PERGAMON

Soil Biology & Biochemistry 32 (2000) 85–91

Soil Biology & Biochemistry

www.elsevier.com/locate/soilbio

Phenolic acids affect transformations of chlorophenols by a *Coriolus versicolor* laccase

Kazuhito Itoh*, Miho Fujita, Kazuo Kumano, Kousuke Suyama, Hiroki Yamamoto

Faculty of Life and Environmental Science, Shimane University, Matsue 690-8504, Japan

Accepted 31 July 1999

Abstract

Partially-purified extracellular laccase of *Coriolus versicolor* transformed chlorophenols in the following order: 2,4,6-trichlorophenol > 2,6-dichlorophenol > 2,4-dichlorophenol, 2-chlorophenol > 2,3-dichlorophenol. On the other hand, 3- and 4-chlorophenols, 2,5-, 3,4- and 3,5-dichlorophenols, 2,4,5-trichlorophenol and pentachlorophenol were recalcitrant to the laccase. Sinapinic acid, a naturally-occurring phenolic acid, enhanced transformations of 2,4,5- and 2,4,6-trichlorophenols, 2,4-dichlorophenol and 4-chlorophenol, whereas ferulic and *p*-coumaric acids inhibited transformations of 2,4,6-trichlorophenol, 2,3-, 2,4- and 2,6-dichlorophenols and 2-chlorophenol. The three phenolic acids have similar chemical structures. Further examination using sinapinic and ferulic acids and 2,4-dichlorophenol revealed that sinapinic acid competitively inhibited the transformation of 2,4-dichlorophenol at the first stage of the reaction, but its reaction intermediates likely enhanced the transformation of 2,4-dichlorophenol. Ferulic acid also competitively inhibited the transformation of 2,4-dichlorophenol at the first stage of the reaction but then its reaction intermediates irreversibly inactivated the laccase.

Keywords: Chlorophenols; *Coriolus versicolor*; Laccase; Phenolic acids

1. Introduction

There has been much concern about the risk to the environment of xenobiotics from industrial activities, therefore studying the fate of these chemicals is important. Chlorinated phenols are hazardous compounds found in manufacturing wastes and as degradation intermediates during microbial metabolism of various pesticides and industrial chemicals. Microorganisms are known to mineralise chlorinated phenols via either catabolic or cometabolic processes to carbon dioxide (Focht, 1993; Harwood and Parales, 1996).

On the other hand, coupling reactions, involving chlorinated phenols and soil humic components and mediated by microbial phenol oxidases, are thought to be major pathways in soil (Bollag, 1992b). These reactions are important in considering the fate of phenolic contaminants in the soil (Bollag and Myers, 1992), humification processes of phenolic compounds through binding to soil humic substances (Bollag and Loll, 1983) and bioremediation of contaminated environments using such enzymes (Bollag, 1992a).

The initial reaction by the phenol oxidases is the transformation of phenolic substrates to the corresponding phenoxy radicals. These radicals are so reactive that coupling reactions can occur between them and with other compounds. Therefore, the coupling reaction and the reaction products are affected by the substrate itself and other compounds. Bollag et al. (1988) reported that addition of naturally occurring more reactive phenolic acids, such as syringic acid, ferulic acid, vanillic acid and *p*-coumaric acid, enhanced the coupling reaction of 2,4-dichlorophenol

* Corresponding author. Fax: +81-852-32-6597.
E-mail address: itohkz@life.shimane-u.ac.jp (K. Itoh).

0038-0717/00/$ - see front matter
PII: S0038-0717(99)00133-9

in the presence of *Rhizoctonia praticola* laccase. On the other hand, it was reported that the presence of catechol, which is also a reactive compound, inhibited the laccase-mediated coupling reaction of 2,4-dichlorophenol (Shuttleworth and Bollag, 1986).

Clarifying these phenomena and understanding the mechanisms of the reactions are important in evaluating the fate of phenolic compounds in the environment and making rational decisions about bioremediation. In this study, we examined the effects of six types of naturally-occurring phenolic acids on coupling reactions involving 12 chlorinated phenols mediated by a partially-purified extracellular laccase of *Coriolus versicolor*.

2. Materials and methods

2.1. Enzyme preparation

C. versicolor (IFO 30340) was grown statically in Erlenmeyer flasks containing glucose–peptone medium at 25°C (Morohoshi and Haraguchi, 1987). After 3 weeks incubation, the growth medium was separated from the mycelia with gauze and filtered through a 0.8 μm membrane filter (type ATTP, Millipore Corp.) under reduced pressure. The filtrate was precipitated by adding solid ammonium sulfate to 90% saturation at 4°C. The precipitate was redissolved in and dialysed against distilled water. Undissolved residues were separated by filtration and discarded.

