CHAPTER III

ROLE OF HUMIC SUBSTANCES ON DEGRADATION OF PENTACHLOROPHENOL BY *TRAMETES VERSICOLOR* LACCASE

3.1 Introduction

Pentachlorophenol (PCP) has been commercially produced for many decades and has been used as bactericide, insecticide, herbicide, and wood preservative (Keith and Telliard, 1979; Crosby, 1981; Wild et al., 1992). It is obvious that its contamination has become a major issue in both human health and environmental protection. Although recently the production and utilization of PCP have decreased and even banned in some countries, it still causes an environmental problem at many locations due to abundant usage in the past and their environmental transportation. For example, chlorophenols have been detected in various environmental samples such as soil, sediment, surface water and wastewater (Choi and Aomine, 1974; Delaune et al., 1983; Butte et al., 1985; Kitunen et al., 1987). Therefore, the knowledge of mechanisms involving the degradation of this aromatic pollutant is critical.

Bioremediation by taking advantage of microorganisms and their enzymes is widespread nowadays. Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) produced as an extracellular enzyme by various white rot fungi belongs to a group of polyphenol oxidases. It catalyzes the reduction of oxygen to water accompanied by the oxidation of a substrate, typically a *p*-dihydroxy phenol or another phenolic compound (Bollag et al., 1997). It was reported that laccase from *Trametes versicolor*

is capable of oxidizing PCP with a high K_m value (Ullah et al., 2000). They stated that the reaction of laccase with PCP must be solely with the phenolic group since they found no detectable free chlorine. This result was confirmed by the work of Roy-Arcand and Archibald (1991). The primary product of PCP reaction with laccase was high molecular weight polymer, which appeared stable under aqueous conditions both in alkaline and acid. Also, trace quantities of highly toxic tetrachlorobenzoquinone were found. Nevertheless, in the actual remediation, the degrading activities of these extracellular enzymes in the environment may be altered due to the interactions with humic substances (HS).

HS including humic acid (HA) and fulvic acid (FA) are up to 70% of soil organic carbon and up to 90% of dissolved organic carbon (Klavins and Serzane, 2000). Even though HS structure is exceeding complex, the major functional groups in HS are reviewed as oxygen containing and include carboxyls, alcoholic and phenolic hydroxyls, carbonyls, and methoxyls (Essington, 2004). Due to the similar functional group of HS and laccase substrate, HS can compete for the oxidation and thus competitively inhibit the transformation of other compounds (Itoh et al., 2000). For example, Zavarzina et al. (2004) estimated inhibition constants for humic acids towards *Panus tigrinus* laccase. The K_i ranged from 0.003 μg/mL for HA from peat soils to 0.025 μg/mL for HA from chernozems. Nevertheless, HS could enhance the degradation rate of aromatic pollutants since it could interact with the pollutant and allowed them to be incorporated into fungal biomass (Nanny et al., 1996). Enzymatic degradation of toxic compounds in the real sites may be far different from in the laboratory experiment due to the types and quantities of HS present.

It is generally presumed that laccase is able to react with soil humic substances (Yavmetdinov et al., 2003). This is supported by the fact that HA can induce laccase

activity and mRNA expression (Scheel et al., 2000). However, the interaction of laccase with HS probably leads both to depolymerization of HS and their synthesis from monomeric precursors. These two processes can be influenced by the nature of the HS (Zavarzina et al., 2004). For example, Fakoussa and Frost (1999) found the decolorization and decrease of molecular weight of HA, accompanied by the formation of FA during the growth of *T. versicolor* cultures producing mainly laccase. Meanwhile, Katase and Bollag (1991) documented the formation of HA in vitro using the same fungus culture. Humic substance can also both stimulate and inhibit laccase activity depending on their origin sources as reported by Clause and Filip (1990).

Another possible role of HS is sorption of chemical including PCP. It has been stated in connection with the structural characterization of HS, that PCP can be trapped and closely intercalated in the void of HS either without or with intermolecular hydrogen bonding between the hydrogen of the protonated carboxylic group (COOH) of HS and the phenolic oxygen of PCP (Schulten, 1996). Several partition coefficients have been determined for PCP with different natural solids and organic solutes. For example, Peuravuori et al. (2001) determined sorption of PCP on dissolved aquatic humic matter isolated from a brown-water lake for very wide concentration range at 60 ng PCP/L – 1 mg PCP/L. They proposed normalized sorption coefficients of 3.19 at pH 5.5. Their results also verify that the conventional HA and FA from the lakes account for the main part of the PCP sorption. Paolis and Kukkonen (1997) found the higher value for log K_{oc} for HA of 3.90 than for FA of 3.00 in PCP binding affinity. Both HA and FA were extracted from water and sediment samples of the River Arno and the Tyrrenhian Sea in Italy. They also suggested that binding efficiency was pH dependent. Only the unionized form of PCP can interact with the humic materials. For example, at pH 5.0, PCP had higher binding

coefficients than at pH 8.0. However, log K_{oc} values of PCP are very large, even a value of 4.5 has been found by Schellenberg (1984). As stated by Peuravuori et al. (2001), these different values were depending on analytical methods, algorithms applied, the nature of humic matters, the acidity and ionic strengths of the solution, resulting in various mechanisms to operate simultaneously in the sorption process.

To understand the mechanisms involving soil decontamination, this research was focus on the role of HS to alter enzymatic degradation of PCP. How HS plays a role in the system was the key question in the whole research. Three general hypotheses for HS' roles were

1. HS disables (denatures/deactivates) laccase by changing the quaternary or tertiary structure of the enzyme protein (Muller-Wegerner, 1988). To test whether HS disables enzyme, laccase activity was measured in the present of HS.

2. HS could compete with PCP for enzyme because it contained numerous functional groups that were known to be oxidized by oxidoreductive enzymes (Kirk et al., 1992). Therefore, HS would disturb the equilibrium of the PCP-laccase reaction and change their kinetic parameters.

3. HS is not a competitive substrate but protects PCP by adsorption (solution-phase interactions) (Berry and Boyd, 1985; Calderbank, 1989; Bollag, 1992; Dec and Bollag, 1997 Peuravuori, 2001). Dialysis-binding experiment was therefore used to test the association of PCP and HS.

