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Degradation of dioxins by cyclic ether degrading fungus, *Cordyceps sinensis*

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Abstract

Use of the cyclic ether degrading fungus, *Cordyceps sinensis* strain A to degrade dibenzo-*p*-dioxin (DD), 2,3,7-trichlorodibenzo-*p*-dioxin (2,3,7-triCDD) and octachlorodibenzo-*p*-dioxin (octaCDD) has revealed a new degradation pathway for dioxins. Catechols and other possible degradation products were synthesized to facilitate the identification, detection and quantification of these products, and phenylboronate was used for the derivatization and analysis of dihydroxylated degradation products. Degradation of DD yielded catechol, which was further metabolized to *cis*,*cis*-muconate. 2,3,7-triCDD yielded mono- and dichloro-catechol as well as catechol itself; and the *cis*,*cis*-muconates were also detected. octaCDD gave mono- to trichloro-catechol as well as catechol, and the *cis*,*cis*-muconates were also found. For all tested dioxin samples dechlorination resulted in replacement of chlorine with hydrogen, and no hydroxylation was observed. It appeared that dechlorination may occur in the degradation of octaCDD to catechols and possibly in the subsequent degradation of chlorinated catechols and/or chlorinated *cis*,*cis*-muconates to further breakdown products.

Keywords: Dibenzo-*p*-dioxin; 2,3,7-Trichlorodibenzo-*p*-dioxin; Octachlorodibenzo-*p*-dioxin; Degradation pathway; Catechol; *cis*,*cis*-Muconate; *Cordyceps sinensis*

1. Introduction

Several studies have been carried out on the bioremediation of dioxin pollution by the use of microorganisms [20]. It is particularly important to degrade dioxins because of their carcinogenic activity [1–3]. Fungi were the most effective degraders of dioxins and core experiments were done by Takada et al. [4] and Valli et al. [5] using the basidiomycete, *Phanerochaete* sp. Valli et al. investigated the degradation pathway of 2,7-dichlorodibenzo-*p*-dioxins. They considered the main degradation product to be monochlorocatechol with a trace amount of trihydroxylbenzene also produced. Further degradation products of monochlorocatechol have been investigated in detail and the degradation pathway and its associated enzymes have been elucidated. Takada et al. have also examined the degradation of highly chlorinated dibenzo-*p*-dioxins, and have identified tetrachlorocatechol as one of the degradation products of octachlorodibenzo-*p*-dioxin (octaCDD). Further degradation has not been revealed in such detail.

When highly polar degradation products have been analyzed by GC/MS, derivatization of the analyte is usually necessary, and trimethylsilyl (TMS) derivatives

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of the hydroxyl groups with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide usually being made. It is a reliable method of derivatization but it does not enhance sensitivity because it will derivatize all compounds containing hydroxyl groups. Phenylboronate has been used for the quantification of ethylene glycol, 1,3-propanediol and other diols [6]. This method derivatizes only diols and diacids and can increase the sensitivity of the analytical method. The method has been used to derivatize a dihydroxylated biphenyl compound [7], but, to our knowledge, it has not been further used for aryl compounds.

We isolated a fungus, provisionally called strain A, as the 1,4-dioxane utilizing microorganism from the garden soil of our laboratory and identified it as *Cordyceps sinensis* from its 18S rRNA sequencing [8]. *C. sinensis*, of the family Ascomycotina, can form fruiting bodies when living on insect larvae, and it has been taken as a herbal medicine in China. Strain A can grow on 1,4-dioxane and several other cyclic ethers for up to 3 days at 30 °C, and it substrate specificity for growth seems quiet broad. The major degradation product of 1,4-dioxane was shown to be ethylene glycol by GC/MS analysis. From the identification of the degradation products of deuterium-labeled 1,4-dioxane and the utilization by strain A of tetramethyl-1,4-dioxane, the proposed degradation pathway involved the hydroxylation of the carbon atom bonded to oxygen in the ether linkage, i.e., a hemiacetal was formed. Thus, it is possible that strain A can degrade other cyclic ethers such as dioxins.