Laccase activity and purity were visualized in native polyacrylamide gel electrophoresis (PAGE) by soaking the gel in 50 mM sodium acetate buffer (pH 5.0) containing 2-methoxyphenol at 2 mM (Coll et al., 1993). As only one brown band appeared due to polymerization of 2-methoxyphenol, this enzyme solution was not purified further. Optimum pH of the laccase was 5.0 in the range 2.2 to 8.0 using 2,4-DCP as a substrate. The procedures are described in Section 2.3.

Laccase activity was determined spectrometrically with a spectrophotometer (UV-160A, Shimadzu Corp.) according to the method described by Dec and Bollag (1990) but using 2-methoxyphenol as a substrate. One unit of the enzyme activity was defined as the amount of the laccase that caused an absorbance change of 1.0 min^{-1} at 468 nm in 3.5 ml of 0.1 M citrate-phosphate buffer (pH 3.6) containing 3.24 μM 2-methoxyphenol.

2.2. Chemicals

Several chlorophenols were used as an anthropogenic substrate for the laccase and phenolic acids were used as a naturally-occurring precursor of humic substances existing in the soil environment (Whitehead, 1964; Shindo et al., 1978; Stevenson, 1982).

2-, 3- and 4-chlorophenol (2-, 3- and 4-CP), 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dichlorophenol (2,4-, 2,5-, 2,6-, 3,4- and 3,5-DCP), 2,4,5- and 2,4,6-trichlorophenol (2,4,5- and 2,4,6-TCP), 4-hydroxybenzoic acid (HBA), 4-hydroxy-3-methoxybenzoic acid (vanillic acid, VA), 4-hydroxycinnamic acid (*p*-coumaric acid, CA), 4-hydroxy-3-methoxycinnamic acid (ferulic acid, FA) and 2-methoxyphenol were purchased from Wako Pure Chemical (Osaka, Japan) and 2,3-dichlorophenol (2,3-DCP) and pentachlorophenol (PCP) were obtained from Tokyo Kasei (Tokyo, Japan). 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid, SyA) was from Sigma Chemical Co. (Tokyo, Japan) and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SiA) was from Aldrich Chemical Co., Inc. (Tokyo, Japan).

2.3. Transformation of chlorophenols by laccase

Each chlorophenol was dissolved in methanol and added to a universal buffer solution (pH 5.0) containing the laccase at 1.8 units ml^{-1}. The final concentration of the substrate was 50 mg l^{-1} in which the molar concentrations ranged from 188 to 389 μM and that of methanol was $<1\%$ (v/v). After static reaction at 30°C for 0, 15, 30 and 45 min, an aliquot of each sample solution was taken and directly analyzed by HPLC. Boiled enzyme solutions were used as control and were inactive. Experiments were conducted at least in duplicate.

2.4. Effect of phenolic acids on transformation of chlorophenols by laccase

In the above study, each phenolic acid dissolved in methanol was added to the enzyme solution. The final concentration of the chlorophenols and phenolic acids were 50 mg l^{-1} (188–389 μM) and 100 mg l^{-1} (446–724 μM), respectively. Reaction and analytical procedures were the same as described above. Transformation of chlorophenols in the absence of phenolic acids was also examined as a control. Relative reactivity of the phenolic acids themselves as a substrate for the laccase was also examined using the buffer solution containing various units of the enzyme.

In some experiments, bovine serum albumin (BSA) dissolved in distilled water was added to the enzyme solution to protect the laccase from inactivation by reaction intermediates. Final concentrations of the protein were 70, 140 and 1400 mg l^{-1}, equivalent to 10-, 20- and 200-fold concentration of the laccase in the reaction solution, respectively. Enzyme concentration was determined using Bio-Rad Protein Assay Kit (Bio-

Rad Lab., Inc., Tokyo, Japan) based on the Bradford method (Bradford, 1976) with BSA as a standard.

