FLUORESCENT ASSAY FOR NEW DIOXIN DEGRADING MICROORGANISM

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Introduction

In recent years, attention has been directed toward the development of technology for the cleanup of these environmental pollutants by bioremediation[1, 3]. Rapid advances have also been made in the analysis of dioxins in the field of environmental biotechnology. However, these analytical methods have found limited applicability in more thorough studies on dioxin degradation due to the complex extraction operations they involve, (which often results in low recovery rates).

We have previously demonstrated that a cell-free extract prepared from cultured cells of *Geobacillus* sp. UZO 3 reductively cleaves diaryl ether bonds of 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD), producing 4',5-dichloro-2-hydroxydiphenyl ether (DCDE) as intermediate and 4-chlorophenol (4CP) produced as the final reaction product[6, 7] (Fig. 1). The detection of DCDE implicated the discovery of an unprecedented dioxin degradation enzyme that reductively cleaves the diaryl ether bonds of 2,7-DCDD similar to glutathione-S-transferase, a reduction cleavage enzyme[5]. The aims of this study are to develop a highly sensitive fluorescent assay for the new dioxin degrading enzyme(s) based on the synthesis of a fluorescent analog of 2,7-DCDD.

In the present study, 2-chloro-4,5-O-(4'-methyl-7', 8'-diphenyl)ether (CMDPE), an analog of 2,7-DCDD, was synthesized and utilized as the substrate. CMDPE consists of a monochlorophenol and a 4-methylumbelliferone (4MU) structural component. We hypothesize that the enzyme(s) from the cell-free extract of *Geobacillus* sp. UZO 3 reductively cleaves the diaryl ether bond of CMDPE, to produce the intermediate 6-chlorophenoxy-4-methylumbelliferone (CPMU) and the final product 4MU, which strongly fluoresces at 450 nm. Using 4MU as analyte will allow for the detection of compounds at concentrations as low as 10 ppb in a conventional spectrofluorometer, and even at a lower concentration of 1 ppt with TLC[6]. Current techniques that use chlorophenol as analyte in a conventional gas chromatography-mass spectrometry (GC-MS) set-up only has a limit of detection of 100 ppt.

This study presents evidence that the enzyme(s) that reductively cleave the diaryl ether bond of chlorinated dioxin in the cell-free extract of *Geobacillus* sp. UZO 3 also reductively cleave the diaryl ether bonds of CMDPE. A highly sensitive and simple new assay of chlorinated dioxin degradation activity is hereby described.

Materials and methods

Synthesis of CMDPE and CPMU.

CMDPE was synthesized from 2,4-dichloronitrobenzene and 4-methylescretin according to the procedure of Lee et al.[4]. CPMU was synthesized from 4-methylescretin and 4-chlorophenylboronic acid according to the procedure of Evans et al.[2].

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 0.1 mM CMDPE 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 (10 µl) from the reaction mixture were fractionated by TLC on the silica gel 60 F_{254} using chloroform:ethyl acetate:formic acid (10:8:1,v/v) as the developing solvent. And 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.

Results and Discussion

Reductively degrading activity of CMDPE to produce 4MU by Geobacillus sp. UZO 3 cell-free extract.

The ethyl acetate extract of the complete reaction mixture containing CMDPE and *Geobacillus* sp. UZO 3 cell-free extract was detected of a compound that strongly fluoresced at 450 nm. Based on the authentic preparation results, the compound was speculated to be 4MU (Fig. 2). Consistently, 4MU and 4CP were detected by GC-MS from the same extract (Fig. 3E, F, d, e). These compounds were not detected in reaction mixtures that contained the cell-free extract (control 1) or CMDPE (control 2) alone. Apparently, the enzyme(s) contained in *Geobacillus* sp. UZO 3 cell-free extract mediated the reductive cleavage of the diaryl ether bonds of CMDPE to produce 4MU in a similar mechanism, as we have previously described for 2,7-DCDD.

Conclusive CPMU identification.

TLC and GC-MS were used to detect production of the CPMU as intermediate in the degradation of CMDPE in *Geobacillus* sp. UZO 3 cell-free extract (Fig. 2, 3). In the conditions employed in this experiment, TLC alone could not detect CPMU production, which may be attributed to the nearly similar behavior of this compound, which has a partially cleaved diaryl ether bond, with CMDPE.

These results provide strong evidence that *Geobacillus* sp. UZO 3 cell-free extract reductively cleaves the diaryl ether bonds of CMDPE in a stepwise fashion to produce 4MU, via the intermediate CPMU. As this stepwise mechanism is reminiscent of the 2-step degradation reaction we have previously reported[6, 7], the degradation of both 2,7-DCDD and CMDPE may be catalyzed by the same enzyme(s) in the cell-free extract. This, however, requires further investigation since other enzymes in the extract may possibly be involved in the degradation of chlorinated dioxin in the cell-free extract.

