DEGRADATION OF CHLORINATED DIBENZO-p-DIOXIN BY A CELL FREE EXTRACT FROM *Geobacillus midousuji* SH2B-J2

Masaya Nakamura,[*] Yuichiro Otsuka,[*] Shojiro Hishiyama,[*] Takeshi Kawakami,[**] Yuzo Suzuki,[**] Yoshihiro Katayama,[**] Sadayori Hoshina,[***] and Atsushi Takahashi[****]

[*] Forestry and Forest Products Research Institute, Tsukuba, Ibaraki 305-8687 Japan
[**]Tokyo University of Agriculture & Technology, Graduate School of BASE, Koganei, Tokyo 184-8588 Japan
[***]Jikei University School of Medicine, Minato-ku, Tokyo 105-8461 Japan
[****]Takasago Thermal Engineering Co. Ltd, Atsugi, Kanagawa 243-0213 Japan

Introduction

Polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) have been widespread environmental contaminants formed unintentionally as a by–products during the bleaching of pulp and paper, the manufacture of pesticides, and the incineration of halogen containing chemicals. PCDDs and PCDFs contamination has led to serious social problem because of their toxicity mutagenic and carcinogenic properties[1,2].

Microbial degradation of model compounds of PCDDs and PCDFs such as non-chlorinated dibenzo-p-dioxin (DD), mono-chlorinated DD and di-chlorinated DD has been studied using some microorganisms[3,4]. *Sphingomonas* sp. Strain RW1, which was isolated using non-chlorinated DD as a sole carbon source, has been studied extremely[5]. The initial reaction of DD degradation occurred by dioxygenation at the angular position of two carbon atoms adjacent to the ether bond. But it could not degrade the highly chlorinated DD. For the past decades, the functions of various microorganisms have been analyzed for the bioremediation of chlorinated dioxins. However, none have been applied for bioremediation.

Recently, we have isolated the bacterial strain *Geobacillus midousuji* SH2B-J2 having the ability to decrease PCDDs from compost under roadside trees in Osaka Japan. This bacterium has been Gram-positive, and thermophilic with an optimal growth temperature of 65 °C [6].

G. midousuji SH2B-J2 have shown to reduce highly chlorinated dioxins in incineration fly ash. Since this bacterium has been capable of reducing highly chlorinated DD even the octa-chlorinated dioxins (OCDD), we have hypothesized that the initial reaction of PCDDs degradation occurred with intra-molecular ether bond cleavage which is common structure regardless of the positions of the chlorine substituents. The main goal of this study is to identify the intermediates of dioxin degradation with 2,7-DCDD as a model dioxin and indicates the degradation pathway of PCDDs by this bacterium.

Materials and Methods

Microorganisms and cell free extract (Crude enzyme)

G. midousuji SH2B-J2 was maintained on triptic soy agar plates (Difco) that has been incubated at 65°C.

Liquid cultures of this bacterial strain were grown at 65°C in triptic soy broth medium (Difco). The log-phase cells were harvested by centrifugation (6,500 *g*; 10 min; 20°C). Cell free extract (Crude enzyme) was isolated from the disrupted bacterial cells by using the French pressure cell press. The disrupted cells was centrifuged(4,000 *g*; 10 min; 20°C) and the supernatant was collected as a crude enzyme.

Chemicals

2,7-DCDD was purchased from Cambridge Isotope Laboratories Inc. (USA). Mono-chlorophenol (2-, 3-, 4-chlorophenol) which were the authentic compounds for degradation products were purchased from Wako Chemical Co. (Japan). 2 hydroxy 4,5'-dichloro biphenyl ether was synthesized from 4-chlorocatechol (Tokyo Kasei, Japan) and 4-chlorophenylboronic acid (Aldrich, USA) according to the procedure of Evans[7].

