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Role of Dissolved Humic Substances and Dissolved Organic Matter on Degradation of Phenanthrene by Crude Ligninolytic Enzymes from *Agrocybe* sp. CU 43

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Abstract: The objective of the study is to clarify the possible roles of dissolved humic substances (HS) and dissolved organic matter (DOM). The possible roles of them are (1) they can deactivate the enzyme responsible for biodegradation (2) they can act as enzymatic substrate and (3) they can sequester the pollutant and protect it from enzymatic degradation. Degradation of phenanthrene using crude fungal ligninolytic enzymes from *Agrocybe* sp. CU 43 was slower with dissolved HS and Dissolved Organic Matter (DOM) addition. Their enzyme activities using catechol assay were active in all conditions; therefore HS could not deactivate the enzymes. Four dissolved HS and DOM showed the capabilities to associate with phenanthrene and protect the contaminant from enzymatic degradation; consequently the phenanthrene bioavailability was decreased. The inhibitory effect of HS and DOM by competitive or linear mixed types suggest that HS and DOM could additionally be substrates for ligninolytic enzyme. Therefore, sorption and inhibitory effects of HS could be the possible mechanisms that govern enzymatic degradation rate of the pollutant in our system. Nature and extent of HS and DOM provide unique degradation potentials for aromatic organic pollutants. Some HS characteristics such as aromatic functional groups and molecular weight showed the propensity to be susceptible to sorption and enzyme degradation phenomena.

Key words: Phenanthrene, ligninolytic enzymes, humic substances, bioremediation

INTRODUCTION

Phenanthrene is one of the most abundant PAHs in the environment and widely used as model PAHs (Cerniglia, 1992). Due to its toxic and persistence, management of phenanthrene is necessary. High cost of abiotic method to remove PAHs in the environment leads to the increase use of biotic method by using microorganisms to decontamination and detoxication of PAHs (Wilson and Jones, 1993). The white rot fungi have been extensively used due to their extracellular enzyme activities such as lignin peroxidase, manganese peroxidase and laccase (Paszczynski and Crawford, 1995). White rot *Agrocybe* sp. CU 43 from yanagni mushroom was first isolated by Chupungars *et al*. (2009). It has previously reported to have high potential in degrading 99.2% of phenanthrene within 21 days (Chupungars *et al*., 2009). However, in aquatic and terrestrial environment, the abilities of the ligninolytic enzymes might be altered due to the presence of dissolved Humic Substances (HS) and Dissolved Organic Matter (DOM). Dissolved HS and DOM are major organic compounds in soil and sediment (Schnitzer, 1978; Stevenson, 1994). They have been widely reported to influence the solubility, mobility and bioavailability of aromatic organic pollutants. However, among the researchers, there are different evidences for the roles of HS and DOM in mineralization and degradation for the aromatic pollutants.

One of the possible role of HS and DOM in aromatic pollutant degradation is they can compete for the

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oxidation of aromatic pollutants and therefore inhibit transformation rate of the pollutant (Itoh *et al*., 2000). Due to the complex structure of HS and DOM, they comprise the numerous function groups, including carboxyls, alcoholic and phenolic hydroxyls, carbonyls and methoxyls (Essington, 2004). Those functional groups could be substrates for nonspecific ligninolytic enzymes. For example, Zavarzina *et al*. (2004) investigated the inhibition effect of Humic Acid (HA) by *Panus tigrinus* laccase. They reported K_i ranged from 0.003 μg mL^{-1} for HA from peat soils to 0.025 μg mL^{-1} for HA from chernozems. Nevertheless, Holman *et al*. (2002) and Bengtsson and Zerhouni (2003) reported the increase in mineralization and biodegradation of organic pollutants with HS addition.

Humic substances are able to react with white-rot ligninolytic enzymes such as manganese peroxidase and laccase (Yavmetdinov *et al*., 2003). The interaction of the enzymes with HS may lead to depolymerization of HS and their synthesis from monomeric precursors. These two processes can be dependent on the nature of HS (Zavarzina *et al*., 2004). For example, decolorization and decrease of HA's molecular weight and the formation of Fulvic Acid (FA) after incubation of the HA with *Trametes versicolor* were reported by Fakoussa and Frost (1999). Contradictorily, with the same culture, the formation of HA was investigated by Katase and Bollag (1991). Humic substances could either stimulate or inhibit enzyme activity dependent on their origin and characteristics (Claus and Filip, 1990). HA and its monomeric constituents either increase (Wang *et al*., 2002) or inhibit oxidoreductases activity (Kang *et al*., 2002). Moreover, Holman *et al*. (2002) proposed that HA can cause inactivation of laccase enzyme.