Four types of HS including Aldrich humic acid (AHA), Leonardite humic acid (LHA), Suwannee River fulvic acid (SRFA), and Waskish peat fulvic acid (WFA) were used in the experiment. The reasons for using those HS as model are (1) they are available worldwide (2) all materials are carefully prepared and homogenized and (3) they are well characterized. These HS are also difference in % aromaticity and

molecular weight; which affecting PCP enzymatic degradation rate and sorption phenomena. HS could be one of the important factors that make us misestimate the transformation rates of pollutants in various soil types. We expected that this novel information would enhance the potential for designing and improving aromatic pollutants bioremediation strategies.

3.2 Materials and Methods

3.2.1 Chemicals

Pentachlorophenol (PCP) was purchased from Aldrich Company, USA. Stock solution containing 1 g/L PCP was prepared in hexane to obtain the absolute dissolution. Purified laccase from *Trametes versicolor* (Fluka Company, USA) was used in the experiment and prepared freshly for each analysis.

Commercial HS supplied by Fluka company and International Humic Substances Society (IHSS) were used in the research to promote the comparisons with other HS studies. Chemical and physical properties of experimental HS were shown in Table 3.1. AHA and LHA were dissolved in 50 μL of 0.5 M NaOH solution and adjusted their volume of 10 mL by distilled water. Final pH for the stock AHA and LHA were 8.27 and 8.61, respectively. SRFA and WFA were dissolved in distilled water. The stock fulvic acids had final pH of 3.19 and 3.29 for SRFA and WFA.

Table 3.1 Chemical and physical properties of particular humic substances

Humic substances	Sources	% Aromaticity	% Carboxyl	% C	% O	% N	MW (Dalton)
Aldrich humic acid (AHA)	Lignite, Aldrich company	41.0[a]	19[a]	65.3[d]	25.1[d]	-	4,731[e]
Leonardite humic acid (LHA)	Gascoyne Mine, North Dakota, USA	58.0[b]	7.5[b]	63.8[b]	31.3[b]	0.8[b]	18,700[f]
Suwannee River fulvic acid (SRFA)	Suwannee River, South Georgia, USA	24.0[c]	12.2[b]	53.0[b]	43.9[b]	0.5[b]	2,519[e]
Waskish peat fulvic acid (WFA)	Pine Island Bog, Minnesota, USA	36.0[b]	-	53.6[b]	38.5[b]	0.3[b]	11,950[g]

a: Ashley, 1996; b: International Humic Substances Society (IHSS); c: Thorn et al., 1989; d: Malcolm and MacCarthy, 1986; e: O'Loughlin et al., 2000; f: Beckett et al., 1987; g: Perminova et al., 2003.

3.2.2 Enzyme activities

Enzyme reaction contained 20 mg/L HS and 15 units laccase in 3 mL 200 mM sodium acetate buffer, pH 5.0 and incubated at 28°C, 200 rpm. The pH 5.0 was selected because it provides the highest activity of laccase enzyme (Ullah et al., 2000). The samples were sacrificed every 24 h for 96 h. Then, laccase activity was measured by adding 100 mM catechol to the sample and observed absorbance change at 440 nm for 1 minute by UV-vis spectrophotometer (Specord 40, Analytik Jena AG). One unit of laccase activity is defined as that which caused a change in absorbance of 1.0 OD/min/mL (Ullah et al., 2000). The experiments were performed in triplication.

3.2.3 PCP-HS sorption and desorption studies

PCP-HS binding experiment using dialysis tubing was modified from O'Loughlin et al. (2000). Dialysis tubing was prepared from Spectra/Por® Biotech Cellulose Ester (CE) dialysis membranes MWCO 500 (Spectrum Laboratories Inc.) and filled with 1 mL of HS solution (20 mg/L) or buffer (for control). The tubes were placed in a 500 mL-beaker containing 200 mL of 200 mM sodium acetate buffer pH 5.0 and a given PCP. The concentrations of PCP were 7.5, 10, 12.5, and 15 mg/L, which ranged from 0.1 – 0.2 times of PCP solubility. To avoid photolysis, beakers were wrapped with aluminum foil. Then, the beakers were wrapped with wrapping film (polyvinylchloride cling film, M Wrap, MMP Packaging Group Co., Ltd) and placed on magnetic stirrer. At equilibrium, aliquots of solution from inside and outside the tubing were removed for PCP analysis.

To study the kinetics of PCP-HS binding, a series of tubes were prepared as described earlier, with each containing 20 mg/L of LHA and 15 mg/L of PCP. The reason to use LHA to study the equilibrium time is LHA has the highest percent aromaticity and molecular weight. These characteristics are believed to be susceptible to binding constants of aromatic pollutions. Sample solutions in the beakers were collected for PCP analysis at regular intervals over a 3-day period.

Desorption experiments were also conducted by equilibrium dialysis method. Dialysis tubing contained 3 mL of 20 mg/L of each HS was placed in 500 mL beaker filled with 15 mg/L of PCP in 200 mL of acetate buffer pH 5.0. The dialysis tubing containing equilibrated PCP-HS solutions were transferred to 17 mL of the fresh acetate buffer in 22 mL test tubes. The samples were shaken at 200 rpm on the shaker. Then, free PCP in the tubes was extracted and analyzed by GC-FID every 24 h for 96 h.

3.2.4 Enzyme kinetics

3.2.4.1 PCP and laccase

K_m and V_{max} for PCP with and without HS were investigated in a vial containing 0.4 mL reaction mixture. Firstly, 30 mg/L of PCP was used as a substrate for 20 units/mL laccase enzyme. 5, 10, 15, and 20 mg/L of each HS were added as competitive inhibitors. PCP concentrations were analyzed every hour for 4 h. Then HS concentrations of 10, 15, 20 mg/L were selected because it provided the decrease in PCP with time linearly.

To determine K_m and V_{max} for PCP, laccase at 20 units/mL was incubated with 10, 20, 25, 30, and 40 mg/L of PCP and the given concentration of each HS. PCP concentrations were measured every hour for 4 h, which was a period of time that the velocity was linear.