Analysis of the reaction products

The 1ml reaction mixture contained 0.15 mM 2,7-DCDD dissolved in dimethyl sulfoxidre (final concentration 0.1%) and the cell free extract. Reactions were carried out at 65°C for 16 – 48 hours. The resulting mixtures were acidified to pH2.0 with 0.1N HCl and extracted with ethyl acetate (three times). Ethyl acetate extracts were dried over anhydrous sodium sulfate and were analyzed gas chromatography-mass spectrometry (GC-MS) after evaporation. GC-MS measurements were carried out by using a JMS-Q1000GC K9 (JEOL, Japan) with a 25-m Chrompack Capillary Column CP-SIL 5CB (GL Science Inc., Japan). The oven temperature was increased from 50°C to 300°C at the rate of 5°C/min.

Results and Discussion

Synthesis of the authentic compound, 2 hydroxy 4,5'-dichloro biphenyl ether.

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 a amino base (5.0 equiv) was added. After stirring the colored heterogeneous reaction mixture for 24 hours at 25°C under ambient atmosphere, the resulting slurry was filtrated, washed with distilled water and organic layer was collected. The diaryl ether was purified by silica gel chromatography (hexane : ethyl acetate = 20 : 1) and analyzed by GC-MS and NMR. This reaction yielded only 23.6% because of insufficient dehydrogenation for the reaction step.

^{1}H-NMR δ (ppm, $CDCl_3$) of 2 hydroxy 4,5'-dichloro biphenyl ether: 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 2 hydroxy 4,5'-dichloro biphenyl ether: 155.0, 148.0, 142.1, 130.0, 129.9, 129.1, 120.7, 119.4, 119.3, 116.8.

Detection and analysis of degradation intermediates from 2,7-DCDD

So far, there have been few reports for detection of degradation products as evidence of PCDDs biodegradation. Our earlier studies showed *G. midousuji* SH2B-J2 have shown to reduce PCDDs in incineration fly ash including OCDD. Extract of the crude enzyme reaction mixture was analyzed by GC-MS. The reaction of the cell free extract with 2,7-DCDD proceeds via formation of some intermediates with a multistep. Due to their characteristic mass spectra resulting from chlorine substituents, the degradation

products from 2,7-DCDD were easily detected by GC-MS. Two intermediates having chlorine atoms (Product I and II) had retention times at 36.50 min. and 18.85 min. on the total ion chromatogram (Fig.1-A). Product I had a molecular ion peak at m/z 326 as a TMS derivative, and the mass fragmentation pattern was the same as that of the authentic compound of 2 hydroxy 4,5'-dichloro biphenyl ether (Fig. 1-B). A molecular ion peak occurred at m/z 200 as a TMS derivative and mass pattern of product II was identical to the authentic compound of 4- chlorophenol (Fig. 1-B). These data support the ether bonds of 2,7-DCDD molecule had been reductively cleaved. In lignin biodegradation, the ether linkage of β-O-4 lignin dimmer was reductively cleaved by the two kinds of the β-etherase with GSH from the bacterium *Sphingobium paucimobilis* SYK-6[8]. *G. midousuji* SH2B-J2 may also have the similar system for ether bond cleavage of PCDD molecule. In this study, these results led us to propose the degradation pathway of 2,7-DCDD by the cell free extract from *G. midousuji* SH2B-J2 (Fig. 2). Further investigation of intermediates from PCDD degradation is needed to clarify the degradation pathway for PCDD biodegradation.

