Effect of humic substances on the enzymatic formation of OCDD from PCP

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INTRODUCTION

The production of chlorophenols (CPs) peaked during the 1970s and has subsequently decreased. Pentachlorophenol(PCP) was commonly used as a fungicidal agent and was extensively applied to farmlands. Moreover, the derivatives of CPs are still used as herbicides in some areas. Therefore CPs remaining in soil constitute an important problem.

Enzymatic or photochemical formation of polychlorinated dibenzo-*p*-dioxins (PCDD) from CPs are attracting attention as a significant natural source of these compounds [1,2]. It is well known that peroxidase catalyzes this transformation in the presence of hydrogen-peroxide[3]. Particularly formation of octachloro-dibenzo-*p*-dioxin (OCDD) from PCP by the enzyme is in far greater quantity than other PCDD/Fs from other CPs[4]. Many kinds of plants and micro-organisms produce peroxidase and it is ubiquitous in the nature. Numerous compounds including CPs can be bound to humic substances through the activity of an enzyme such as laccase, peroxidase, or tyrosinase[5,6,7], and to assess the fate of CPs in soil, it is important to consider the participation of humic substances in that process. However, this is very difficult because humic substances are complex and heterogeneous. Model experiments using simple humus constituents are very useful and provide information concerning the fate of CPs. It is known that various phenolic derivatives such as *p*-coumaric acid, *p*-hydroxybenzoic acid, ferulic acid, vanillic acid ,*etc* are present in the environment[8,9,10,11]. They are precursors of humic substances originating from lignin[12,13]. Hence these phenolic acids can be used as model compounds to assess the enzymatic reactions in soil.

In this paper, we investigated the effect of these phenolic acids on the transformation of PCP and the production of OCDD catalyzed by horseradish peroxidase (HRP).

MATERIALS AND METHODS

Materials

Pentachlorophenol, 2,4,6-tribromophenol (TBP), *p*-coumaric acid and ferulic acid were purchased from Nacalai tesque (Kyoto, Japan). ^{13}C-octachloro-dibenzo-*p*-dioxin (^{13}C-OCDD) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, Massachu-

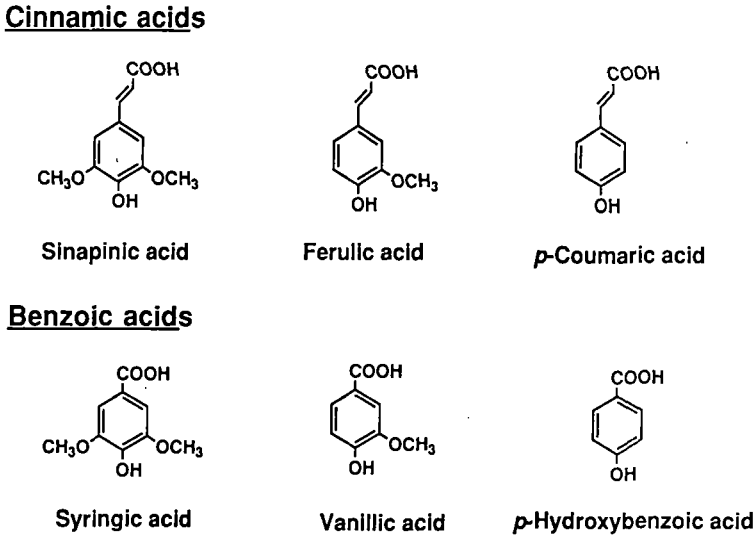

Fig.1 Structures of Phenolic acids used in this investigation

setts, USA). Sinapinic acid, vanillic acid and syringic acid were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Vanillic acid was recrystallized from hot water. *p*-Hydroxybenzoic acid and HRP(horseradish peroxidase) were obtained from Wako chemicals (Tokyo, Japan).

Incubation

Reactions were carried out in McIlvaine buffer (sodium phosphate dibasic and citric acid), pH 5, containing 50 μM PCP, 200 μM phenolic acids, 0.1 units HRP ml^{-1} and 500 μM hydrogenperoxide. When reaction was done without phenolic acids, the concentration of hydrogenperoxide was 100 μM. The reaction mixture was incubated at 28 °C under aerobic conditions using a water bath shaker (TAITEC PERSONAL-11, Tokyo Japan).