We hereby described a simple and sensitive quantitative assay to detect and monitor enzymatic activity for the degradation of chlorinated dioxin. This method presents a powerful tool in our current efforts to isolate and purify enzyme(s) in the cell-free extract of *Geobacillus* sp. UZO 3 involved in the reductive cleavage of chlorinated dioxins. Further, this method may find potential application in: (1) uncovering the intracellular locus of the enzyme(s) in *Geobacillus* sp. UZO 3 that reductively cleave the diaryl ether bonds of 2,7-DCDD, (2) analyzing their substrate specificity to PCDDs, and (3) measuring these enzymatic activities in the contaminated environment.

References:

1. Armengaud J, Happe B, Timmis K. N. (1998); *J. Bacteriol.* 180: 3954-3966.
2. Evans A. D, Katz L. J, West R. T. (1998); *Tetrahedron letters.* 39: 2937-2940.
3. Habe, H, Chung, J. S, Lee, J. H, Kasuga, K, Yoshida, T, Nojiri, H, Omori, T. (2001); *Appl. Environ. Microbiol.* 67: 3610-3617.
4. Lee H. H, Denny W. A. (1990); *J. Chem. Soc. Perkin. Trans.* 1:1071-1074.
5. Masai, E, Katayama, Y, Kawai, S, Nishikawa, S, Yamasaki, M, Morohoshi, N. (1991); *J. Bacteriol.* 173: 7950-7955.
6. Nakamura M, Otsuka Y, Hishiyama S, Kawakami T, Suzuki Y, Katayama Y, Hoshina S, Takahashi A. (2008); *Organohalogen Compounds*. 70: 1920-1923.
7. Suzuki Y, Nakamura M, Otsuka Y, Suzuki N, Ohyama K, Kawakami T, Sato K, Kajita S, Hishiyama S, Fujii T, Takahashi A, Katayama Y. (2011); *Chemosphere*. 83: 868-872.
8. Suzuki Y, Nakamura M, Otsuka Y, Suzuki N, Ohyama K, Kawakami T, Sato K, Kajita S, Hishiyama S, Takahashi A, Katayama Y. (2012); *Environ. Toxicol. Chem.* 31: 1072-1075.

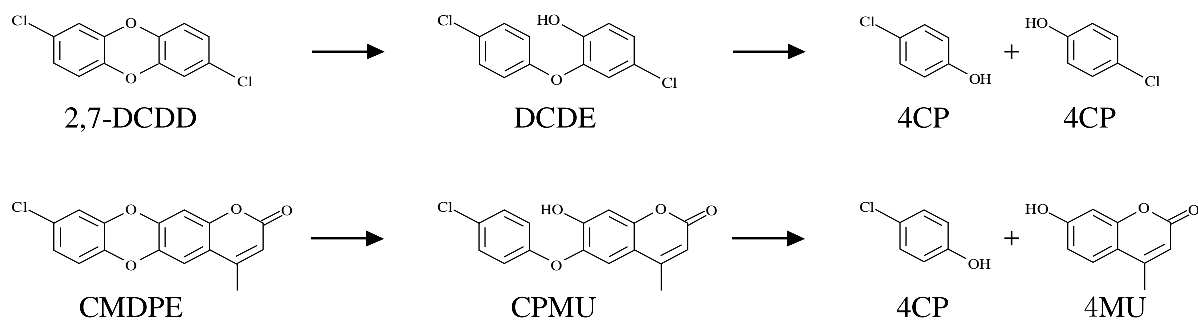

Fig. 1. Proposed degradation pathway for 2,7-DCDD and CMDPE by the *Geobacillus* sp. UZO 3 cell-free extract.

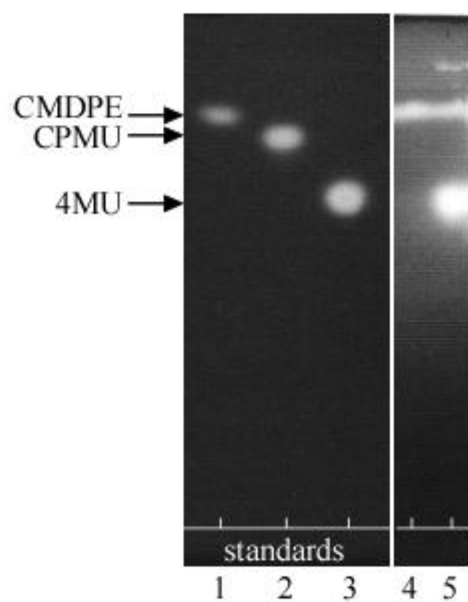

Fig. 2. TLC analysis of the reaction milieu for CMDPE degradation mediated by *Geobacillus* sp. UZO 3 cell-free extract. Lanes 1, 2 and 3: authentic compound, CMDPE, CPMU, and 4MU, respectively; lane 4: control culture containing no cell-free extract; lane 5: reaction milieu.

