DEGRADATION OF DIBENZO-*P*-DIOXIN WITH FUNGAL MANGANESE PEROXIDASE IN THE PRESENCE OF UNSATURATED FATTY ACID

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Introduction

Various methods for treatment of dioxins released to the environment were proposed. It may be suitable to apply a pollutant-degradable microorganism to treatment of wide-range-contaminated soil or water containing lower concentration of dioxins.

White rot fungi (basidiomycetes) such as *Phanerochaete chrysosporium* have been known to possess a highly nonspecific battery of extracellular enzymes that allows them to degrade the plant polymer lignin [1]. The random nature of the structure of lignin requires lignin degradation to function in a nonspecific manner. It is generally thought that the major enzymes involved in lignin biodegradation by fungi are two extracellular heme-containing peroxidases: lignin peroxidase (LiP) [2] and manganese peroxidase (MnP) [3]. These enzymatic systems can degrade other compounds that have an aromatic structure, such as many xenobiotic compounds. LiP directly oxidizes polycyclic aromatic hydrocarbons [4], dibenzo-*p*-dioxin [5] and 2,7-dichlorodibenzo-*p*-dioxin [6]. MnP is unique enzyme that oxidizes Mn(II) to Mn(III), which attack to phenolic lignin and organopollutants. The MnP system can not degrade nonphenolic compounds. However, there are MnP-producing white rot fungi that evidently lack LiP, which nevertheless degrade non-phenolic lignin structures efficiently. Recently, it is reported that the MnP system is capable to mineralize nonphenolic compounds in the presence of an unsaturated lipid [7,8].

In this study, we report that the MnP system in the presence of unsaturated fatty acid can degrade dibenzo-*p*-dioxin.

Methods and Materials

Phanerochaete sordida YK-624 (ATCC 90872) were used for MnP preparation. A carbon and nitrogen-limited medium as described by Kondo et al. was used [9]. The fungus was cultivated in 500-ml Erlenmeyer flasks containing 200 ml of liquid culture medium. One liter of this medium contained 10 g of glucose, 0.221 g of ammonium tartrate, 1.64 g of sodium acetate, 1.0 g of Tween 80, and Kirk's trace elements and salts [10]. Flasks were shaken at 30°C and 150 rpm for 7 days. On day 3, 0.25 g of veratryl alcohol was added to the cultures. The flasks were purged with oxygen for about 1 hour every day. After the incubation for 7 days, the supernatant was separated from the mycelium by filtration through glass fiber. The liquid was frozen at -20°C and thawed.

After removing the extracellular glucan by filtration, the supernatant was dialyzed against 10 mM sodium acetate buffer (pH 4.5) for 24 h. The crude enzyme solution was concentrated by ultrafiltration (10-kDa molecular weight cut-off membrane; ADVANTEC). Enzyme solution containing MnP from *P. sordida* YK-624 was used for following enzymatic reactions.

The volume of reaction mixture was 5 ml. Reactions contained 25 U MnP, 50 mM sodium malonate buffer (pH 4.5), 1 mM Mn(II) (as $MnSO_4$), 2 mM unsaturated fatty acid, 0.5 mM glucose and 1 U glucose oxidase. 50 µl (final concentration; 50 µM) of 5 mM dibenzo-*p*-dioxin (DD) dissolved in dimethylformamide (DMF) was added to the reaction mixture. Each tube (30 mm X 200 mm) was sealed with a glass stopper and sealing tape. The tubes were shaken at 37°C and 150 rpm for 24 h.
After incubation, 50 µl of 4 M H_2SO_4 and 500 µl of biphenyl in DMF solution (internal standard, 2 mM) were added to the reaction mixture. Mixtures were extracted with ethyl acetate (two 10-ml portions). The organic phases were evaporated under reduced pressure. The extracts were analyzed by high-performance liquid chromatography (HPLC) on a reversed-phase column (µBONDASPHERE C18, 3.9 X 150 mm, Waters). HPLC was done on a Waters Model 2690 with a diode array detector. The column was eluted at 1 ml min^{-1} with acetonitrile-water (70 : 30). The elution was monitored at 230 nm.

To detect metabolites of DD formed by MnP system, reaction products were reduced with sodium dithionite, extracted with ethyl acetate, evaporated under reduced pressure and trimethylsilylated with N,O-bis(trimethylsilyl) acetamide. The trimethylsilylate derivatives were analyzed by GC-MS (TurboMass GC Mass Spectrometer, Perkin Elmer).