In this study we investigated the degradation of the aryl cyclic ethers, dibenzo-*p*-dioxin (DD), 2,3,7-trichlorodibenzo-*p*-dioxin (2,3,7-triCDD), and octaCDD, and analyzed their degradation products using phenylboronate derivatization. Analytical standards of possible degradation products, chlorocatechols and chlorinated *cis*,*cis*-muconates, were synthesized and were used for the quantification of the breakdown products. We propose a pathway for the degradation of dioxins.

2. Materials and methods

2.1. Microorganisms, cultivation conditions and degradation tests

C. sinensis strain A [8], which has 1,4-dioxane degrading activity, was used in these experiments. A basal salt medium (BSM) with 0.3% 1,4-dioxane or glucose [9] was employed. The pH was adjusted to 6.8 with HCl and/or NaOH. For the preparation of a solid medium, 1.5% agar was added to the BSM. Strain A was maintained on solid medium composed of BSM containing 0.3% 1,4-dioxane without other carbon sources. Before the degradation experiments, strain A was pre-cultivated on 1,4-dioxane or glucose supplied to the solid medium in order to decrease the effect of the 1,4-dioxane and any unexpected metabolite in the stock culture. It was then cultured in liquid medium for each substrate but without dioxins. For the degradation tests, the dioxins dissolved in ethyl acetate were placed in a 15 ml screw-capped vial with a Teflon inner liner and the solvent removed under a stream of nitrogen at 40 °C. An aliquot of the culture medium (3 ml) was then added and the tube autoclaved for 15 min at 121 °C. Glucose was added to the medium before sterilization. Filter sterilized 1,4-dioxane was added to the medium after sterilization. After inoculation with 30 μl of pre-culture (cultured for 3 days), cultures were grown at 30 °C with constant shaking (150 rpm). Growth was estimated from protein concentrations in the culture media by the Bradford method [10]. Bovine serum albumin (BSA) was used as the calibration standard.

2.2. Analytical procedure

One volume of culture medium was mixed with an equal volume of methanol and centrifuged for 5 min (12,000 rpm) at room temperature. The supernatant was removed and an equal volume of 80 mM methanolic phenylboronate was added to it to derivatize the degradation products [6]. The mixture was kept for 5 min with shaking at room temperature and then dried by the addition of anhydrous Na_2SO_4. The dried sample was analyzed by gas chromatography/mass spectrometry (GC/MS) (GC-MATE, JEOL Ltd., Tokyo, Japan, and a HP 6890, Agilent Technologies, CA, USA). Separations were carried out on a DB-17MS column (30 m × 0.25 mm i.d., J&W Scientific, CA, USA), and the analytes were ionized in EI mode. The oven temperature program was: 80 °C (hold 2 min), 150 °C (20 °C/min) and 300 °C (10 °C/min). Internal standards were not used because of overlap of the peaks of catechols and *cis*,*cis*-muconates and calibration was done with authentic standards.

The clean-up method for the dioxins was as formerly used [11].

2.3. Chemicals

DD, 2,3,7-triCDD, and octaCDD were synthesized by the method of Gray et al. [12] and Kulka [13]. Non-chlorinated catechol and tetrachlorocatechol were purchased from Wako Pure Chem. Ind. Ltd., Osaka, Japan, and Lancaster Synthesis Ltd., Morecanbe, UK, respectively. Monochloro- to tetrachlorocatechols were synthesized by Gray's method [12]. Nonchlorinated- to tetrachloro-*cis*,*cis*-muconate were synthesized by the method of Pandell [14] and Wacek [15]. Other chemicals were of laboratory grade.

