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Novel enzymatic activity of cell free extract from thermophilic *Geobacillus* sp. UZO 3 catalyzes reductive cleavage of diaryl ether bonds of 2,7-dichlorodibenzo-*p*-dioxin

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ABSTRACT

We characterized the ability of the cell free extract from polychlorinated dibenzo-*p*-dioxins degrading bacterium *Geobacillus* sp. UZO 3 to reduce even highly chlorinated dibenzo-*p*-dioxins such as octachlorodibenzo-*p*-dioxins in incineration fly ash. The degradation of 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD) as a model dioxin catalyzed by the cell free extract from this strain implicates that the ether bonds of 2,7-DCDD molecule undergo reductive cleavage, since 4′,5-dichloro-2-hydroxydiphenyl ether and 4-chlorophenol were detected as intermediate products of 2,7-DCDD degradation.

1. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are among the most problematic by-products of pulp and paper bleaching, pesticide manufacturing, and incineration of halogen-containing chemicals. These widespread and persistent environmental contaminants present serious public health issues because of their toxicity, and mutagenic and carcinogenic properties (Meharg and Osborn, 1995; Kaiser, 2000).

The past few decades have been marked with breakthroughs in discovering biological agents for remediation of these pollutants. A number of bacteria capable of mineralizing non-chlorinated dibenzo-*p*-dioxin (DD) and/or non-chlorinated dibenzofuran (DF) have been isolated by enrichment culture methods using carbon-free mineral medium supplemented with DD or DF as sole carbon and energy sources. The degradation of these compounds by aerobic microorganisms including *Sphingomonas wittichii* RW1, *Terrabacter* sp. DBF63, and *Pseudomonas* sp. CA10 strain has been studied using molecular techniques (Wittich et al., 1992; Monna et al., 1993; Wilkes et al., 1996; Sato et al., 1997; Armengaud et al., 1998; Nojiri et al., 1999; Habe et al., 2001; Kasuga et al., 2001; Hong et al., 2002; Nam et al., 2006).

Armengaud et al. (1998) succeeded in cloning a gene (*dxnA1A2*) from *S. wittichii* RW1 that codes for angular dioxygenase, a dioxin-degrading enzyme. They demonstrated that this enzyme introduces two hydroxyl groups in angular positions neighboring the dioxin diaryl ether bonds for subsequent degradation through an unstable intermediate acetal structure (Armengaud et al., 1998). Habe et al. (2001) also isolated angular dioxygenase genes from *Terrabacter* sp. DBF63 and *Pseudomonas* sp. CA10 strain encoding dioxin-degrading enzymes that act in the same oxygenation mechanism. However, neither the crude enzyme extracts prepared from these microorganisms nor the angular dioxygenase obtained by genetic recombination of the isolated genes (*dbfA1A2*, *carAaAcAd*) has demonstrated degradative activity on the diaryl ether structure of PCDDs such as mono- and di-chlorodibenzo-*p*-dioxin (Sato et al., 1997; Nojiri et al., 1999; Habe et al., 2001; Kasuga et al., 2001). Nam et al. (2006) recently used resting cells to investigate degradation products from PCDDs, 1,2,3-tri, 2,3,7-tri, 1,2,3,4-tetra, 1,2,3,7,8-penta and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin, but no 2,3,7-tri and 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin degradation activity was found in these cells. Despite numerous studies on the microbial degradation of chlorinated dioxins, there is still

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a lack of information concerning the enzyme-catalyzed degradation of these compounds.

In 1995, we isolated the bacterial strain *Geobacillus* sp. UZO 3 from a forest compost pile and discovered its ability to deplete PCDDs, suggesting its potential application in bioremediation. The bacterium is Gram-positive and thermophilic, with an optimal growth temperature of 65 °C. By the transformation experiments that monitored the disappearance of PCDDs in fly ash, we have demonstrated the ability of *Geobacillus* sp. UZO 3 to deplete highly chlorinated dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), hexachlorodibenzo-*p*-dioxin, and even octachlorodibenzo-*p*-dioxin (Hoshina et al., 2002). Since this bacterium has been known to reduce even highly chlorinated dibenzo-*p*-dioxins, we hypothesized that the initial catalytic step in the degradation of PCDDs involves cleavage of the intra-molecular ether bond which is a common structure regardless of the position of the chlorine substituents.

In the present study, we have investigated the capacity of the cell-free enzyme extract from *Geobacillus* sp. UZO 3 to degrade chlorinated 2,7-DCDD, and successfully detected its chlorinated dioxin-degrading activity. This novel finding further elucidate that the enzymatic catalyzes the reductive cleavage of the diaryl ether bonds of 2,7-DCDD to produce 4′,5-dichloro-2-hydroxydiphenyl ether (DCDE) and 4-chlorophenol (4CP).

2. Materials and methods

2.1. Chemicals

2,7-DCDD was purchased from Accu Standard (New Haven, Conn.). Mono-chlorophenol (2-, 3-, 4- chlorophenol), $Cu(OAc)_2$, CH_2Cl_2, $CDCl_3$, and tetramethylsilane were purchased from Wako Pure Chemical (Osaka, Japan). N,O-Bis(trimethyl silyl)trifluoroacetamide (BSTFA) and 4-chlorocatechol were purchased from Tokyo Kasei (Tokyo, Japan). 4-chlorophenylboronic acid was purchased from Sigma–Aldrich (Steinheim, Germany). Purities of these chemicals range from 96.6% to 100%. All other chemicals used were of analytical grade and of the highest purity available.