3.2.4.2 HS and laccase

To ensure that HS can be substrates for enzyme, K_m and V_{max} for HS were studied similar to the above experiment. 10, 15, 20, 25, 30, and 40 mg/L of HS were incubated with 15 units/mL of laccase in 200 mM sodium acetate buffer (pH 5.0). They were incubated at 28°C and shaken continuously at 200 rpm. Absorbance at 465 nm was measured by UV-vis spectrophotometer every 24 h for 96 h. The absorbance at 465 nm is useful in HS characterization (Zavarzina et al., 2002, 2004). Moreover, laccase did not absorb at 465 nm. This wavelength could be used to observe the changes in HS. The samples were performed in triplication.

3.2.5 PCP determination

PCP concentrations were monitored from the samples sacrificing at intervals throughout the time for reaction. At each measurement, hexane was added to the samples for extraction and shaken at 200 rpm for 1 h. The solvent phase was then

removed and mixed with sodium sulfate to dewater the samples. The sample was aliquoted for GC-FID analysis. The PCP concentrations were determined by gas chromatography (6890N, Agilent Technologies) equipped with flame ionization detector. The following instrument parameters were used: column, HP 5 MS (30 m x 0.25 mm id x 0.25 μm); carrier, helium 33 cm/sec constant flow; oven, 35°C for 5 min 35 - 220°C at 8°C/min; injector, 5 μL splitless 250°C, retention time, 13.5 min. Percent recovery by this procedure was 95 - 98 %.

3.3 Results and Discussion

3.3.1 PCP degradation rate

Laccase from *Trametes versicolor* has previously known to remove PCP from solution at pH 5, depending on initial PCP concentration and amount of laccase (Ullah et al., 2000). In this research, PCP was enzymatically degraded by laccase both in the presence and absence of HS. PCP 10, 20, 25, 30, 40 mg/L were mixed with 20 units/mL of laccase. PCP concentrations were analyzed at initial time reaction of 0, 1, 2, 3, 4 h. Each HS 10, 15, 20 mg/L were added as inhibitors for the reaction. Initial reaction of PCP enzymatically degraded by laccase enzyme with and without HS was demonstrated in supplemental information. Since the degradation rate of PCP was fitted to straight line, pseudo first order reaction was assumed in the system. The pseudo first order reaction can be represented as:

$$dC/dt = k'[C]$$

where k′ is equal to the true rate constant, k, multiplied by HS concentration (mg/L) and [C] is the PCP concentration (M). The k′ values are concluded in Table 3.2. We found that as HS concentrations increased, k′ was decreased in non-linear fashions (Figure 3.1). This demonstrates that the presence of HS decreased PCP degradation

rate in the system of interest. The non-linear behavior also suggested that HS might interact with PCP and/or HS might interact with laccase enzyme.

Table 3.2 k′ degradation rate constants (hr^{-1})

HS concentration	0 mg/L	10 mg/L	15 mg/L	20 mg/L
Sample				
PCP	2.23×10^{-1}	na	na	na
PCP + AHA	na	1.62×10^{-1}	1.03×10^{-1}	7.75×10^{-2}
PCP + LHA	na	4.77×10^{-2}	4.15×10^{-2}	4.04×10^{-2}
PCP + SRFA	na	1.35×10^{-1}	6.87×10^{-2}	5.79×10^{-2}
PCP + WFA	na	1.09×10^{-1}	8.29×10^{-2}	7.48×10^{-2}

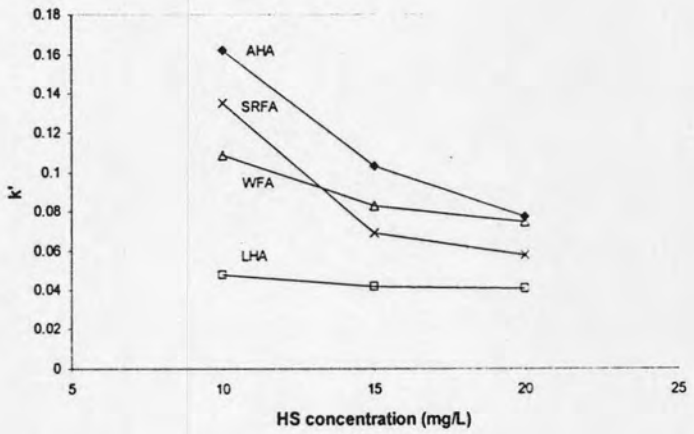

Figure 3.1 Relationship between k′ and HS concentration (mg/L). Symbols represent HS, ♦: AHA, □: LHA, Δ: SRFA, ×: WFA

3.3.2 Enzyme activity

Laccase was fully active throughout the analysis for all HS with concentrations ranging from 0 – 40 mg/L (Figure 3.2) as analyzed by catechol assay. Therefore, the hypothesis stating HS can disable enzyme was invalidated in our study. This is probably because the high amount of laccase used here which could overcome the deactivation effects of HS.