3. Results and discussion

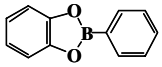
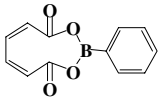
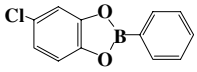
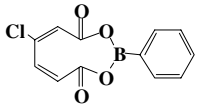
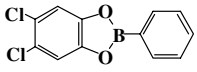
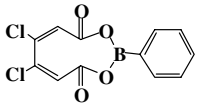
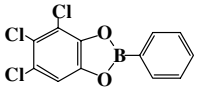
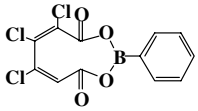
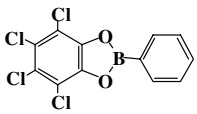
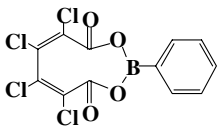
3.1. Derivatization of standard chemicals

All standard chemicals could be derivatized by phenylboronate (Table 1). The parent mass of each compound almost matched with molecular weight of the phenylboronate derivatives. But highly chlorinated compounds have cluster-like fragmentation patterns, which were generally found for the chlorinated compounds, with the most intense peak within the cluster being 2 or 3 mass units higher than the calculated molecular mass of the relevant phenylboronate derivative. The retention times of each derivative, especially those of the chlorocatechols and chloro-*cis*,*cis*-muconates were very similar and they also had similar parent *m*/*z* values. They were, however, distinguished by the specific target mass shown in Table 1. When these compounds were analyzed on the DB-5MS or DB-1MS column (30 m × 0.25 mm i.d., J&W Scientific, CA, USA), the adsorption of compounds with 2 to 4 chlorine substituents on to the column was observed and peaks for individual compounds could not be discerned. The DB-17MS column has a higher polarity and is suitable for the analysis of the compounds. Using this information, we investigated the degradation products from dioxins by strain A.

3.2. Dioxin degradation experiments

Without dioxins, strain A will grow on glucose or 1,4-dioxane and reach the stationary growth phase within 4 days, but when strain A was cultivated with 0.03% DD or 2,3,7-triCDD on 0.3% glucose or 1,4-dioxane it took 7 days or more to reach stationary phase. It did, however, grow well on 0.003% of each of the three experimental dioxins and reached the stationary phase within 4 days. Therefore we chose the standard cultivation conditions for the degradation experiments to be 0.003% of dioxin and cultivation for 4 days.

Table 1
Target mass and retention time on GC/MS chromatogram of derivatized catechols and *cis*,*cis*-muconates

Catechol derivatives	MW	*m*/*z*	RT (min)	*cis*,*cis*-Muconate derivatives	MW	*m*/*z*	RT (min)
	196.0	<u>196.0</u>	10:37		228.0	<u>229.0</u>	12:49
	230.4	<u>230.9</u>	12:52		262.4	264.6[a] 267.0 <u>268.0</u>	14:46
	264.9	<u>265.5</u>	14:45		296.9	<u>297.0</u>, 298.0[a] 299.0, 300.0 301.0, 302.0	16:40
	299.3	<u>299.8</u>	16:42		331.4	<u>330.9</u>, 331.9 332.9, 333.9[a] 334.9, 335.9	18:18
	333.8	<u>334.8</u>	18:16		365.8	365.8 <u>367.8</u>[a] 369.8 371.8 373.8	20:54

Target mass is underlined.
m/*z*; major fragment(s) included in cluster of parent mass.
RT; retention time.
[a] The highest peak of the cluster.

