Peroxidase catalyzed co-polymerization of pentachlorophenol and a potential humic precursor

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Abstract

The transformation of xenobiotics in the soil was studied in a model experimental system using pentachlorophenol (PCP), an enzyme, and a potential humic precursor. It has been shown that potential humic precursors such as \( p \)-coumaric acid greatly accelerated the peroxidase-catalyzed reaction of PCP (Morimoto K. and Tatsumi K., 1997. Effect of humic substances on the enzymatic formation of OCDD from PCP. *Chemosphere* 34, 1277–1283). About 90% of the PCP reacted but almost no transformation products were detected by GC/MS analysis. Most of the transformed PCP was detected in the polymeric products by the determination of the adsorbable organic halide (AOX) in gel permeation chromatography (GPC) fractions. Pyrolysis gas chromatography/mass spectrometry (py-GC/MS) of the polymers showed that tetrachlorophenol (TeCP) was the major pyrolysis product. This accords with the ion chromatography observation that about 20% of the PCP chlorine was released as chloride ion. However, nitrobenzene oxidation produced only a small amount of PCP and no TeCP. Based on the selectivity of the two degradation methods, it is concluded that TeCP is bound to the polymer by a diphenylether linkage, and PCP by an ether linkage to the aliphatic side chain. Thus, it was concluded that the major part of the PCP was incorporated into the polymer from \( p \)-coumaric acid by the release of a chlorine atom from the PCP molecule to form a diphenylether linkage with the \( p \)-hydroxyl group of the \( p \)-coumaric acid polymer. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Large amounts of toxic chemicals have been applied as agricultural chemicals or discharged as industrial effluent into the environment. Their fate and the possibility of their bioremediation have been an active concern.

The transformation of phenolic substances by white rot fungi may provide a solution for part of this problem. The fungi excrete extracellular phenol-oxidizing enzymes such as laccase or peroxidase, which bring about the transformation. Peroxidase is a common extracellular enzyme of white rot fungi but also occurs elsewhere, e.g. in lignin synthesis, in plants or in the reduction of superoxide in various organisms (Everse et al., 1991). It is known to produce phenoxy radicals by dehydrogenation of phenolic substances in the presence of hydrogen peroxide, leading to coupling reactions which form phenolic polymers such as lignin.

This enzyme also has an important role in humification reactions in soils (Ladd and Butler, 1975; Sulfita and Bollag, 1981), a complex process including both degradation and synthesis. The ability of the enzyme to cause phenolic coupling is also likely to allow the immobilization of toxic phenolic substances such as chlorophenols (Bollag et al., 1987; Ruttimann-Johnson and Lamar, 1997). In any study of such immobilization, it is easier to use the isolated enzyme rather than fungal cultures, with their attendant growth restrictions. If the process is to become an instrument...
for bioremediation, it will also be safer to use the isolated enzyme rather than whole micro-organisms.

Pentachlorophenol (PCP) has commonly been used as a herbicide and it is still used for wood preservation in some countries (Tanjore and Viraraghavan, 1994). It is well known that peroxidase catalyzes the transformation of PCP in the presence of hydrogen peroxide. This reaction is thought to involve the polymerization of PCP but also yields octachlorodibenzo-p-dioxin (OCDD) as a by-product (Oberg et al. 1990; Oberg and Rappe, 1992). However, this result was obtained in an experiment where no humic substances were present. To assess the fate of PCP in soil, it is necessary to consider the participation of humic substances in any transformation process (Tatsuji et al., 1994a, 1994b; Roper et al. 1995; Rissemann-Johnson and Lamar, 1996). Model experiments (Morimoto and Tatsuji, 1997) have shown that the oxidation of PCP in the presence of potential humic precursors such as p-coumaric acid or p-hydroxybenzoic acid can remove up to 90% of the PCP. This reaction also significantly reduced the yields of chlorinated quinones or dimers, such as chlorinated dioxins or phenoxyphenols. However, the details of the PCP reaction pathway are not clearly understood.

We studied the dechlorination and co-polymerization of PCP with p-coumaric acid, and the resulting chlorine balance. From analyses using alkaline nitrobenzene oxidation and pyrolysis GC/MS, the chemical structure of the polymeric product is discussed.

2. Materials and methods

2.1. Materials

Pentachlorophenol and p-coumaric acid were purchased from Nacalai Tesque (Kyoto, Japan). Nitrobenzene was obtained from Aldrich Chemical (Milwaukee, WI, USA). Horseradish peroxidase (HRP) was obtained from Wako Pure Chemical Industry (Osaka, Japan).