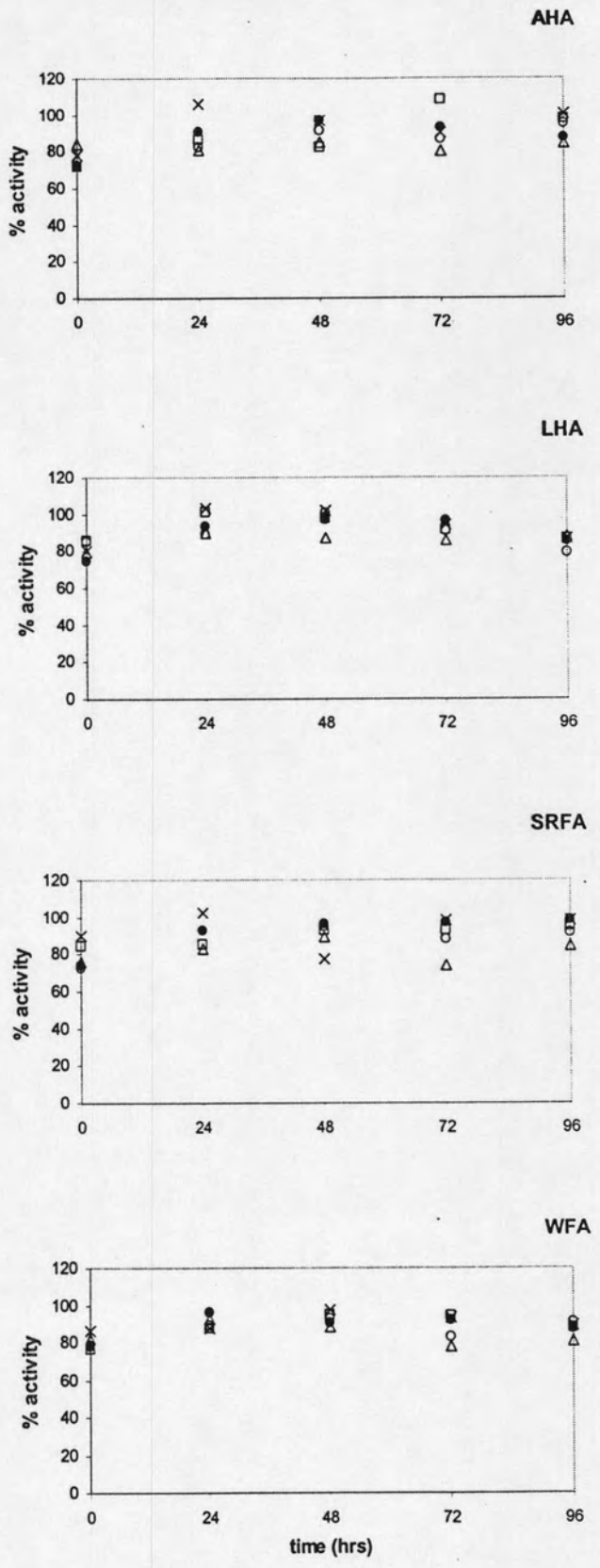

Figure 3.2 Percent enzyme activity for each HS. Activity of laccase in oxidation of catechol without HS is 100%. Symbols represent HS concentration, □: 10 mg/L, Δ: 20 mg/L, ×: 25 mg/L, o: 30 mg/L, •: 40 mg/L

3.3.3 Enzyme kinetics for PCP

K_m value of PCP with HS present was about 1.5 - 6 times higher than in the absence of HS. As HS concentration increased, K_m of PCP was increased. Values of K_m and V_{max} with and without HS addition were shown in Table 3.3. Strong linear relationship of K_m and HS concentration was found (Figure 3.3a). This implied the inhibition of laccase by HS. HS could act as competitive substrate for PCP degraded by laccase enzyme as well as sequester PCP from enzymatic degradation. Since V_{max} remained unchanged upon HS concentration (Figure 3.3b) implies the competitive type of HS for laccase enzyme. Functional groups of complex HS were known to be substrates for oxidoreductive enzyme, including laccase (Kirk et al., 1992).

Table 3.3 K_m and V_{max} of PCP and of when HS were added as inhibitors

HS	[HS] (ppm)	K_m (mM)	V_{max} (μM/min)
Control (PCP)	-	0.21 ± 0.03	1.16 ± 0.19
AHA	10	0.30 ± 0.02	0.86 ± 0.27
	15	0.63 ± 0.17	1.16 ± 0.13
	20	0.82 ± 0.12	1.05 ± 0.16
LHA	10	0.99 ± .04	1.03 ± 0.04
	15	1.04 ± 0.19	0.99 ± 0.15
	20	1.30 ± 0.05	1.14 ± 0.04
SRFA	10	0.68 ± 0.23	1.59 ± 0.23
	15	0.72 ± 0.06	0.86 ± 0.15
	20	1.17 ± 0.06	1.18 ± 0.11
WFA	10	0.51 ± 0.03	1.21 ± 0.07
	15	0.59 ± 0.09	0.90 ± 0.09
	20	0.77 ± 0.11	1.23 ± 0.11

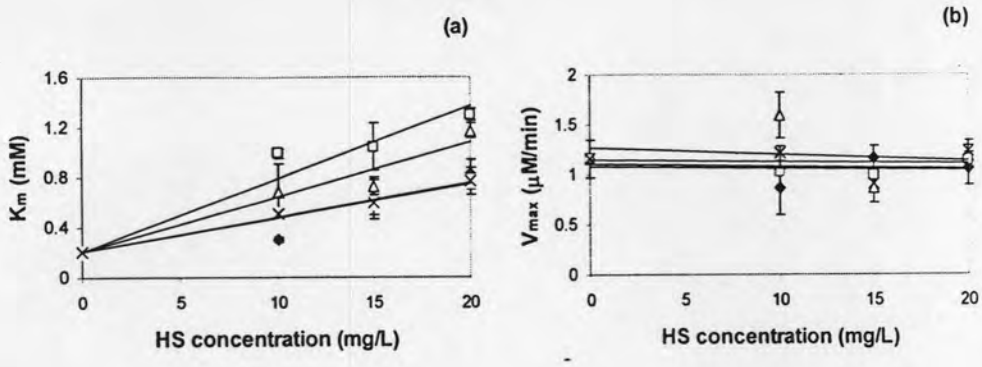

Figure 3.3 Effect of HS concentration; ♦: AHA, □: LHA, Δ: SRFA, ×: WFA, on (a) K_m and (b) V_{max} values of purified laccase from *Trametes versicolor* on PCP degradation. r^2 for AHA, LHA, SRFA, and WFA of K_m were 0.85, 0.93, 0.94, and 0.99, respectively and of V_{max} were 0.03, 0.09, 0.04, and 0.01, respectively

3.3.4 Inhibitory effect of HS on Laccase

K_i is an inhibitor binding constant. It can be calculated from

$$K_i = I / [(K_m' / K_m) - 1]$$

where, K_m' represents K_m obtained from the data set when competitive inhibitor is added. I is the concentration of competitive inhibitor. A stronger inhibitor has a lower K_i. K_i of each HS is shown in Table 3.4. Among HS group, K_i for LHA < AHA < SRFA < WFA was found. The inhibitory effect of HS might be due to their ability to bind with PCP as well as to act as analogous substrate for laccase. The inhibitory effect of HS was the competitive type because as HS concentration increased, K_m increased and V_{max} remained stable. The inhibitory effect of HS was in agreement with the results of Gianfreda and Bollag (1994) and Zavarzina et al. (2004). They reported a linear relationship between the dissolved organic matter content and its inhibitory effect on activities of laccase. The competitive inhibitor properties were

shown for other enzymes incubated with HS such as lignin peroxidase (Wondrack et al., 1989), pronase, trypsin and carboxypeptidase (Ladd and Butler, 1971).