A main fate of nonvolatile, nonionic organic contaminants such as phenanthrene is sorption to soil or sediment organic matter (Schwarzenbach *et al*., 2002). A number of sorption studies have grown to progress the more understanding of phenanthrene behaviors in environmental fate. For example, sorption coefficient (Log K_{oc}) of various HS to phenanthrene ranging from 4.15 to 4.67 was reported (Salloum *et al*., 2001). Vacca *et al*. (2005) reported phenanthrene sorption coefficient indicated as K_p = 33 to Aldrich humic acid ($K_p = K_{oc}.f_{oc}$, f_{oc} = fraction of organic carbon in HS).

To date there has been no clear explanation for mechanisms of which how dissolved HS and DOM play a role in organic pollutant degradation. To identify the possible role of dissolved HS and DOM on enzymatic degradation rate of aromatic pollutants, degradation of phenanthrene, a model compound, by crude ligninolytic enzymes from *Agrocybe* sp. CU 43 was studied. Three hypotheses of dissolved HS and DOM's role were (1) deactivate enzymes (2) compete with aromatic pollutants for enzyme (3) are inert and protect aromatic contaminant.

MATERIALS AND METHODS

The project was conducted in Chulalongkorn University, Bangkok, Thailand during 2007-2008.

Chemicals: Phenanthrene, 98% purity, (Sigma-Aldrich) was dissolved in hexane to produce a stock solution of 1 g L^{-1}. Commercial HS were used in the research to promote the comparisons with other HS studies. Chemical and physical properties of experimental HS were shown in Table 1. Aldrich Humic Acid (AHA) (Fluka) and Leonardite Humic Acid (LHA) (International Humic Substance Society, IHSS, St. Paul, MN, USA) were dissolved in 50 μL of 0.5 M NaOH solution and adjusted its volume to 10.0 mL with Nanopure water (18.2 ÙM, Barnstead) to final concentration of 1.0 g HA/L with pH values of 8.27 and 8.61, respectively. Suwannee River Fulvic Acid (SRFA) (IHSS) and Waskish Peat Fulvic Acid (WFA) (IHSS) were dissolved in nanopure water (1.0 g FA/L, 10 mL). The stock fulvic acids had final pH of 3.19 and 3.29 for SRFA and WFA. 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 phenanthrene enzymatic degradation rate and sorption phenomena.

Dissolved organic matter

Soil sample collection, preparation and characterization: Soil sample used in these experiments was from paddy field in Na-Klang District, Nongbualumphu province, Thailand. Surface soil was collected from 10-15 cm depth. The paddy field soil was selected in the experiment

Table 1: Chemical and physical properties of particular humic substances

Humic substances	Sources	Aromaticity	Carboxyl	C	O	N	Weight average MW
		(%)					(dalton)
Aldrich humic acid (AHA)	Lignite, Aldrich company	41.0[a]	19.0[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]	ND

[a]Ashley (1996), [b]International Humic Substances Society (IHSS); [c]Thorn *et al*. (1989), [d]Malcolm and MacCarthy (1986), [e]Loughlin *et al*. (2000) and [f]Beckett *et al*. (1987). ND: No determination

because it was widespread and could be a soil representative in Thailand. The sample was air-dried and homogenized by sieving (<2 mm). The soil samples were analyzed for physiochemical properties including soil texture (sieve analysis), pH (soil:water, 1:1), Cation Exchange Capacity (CEC) (Ammonium saturation and distillation method) and total organic carbon by TOC analyzer (Analytik Jena, Multi N/C 2100). The paddy field soil contained 21.8% sand, 25.8% silt and 52.4% clay. It had pH of 5.20, CEC of 14.6 cmol kg^{-1} and TOC of 1.52%.

Preparation of dissolved organic matter (DOM): The soil was stored at -20°C and thawed at 4°C overnight. Then, the soil was extracted for 2 h with deionized water pH 7.0 using a soil:water ratio of 1:2 (by weight). The suspension was centrifuged for 30 min at 3600 g (Sorvall® Biofuge Stratos) and filtered through a 0.45 µm micro fiber filter (GF/C, Whatman®, Schleicher and Schuell). The solution was analyzed for DOM by TOC analyzer (Analytik Jena, Multi N/C 2100). The soil comprised 63.8±0.07 mg L^{-1} DOM. The extract was stored at 4°C in the dark no longer than 5 days.