2.5. Effect of reaction intermediates of SiA and FA by laccase on transformation of 2,4-DCP

After static incubation of SiA and FA at 30°C for 0, 0.5, 1, 2, 3, 4 and 5 h, 2,4-DCP was added at 50 mg l^{-1} (307 μM). The mixtures were reacted under the same conditions and an aliquot of each sample solution was taken for HPLC analysis at 0, 15, 30 and 45 min after the application of 2,4-DCP. Transformation of 2,4-DCP in the absence of phenolic acids was also examined. Concentrations of the laccase were adjusted to give the same reaction rate between SiA and FA, that is 1.8 and 5.4 units ml^{-1}, respectively. Concentrations of the phenolic acids were 100, 200, 400 and 800 mg l^{-1} (446 μM–3.57 mM for SiA and 515 μM–4.12 mM for FA) to change the reaction rate of the phenolic acids by the laccase.

2.6. HPLC analysis

HPLC was performed with a Shimadzu model LC-10AT liquid chromatograph equipped with a SUMIPAX ODS-A212 column (6 mm i.d. × 15 cm, Sumika Analytical Service Ltd., Osaka, Japan), using methanol: 0.1% formic acid (7:3, v:v) as a mobile phase. The chlorophenols and phenolic acids were detected by a Shimadzu model SPD-10A UV-VIS detector at 254 nm. Chromatographic peaks were quantified using a Shimadzu model C-R6A integrator.

3. Results

3.1. Transformations of chlorophenols by laccase

Transformations of chlorophenols by the laccase are shown in Fig. 1. Rates of transformation were dependent on the number and position of the chlorine atoms. Among the monochlorophenols, 19% 2-CP was transformed during 45 min of reaction, whereas 3-CP and 4-CP were not transformed at all. Among the dichlorophenols, 2,3-, 2,4- and 2,6-DCP were susceptible substrates for the laccase with 16%, 32% and 46% transformed, respectively. The other three dichlorophenols were not transformed. 2,4,6-TCP was the most susceptible of the other chlorophenols examined and 74% was transformed during the reaction period. 2,4,5-TCP and PCP were not affected by the laccase.

3.2. Effect of phenolic acids on transformations of chlorophenols by laccase

The disappearance of 2,4-DCP with or without each phenolic acid is shown in Fig. 2. Transformation of 2,4-DCP was enhanced when SiA was added to the laccase reaction buffer. On the other hand, addition of VA, FA and CA inhibited the transformation of 2,4-DCP. HBA and SyA had no effect on the transformation of 2,4-DCP.

The reactivity of the phenolic acids in the presence of laccase were variable. SiA and FA were reactive substrates and were not detected after 15 min of reac-

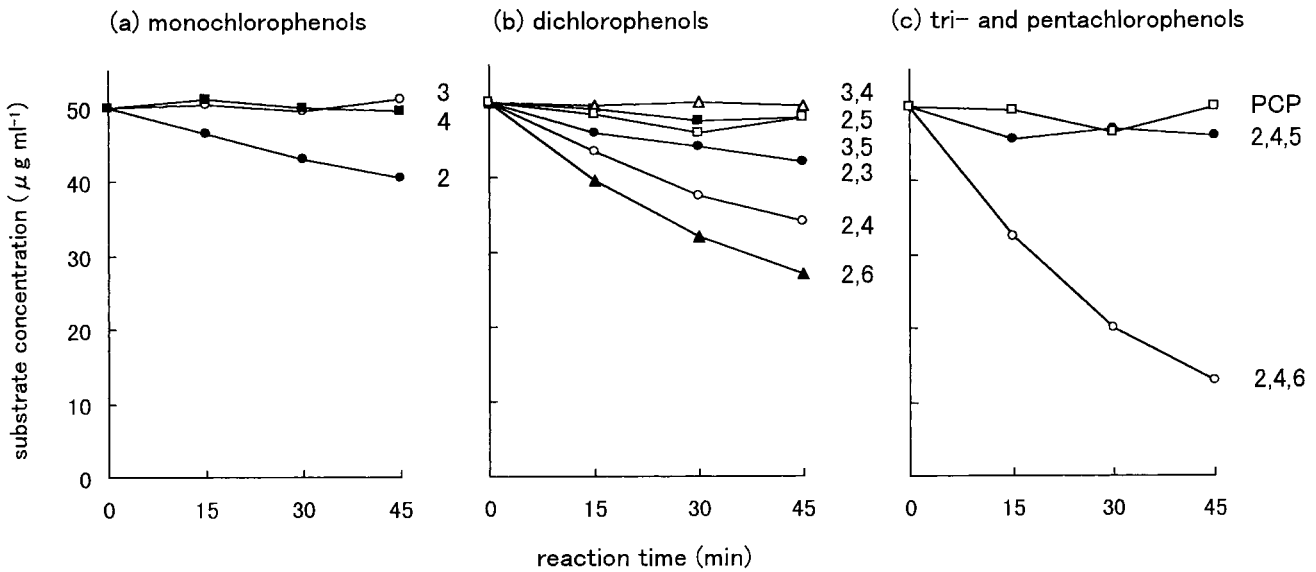

Fig. 1. Transformations of (a) monochlorophenols, (b) dichlorophenols and (c) trichloro- and pentachlorophenols by *C. versicolor* laccase. Numbers indicate the positions of chlorine substitutions. Results are means of two replicated experiments.