Table 3.4 K_i for each HS

Humic substances	K_i (μg/mL)
LHA	3.63
AHA	5.59
SRFA	5.99
WFA	6.47

3.3.5 Relationship between K_i and physical properties of HS

The inhibitory effect of HS accorded more with % aromaticity ($r^2 = 0.74$) than molecular weight ($r^2 = 0.46$) (Figure 3.4). This suggested PCP interaction with HS in addition to HS acting as a substrate. We proposed that aromatic group was an important factor in inhibitory effect of HS, including AHA, LHA, SRFA, and WFA, in the systems containing laccase enzyme and PCP. Zavarzina et al. (2004) reported the more hydrophobic HA were stronger inhibitors. Their early work of Zavarzina et al. (2002) also suggested that the hydrophobicity of HA may be due to the presence of aromatic structures (e.g., aromatic rings). This was correlated to our results that higher aromatic HS occupied stronger inhibitors. Moreover, Zavarzina (2004) suggested that MW of HS would not affect inhibitory effect as significant as those hydrophobic properties.

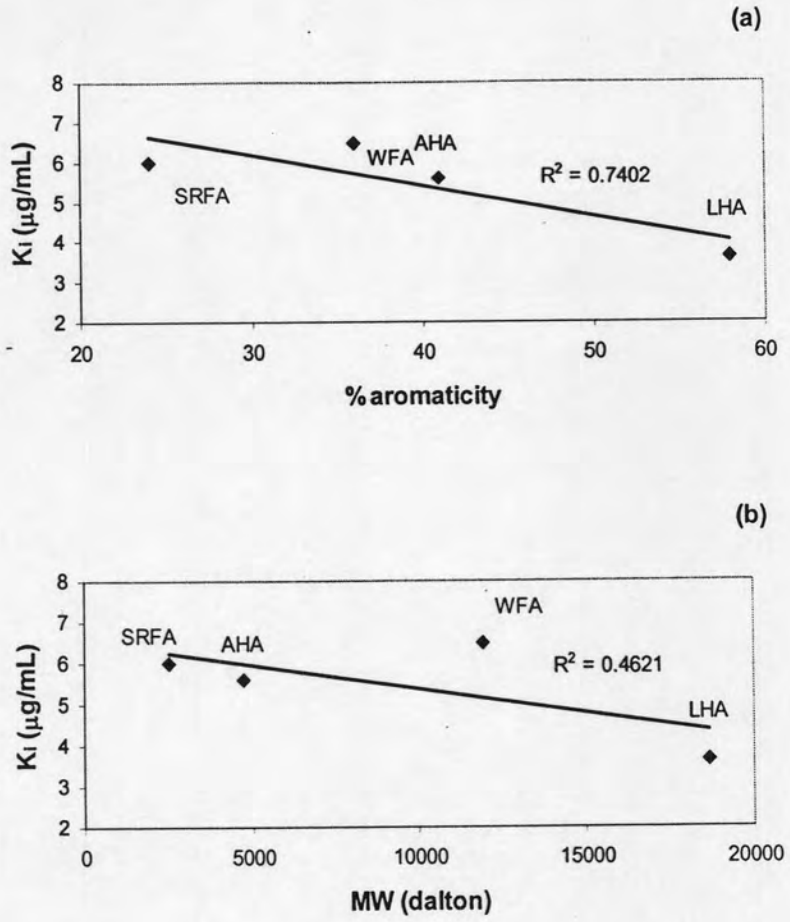

Figure 3.4 Relationship between K_i and (a) %aromaticity ($r^2 = 0.74$), (b) molecular weight ($r^2 = 0.46$)

3.3.6 Enzyme kinetics for HS

This section tested the hypothesis that HS act as a competitive substrate with laccase. We found that K_m of HS ranged from 13.59 – 89.47 mg/L (Table 3.5). After the incubation of HS with laccase, decrease in the absorbance of HS at 465 nm was found. Therefore we could conclude that HS could be substrates for the enzyme. Visible light absorption at 465 nm wavelength represents degree of humification (Tan, 2003), especially in an initial humification stage (Dębska et al., 2002). Absorbance at this wavelength indicates the presence of heterocyclic, aromatic, carboxylic, and monoester functional groups in HS (Olk, 2006). In addition, the 465 nm wavelength is

useful to quantify 0 – 5 mg/L of dissolved HA extracted from lake (Mazzuoli et al., 2003). Our result also showed the slow degradation of all HS and the smaller molecular weight of SRFA had a faster degradation than the higher molecular weight HS like LHA. However, the relationship between K_m of HS and their molecular weight is insignificant ($r^2 = 0.50$) (Figure 3.5 (a)). A strong relationship between HS % aromaticity ($r^2 = 0.90$) and K_m (Figure 3.5 (b)) was expected since the aromatic functional groups are substrates for laccase.