2.2. Reaction conditions

To observe any PCP decrease and dechlorination, the reaction solution contained 50 μM PCP, 200 μM p-coumaric acid, 1 unit HRP ml⁻¹, 0.5 mM H₂O₂. The pH was adjusted to 5, and the mixture was reacted at 28°C under aerobic conditions using a water bath shaker. PCP and chloride ion concentrations in the solution were monitored during the reaction.

To obtain sufficient polymeric product for structure analysis, the concentrations of the reactants were raised in a second experiment to 150 μM PCP, 300 μM p-coumaric acid, 6 units HRP ml⁻¹, 0.9 mM H₂O₂. To dissolve this quantity of PCP, acetone was added (25% v/v) to the solution.

2.3. Sample preparation

The reaction solution was fractionated by gel permeation chromatography (GPC) after 0, 5 and 30 min incubation. Fractions (15 ml) were collected and the adsorbable organic halide (AOX, see below) of each fraction was measured as an index of the total organic chloride.

In a further reaction experiment (500 ml volume), 50 ml samples were taken at time points 0, 5, 15, 30, 60, 120 and 240 min. The pH of each sample was raised to 9 with NaOH to stop the reaction and to dissolve the transformation products. Then 50 μl samples from these solutions were used to determine AOX, and to obtain the polymeric products and the remainder was dialyzed against water in cellulose tubing (mean pore diameter 2.4 nm). The AOX of the dialyzed reaction solution was measured to evaluate the quantity of PCP that was incorporated into the enzymatic polymerization, and the remaining solution was taken to dryness and analyzed by pyrolysis-GC/MS (Meier and Faix, 1992). A further 2 l of solution was similarly reacted to obtain 20 mg of polymer products for alkaline nitrobenzene oxidation analysis.

2.4. Analytical methods

PCP in the reaction solution was monitored by HPLC using a Jasco PU-980 (Japan Spectroscopic, Tokyo, Japan) provided with a UV detector and an integrator. A reverse phase column, Cosmosil 5C18- SL(4.6 mm i.d. × 25 cm, Nacalai tesque, Kyoto, Japan), was used with a 10:90 methanol/0.08% phosphate buffer mobile phase (flow rate 1.0 ml min⁻¹).

The concentration of chloride ions released from PCP was measured in a DIONEX series 4000i ion chromatograph using a HPIC AS12A column with 0.3 mM NaHCO₃/2.7 mM Na₂CO₃ as eluant. GPC was carried out on Biogel P-30 polyacrylamide gel (Bio-Rad, California, USA) packed in a 2.5 × 50 cm column using 50 mM NaOH/50 mM Na₂B₄O₇ as eluant.

To evaluate the quantity of total organic chlorine, the reaction solution was injected into a 3 × 40 mm column of activated carbon powder (40 mg). After washing the column repeatedly with KNO₃ to remove inorganic halide ion, the total chloride concentration of this column was measured by a TOX-10Σ instrument (Mitsubishi Kasei, Tokyo, Japan). The chloride concentration measured as above is called adsorbable organic halide (AOX) that is used as an index of total organic halide.

Following dialysis, the polymeric reaction product
structure was analysed by pyrolysis-GC/MS and by nitrobenzene oxidation, using syringaldehyde as an internal standard. About 100 μg of the material was pyrolyzed at 500°C for 5 s in a JHP-3 curie point pyrolyzer (Japan Analytical Industry Co. Ltd., Mizuho, Tokyo, Japan). The pyrolyzate was directly injected into an HP5972 / HP5890 series II GC/MS (Hewlett Packard, Palo Alto, CA, USA) equipped with a HP-1 fused silica capillary column (0.2 mm x 25 m). The temperature of the injector and the detector transfer line was kept at 300°C. The column oven temperature was held at 50°C for 5 min and then raised to 300°C at a rate of 8°C min⁻¹.

The polymer (20 mg) was also oxidised in alkaline nitrobenzene at 170°C for 2.5 h in an oil bath shaker (Chen, 1992). The resultant mixture was filtered and the acidified filtrate was extracted with diethylether. The ether-soluble products were analyzed by GC/MS as described above.

3. Results and discussion

3.1. Incubation analysis

Morimoto and Tatsumi (1997) have shown that horseradish peroxidase can effectively catalyze the transformation of PCP when reacted with p-coumaric acid. Almost no chlorinated monomeric or dimeric products were detected. This suggests that the PCP was incorporated into polymeric structures or further degraded by dechlorination and ring cleavage. To quantify the degradation of PCP, its concentration during the reaction was determined, along with the chloride ion released (Fig. 1). This chloride was expressed as a percentage of the total chlorine contained in the PCP in the initial solution. On this basis, more than 80% of the PCP disappeared within the first 5 min of reaction and more than 90% within 20 min. Along with the PCP decrease, 18% of the chlorine initially contained in PCP was released as chloride ion in the first 5 min, but thereafter the process slowed with the maximum being near 20%, corresponding to the release of one chlorine atom from each PCP molecule. However, about 70% of the chlorine in the initial PCP was not detected in the reaction solution. Its incorporation in the polymeric products is the only remaining explanation for its fate.