For 4 days from the start of cultivation, sampling was carried out every day, and the concentrations of dioxins and degradation products in the cultures were determined. A blank was made up without inoculation with strain A and no decrease in dioxin concentration and formation of degradation products in the culture medium were observed after 4 days of cultivation at 30 °C (data not shown). Figs. 1 and 2 show that decreases of about 50% of the initial concentrations of the dioxins were observed for each compound. The rate of degradation and the proportions of the degradation products were not much affected by the nature of the substrate. This might suggest that the enzymes required to degrade the dioxins are induced the dioxins themselves. About 2% of the added dioxins were detected as the catechols and 0.2% to *cis*,*cis*-muconates after 3 days of cultivation. Products resulting from *meta*-cleavage of the dioxins were not observed by GC/MS using the TMS derivatization method (data not shown). It appeared that the rate-determining step in the degradation pathway for the dioxins was the production of catechols and that the further degradation products of the catechols and *cis*,*cis*-muconates degrade more rapidly.

3.3. Proposed degradation pathway of dioxins by C. sinensis *strain A*

Fig. 3 shows the proposed degradation pathway for dioxins. Dechlorinated dioxins, even those possessing hydroxyl substituents were not found as a result of the degradation of chlorinated dioxins (data not shown). Thus, hydroxylase activity, which was found for *Beijerinickia* sp. and/or *Phanerochaete chrysosporium* [16,17] was not detected in this fungus (strain A). The only possible reaction products were catechols. This suggested that the first reaction in the breakdown of the dioxins was ring cleavage of the ether bonds. When 1,4-dioxane was degraded by strain A, hemiacetal formation was proposed [8]. Cleavage of the ether linkage in DD by the bacterium, *Pseudomonas* sp. strain HH69 has been reported [18–20], and its reaction involved hemiacetal formation. However, the reaction occurred on only one of the four carbons involved in the two ether linkages and catechols were produced as minor reaction products. Possibly the mechanism of degradation by strain A involves the formation two hemiacetal groups on one benzene ring.