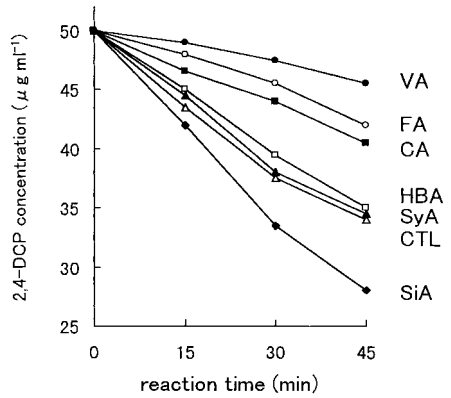

Fig. 2. Effect of phenolic acids on transformations of 2,4-DCP by *C. versicolor* laccase. Transformations of 2,4-DCP by laccase in the presence of coumaric acid (CA, -■-), ferulic acid (FA, -○-), sinapinic acid (SiA, -◆-), 4-hydroxybenzoic acid (HBA, -□-), vanillic acid (VA, -●-) and syringic acid (SyA, -▲-). Transformations of 2,4-DCP in the absence of phenolic acids (-△-). Results are means of two replicated experiments.

tion time. VA and CA were less reactive than SiA and FA and 39% and 46% of the initial amounts remained after 15 min of reaction, respectively. SyA (84% remaining) and HBA (97% remaining) were the least reactive substrates among the phenolic acids used in this study. In order to determine the relative reactivity for the phenolic acids, concentration of laccase was reduced to 6.25–1.56% with distilled water in the reaction for SiA and FA. The relative reactivity of the phenolic acids by the laccase was as follows: SiA (75) > FA (25) > VA (8) > CA (7) > SyA (1) > HBA (< 1), which means for example that SiA was 75 times more reactive than SyA.

Effects of CA, FA and SiA on the transformation of chlorophenols are summarized in Table 1. CA and FA inhibited the transformation of 2-CP, 2,3-, 2,4- and 2,6-DCP and 2,4,6-TCP, whereas SiA enhanced the transformation of 4-CP, 2,4-DCP, 2,4,5- and 2,4,6-TCP.

The presence of BSA in the reaction medium enhanced the transformation of 2,4-DCP by the laccase and reduced the inhibiting effect of FA. At 200 times concentration of BSA to laccase, 2,4-DCP was similarly transformed with and without FA.

3.3. Effect of reaction intermediates of SiA and FA by laccase on transformation of 2,4-DCP

Disappearance of 2,4-DCP, added during the course of the transformation of SiA, depended on the concentration and the reaction time with SiA (Fig. 3). The most enhanced transformation of 2,4-DCP was observed when 2,4-DCP was added to the enzyme solution after 0.5–1 h of incubation of SiA (100 mg l^{-1}). At 200, 400 and 800 mg l^{-1} of SiA, the most enhanced transformation of 2,4-DCP was observed after 0.5–1, 1 and 3 h of exposure, respectively. On the other hand, inhibition of the transformation of 2,4-DCP was observed when 2,4-DCP was added at early stage of the reaction with SiA. The higher the concentrations of SiA, the longer the period of reaction was observed.

In contrast to the effect of SiA, FA inhibited the transformation of 2,4-DCP whenever 2,4-DCP was added during the course of the transformation of FA (Fig. 4). Greater inhibition was observed at higher concentration of FA and 2,4-DCP was hardly degraded at the early stage of the reaction of FA at 800 mg l^{-1}.

