Enzymatic Activity of Cell Free Extract from *Geobacillus* sp. UZO 3 Catalyzes Reductive Cleavage of Diaryl Ether Bonds of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

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Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) are readily produced in the process of manufacturing herbicides and pesticides as well as the incineration process of chlorinated compounds to produce 75 and 135 different isomers, respectively, depending on the position of chlorination. Since these environmental chemicals, PCDD and PCDF, are chemically stable and difficult to biodegrade, they accumulate at high concentrations in organisms relative to the food chain. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) is the highest toxicity in dioxin isomers[5, 6, 7].

In recent years, attention has been directed toward technology for the degradation of these environmental chemicals by microorganisms and for the clean-up of pollutants. Many reports have been published on the microbial degradation of PCDDs and PCDFs[1, 2, 4, 8, 9, 10, 11, 12]. An aerobic bacterium, *Sphingomonas wittichii* RW1, which is the dibenzo-*p*-dioxin- and dibenzofuran- mineralization strain, has a dioxin dioxygenase system [1, 4, 9, 11, 12]. Bunge et al. reported that an anaerobic bacterium *Dehalococcoides* sp. strain CBDB1 is capable of conversion of 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin to 2,7- or 2,8-dichlorodibenzo-*p*-dioxin by dehalogenase[2]. Despite numerous studies on the microbial degradation of chlorinated dioxins, there is still a lack of information concerning the enzyme-catalyzed 2,3,7,8-TCDD degradation. Thus, the discovery or generation of a 2,3,7,8-TCDD-metabolizing enzyme and its overexpression in microorganisms would be promising for bioremediation uses.

In previous studies, we demonstrated that a cell-free extract prepared from cultured cells of *Geobacillus* sp. UZO 3 reductively cleaved diaryl ether bonds of 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD). 4',5-dichloro-2-hydroxydiphenyl ether (DCDE) was produced as a degradation intermediate, and 4-chlorophenol (4CP) was produced as the final reaction product (Fig. 1) [8, 10]. In the detection of DCDE, which is a degradation intermediate, an enzyme that reductively cleaves the diaryl ether bonds of 2,7-DCDD is an unprecedented new dioxin degradation enzyme which may be a type of reduction cleavage enzyme similar to GST.

In the present study, we have investigated the capacity of the cell-free enzyme extract from *Geobacillus* sp. UZO 3 to degrade 2,3,7,8-TCDD, 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 2,3,7,8-TCDD to produce 3',4',4,5-tetrachloro-2-hydroxydiphenyl ether (TCDE) and 3,4-dichlorophenol (DCP).

Materials and methods

Chemicals.

2,3,7,8-TCDD was purchased from Cerilliant (Texas, USA). DCP, $Cu(OAc)_2$, CH_2Cl_2, $CDCl_3$, tetramethylsilane and N,O-Bis(trimethyl silyl)trifluoroacetamide (BSTFA) were purchased from Wako Pure Chemical (Osaka, Japan). 4,5-dichlorocatechol and 3,4-dichlorophenylboronic 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.

Synthesis of TCDE.

TCDE was synthesized from 4,5-dichlorocatechol and 3,4-dichlorophenylboronic acid according to the procedure of Evans et al.[3]. A flask was charged with 4,5-dichlorocatechol (1.0 equiv), $Cu(OAc)_2$ (1.0 equiv), 3,4-dichlorophenylbolonic 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 TCDE: 5.51 (H, s, OH), 6.89 (1H, dd, J=2.4 Hz), 6.94 (1H, d, J=8.5 Hz), 7.17 (1H, d, J=8.5 Hz), 7.26(1H, d, J=8.5 Hz), 7.44 (1H, dd, J=8.5 Hz). ^{13}C-NMR δ (ppm, $CDCl_3$) of TCDE: 154.7, 146.4, 142.0, 133.8, 131.4, 128.6, 128.2, 123.6, 120.2, 120.0, 118.1, 117.6.

Microorganism, culture condition and cell free extracts.

Geobacillus sp. UZO 3 was maintained in tryptic soy agar plates (Difco). *Geobacillus* sp. UZO 3 and 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), 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).

Enzymatic reaction assays.

The assay was performed at 65 °C for 18 h in glass tubes sealed with teflon caps. The 1mL reaction mixture contained 0.15 mM 2,3,7,8-TCDD dissolved in dimethyl sulfoxidre (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 with 12 M HCl and extracted with 1mL 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.

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 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.

Results and Discussion

Synthesis of TCDE.

