivate its activity.

Membrane enzyme: On the other hand, the membrane enzyme demonstrated the 2,3,7,8-TCDD degrading activity shown in figure 5. The activity was higher when Fe^{2+} was added to the membrane enzyme. Therefore, 2,3,7,8-TCDD-degrading enzyme was also found to exist in the membrane enzyme of *P. boydii*, like the OCDD-degrading enzyme and the DD-degrading enzyme[4]. From past studies, for example, white-rot fungi[6], it is evident that the external cell enzyme seems to be related to dioxins degradation. Therefore, the dioxin degradation mechanism of *P. boydii* seems to be difference from that of white-rot fungi.

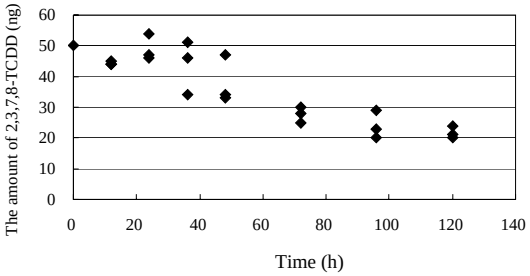

Figure 3 2,3,7,8-TCDD-Degradation by living cell of *P.*

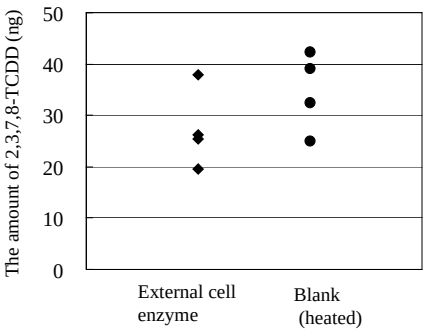

Figure 4 2,3,7,8-TCDD-degrading activity of external cell enzyme

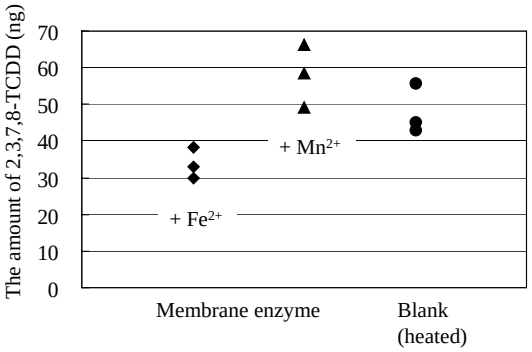

Figure 5 2,3,7,8-TCDD-degrading activity of membrane enzyme

Acknowledgments
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