Table 3.5 K_m and V_{max} of laccase for HS

Humic substances	K_m (mg/L)	V_{max} (mg/hr)
AHA	46.61 ± 2.03	0.22 ± 0.02
LHA	89.47 ± 3.91	0.20 ± 0.00
SRFA	13.59 ± 0.08	0.78 ± 0.02
WFA	17.16 ± 0.04	0.23 ± 0.00

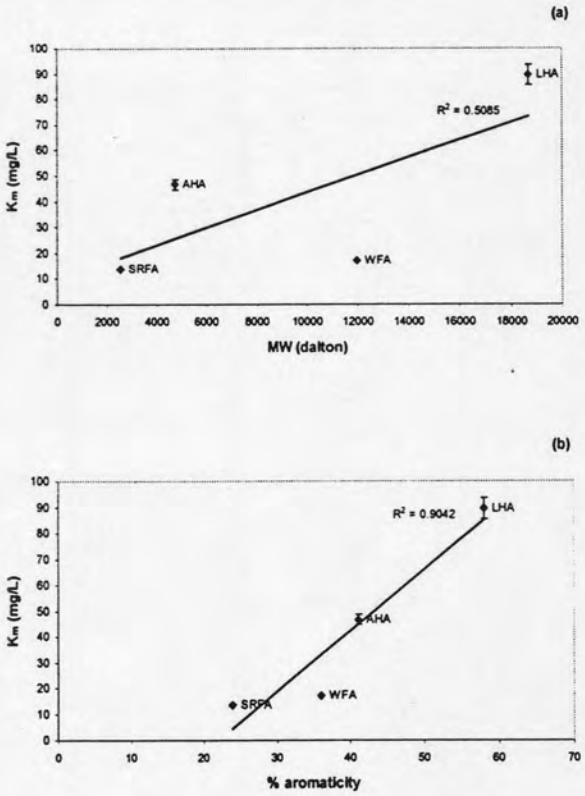

Figure 3.5 Relationship between K_m of HS and (a) molecular weight ($r^2 = 0.50$), (b) % aromaticity ($r^2 = 0.90$)

3.3.7 PCP-HS binding studies

This section was to specifically address the third hypothesis: HS protect PCP from enzymatic degradation. It was reported that PCP can bind to HS (Paolis and Kukkonen, 1997; Schellenberg, 1984; Peuravuori et al, 2001). The amount of PCP diffusion across the dialysis membrane was used to determine the amount of PCP bound by HS. Firstly, the kinetics of PCP-LHA association was carried out to determine the equilibrium time. Approximately 70 % of the total PCP was diffused into dialysis tubing and bound by LHA during the first 24 h and the equilibrium was attained within 3 days. Therefore, 3-day equilibrium dialysis experiments were performed to test that PCP could be sorbed into any HS. Sorption isotherms were shown in Figure 3.6 and Table 3.6. The relationship between bound and free PCP was linear over the concentration range examined. The amount of PCP bound to the HS was determined as described in O'Loughlin et al. (2000) using the relationship

$$C_{dom} = \frac{C_t - C_w}{C_{HS}} \times 10^6 \text{ mg/kg}$$

where C_{dom} is the amount of PCP bound per unit mass of HS expressed in terms of DOM (mol/kg dissolved organic mass, [DOM]), C_t is the total concentration of PCP (free and bound) in the dialysis bag, C_w is the concentration of free PCP (as determined by measuring PCP in the solution outside the dialysis bag), and C_{HS} is the humic substances present in the system (mg/L). All of the isotherms were linear, with an average calculate log K_{dom} (DOM normalized binding constant, $K_{dom} = C_{dom} / C_w$) value range from 4.09 to 4.81 (Table 3.6). Binding constants were SRFA < WFA < AHA < LHA. It appeared that the group of aquatic fulvic acids (WFA, SRFA) bound less strongly than that of terrestrial humic acids (AHA, LHA).

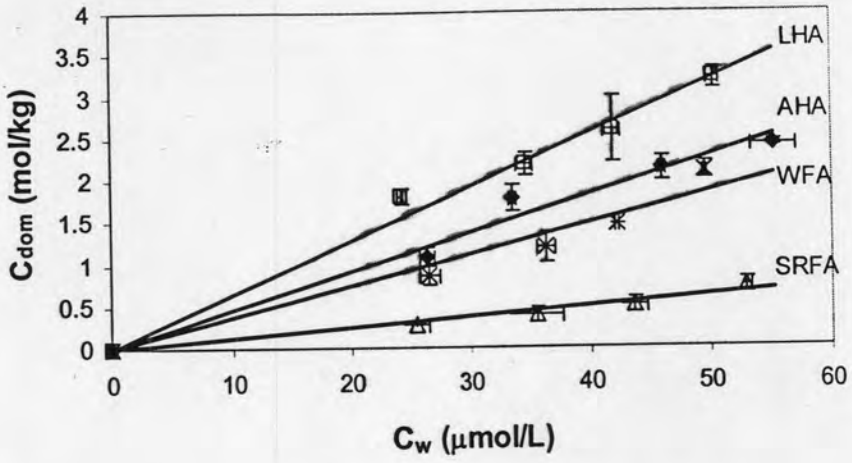

Figure 3.6 Sorption isotherms for PCP and HS, ♦: AHA, □: LHA, Δ: SRFA, ×: WFA. Error bars represent one standard deviation. C_{dom} = amount of PCP per unit mass of each HS, determined by $C_{dom} = (C_t - C_w) / C_{HS}$, C_w = concentration of free PCP

Table 3.6 Effects of PCP concentration on PCP binding by HS

Humic substances	K_{dom} ([L/kg OC*] x 10^4)	Log K_{dom}	r^2
AHA	4.49 ± 0.32	4.65	0.98
LHA	6.44 ± 0.24	4.81	0.99
SRFA	1.22 ± 0.17	4.09	0.96
WFA	3.59 ± 0.23	4.56	0.87

* OC means the total mass of the organic humic substance.

3.3.8 Relationship of K_{dom} and physical properties of HS

Among the HS used in this study, we found direct relationship between physicochemical properties of the HS and the degree of PCP binding as shown in Figure 3.7. We observed the strong relationship between PCP binding coefficients and aromatic carbon in the humic material (r^2 = 0.97, Figure 3.7 (a)). The same trend was also reported by Chin (1997), Uhle (1999), Perminova (1999), and Gadad (2007). One reason for this more preferable partition is the increased polarizability of the aromatic-

rich HS (Gauthier, 1987; Chin, 1997). An increase in the polarizability of the HS will result in an increase in van der Waals interactions between the solute and substrate. In an aromatic-rich environment such as LHA, PCP would be susceptible to these interactions. In contrast, SRFA is significantly less aromatic and would presumably be less polarizable. Thus, as observed in our work, the magnitude of the PCP K_{dom} values is dependent upon the aromatic nature of HS substrates.