For the evaluation of PCP degradation the reaction solution was measured by HPLC. In order to determine the products, reaction solutions were extracted with hexane after two hours of incubation and analyzed by GCMS. TBP (20 μM) was added into extracts as an internal standard to quantify monomer products, as ^{13}C-OCDD (0.042 μM) was used for dimer products, particularly OCDD.

Analytical methods

The disappearance of PCP was monitored by HPLC using a Jasco PU-980 (Japan Spectroscopic, Tokyo, Japan) provided with an UV detector (Jasco 875-UV) and an integrator (Jasco 807-IT). A reverse phase column, Cosmosil 5C18-SL(4.6 mm i.d. x 25 cm, Nacalai tesque, Kyoto, Japan), was used. The mobile phase was (flow rate 1.0 mL/min) consisted of methanol and 0.08 % phosphate buffer and the ratio of methanol to the buffer

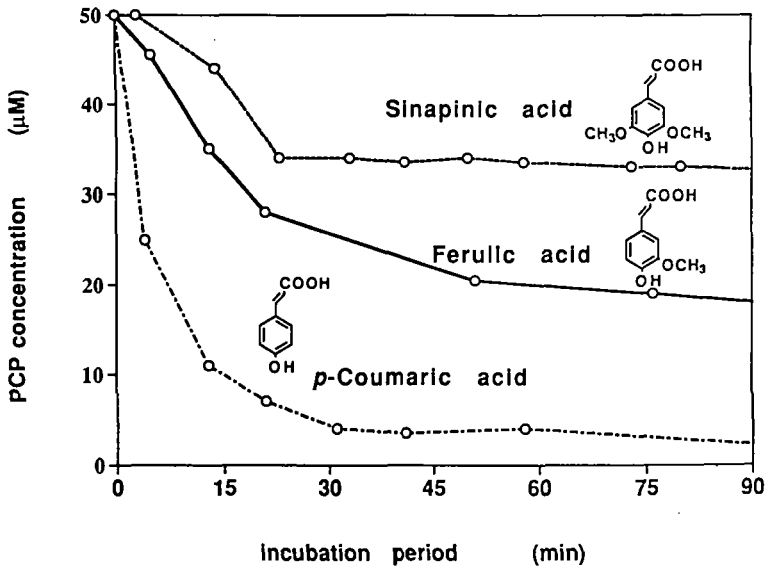

Fig.2 PCP reduction by HRP in the presence of cinnamic acids

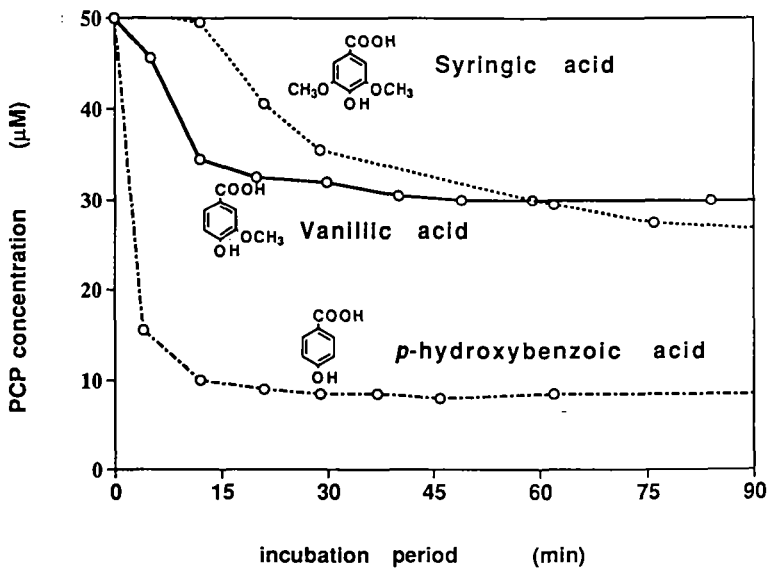

Fig.3 PCP reduction by HRP in the presence of benzoic acids

was 95:5.

Products formed were analyzed by a GCMS instrument ,HP5971 / HP5890 series II (Hewlett Packard, Palo Alto, CA, USA). An HP-5 fused silica capillary column (0.2 mm X 25 m) coated with 5 % phenyl methyl silicon underwent a gradient temperature change; 65 °C for 1.5 min, 65-120 °C at 35 deg/min, 120-300 °C at 7 deg/min and then 300 °C for 5 min.