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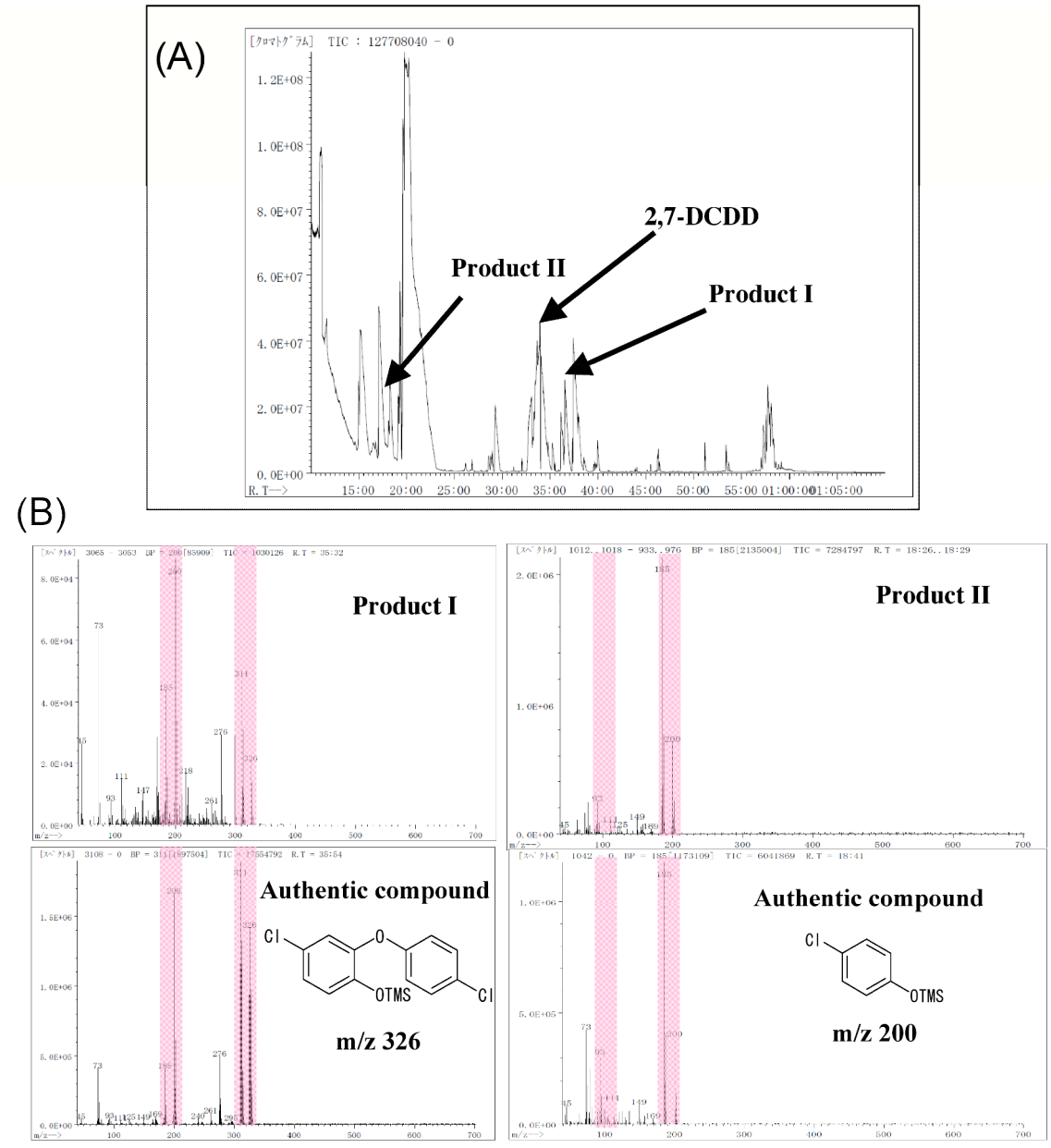

Fig.1. GC-MS analysis of the degradation intermediates by the cell free extract with 2,7-DCDD
(A) The total ion chromatogram of the reaction mixture.
(B) MS spectra of the GC peaks corresponding to the product I and II

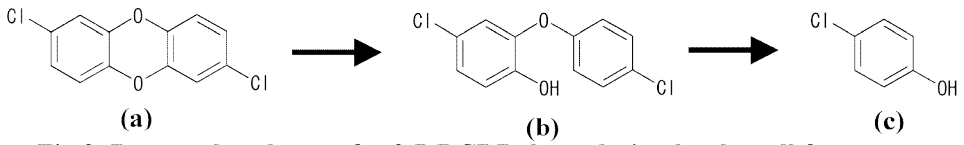

Fig.2. Proposed patheway for 2,7-DCDD degradation by the cell free extract
(a) 2,7-DCDD, (b) 2hydroxy 4,5'-dichlorobiphenyl ether, (c) 4-chlorophenol.