We found a lack of the strong linear relationship between K_{dom} values and molecular weight of HS ($r^2 = 0.65$) as shown in Figure 3.7 (b). A weak correlation between K_{dom} values and molecular weight of HS might be due to heterogeneity and complex structure intrinsic to humic molecules as explained by MacCarthy (1988). Consequently, these humic macromolecules might have a large number of main structural fragments that affect to their ability to partition PCP. This contrasted to the finding of Chin (1994) who reported the strong positive relationship between molecular weight and binding affinity of HS for PAHs.

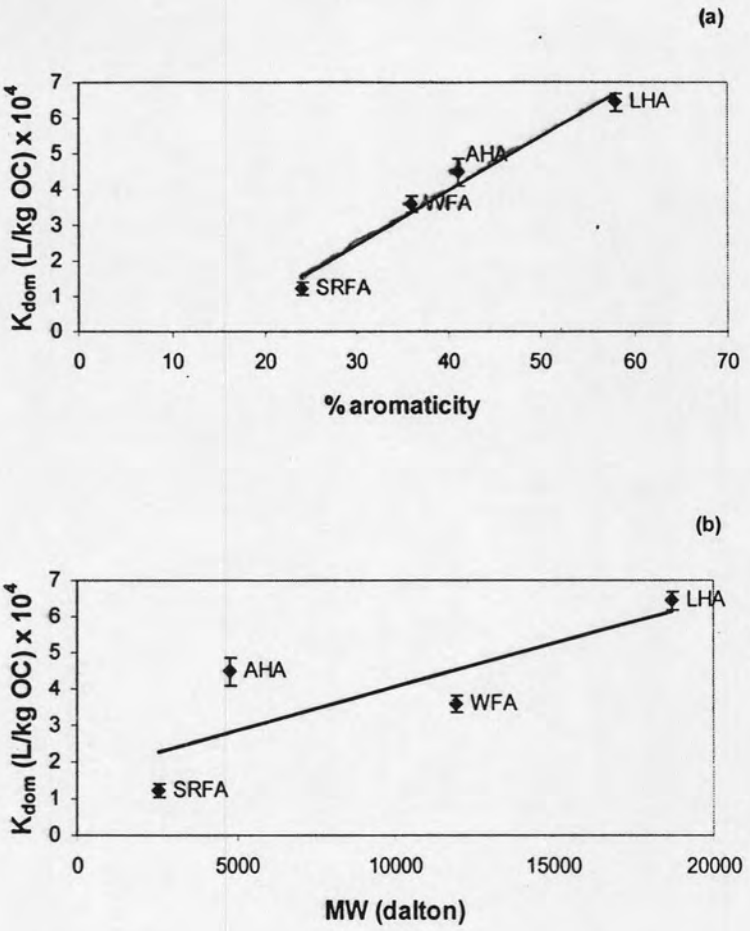

Figure 3.7 Linear relationship between K_{dom} and (a) % aromaticity (r^2 = 0.97), (b) molecular weight (r^2 = 0.65)

3.3.9 PCP-HS desorption studies

Bound PCP could relatively slowly release from HS. If a non-equilibrium system exists, PCP would not be immediately released from HS as the free-phase PCP is degraded by laccase. The concentration of PCP desorbed from HS seemed to reach equilibrium after 48 h. Percent desorption could be calculated from (C_w / C_t) x 100 %. Percent desorption of PCP from LHA < AHA < WFA < SRFA (Figure 3.8) was found. The result was correlated to binding capabilities of HS to PCP as shown by log K_{doc}. A group of terrestrial HA had a stronger potential to bind with PCP than of aquatic FA, resulting in HA released less PCP.

It is valuable to note that the rate of PCP desorption from HS must be significantly slower (by the index of 10) than the degradation rate of freely dissolved PCP. Otherwise, if the PCP desorption rate from HS is on the same order of magnitude as the degradation rate of freely dissolved PCP, we will not be able to correlate measured degradation rates in the presence of HS, and the amount of freely dissolved PCP. It is because as soon as a freely dissolved PCP molecule is degraded by laccase, a HS-bound PCP molecule will be released into solution as a freely dissolved molecule. The degradation rate of PCP (dC/dt) can be calculated from dC/dt = k′[C], where k′ was of HS at 20 mg/L and [C] was 15 mg/L, which was a PCP concentration used in desorption experiment. The calculated PCP degradation rates for AHA, LHA, SRFA, and WFA were 4.36×10^{-6}, 2.26×10^{-6}, 3.24×10^{-6}, and 4.19×10^{-6} M/hr, respectively. The desorption rate of PCP from HS can be calculated by using K_{dom}, and %desorption, including default values of 20 mg/L of HS and 15 mg/L of PCP concentration. We found desorption rates of AHA, LHA, SRFA, and WFA were 2.77×10^{-7}, 2.24×10^{-7}, 2.43×10^{-7}, and 3.13×10^{-7} M/hr, respectively (See appendix E for calculation). The desorption rate was about ten times slower than PCP degradation rate by laccase enzyme. Therefore, we can assume that the amount of freely dissolved PCP and the HS – modified degradation rate are correlated. The degradation rate of freely dissolved PCP available from PCP bound to HS could be compared to experimental PCP enzymatic degradation rate.