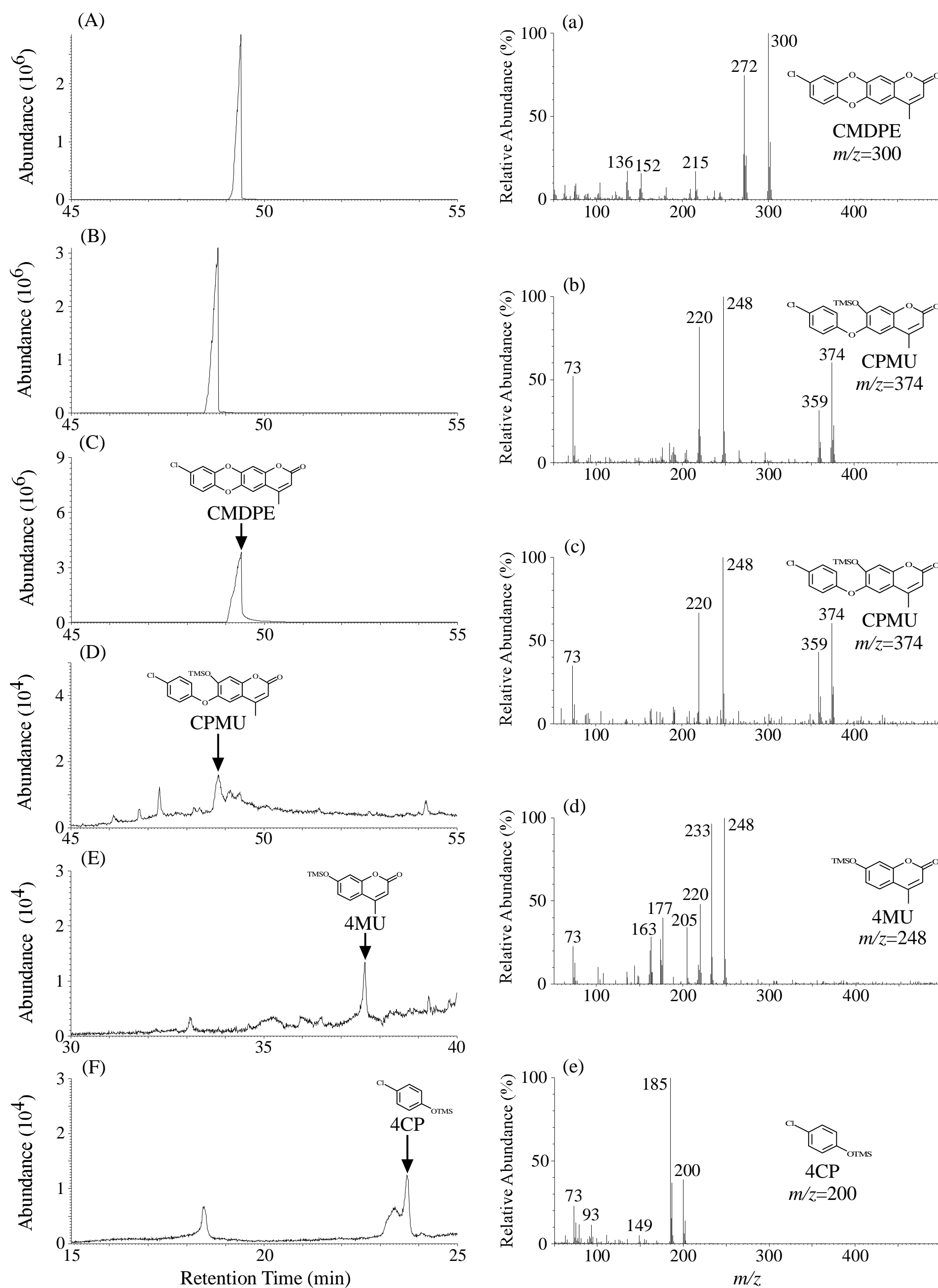

Fig. 3. GC-MS analysis of the reaction milieu for CMDPE degradation mediated by *Geobacillus* sp. UZO 3 cell-free extract. The SIM chromatogram of the detected substrate CMDPE at *m/z*=300 (C) was compared to that of the authentic compound (A) and its corresponding MS spectrum (a). The SIM chromatograms of the detected intermediate CPMU at *m/z*=374 (D), 4MU at *m/z*=248 (E) and 4CP at *m/z*=200 (F) and their corresponding MS spectrum (TMSi derivative) (c, d and e, respectively). D and c were compared to authentic, CPMU (TMSi derivative) (B and b).