3.2. Molecular weight distribution of the chlorinated reaction products

Dialysis and GPC were used to detect co-polymerization of PCP as a possible fate of the unknown 70% chlorine. To obtain more products for fractionation, the concentration of PCP in the reaction solution was raised three-fold. This decreased the transformation yield and 15% of the initial PCP was left unchanged for 30 min after the reaction started.

Fig. 2 shows the AOX change in the reaction solution and the dialysis residue along with the residual PCP concentration. The AOX of the reaction solution was about 20–30% less than the initial concentration of PCP, corresponding to a dechlorination of nearly 20%. The AOX of the dialysis residue was slightly more than 50%. Since this fraction cannot pass through the 2.4 nm pore-sized dialysis tubing, these molecules should contain at least 5 benzene rings. The difference in AOX between the reaction solution and the dialysis residue should correspond to the amount of organic chlorine (including that of residual PCP) in products smaller than 2.4 nm.

Dialysis showed most of the transformed PCP was coupled with p-coumaric acid to form oligomeric or polymeric products. GPC was used to determine the molecular size distribution of these products in the reaction solution and the AOX of each GPC fraction is shown in Fig. 3. Most of the organic chlorine was detected in two peaks, corresponding to the exclusion limit and low molecular weight peaks. The latter peak was found to be residual PCP by comparing AOX with the PCP concentration of the same fractions.
measured by HPLC, using the AOX content of 150 μM PCP solution as a standard. Another peak of almost the same AOX content appeared at the exclusion volume, which corresponds to a 40,000 Da protein standard. Allowing for the physical differences between phenolic polymers and protein molecules, this peak suggests that the polymer contained more than 200 monomer units, and the detection of AOX from this fraction indicates the incorporation of PCP into that polymer, since PCP was the only chlorine source in the solution mixture.

HPLC analysis showed the total absence of p-coumaric acid after 2 min of reaction, at the point where PCP just started to decrease. In addition, preliminary experiments showed that when PCP was reacted with the polymer resulting from a 10 min previous reaction of p-coumaric acid, the PCP reduction rate was almost the same or even a little faster than with the p-coumaric acid monomer. These results suggest that p-coumaric acid first polymerized and then the PCP was bound to that polymer, a mechanism that also explains the absence of dimeric or trimeric products including chlorine. No oligomers or dimers were present even in the very early stages of the reaction, despite several attempts to obtain dimeric or trimeric product.

3.3. Characterization of the polymerized product

Chromatograms of the nitrobenzene oxidation products from the 30 min-reaction product are shown in Fig. 4(a) and the pyrogram is shown in Fig. 4(b). The only chlorinated substance produced by nitrobenzene oxidation was PCP and the yield was 2% of the initial solution. In contrast, pyrolysis produced a considerable amount of tetrachlorophenol (TeCP) and also a small amount of trichlorophenol (TrCP) and PCP, the PCP is almost the same yield as in the nitrobenzene oxidation. The detection of TeCP means one chlorine atom of PCP was lost at the binding site where PCP forms a linkage with the polymer.

Fig. 5 shows the variation of each chlorinated pyrolysis product in the dialyzed reaction solution while Fig. 2 shows the AOX variation in the same incubation. The total chlorophenol recovery at 120 min was about 18% of the initial solution and 35% of the dialysis residue AOX. This yield is appropriate considering that the recovery of phenolic substances from lignin in general is about 30% (Lam et al., 1990; Kuroda et al., 1994).

TeCP was the major organohalogen detected, corresponding to the observation that nearly 20% of the chlorine was released as chloride ion from the transformed PCP. The concentration of pyrolysis products increased for the first 2 h of reaction, but decreased afterwards. However the AOX of the dialysis residue showed no such decline (Fig. 2). One possible explanation is that the polymer became more resistant to pyrolysis as the reaction proceeded, indicating a continuous condensation within the polymer structure even after most of the monomers were consumed.

Phenol oxidizing enzymes such as peroxidase produce phenoxy radicals through dehydrogenation of the hydroxyl group (Courteix and Bergel, 1995a, 1995b). This radical is resonance-stabilized as shown in Fig. 6 for p-coumaric acid. Here radical coupling occurs at the p-hydroxyl group, the m-position of the benzene ring and the β-carbon of the side-chain.

