ENZYME PURIFICATION FROM A DIOXIN-OXIDIZING FUNGUS

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Introduction

In Japan, soil contamination caused by dioxins produced at incinerators for municipal solid waste (MSW) has been a cause of concern. 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.

We have developed bioremediation technologies for dioxin-contaminated water and soil[1-3], because biological methods are inexpensive and have little potential to produce toxic by-products. We showed that a fungus isolated from activated sludge treating wastewater that contained dioxins would degrade highly chlorinated dioxins[2]. A reaction product of octachlorinated dibenzo-p-dioxin (OCDD) was identified as heptachlorinated dibenzo-p-dioxin. Therefore, one of the pathways for degradation of OCDD by this fungus was predicted to be as follows: OCDD would be transformed by dechlorination and one of the remaining aromatic rings would be oxidized. In other words, the fungus may produce enzymes capable of both dechlorination and oxidation. Therefore, we focused on purification of the oxidases, as the first step toward elucidating the degradation mechanisms of dioxins by the fungus.

In general, dioxin-degrading enzymes of fungi include the P-450 mono-oxygenases and peroxidases of white-rot fungi[4]. However, relatively little is known about methods for purification of these dioxin-degrading enzymes. In addition, the enzymes in which we are interested may be new enzymes and may not be able to be purified by existing methods. Therefore, the objective of this study was to develop a reproducible and effective method for purifying enzymes that degrade dibenzo-p-dioxin (DD), by measurement of DD in enzyme assays.

Materials and Methods

Fungus

The fungus used has been isolated from activated sludge in a leachate treatment facility associated with MSW landfill sites in Japan[2]. The leachate was found to contain dioxins. This fungus has the ability to decolorize lignin.

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; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCO_3$, 0.2 g and 0.1 mL of a trace element solution ($FeSO_4 \cdot 7H_2O$, 0.01 g; $MnCl_2 \cdot 4H_2O$, 0.01 g and $ZnSO_4 \cdot 7H_2O$, 0.01 g per 10 mL of distilled water) per 100 mL of distilled water. In addition, we added 0.1 mL of 0.1% sodium resazurin solution to enhance the activity of oxidases, and carboxymethyl cellulose (0.3 g) to prevent the fungus from turning into a solid mass. After pre-cultivation was carried out in a 500-mL Erlenmeyer flask for two days at 30 °C, cultivation at 30 °C in an 8-L fermentor was continued until the resazurin was decolorized.

18S rDNA analysis

The DNA was extracted from the cultured cells using a DNA extraction kit Dr.GenTLE for Yeast

(Takara, Japan). PCR was then performed according to White[5]. The sequence of the PCR product was analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Enzyme purification

After cultivation of the fungus, the cells were collected from the culture medium by centrifugation at 10,000 rpm and 4 °C for 15 min. (Himac CR21F and R12A3, Hitachi, Japan). We collected and used about 2 kg of cells for separation of the enzyme. The collected cells were processed, in 80 g batches, by homogenizing in an ultrasonic liquid processor (Digital Sonifier, Branson) in 800 mL of buffer A for 10 min. Buffer A is a citric acid – trisodium citrate buffer (pH 3.5) that includes 0.6 % n-octyl-b-D-glucoside as a surfactant. It was kept on ice during the homogenization step. After removal of the broken cells by centrifugation, the supernatant was used again as the medium to homogenize the next batch of cells to concentrate enzymes of our interest into the buffer. This and all of the subsequent operations required for separation and purification of the enzyme were carried out at 4 °C.

The proteins in the supernatant were separated by ammonium sulfate fractionation (ASF). The concentrations of ammonium sulfate were 30 %, 40 %, 50 %, 60 % and 80 % of the saturated concentration (767 g/L).

The fractions obtained were desalted and then concentrated to about 5 mL by ultrafiltration with a 10,000 MW cut-off membrane filter (UK-10, Advantec). The fraction was then subjected to anion-exchange chromatography (AEC). The electrical conductivity of each fraction 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 desalted and concentrated fraction was injected, the column was eluted with 100 mL of buffer B (about 1 mL/min) in which the NaCl concentration was progressively increased from zero to 0.35 M in 0.05 M increments. Eeach eluted fraction was concentrated to about 5 mL by ultrafiltration.

Those AEC fractions that had activity toward DD degradation were combined and cleaned up by molecular sieve chromatography (MSC) on an open column (50 x 900 mm) filled with Toyopearl HW-55F (Toso, Japan) that had been equilibrated with buffer A overnight. Following application of the combined sample, the column was eluted with buffer B at about 1 mL/min. The elute was collected in 150-drop fractions.

Gel electrophoresis

Some fractions selected from the MSC fractions on the basis of enzyme assays were submitted to Native-PAGE and SDS-PAGE to confirm the purity of the enzymes. Native-PAGE was performed by the method of Davis[6] on a 7.2 % acrylamide gel (pH 9), whereas SDS-PAGE was performed by the method of Laemmli[7] on a 12.5 % acrylamide gel containing 0.1 % sodium dodecyl sulfate. In both cases, gel staining was carried out with a Silver Stain II Kit (Wako, Japan). The molecular masses of enzymes were estimated with the standard protein 10-kDa protein ladder (GIBCO BRL).