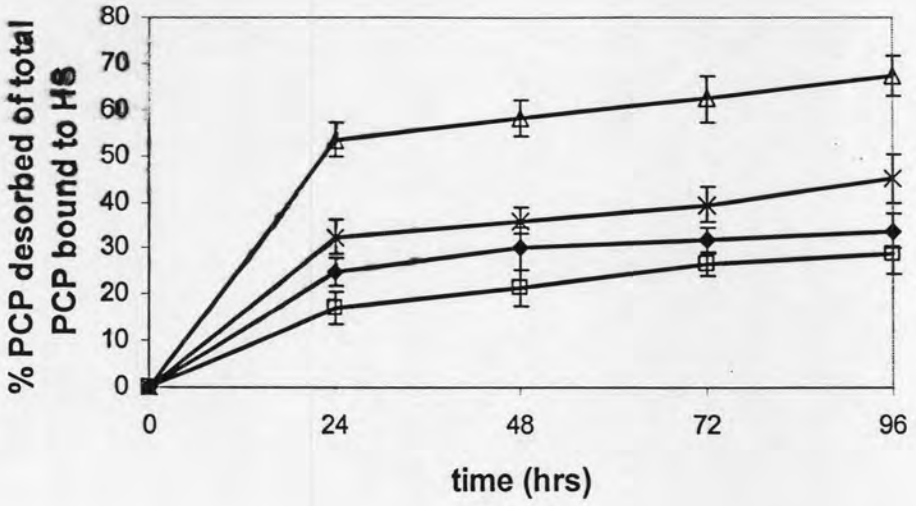

Figure 3.8 Percent PCP desorbed from HS, ♦: AHA, □: LHA, Δ: SRFA, ×: WFA.

3.3.10 The effect of HS on PCP enzymatic degradation rate

So far we could only disprove hypothesis 1 stated that HS could deactivate enzyme. We also found that HS could show a competitive type inhibitory effect on PCP degradation. Furthermore, sorption of PCP on HS could occur in the system. In this section we need to clarify by which mechanism could take place and affect PCP enzymatic degradation rate. Firstly, we have to prove whether only freely dissolved PCP is degraded by laccase. The calculations of 1) the experimental degradation rates of PCP in the presence of HS (k'[C]), 2) the calculated concentration of freely dissolved PCP (assume total PCP concentration of 15 mg/L because it was the highest PCP concentration used in binding experiment and using K_{dom} from table 3.6 for calculation), and 3) the calculated expected PCP degradation rate for 15 mg/L of PCP at 4 hr initial reaction were made. Four hrs reactions were used because PCP degradation rate exhibited linear fashion. Then, we can compare the experimental PCP degradation rate and the calculated PCP degradation rate. If this ratio = 1, it means only freely dissolved PCP is degraded by laccase. If the ratio < 1, it indicates

that the degradation of HS by laccase does influence the overall PCP degradation rate. If the ratio > 1, it suggests that PCP bound to HS is available to some extent for laccase degradation; either HS – associated PCP is rapidly released or HS – associated PCP is accessible to laccase. The experimental and calculated PCP degradation rate constants were shown in Table 3.7 (See appendix F for detailed calculation).

We found that all types and concentrations of HS provide experimental degradation rate and calculate degradation rate ration less than 1. This imply that the degradation of laccase is slower than expected and might be due to HS could act as another substrate and compete with PCP for laccase enzyme. This confirms the results that PCP could bind to HS in PCP binding experiment, and act as another substrate and compete to PCP enzymatic degradation. If not considered AHA, which is the least homogeneous sample, (A)/(C) ratio of LHA < SRFA < WFA was correspond to K_i values of LHA < SRFA < WFA. The lowest K_i of LHA implied that LHA was the strongest inhibitor and competed to PCP for laccase degradation, resulting in the least (A)/(C) ratio. We would like to propose the possible mechanism of HS influencing PCP enzymatic degradation rate by laccase enzyme. Firstly, HS could sorb PCP and protect PCP from enzymatic degradation. Then, freely dissolved PCP would be degraded while HS could compete with PCP for enzyme degradation. We also discover that the higher amount, higher molecular weight and higher aromatic functional groups of HS affect the higher propensity to bind to PCP, resulting in decreasing PCP degradation rate and providing longer time for remediation.

This is the first time to clarify the mechanisms by which HS interact with laccase enzyme and change PCP enzymatic degradation rate. In application of bioremediation, we can use this predictive information in designing for bioremediation strategies such as % aromaticity and preliminary experiment on

sorption efficiency. The next research may conduct experiment on various PAHs with different K_{ow} react with HS with different % aromaticity. Then, the relationship of enzymatic degradation rate and those factors can be correlated. However, in the actual applications in remediation technology, other factors such as existing and degradative ability of the microorganism strains present in the environment, and characteristics and amount of organic content in a complex HS located in the site must be significant variations needed to be further studied. Our goal is to highlight that HS which is the most widespread and ubiquitous natural nonliving organic materials in terrestrial and aquatic environments and represent a major fraction of soil organic matter should be more concerned in bioremediation technique.

Table 3.7 Experimental and calculated PCP degradation rate constants

Sample	Experimental PCP degradation rate constant (k'[C]) (M/hr) (A)	Calculated freely dissolved PCP concentration (M) (B)	Calculated PCP degradation rate considering only freely dissolved PCP (M/hr) (C)	Ratio (A)/(C)
PCP	1.26×10^{-5}	na	na	na
PCP + AHA				
10 mg/L	9.12×10^{-6}	3.89×10^{-5}	9.71×10^{-6}	0.94
15 mg/L	5.80×10^{-6}	3.36×10^{-5}	8.41×10^{-6}	0.69
20 mg/L	4.36×10^{-6}	2.95×10^{-5}	7.38×10^{-6}	0.59
PCP + LHA				
10 mg/L	2.69×10^{-6}	3.42×10^{-5}	8.56×10^{-6}	0.31
15 mg/L	2.34×10^{-6}	2.86×10^{-5}	7.16×10^{-6}	0.32
20 mg/L	2.27×10^{-6}	2.46×10^{-5}	6.15×10^{-6}	0.37
PCP + SRFA				
10 mg/L	7.60×10^{-6}	5.02×10^{-5}	1.25×10^{-5}	0.60
15 mg/L	3.87×10^{-6}	4.76×10^{-5}	1.19×10^{-5}	0.33
20 mg/L	3.26×10^{-6}	4.53×10^{-5}	1.13×10^{-5}	0.29
PCP + WFA				
10 mg/L	6.14×10^{-6}	4.14×10^{-5}	1.04×10^{-5}	0.59
15 mg/L	4.67×10^{-6}	3.66×10^{-5}	9.14×10^{-6}	0.51
20 mg/L	4.21×10^{-6}	3.28×10^{-5}	8.20×10^{-6}	0.51

3.4 Acknowledgements

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