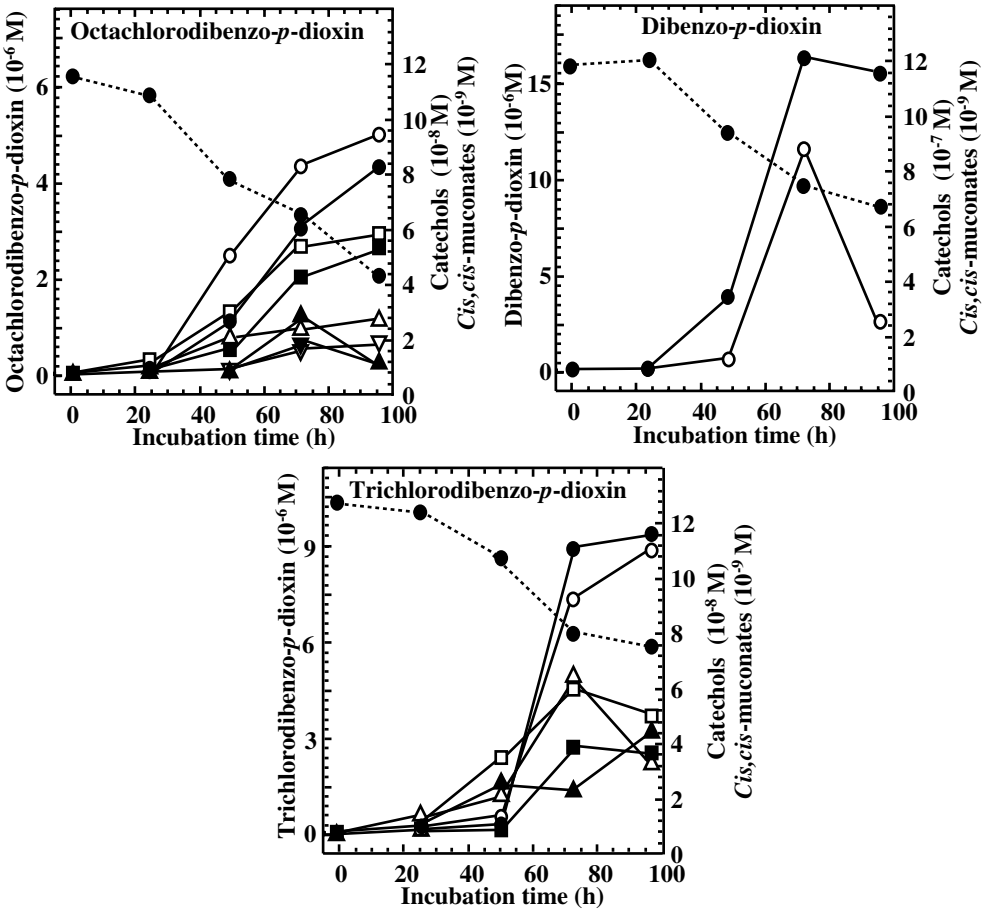


Fig. 1. Effect of 1,4-dioxane on the degradation of dioxins. Symbols: dotted line, dioxins; circle, non-chlorinated chemicals; square, monochlorinated chemicals; triangle, dichlorinated chemicals; inverted triangle, trichlorinated chemicals. Filled symbols represent *cis*,*cis*-muconates; unfilled symbols represent catechols. Each point is the mean of three replicates.

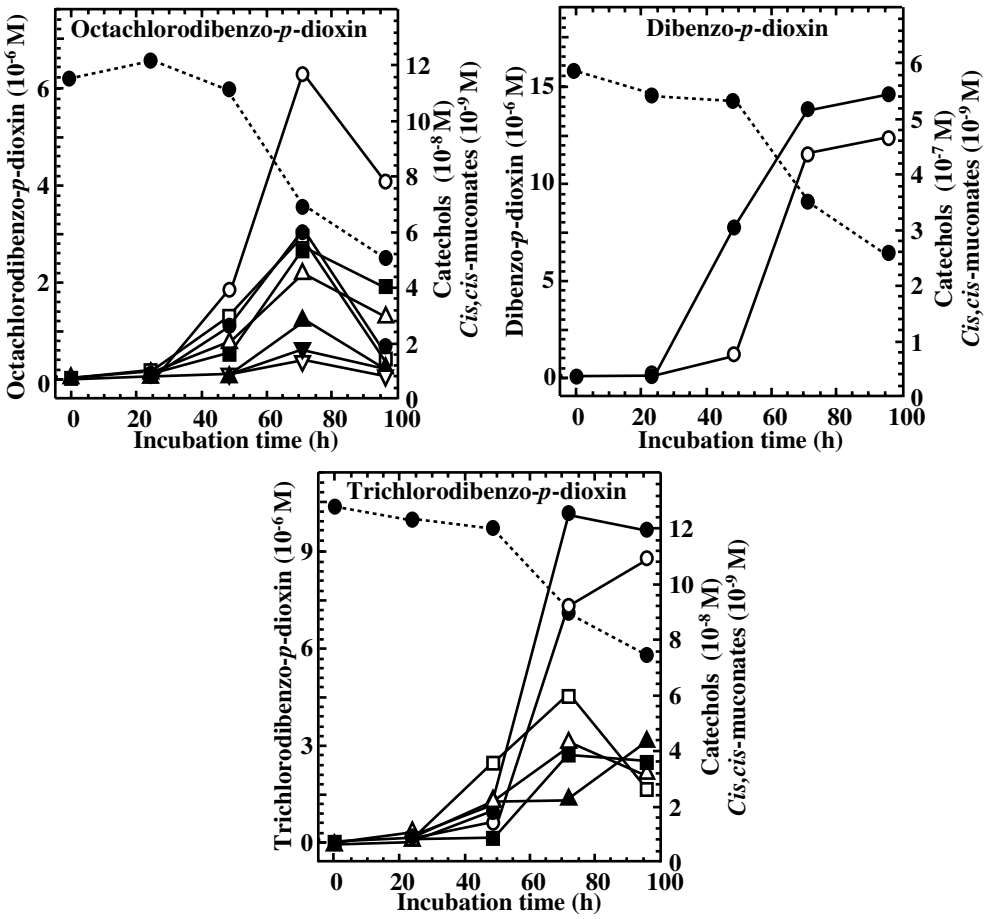


Fig. 2. Effect of glucose on the degradation of dioxins. Symbols as for Fig. 1.