2.2. Synthesis of DCDE

DCDE was synthesized from 4-chlorocatechol and 4-chlorophenylboronic acid according to the procedure of Evans et al. (1998). A flask was charged with 4-chlorocatechol (1.0 equiv), $Cu(OAc)_2$ (1.0 equiv), 4-chlorophenylbolonic acid (1.0–3.0 equiv) and powdered molecular sieves. The reaction mixture was diluted with CH_2Cl_2, and pyridine as the amino base (5.0 equiv) was added. After stirring (24 h, 25 °C) the colored heterogeneous reaction mixture for under ambient atmosphere, the resulting slurry was filtered, washed with distilled water and the organic layer was collected. The diaryl ether was purified by silica gel chromatography (hexane/ethyl acetate, 20:1 [vol/vol]) and analyzed by gas chromatography–mass spectrometry (GC–MS) and NMR. This reaction yielded only 23.6% because of insufficient dehydrogenation during the reaction step. ^{1}H NMR δ (ppm, $CDCl_3$) of DCDE: 5.57 (H, s, OH), 6.78 (1H, d, J = 8.5 Hz), 6.83 (1H, dd, J = 8.5, 2.4 Hz), 6.95 (2H, d, J = 8.6 Hz), 7.06(1H, dd, J = 2.4 Hz), 7.31 (2H, dd, J = 8.6 Hz). ^{13}C NMR δ (ppm, $CDCl_3$) of DCDE: 155.0, 148.0, 142.1, 130.0, 129.9, 129.1, 120.7, 119.4, 119.3, 116.8.

2.3. Microorganism, culture condition and cell free extracts

Geobacillus sp. UZO 3 was maintained in tryptic soy agar plates (Difco). *Geobacillus* sp. UZO 3 was routinely cultivated in liquid cultures of tryptic soy broth medium (Difco) at 65 °C with vigorous shaking. When the optical density at 600 nm reached 1.2, cells were harvested by centrifugation, washed twice with 100 mM phosphate buffer (pH 7.0), and resuspended in a reduced volume of the same buffer. Cell free extracts (Crude enzyme) was prepared from the disrupted bacterial cells using the French pressure cell press (Ohtake Co. Ltd., Tokyo, Japan). The disrupted cells were centrifuged at 25 000 g for 30 min (25 °C) to remove intact cells and the supernatant was collected as cell free extracts (Crude enzyme).

2.4. Enzymatic reaction assays

The assay was performed at 65 °C for 18 h in glass tubes sealed with Teflon caps. The 1 mL reaction mixture contained 0.1 mM 2,7-DCDD dissolved in dimethyl sulfoxide (final concentration 2.5%) and the cell free extracts. Control tubes were prepared: cell free extracts without the substrate (Control 1); and substrate without the cell free extracts (Control 2). The mixtures were acidified to pH 2.0 with 12 M HCl and extracted with 1 mL ethyl acetate three times. Ethyl acetate extracts were dried over anhydrous sodium sulfate and the solvent was volatilized by nitrogen gas. The dried enzyme reactant was derivatized by BSTFA prior to GC–MS analysis.

2.5. Analytical methods

All ^{1}H and ^{13}C NMR spectrum were determined with a JEOL JNM-A500 spectrometer (JEOL, Ltd., Tokyo, Japan) operated at 500 MHz, using tetramethylsilane as an internal standard. GC–MS analyses were performed with a model JMS-Q1000GC (JEOL, Ltd., Tokyo, Japan) fitted with a fused-silica chemically bonded capillary column (DB-5; 0.25 mm i.d by 25 m; 0.25 μm film thickness; Agilent J&W, Inc. CA. USA). Each sample was injected into the column at 50 °C in the splitless mode. After 10 min at 50 °C, the column temperature was increased at 5 °C min^{-1} to 300 °C. Mass spectrum were obtained under 70 eV of the electron accelerating voltage. Products were identified by comparison of their retention times on GC and their mass fragmentation patterns with authentic compounds.

3. Results

3.1. 2,7-DCDD-degrading activity of Geobacillus sp. UZO 3 cell free extract

To investigate the degradation activity of the crude enzyme from *Geobacillus* sp. UZO 3 on 2,7-DCDD *in vitro*, GC–MS analysis was conducted on the ethyl acetate extract (TMSi derivative) from the enzyme reaction solution. Since chlorinated compounds show characteristic mass spectrum, chlorinated compounds produced by the enzymatic reaction of 2,7-DCDD can be readily detected by GC–MS. In addition to the substrate 2,7-DCDD at retention time 33.83 min, two additional peaks were detected at retention times 18.70 min (Product I) and 35.53 min (Product II) particularly corresponding to chlorinated compounds (Fig. 1B, C, D, b, c). These products were not detected in the ethyl acetate extract of the control culture containing no bacteria. Product I had a molecular ion peak of m/z = 200 and an isotope peak intensity ratio (202/200 = 0.358) characteristic of compounds with one chlorine substitution (M + 2/M = 0.326). Compared with the prepared authentic candidate compounds, Product I correspond exactly with 4CP (Fig. 1A, a). Product II had a molecular ion peak of m/z = 326 and an isotope peak intensity ratio (328/326 = 0.650, 330/326 = 0.128) characteristic of compounds with two chlorine substitutions (M + 2/M = 0.653, M + 4/M = 0.106). Based on MS analysis, the Product II detected corresponds to DCDE, which may be produced when one of the two diaryl ether bonds of 2,7-DCDD is reductively cleaved (Fig. 1D, c).