RESULTS AND DISCUSSION

The effect of six phenolic acids (Figure 1) on the enzymatic reaction of PCP was investigated. Three of the acids are substituted cinnamic acids while the rest are substituted benzoic acids. They are known to be found in the nature as humic precursors. When PCP was incubated in the absence of humic precursors, about 60% of PCP was transformed in the first 4 minutes.

Figure 2 shows the decrease of PCP concentration in the reaction solutions in the presence of substituted cinnamic acids. The phenolic acids were transformed more rapidly than PCP and thoroughly disappeared in 5 minutes (data not shown). The final concentration of PCP in the presence of ferulic acid was almost the same as that in the case of PCP alone. Although the degradation rate during the first 30 minutes was slower than that of PCP alone. The possible explanation is that HRP was at first mainly used for degrading ferulic acid. Degradation of PCP was accelerated in the presence of *p*-coumaric acid and more than 90% of PCP was degraded within 30 minutes. However, PCP degradation was interfered by sinapinic acid and only 25 % was degraded. Figure 3 shows the time course of the disappearance of PCP in the presence of benzoic acids. In this case PCP degradation was accelerated in the presence of *p*-hydroxybenzoic acid and the degradation rate was more than 80 %, whereas the addition of other substituted benzoic acids caused a decrease in degradation of PCP.

Five compounds were detected from the reaction solution of PCP by a GCMS. Three of them have a molecular weight of 248, and the spectrum shows that they are tetrachloro compounds. One of them was identified as tetrachloro-hydroquinone when it was compared with the authentic sample. Other two compounds might possibly be the isomers. Another compound was found to be OCDD in comparison with an authentic sample. Molecular weight of the last one was 496 with nine chlorines according to the isotopic pattern of its mass spectrum. This should be nonachloro-phenoxyphenol.

Concentrations of the products in the reaction solutions were measured after 2 hours of incubation (Figure 4). Approximately 30 μM of PCP was degraded and 5 % of the degraded PCP was converted to tetrachloro-hydroquinone and 1.4 % was converted to OCDD, while in the presence of phenolic acids these products were formed in minor quantities. Almost no quinones were detected in the presence of phenolic acids. OCDD production decreased by 63 % in the presence of sinapinic acid, 32 % in ferulic acid, 5 % in *p*-coumaric acid. OCDD and phenoxy-phenol are known to be formed by oxidative coupling of two PCPs. But if more reactive humic precursors are present, the probability of this coupling should be reduced and most of PCP may be linked to the polymer produced in oxidative coupling of humic precursors.

p-Coumaric acid and *p*-hydroxybenzoic acid are known to be more abundant in soils and sediments than other humic precursors. It was reported that river sediments contain

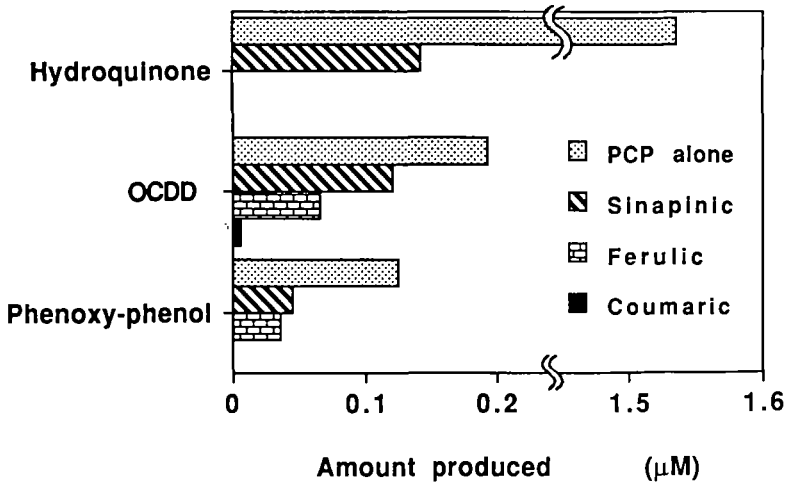

Fig.4 Reaction products of PCP by HRP with phenolic acids

110 ppm [9] and the soil of crop fields[10] contain 5 ppm of *p*-Coumaric acid as a monomer. Our experimental results suggest less formation of PCDDs or other products in the environment than could be estimated on the basis of enzymatic reactions of chlorophenols alone.

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