In a previous report, we confirmed the production of DCDE, an intermediate that partially and reductively cleaved one of the two diaryl ether bonds that exist in dioxin molecules, in a crude enzyme reaction of 2,7-DCDD by *Geobacillus* sp. UZO 3 cell-free extract [8, 10]. In the crude enzyme reaction of 2,3,7,8-TCDD, TCDE is predicted to be produced as the intermediate that partially and reductively cleaves the diaryl ether bonds (Fig. 1).

We synthesized TCDE according to the method of Evans et al[3]. The results of GC-MS analysis of the synthesized compound (TMSi derivative) are shown in Fig. 2E and d. The molecular ion peak of this compound corresponds to the estimated molecular weight of TCDE (*m/z*=396). ^{1}H-NMR and ^{13}C-NMR analyses confirmed this compound to be TCDE.

Reductively degrading activity of 2,3,7,8-TCDD to produce TCDE and DCP by *Geobacillus* sp. UZO 3 crude enzyme.

We analyzed the 2,3,7,8-TCDD-degrading activity of *Geobacillus* sp. UZO 3 cell-free extract. GC-MS analyses were used to investigate whether TCDE and DCP are produced as a degradation intermediate by the crude enzyme reaction of 2,3,7,8-TCDD in *Geobacillus* sp. UZO 3 cell-free extract. In addition to the substrate 2,3,7,8-TCDD (Retention time 47.08 min), TCDE (Retention time 46.88 min) and DCP (Retention time 26.12 min) peaks were detected as reaction products (Fig. 2B, D, b, c). GC-MS analysis results of ethyl acetate extracts for the controls (1 and 2) showed no peaks for TCDE and DCP. These results show that the chlorinated dioxin degradation enzymes contained in *Geobacillus* sp. UZO 3 cell-free extract reductively cleaves diaryl ether bonds of 2,3,7,8-TCDD with the same mechanism as 2,7-DCDD degradation, allowing generation of TCDE and DCP.

***Geobacillus* sp. UZO 3 cell free extract reductively degrades TCDE to DCP.**

We investigated whether TCDE was a reaction intermediate during 2,3,7,8-TCDD degradation to DCP. With TCDE as the substrate, we analyzed the resulting ethyl acetate extract degraded by the crude enzyme by GC-MS. DCP was detected in the ethyl acetate extract (data not shown), showing that the *Geobacillus* sp. UZO 3 cell free extract-mediated reductive cleavage of the two diaryl ether bonds in 2,3,7,8-TCDD proceeds in a sequential fashion. That is, TCDE is first produced as a reaction intermediate which is then converted to DCP (Fig. 1).

In the present study, we demonstrated that *Geobacillus* sp. UZO 3 cell-free extract which can reductively cleave the diaryl ether bonds of 2,7-DCDD also reductively cleaves the diaryl ether bonds of 2,3,7,8-TCDD and transforms it to the final product DCP via the intermediate TCDE in a sequential fashion. This is the first report that 2,3,7,8-TCDD-degrading enzymatic activity from bacterium are detected. Work is in progress to investigate clone the genes of the enzymes affecting 2,3,7,8-TCDD. Our future studies will involve isolation of the degrading enzymes and elucidation of the response mechanism of chlorinated dioxin reduction to the enzyme isolates, and mapping of the genes coding for the enzymes in view of reducing the contamination of substituted PCDDs including 2,3,7,8-TCDD in the environment.

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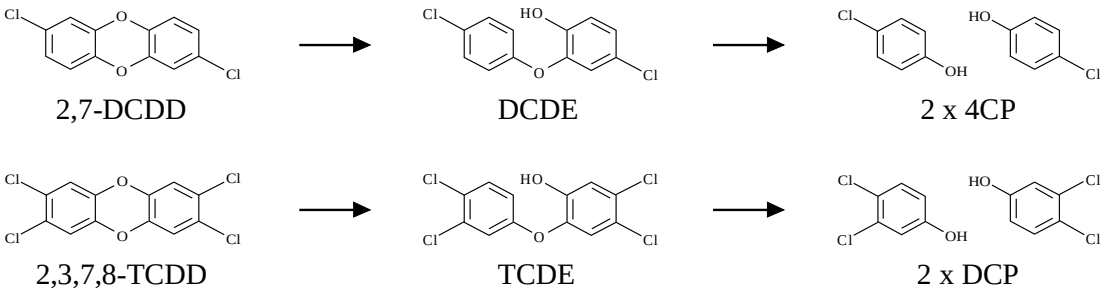

Fig. 1. Proposed degradation pathway for 2,7-DCDD and 2,3,7,8-TCDD by the *Geobacillus* sp. UZO 3 cell free extract.

