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ENZYMATIC INCORPORATION OF PENTACHLOROPHENOL INTO HUMIC SUBSTANCES

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Introduction

Large amounts of toxic chemicals have been applied as agricultural chemicals or discharged as industrial effluent. Their fate in the environment and also the possibility of their on-site bioremediation have been the subject of active concern.

Humification is a natural process in which substances newly introduced into the soil mature and become a part of the humic substances.Chemicals introduced into the soil also participate in this process. Humification is mainly the enzymatic transformation of immature substances which then are incorporated with humic substances in the soil. One of the most important enzymes catalyzing this process is peroxidase.

Pentachlorophenol(PCP) had commonly been used as a herbicide and it is still presently used for wood preservation in some countries. It is well known that peroxidase catalyzes the transformation of PCP in the presence of hydrogen-peroxide[1]. This reaction is known to produce a fairly high amount of octachlorodibenzo-*p*-dioxin (OCDD) as a byproduct[2]. However, this result was obtained by an experiment in which no humic substances were present. To assess the fate of PCP in soil, it is necessary to consider the participation of humic substances in the transformation process. Model experiments using simple humus constituents showed that humic precursors without a methoxyl group, ie. *p*-coumaric acid or *p*-hydroxybenzoic acid, accelerated

PCP transformation remarkably[3]. Resuls of up to 90% removal were observed.Association of these acids also decreased the production of GC detectable oligomers such as dioxin to almost zero.However,as for the fate of PCP through this reaction there are many points that were remained unknown.

This paper reports on the measurement of the dechlorination and polymerization of PCP and the resulting chlorine balance is presented. Also analysis of the polymer by pyrolysis GC/MS is reported.

Materials and Methods

Materials : Pentachlorophenol, *p*-coumaric acid and ferulic acid were purchased from Nacalai Tesque (Kyoto, Japan). horseradish peroxidase (HRP) were obtained from Wako Pure Chemical Industry (Osaka, Japan). Biogel P30 was obtained from Bio-Rad Laboratories (California, USA), Pyrofoil was obtained from Japan Analytical Industry (Mizuho,Tokyo,Japan).

Incubation : Reaction solution contains 150 μ M PCP, 300 μ M *p*-Coumaric acids,6 unit HRP mL^{-1}, 0.9mM H_2O_2 and adjusted to pH5. The reaction mixture was incubated at 28 °C under aerobic conditions using a water bath shaker.

For the evaluation of PCP transformation the reaction solution was measured by HPLC. The concentration of chloride ions released from PCP was measured by ion chromatography. After incubation the reaction solution was fractionated by gel chromatography and the adsorbable organic halogen (AOX) was measured for each fraction to determine the amount of PCP bound into the polymer.

The polymer was analyzed by pyrolysis GC/MS after dialysis using 24 Å cellulose tube.

Analytical methods : Disappearance of PCP 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. The mobile phase (flow rate 1.0 mL/min) consisted of methanol and a 0.08 % phosphate buffer. The ratio of methanol to the buffer was 90:10

The concentration of chloride ions dechlorinated from PCP in the reaction solution was measured by ion chromatography (DIONEX series 4000i, Dionex,California,USA).

Polymerization of PCP was detected by gel chromatography using biogel P-30 poly acrylic

amide gel packed in a 2.5 × 50 cm column (Bio-Rad,California,USA). Fractions of 15mL were collected and organic chloride concentration of each fraction was measured asAOX using TOX-10 Σ (Mitsubishi Kasei Co, Tokyo, Japan).

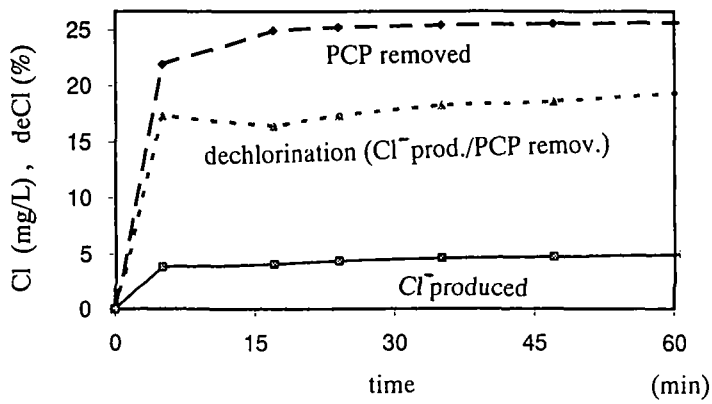
Produced polymers were pyrolyzed at 500°C, 5sec.by JHP-3 curie point pyrolyzer (Japan Analytical Industry Co.,Ltd.,Mizuho,Tokyo,Japan). The pyrolysis products were directly injected into a GCMS instrument, HP5971 / HP5890 series II (Hewlett Packard, Palo Alto, CA, USA). An HP-1 fused silica capillary column (0.2 mm × 25 m) coated with methyl silicon gam underwent a gradient temperature change; 50°C for 5 min, 8 deg/min and then 300 °C for 5 min.