Fig. 7 shows the probable linkage in an analogous lignin structure and the possibility of their cleavage by nitrobenzene oxidation and pyrolysis. L2 and L4 are C–C linkages, presumed to form enzymatically, between PCP and p-coumaric acid. Here radical coupling occurs at the p-hydroxyl group, the m-position of the benzene ring and the β-carbon of the side-chain. On the other hand, would TeCPs detected only by pyrolysis were probably derived from a diphenylether linkage, because if they were on the side-chain they must also be cleaved and detected by nitrobenzene oxidation. Pyrolysis of the diphenylether linkage is thought to be difficult and has not been studied in detail. However, when pyrolyzed, some lignin dipheny-
lethers were cleavable to a remarkable extent. In our hands, 52% of 2-(4-carboxy-2-methoxyphenoxy)-4,5-dimethoxybenzoic acid was cleaved at 500°C, and around 32% of 3-(4-carboxy-2-methoxyphenoxy)-4,5-dimethoxybenzoic acid. Diphenylethers including a polychlorinated phenol such as PCP may cleave readily due to the reduction of the ring aromaticity by the chlorine substituents.

The oxygen of the ether linkage connecting TeCP to the polymer must have come from p-coumaric acid or its polymer, because any linkage through the hydroxyl group of PCP will cleave to form PCP and not TeCP.

Fig. 4. GC/MS chromatogram of the oxidation products from the polymer produced by the peroxidase catalyzed transformation of PCP and p-coumaric acid. The degradation methods were (a) nitrobenzene oxidation, and (b) pyrolysis.
The phenolic \( p \)-hydroxyl group (L5) is the only point where \( p \)-coumaric acid or its polymer can supply the phenoxy radical to form a diphenylether. This corresponds with the observation (Morimoto and Tatsumi, 1997) that the enzymatic transformation of PCP was highly affected by a methoxyl group at the \textit{meta} position of the humic precursors. This can be explained by the steric hindrance between the \textit{m}-methoxyl groups and any incoming PCP molecule approaching the \( p \)-hydroxyl group.

Thus, we can conclude that the major part of the PCP enzymatically transformed with \( p \)-coumaric acid was bound to the polymer through diphenylether linkages at the \( p \)-hydroxyl groups of \( p \)-coumaryl subunits and the remaining PCP was bound to their aliphatic side chains by an ether linkage.

From the direct measurement of chemicals in soil, it is known that the major part of PCP applied to farmland is retained in the soil and does not easily leach out (Weiss et al., 1982). However the mechanism of retention is not known but adsorption to the soil organic matter is thought to be one of the major causes (Christodoulatos et al., 1994; Christodoulatos and Mohiuddin, 1996). The enzymatic binding of xenobiotics into humic substances has also been highlighted (Bollag et al., 1992), but in the case of PCP, enzyme catalyzed association with ferulic or syringic acid was quantitatively not so effective. Ruttimann-Johnson and Lamar (1996) reported on the ability of manganese peroxidase to polymerize PCP with ferulic acid, but little attention was paid to \( p \)-coumaric acid although it is the most abundant humic precursor in various kinds of soils and sediments (Shindo et al., 1978; Matsumoto and Hanya, 1980). Our research shows that peroxidase can effectively catalyze the linkage of PCP to \( p \)-coumaric acid polymer, principally through a diphenylether linkage between the \( p \)-hydroxyl group of \( p \)-coumaric acid and the benzene ring of PCP by dechlorination at the point of linkage. These results reinforce the importance of enzymatic coupling of PCP with humic substances in soil as an immobilization process.

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### Figure 5

The amounts of pyrolysis products from the dialyzed reaction solution and their change with incubation.

### Figure 6

Resonance stabilization of the dehydrogenation product from \( p \)-coumaric acid.

### Figure 7

Structural analysis of the polymeric product from \( p \)-coumaric acid and PCP. PCP detected by nitrobenzene oxidation and pyrolysis GC/MS; TeCP detected only by pyrolysis GC/MS.

Lamar (1996) reported on the ability of manganese peroxidase to polymerize PCP with ferulic acid, but little attention was paid to \( p \)-coumaric acid although it is the most abundant humic precursor in various kinds of soils and sediments (Shindo et al., 1978; Matsumoto and Hanya, 1980). Our research shows that peroxidase can effectively catalyze the linkage of PCP to \( p \)-coumaric acid polymer, principally through a diphenylether linkage between the \( p \)-hydroxyl group of \( p \)-coumaric acid and the benzene ring of PCP by dechlorination at the point of linkage. These results reinforce the importance of enzymatic coupling of PCP with humic substances in soil as an immobilization process.
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References