Enzyme assays

The DD-degrading activity of the enzyme-containing fractions was assayed at each step during the enzyme purification. In preliminary experiments with resazurin, a basic reaction solution was determined that contained 1 mM NAD and 1 mM Mn in buffer A. One mL of 10 ng/L DD dissolved in toluene was injected into a test tube. Then 1 mL of sample including enzymes and 9 mL of the reaction solution were added to the test tube. The test tube was shaken slowly at 30 °C for 12 hours. After 1 mL of 10 ng/L labeled DD (^{13}C) and 100 mL of 10 M NaOH were added to the tube, DD in the reaction solution was extracted with 0.3 mL of toluene, three times. The toluene phase was evaporated to 0.1 mL and was submitted to GC-MS analysis. In this study, the DD-degrading activity of the enzymes was represented as the degradation ratio of DD, which was defined as (1- (remaining DD)/(spiked DD))x100, according to this procedure.

Analytical methods

The protein concentration was determined by its absorbance at 280 nm, using a spectrophotometer U-2001 (Hitachi, Japan). The concentration of DD was determined using a GC-MS apparatus (ThermoQuest GCQ plus ion trap mass spectrometer and a TRACE GC 2000 gas chromatograph) on a DB5 column (0.25 mm×30 m). The oven was held at 100 °C for 1 min., and subsequently the temperature was programmed to increase by 10 °C /min to 190 °C , then to increase at 12.5 °C /min to 290 °C . It was then held at 290 °C for 10 min. The injector temperature was kept at 280 °C, and the ion volume temperature at 200 °C. The MS condition was full-scan mode from 100 to 350 amu.

Chemicals

For the quantification of DD, labeled and unlabeled dibenzo-p-dioxins (CIL, Inc.) were used and all other chemicals were laboratory grade.

Results and Discussion

Identification of the fungus

Morphological and 18S rDNA analysis revealed that the fungus is *Pseudallescheria boydii.*

Enzyme purification

Figure 1 shows the degradation ratio of DD for each of the fractions separated by ASF. Since the fractions in which the concentrations of ammonium sulfate were 30 % and 80 % of its saturated concentration had no activity, the protein precipitation obtained in 30% of the saturated concentration was removed, and the remaining solution was submitted to AEC.

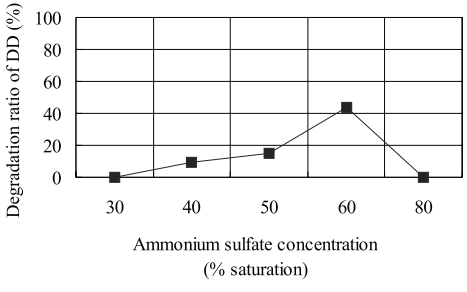

Figure 1. Activity in each fraction separated by ammonium sulfate fractionation

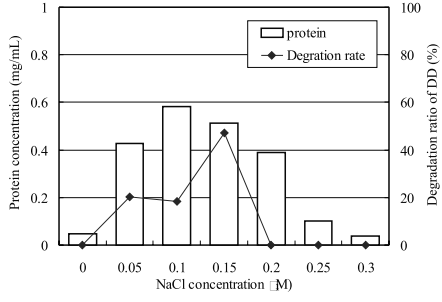

Figure 2. Protein fraction chromatographed on Toyopearl DEAE-650M and the activity of each fraction

Figure 2 shows the protein concentration and the degradation ratio of DD for each fraction resulting from AEC. Since no protein was observed in the fraction in which the concentration of NaCl was 0.35 M, the elution was stopped at that point. The results revealed that the fractions from 0.05 to 0.15 M NaCl concentration showed the DD-degrading activity. Therefore, these fractions were pooled and submitted to MSC.

Figure 3 shows a chromatogram of absorbance at 280 nm, representing the concentration of proteins, obtained from MSC. Since it is very difficult and time-consuming to determine the activity of each fraction toward DD and clear peaks could not be obtained through this procedure, fractions were pooled together to give a total of five fractions, as shown in Figure 3. For the five fractions, the activity toward DD was determined. The DD analysis revealed that there was activity only in the first fraction and the degradation ratio of DD was 32.5%.

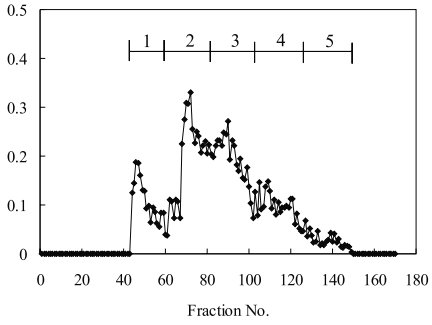

Figure 3. Protein fraction by Molecular sieve chromatography

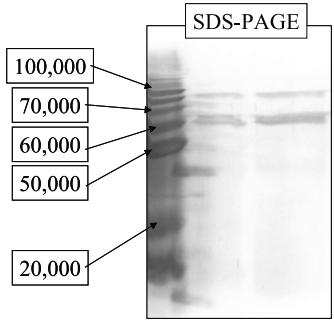

Figure 4. SDS-PAGE of the MSC-purified enzymes

Molecular mass of purified enzymes

Native-PAGE of the purified enzyme gave two protein bands (not shown). The same purified enzymes were submitted to SDS-PAGE, which also showed two protein bands, as seen in Figure 4. The molecular masses of the two enzymes were estimated as approximately 61 kDa and 83 kDa. Currently, we are analyzing N-terminal sequences of these protein bands.

Reaction products

During assay of DD-degrading activity, a peak that might be that of a reaction product, was detected by GC-MS. Its molecular mass was 200, which implied that DD had been hydroxylated (the molecular mass of DD is 184).

Acknowledgments

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