Results and Discussion

In the previous paper, we showed that horseradish peroxidase catalyzed PCP transformation very effectively when it was incubated with *p*-coumaric acid. And almost no reaction products were detected by GC/MS. This suggests that transformed PCP could be either degraded or polymerized.

Dechlorination of PCP : Fig.1 shows the change of PCP concentration measured by HPLC and chloride ions concentration measured by ion chromatography during incubation.

Fig.1 Time course of dechlorination at enzymatic transformation of PCP and *p* -coumaric acid



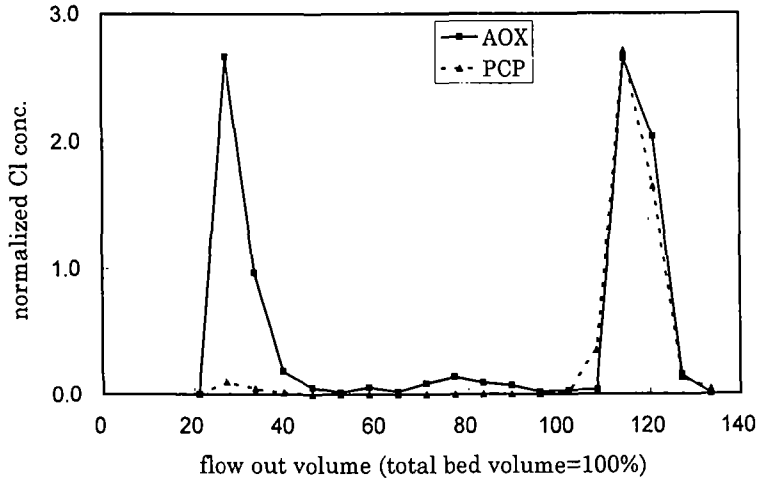
About 18% of the chlorine originally contained in PCP was released as chloride ions in the first 5 minutes, but thereafter process slowed with the maximum being near 20 %. It corresponds to the release of one chlorine atom from each PCP molecule.

Detection of PCP polymerizartion : The reaction solution was fractionated by gel chromatography using bio-gel P30. Adsorbable organic halogen of each fraction was measured

as an indicater of organochlorine. Therefore the appearance of a peak means the detection of an organic chloride originating from PCP.

Fig.2 shows the molecular size distribution of the reaction solution after 5 minutes incubation. Organic chloride was mostly detected in two of the fractions, an exclusion size fraction and a low molecular weight fraction. The latter fraction was found to be residual PCP from a comparison with PCP concentration of the same fraction measured by HPLC. Another peak of almost the same strength appeared at the exclusion size volume. The result shows that a good amount of PCP was bound to the polymer larger than this size. Exclusion size of P30 measured by dextran standard is 10,000. Oligomers or polymers including chlorine and smaller than this size were not detected, suggesting that PCP was combind with the polymers made from *p*-coumaric acid rather than *p*-coumaric acid itself.

Fig.2 AOX of Biogel P30 Gel chromatography fractions of 5min reaction solution



Chlorine balance was obtained from this result (Table1). Amount of chloride added as PCP at the beginning of incubation is considered to be 100%. Besides the 20% experimental loss 20% of chloride was detected as chloride ions and remaining 60% was AOX. The AOX was consist

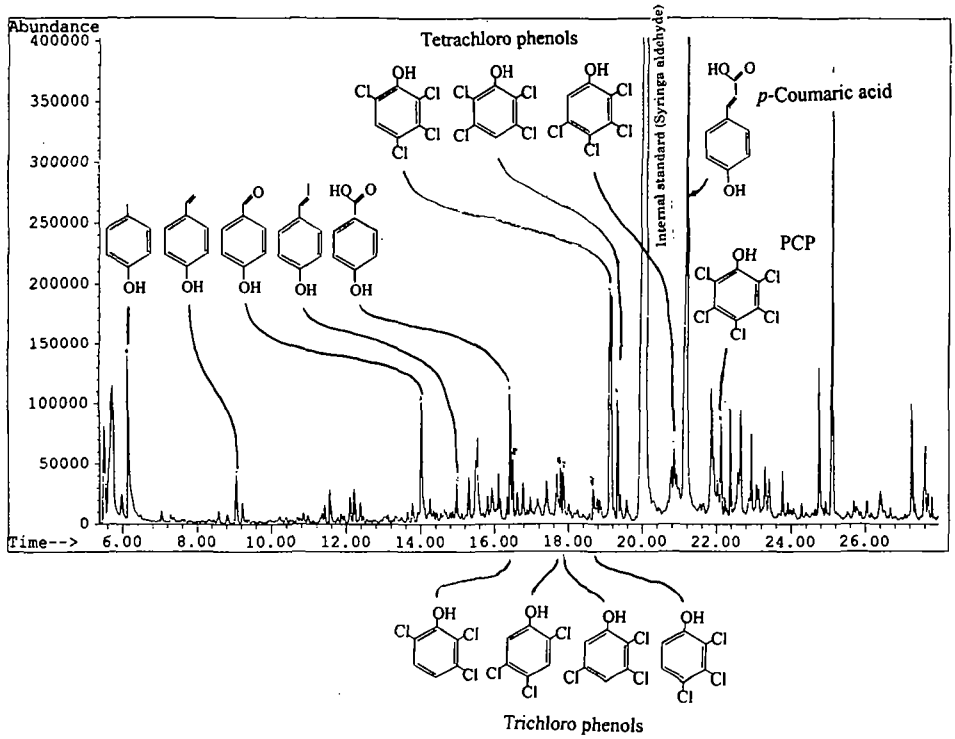
Table1 Chlorine balance of enzymatic ransformation of PCP with *p*-coumaric acid