Results and Discussion
MnP reactions were done in the presence of linoleic acid (2 mM) as a unsaturated fatty acid under various conditions. Decrease in DD by MnP system required linoleic acid and active MnP (Table 1). MnP system also decreased DD by 8.7% when H_2O_2-producing enzyme system (glucose oxidase and glucose) is not added to the reaction mixture. It is reported that H_2O_2 is produced by conversion of malonate, Mn (III)-chelator in the MnP reaction [11]. Addition of H_2O_2-producing enzyme system (glucose oxidase and glucose) to the reaction mixture improved the decrease in DD. When the reaction was done in the presence of various concentration of linoleic acid, the extent of decrease in DD was dependent on the concentration of linoleic acid (Figure 2). The addition of Mn(II) at a concentration from 0.1 to 1.0 mM did not affect the extent of decrease in DD (Figure 3).

Treatments of DD by the MnP system in the presence of seven unsaturated fatty acids (2 mM) were tested (Table 2). All MnP systems reduced DD. The addition of fatty acid containing higher unsaturated linkage increased in the extent of DD decrease. In the presence of 4, 7, 10, 13, 16, 19-docosahexanoic acid (DHA, 22 : 6), MnP system decreased DD by about 40% for 24 h. It has been reported that MnP supports the Mn-dependent peroxidation of unsaturated fatty acids [7,8]. The oxygen-centered radicals produced during lipid peroxidation are known to trigger xenobiotic cooxidations [12]. Some white rot fungi produce unsaturated fatty acids [13], but there are no reports

that white rot fungi produce higher unsaturated fatty acids such as EPA and DHA. Co-culture of white rot fungi and higher unsaturated fatty acid-producing organisms may be required for biodegradation of dioxins by the MnP system.

To detect metabolites of DD formed by MnP system in the presence of DHA, reaction products were reduced with sodium dithionite, extracted with ethyl acetate, trimethylsilylated with N,O-bis(trimethylsilyl) acetamide, analyzed by GC-MS. The trimethylsilylate derivative of catechol for a metabolite of DD was detected. Now, detection of other metabolites and degradation of chlorinated dibenzo-*p*-dioxin by MnP system are being done.

Table 1 DD decrease by MnP system under various conditions.

Reaction conditions	DD decrease (%)
Complete	16.1
With boiled MnP	0
Minus MnP	2.8
Minus linoleic acid	1.1
Minus H_2O_2-producing enzyme	8.7

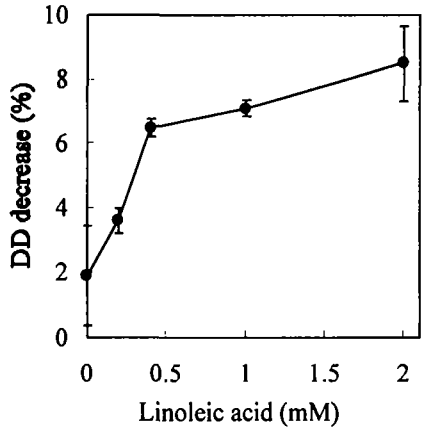

Figure 1 Effect of linoleic acid on treatment of DD by MnP system.

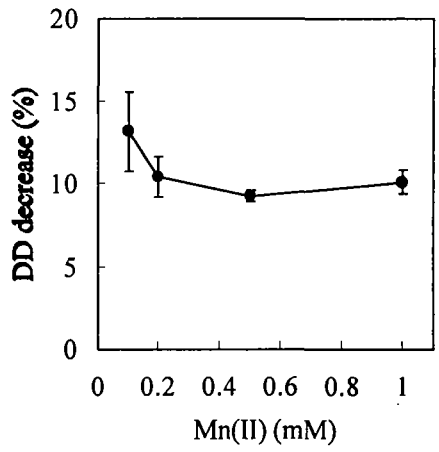

Figure 2 Effect of Mn (II) on treatment of DD by MnP system.

Table 2 Treatment of DD by MnP system in the presence of various fatty acids.

Fatty acids (C No.: C=C No.) [a]	DD decrease (%)
Palmitoleic acid (16:1)	7.7
Oleic acid (18:1)	8.3
Linoleic acid (18:3)	18.9
Arachidonic acid (20:4)	13.3
EPA (20:5)	25.0
DHA (22:6)	41.5

[a] EPA, 5,8,11,14,17-icosapentaenoic acid;
DHA, all *cis*-4,7,10,13,16,19-docosahexaenoic acid.

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