Table 1
Substrate specificity of laccase from *C. versicolor* for chlorophenols and effect of coexisting phenolic acids on the degradation of chlorophenols[a]

Chlorophenols	Substrate remaining (%)[b]			
	Control	+CA	+FA	+SiA
2-CP	81	87	91	80
3-CP	102	101	99	95
4-CP	99	104	93	85
2,3-DCP	84	94	94	91
2,4-DCP	68	81	84	56
2,5-DCP	96	91	93	91
2,6-DCP	54	74	71	56
3,4-DCP	99	103	96	97
3,5-DCP	96	102	96	99
2,4,5-TCP	92	86	93	69
2,4,6-TCP	26	52	54	5
PCP	100	99	103	98
2,4-DCP + BSA (×10)	56	ND[c]	75	ND
2,4-DCP + BSA (×20)	56	ND	73	ND
2,4-DCP + BSA (×200)	52	ND	61	ND
2,4-DCP (×3 laccase)	28	ND	38	ND

[a] Results are means of two replicated experiments. (CA: *p*-coumaric acid, FA: ferulic acid, SiA: sinapinic acid, CP: chlorophenol, DCP: dichlorophenol, TCP: trichlorophenol, PCP: pentachlorophenol, BSA: bovine serum albumin.).

[b] % of the initial dose after 45 min reaction with laccase at 1.8 units ml^{-1}.

[c] Not determined.

4. Discussion

As extracellular laccases are often composed of isozymes, purification using ammonium sulfate fractionation and chromatographic techniques such as ion-exchange and molecular-size gel filtration is required for their study (Morohoshi and Haraguchi, 1987; Coll et al., 1993; Perez et al., 1996). In the experiments described here, only ammonium sulfate precipitation was necessary to obtain a single band laccase identified by activity-staining native PAGE, therefore, this

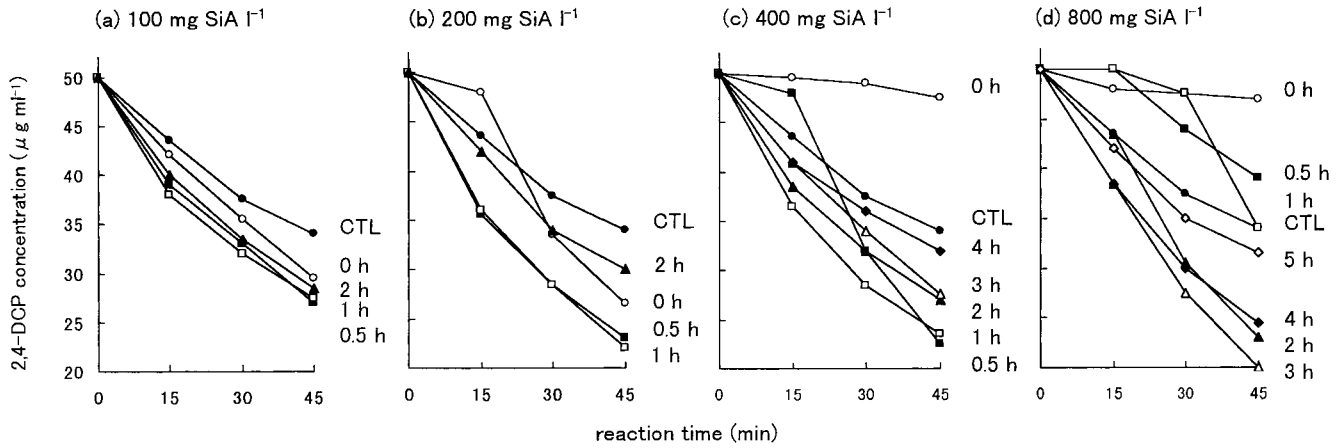

Fig. 3. Effects of prior exposure to sinapinic acid (SiA) on transformations of 2,4-DCP by *C. versicolor* laccase. 2,4-DCP added after prior reaction of SiA with laccase for 0 h (-○-), 0.5 h (-■-), 1 h (-□-), 2 h (-▲-), 3 h (-△-), 4 h (-◆-) and 5 h (-◇-) and continuously reacted for 45 min. Transformation of 2,4-DCP without SiA (-●-). Concentrations of SiA were (a) 100 mg l^{-1}, (b) 200 mg l^{-1}, (c) 400 mg l^{-1} and (d) 800 mg l^{-1}. Results are means of two replicated experiments.

enzyme solution preparation was used without further purification. There was no loss of laccase activity as a result.

The optimum pH of the purified laccase from *C. versicolor* used in this study was 5.0, comparable with the value of 4–6 reported by Leonowicz et al. (1984); Hoff et al. (1985); Dec and Bollag (1990), but different from the laccase of *R. praticola* (pH 7.0–7.5) used by Leonowicz et al. (1984); Hoff et al. (1985); Dec and Bollag (1990) and *Polyporus pinsitus* (pH 3.0–4.0) (Kim et al., 1997). Leonowicz et al. (1984) reported that routes of reactions of VA and SyA by laccase from *R. praticola* and *T. versicolor* were pH-dependent. Though transformation products at pHs other than at the optimum pH (5.0) might be different, these were not examined.