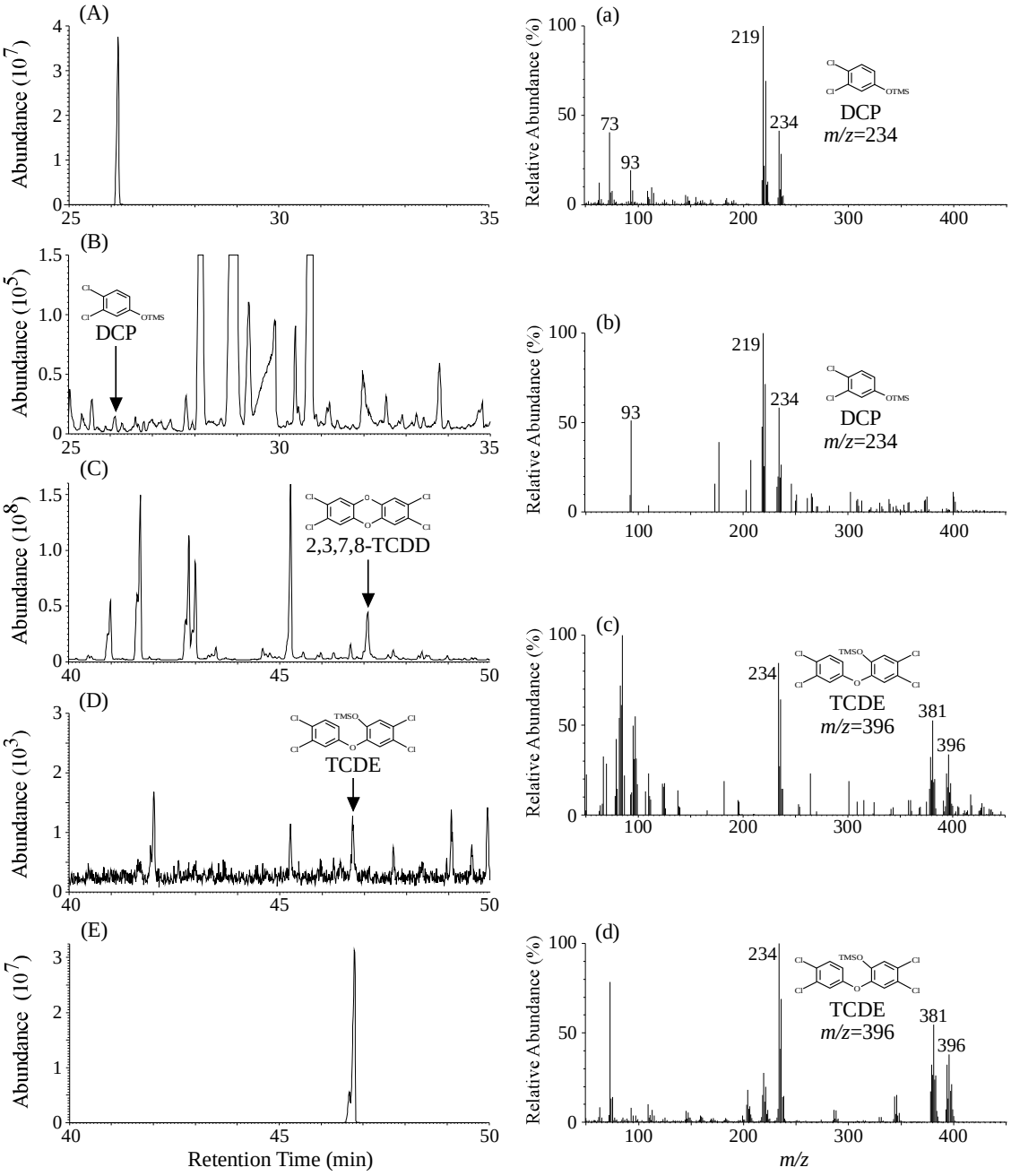

Fig. 2. Full scan run of the (C) reaction milieu for 2,3,7,8-TCDD degradation mediated by *Geobacillus* sp. UZO 3 cell free extract. The SIM chromatograms of the detected intermediate (B) DCP at *m/z*=234 and (D) TCDE at *m/z*=396 and corresponding MS spectrum (b and c, respectively) were compared to authentic compound, DCP (A, a) and TCDE (E, d).