dechlorination	AOX recovered from Gel chromatography		
20%	60%	polymer	PCP
5min incubation		30%	30%
30min incubation		45%	15%

of remaining PCP and polymer. Polymer content increased with time from 30% after 5min to 45% after 30min incubation. This result shows that the main reaction product was the polymer.

Analysis of polymerized product using pyrolysis GC/MS : Reaction products larger than 24 Å was measured by pyrolysis GCMS. A pyrogram of 30 min inubation product is shown in Fig.3. Chlorinated phenols originated from PCP, methyl,ethenyl and propenyl phenols,phenolic acid and aldehyde came from *p*-coumaric acid.

Fig.3 Pyrogram of dialyzed reaction solution after 30min incubation



The quantity of each substances at each incubation time was shown in Fig.4. More than 80% of the organic chloride was TeCP which corresponds to the fact that 20% of the chlorine was released as chloride ions.

Pyrolysis products increased for the first 2hrs of incubation, but decreased afterwords. However AOX of dialysis residue showed no such decline. Possible explanation is that the polymer became stronger with incubation causing less recovery of pyrolysis. The same explanation can be adopted to the ratio of the PCP resulting substances to those of the p-coumaric

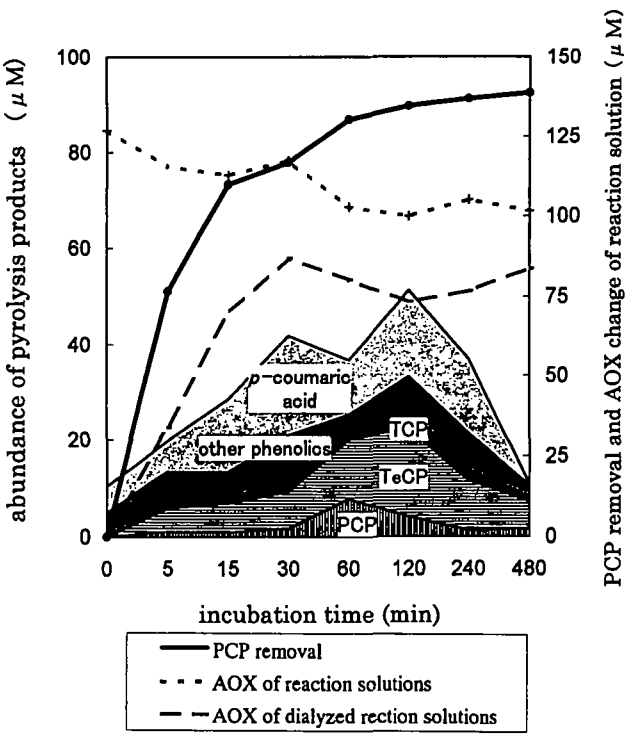


Fig.4 Change of polymer contents during incubation analyzed by GC/MS with pyrolyser along with PCP and AOX change of reaction solution

acid. At the start of incubation *p*-coumaric acid was added twice as much as PCP. But the sums of the pyrolysis products resulting from PCP and *p*-coumaric acid were almost the same. The larger recovery of PCP suggests that the PCP was bound to the surface of the core polymer made from mainly *p*-coumaric acid. It corresponds to the assumption stated earlier that PCP may be bound to the polymer rather than *p*-coumaric acid itself. And also it can be concluded that reconstruction of polymer continues even after most of the monomers were consumed.

From the direct measurement of chemicals in soil, it was known that the major part of PCP applied to the farmland remains in the soil nearby and not so easily flows out. However the mechanism of retension was not known and adsorption was thought to be one of the main cause. This paper showed that covalent bonding to the humic substances in soil through enzymatic process is thought to be another mechanism of retension and this is more important from the stand point of self-purification process of toxic chemicals in nature.

References

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