As previous investigators used different laccases and only a few chlorophenol substrates (Shuttleworth and Bollag, 1986; Dec and Bollag, 1990), it is difficult to make comparisons. The order of the reactivity by laccases from *R. praticola* and *T. versicolor* reported for the previous studies were 2,4-DCP > 2-CP, 4-CP > 3-CP, 2,6-DCP, whereas the order in our study was 2,4,6-TCP > 2,6-DCP > 2,4-DCP > 2-CP > 2,3-DCP > 3-CP, 4-CP. Phenols with chlorine atoms at *ortho* and *para* positions were more susceptible to the laccase and increased reactivity was recorded as the number of the chlorine atoms increased. In contrast, the chlorine

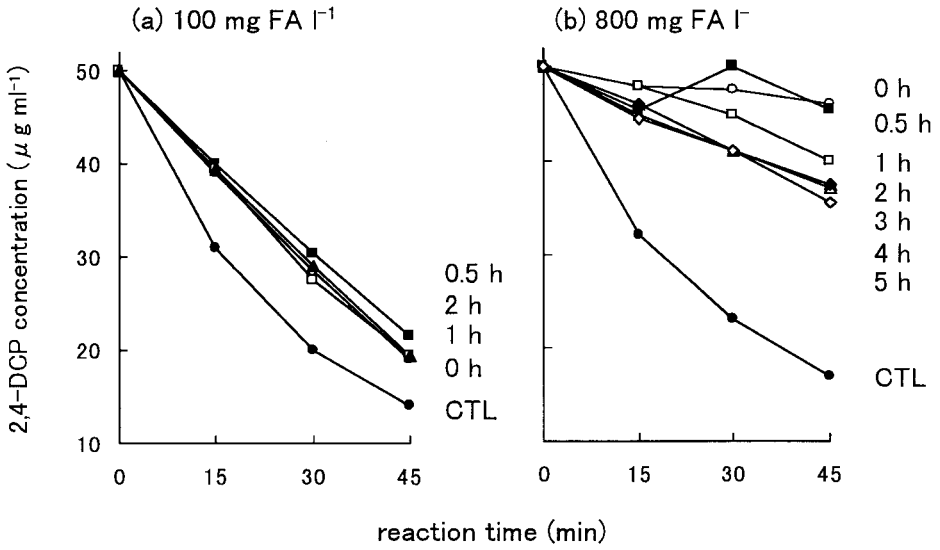

Fig. 4. Effects of prior exposure to ferulic acid (FA) on transformations of 2,4-DCP by *C. versicolor* laccase. 2,4-DCP added after prior reaction of FA with laccase for 0 h (-○-), 0.5 h (-■-), 1 h (-□-), 2 h (-▲-), 3 h (-△-), 4 h (-◆-) and 5 h (-◇-), 2,4-DCP and continuously incubated for 45 min. Transformation of 2,4-DCP without FA (-●-). Concentrations of FA were (a) 100 mg l^{-1} and (b) 800 mg l^{-1}. Results are means of two replicated experiments.

atoms at *meta* position decreased reactivity (Fig. 1 and Table 1). The increase in the number of chlorine atoms at the aromatic ring is expected to reduce the rate of transformation due to their electron-withdrawing property. Therefore, it is suggested that the reactivity is due to the substrate specificity of the laccase rather than the chemical property of the substrates.

The laccase showed higher reactivity to cinnamic acid-type phenolic acids (SiA, FA and CA) than to benzoic acid-type phenolic acids (SyA, VA and HBA), but there was no clear relationship between the reactivity and the number of methoxyl groups. SiA which was the most reactive phenolic acid enhanced the transformation of 2,4-DCP, whereas moderate reactive FA, CA and VA inhibited the reaction. HBA and SyA which were recalcitrant substrates for the laccase showed no effect on the transformation of 2,4-DCP. Therefore, the effect of the phenolic acids on the transformation of 2,4-DCP seemed to depend on the reactivity of the phenolic acids rather than their chemical structures. However, the transformation of 2,4-DCP was also inhibited by FA even at the same reaction rate as that of SiA by treating FA with 3-fold higher units of the laccase (Table 1). It seems that the effect depends on the reactivity between 2,4-DCP and the phenoxy radicals produced from SiA and FA rather than the reaction rate of the phenolic acids per se. Concerning the effect of phenolic acids on the transformation of chlorophenols, Shuttleworth and Bollag (1986) reported that the transformation of 2,4-DCP was enhanced in the presence of FA, SyA or VA by the laccase of *R. praticola*. These opposite effects might be caused by some properties of the enzymes because both laccases are expected to produce the same phenoxy radicals through their reactions. Davin et al. (1997) reported the presence of a guiding protein which leads a stereospecific coupling reaction of phenoxy radicals of coniferylalcohol in lignin biosynthesis. Fungal laccases might have some kind of properties leading phenoxy radical intermediates to specific coupling reactions.