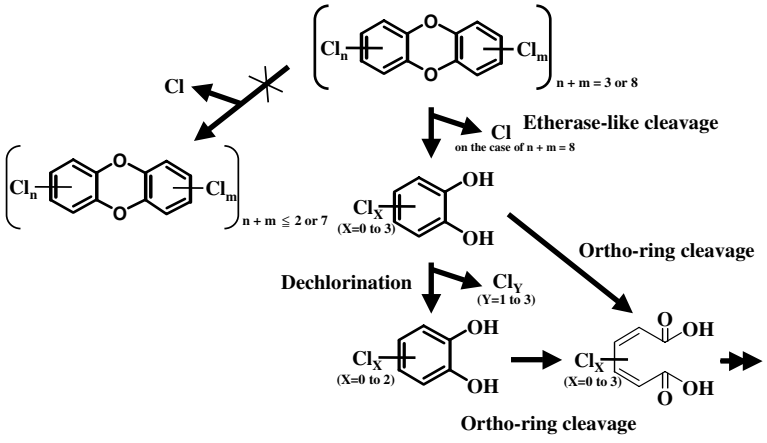


Fig. 3. Proposed degradation pathway of dioxins by strain A. The crossed arrow indicates that dechlorination of intact dioxins does not occur.

Tetrachlorocatechol was not detected among the degradation products of octaCDD. It has been identified as a breakdown product of dioxin treatment by *Phanerochaete* sp. [4], but only non-chlorinated to trichlorinated catechols can be detected after 2 to 4 days in strain A culture medium. Possibly, removal of the chlorine in the 1-position on octaCDD occurred upon cleavage of the ether link.

Non-chlorinated to dichlorinated catechols were detected as degradation products of 2,3,7-triCDD. Possibly dechlorination of the catechols occurred. This reaction did not produce quinones, which have been reported to result from the dechlorination of the aromatic rings of tetrachloroguaiacol and 2,4,6-trichlorophenol by *Coriolus versicolor* and *Pseudomonas pikettii* [21,22]. From octaCDD and 2,3,7-triCDD, trichloro *cis*,*cis*-muconates

and dichloro *cis*,*cis*-muconates, respectively, were detected. This indicated that *ortho*-ring cleavage occurred on all the observed catechols. Formation of *cis*,*cis*-muconates from chlorocatechols was also reported for *Rhodococcus erythroplis* 1CP [23,24]. It was not proposed that dechlorination yielded the *cis*,*cis*-muconates. Dechlorination by strain A may not occur during the ring cleavage reaction. A possible mechanism for the dechlorination of chlorinated *cis*,*cis*-muconates by *Pseudomonas putida* involving chloromuconolactone as an intermediate has been reported by Kaulmann et al. [25] but these compounds were not detected in the culture medium. Further study will be necessary for the elucidation of the detailed mechanisms of the dechlorinations. Possibly UV radiation could be involved in the dechlorination of a destabilized aromatic ring. Dechlorination of chlorinated catechols and chlorinated *cis*,*cis*-muconates may be assisted by any enzymatic reaction that can destabilize the aromatic ring. Reductive dechlorination by fungi [11] and the ability of basidiomycetes to dechlorinate halomethanes by the action of lignin-degrading enzymes [26] have been reported. Removal of the chlorines on catechols and *cis*,*cis*-muconates that are less stable than the chlorines on DD might be catalyzed by such an enzyme. Finally, the concentrations of the *cis*,*cis*-muconates decreased in the culture medium; possibly they were further metabolized by incorporation into the TCA cycle.

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