Although CA, FA and SiA have similar chemical structures, their effects on the transformation of chlorophenols were different (Table 1). For further examination, we selected FA and SiA, which were highly reactive with the laccase and showed the opposite effect on the transformation of chlorophenols. When 2,4-DCP was added to the SiA solution after 0.5–1 h of incubation with the laccase, the most enhanced transformation of 2,4-DCP was observed (Fig. 3a). As SiA disappeared within 15 min in the reaction solution, reaction intermediates of SiA likely enhanced the transformation of 2,4-DCP. At higher concentrations of SiA, the rate of transformation of SiA decreased and the most enhanced transformation of 2,4-DCP was observed after a longer incubation of SiA (Fig.3b–d), supporting the presence of reactive intermediates of SiA with 2,4-DCP. As SiA is a more reactive substrate with the laccase than 2,4-DCP, competitive inhibition of the transformation of 2,4-DCP by SiA was observed during the early stage of SiA incubation (Fig.3b–d) when SiA still remained in the reaction solution (data not shown).

The presence of FA caused an inhibitory effect on the transformation of 2,4-DCP and the inhibition was not reversed even when 2,4-DCP was added after 2 and 5 h of reaction of FA with the laccase at 100 and 800 mg l^{-1}, respectively (Fig. 4). This irreversible inhibition seems to be inactivation of the laccase by FA

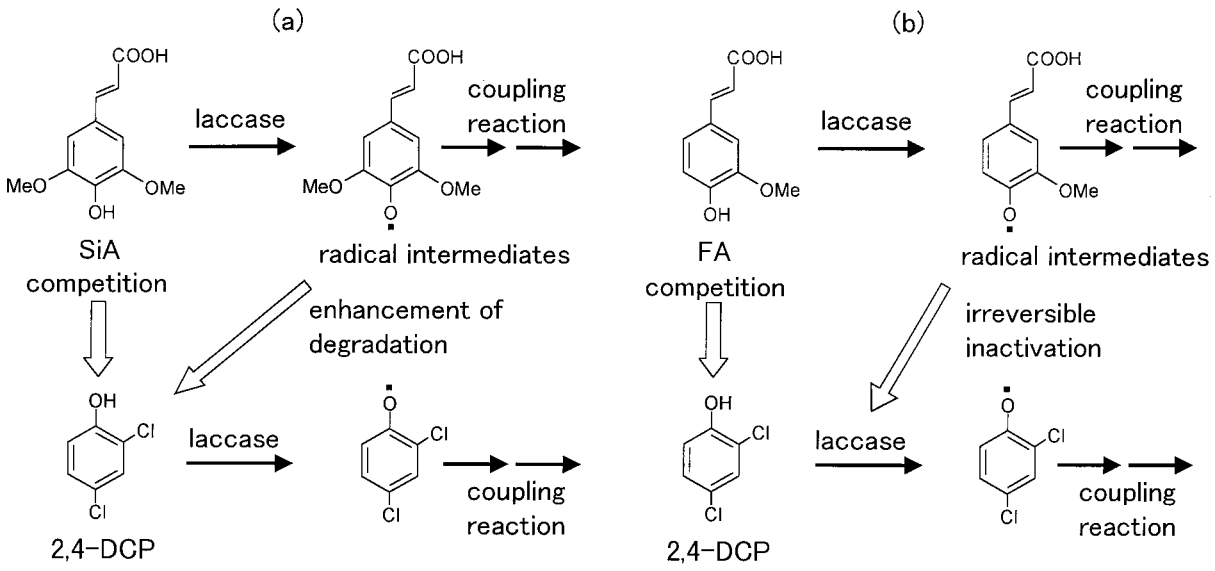

Fig. 5. Proposed reaction schemes of laccase-mediated transformation of 2,4-DCP with (a) sinapinic acid and (b) ferulic acid.

reaction intermediates such as FA phenoxy radicals. Addition of BSA to the reaction solution reduced the inhibition, suggesting that excess amounts of the added protein act as a scavenger to the reactive intermediates of FA. Competitive inhibition of the transformation of 2,4-DCP by FA was also observed during the early stage of FA incubation at 800 mg l^{-1}.

The transformation of 2,4-DCP by laccase was affected in different ways by FA and SiA, although they have similar chemical structures, indicating the fate of chlorinated phenols in the natural environment seems to be complex. Estimated reaction schemes are proposed in Fig. 5 based on the results we obtained. The different effects of FA and SiA seem to be due to different reactivities of their transformation intermediates produced by the laccase. Further studies to identify the reactive intermediates and their reaction products could elucidate the complex phenomena of laccase-mediated transformation of phenolic compounds.

References

Bollag, J.-M., 1992a. Decontaminating soil with enzymes. Environmental Science and Technology 26, 1876–1881.

Bollag, J.-M., 1992b. Enzymes catalyzing oxidative coupling reactions of pollutants. In: Sigel, H., Sigel, A. (Eds.), Metal Ions in Biological Systems, vol. 28. Marcel Dekker, New York, pp. 205–217.

Bollag, J.-M., Loll, M.J., 1983. Incorporation of xenobiotics into soil humus. Experientia 39, 1221–1231.

Bollag, J.-M., Myers, C., 1992. Detoxification of aquatic and terrestrial sites through binding of pollutants to humic substances. The Science of the Total Environment 117/118, 357–366.

Bollag, J.-M., Shuttleworth, K.L., Anderson, D.H., 1988. Laccase-mediated detoxification of phenolic compounds. Applied and Environmental Microbiology 54, 3086–3091.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72, 248–254.

Coll, P.M., Fernandez-Abalos, J.M., Villanueva, J.R., Santamaria, R., Perez, P., 1993. Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). Applied and Environmental Microbiology 59, 2607–2613.

Davin, L.B., Wang, H.B., Crowell, A.L., Bedgar, D.L., Martin, D.M., Sarkanen, S., Lewis, N.G., 1997. Stereoselective bimolecular phenoxy radical coupling by an auxiliary (dirigent) protein without an active center. Science 275, 362–366.

Dec, J., Bollag, J.-M., 1990. Detoxification of substituted phenols by oxidoreductive enzymes through polymerization reactions. Archives of Environmental Contamination and Toxicology 19, 543–550.

Focht, D.D., 1993. Microbial degradation of chlorinated biphenyls. In: Bollag, J.-M., Stotzky, G. (Eds.), Soil Biochemistry, vol. 8. Marcel Dekker, New York, pp. 341–407.

Harwood, C.S., Parales, R.E., 1996. The β-ketoadipate pathway and the biology of self-identity. Annual Review of Microbiology 50, 553–590.

Hoff, T., Liu, S.-Y., Bollag, J.-M., 1985. Transformation of halogen-, alkyl- and alkoxy-substituted anilines by a laccase of *Trametes versicolor*. Applied and Environmental Microbiology 49, 1040–1045.

Kim, J.E., Fernandes, E., Bollag, J.-M., 1997. Enzymatic coupling of the herbicide bentazon with humus monomers and characterization of reaction products. Environmental Science and Technology 31, 2392–2398.

Leonowicz, A., Edgehill, R.U., Bollag, J.-M., 1984. The effect of pH on the transformation of syringic and vanillic acids by the laccases of *Rhizoctonia praticola* and *Trametes versicolor*. Archives of Microbiology 137, 89–96.

Morohoshi, N., Haraguchi, T., 1987. Degradation of lignin by the extracellular enzymes of *Coriolus versicolor*. III. Degradation of various substrates by the phenol oxidase fraction. Mokuzai Gakkaishi 33, 143–150.

Perez, J., Martinez, J., de la Rubia, T., 1996. Purification and partial characterization of a laccase from the white rot fungus *Phanerochaete flavido-alba*. Applied and Environmental Microbiology 62, 4263–4267.

Shindo, H., Ohta, S., Kuwatsuka, S., 1978. Behavior of phenolic substances in the decaying precess of plants. IX. Distribution of phenolic acids in soils of paddy fields and forests. Soil Science and Plant Nutrition 24, 233–243.

Shuttleworth, K.L., Bollag, J.-M., 1986. Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. Enzyme and Microbial Technology 8, 171–177.

Stevenson, F.J., 1982. Humus Chemistry: Genesis, Composition, Reactions. Wiley, New York.

Whitehead, D.C., 1964. Identification of *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids in soils. Nature 202, 417–418.