Ligninolytic enzymes

Fungal cultivation and enzyme induction: *Agrocybe* sp. CU 43 was cultivated and induced for ligninolytic enzymes following Chupungars *et al*. (2009). Inoculums were prepared by growing the fungus on malt extract agar for 10 days at room temperature. Then, 5-6 pieces of 1×1 cm agar containing mycelia were transferred to 100 mL malt extract broth in a 500 mL Erlenmeyer flask and incubated at 28°C on an orbital mixer incubator (Ratek) at 120 rpm for 14 days. The cultured broth was transferred to a sterilized centrifuge tube and centrifuged at 10000 g for 10 min and washed twice with sterilized deionized water. The supernatant was decanted and the fungal pellet was weighed. Ten grams of the pellet was transferred to N-limiting medium 100 mL in 500 mL Erlenmeyer flask and incubated at 28°C on an orbital mixer incubator at 120 rpm for 7 days. Then, 100 mg L^{-1} of phenanthrene was added and incubated at the same condition. At the cultivated time, 5 mL of the broth was centrifuged (Sorvall® Biofuge Stratos) at 10000 g for 20 min. The supernatant which contained ligninolytic enzymes and the remaining phenanthrene was tested for their activities using catechol assay.

Reaction of crude fungal ligninolytic enzyme with Phenanthrene: Lineweaver-Burke Plot was used to calculate K_m, the Michaelis-Menten constant and V_{max}, the maximum velotcity, for the reaction of crude fungal ligninolytic enzyme with phenanthrene. Crude fungal enzyme 6 U was incubated with the 10, 15, 20 mg phenanthrene/L in 22 mL glass vials with a 3 mL of 200 mL sodium acetate buffer pH 5.0. 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 reaction mixtures were sealed with Teflon caps, incubated at 28°C for 96 h while shaken at 200 rpm simultaneously. The vials were covered with aluminum foil to avoid photodegradation. Then, Phenanthrene concentrations were analyzed by GC-FID every 24 h, giving the linear line of phenanthrene degradation. One of the given dissolved HS and DOM at the concentration of either 10 or 15 mg L^{-1} was added as a competitive inhibitor. The concentrations of HS and DOM were preliminary experimented to lower phenanthrene degradation rate linearly. The same experiment was carried out, then, the results of K_m and V_{max} of the conditions with and without HS and DOM were compared. Samples for every analysis were experiment in triplicate.

Reaction crude fungal ligninolytic enzyme with dissolved HS and DOM: This experiment was to test whether HS and DOM could be substrates for ligninolytic enzyme. HS and DOM (10-40 mg L^{-1}) were mixed with 6 U of the enzyme. Sodium acetate buffer (200 mM) pH 5.0 was added to adjust their volume to 3 mL. The glass vials were cover with aluminum foil to avoid photo bleaching. The samples were shaken at 200 rpm at 28°C until their 465 nm light absorptions by UV-vis spectrophotometer (Specord 40, Analytik Jena AG) were performed every 24 h for 96 h. The absorbance at 465 nm has been used to monitor the degree of humification in humic acids (Tan, 2003), particularly the early stages of humification (Dêbska *et al*., 2002) and to examine humic acid concentration from lake (Mazzuoli *et al*., 2003). Ligninolytic enzymes exhibited their absorption at 465 nm of 0.065±0.00.

Sorption and desorption of phenanthrene to dissolved HS and DOM: Sorption experiment was performed by equilibrium dialysis method. Firstly, equilibrium time needed to be investigated. Dialysis tubing was prepared from Spectra/Por® Biotech Cellulose Ester (CE) dialysis membranes MWCO 500 (Spectrum Laboratories Inc.) and filled with 3 mL of 15 mg L^{-1} of LHA solution. The tubes were placed in a 500 mL-beaker containing 200 mL of 200 mM sodium acetate buffer pH 5.0 and 20 mg L^{-1} of phenanthrene. The beakers were covered with aluminum foil to avoid photolysis. LHA was selected for equilibrium time experiment because it occupied the highest percent aromaticity and molecular weight. These characteristics are believed to be susceptible to binding capacities of aromatic pollutants. The LHA 15 mg L^{-1} and

phenanthrene 20 mg L^{-1} were the highest concentration used for enzyme kinetics experiment. Phenanthrene concentration was about 20 times higher than its water solubility. However, this HS concentration represented the upper range of HS concentrations previously reported to enhance the solubility of hydrophobic compounds (Chien and Bleam, 1997; Chien *et al*., 1997; Vacca *et al*., 2005). To avoid photolysis, the beakers were wrapped with aluminum foil. Then, they were closed with wrapping film (polyvinylchloride cling film, M Wrap, MMP Packaging Group Co., Ltd) and placed on magnetic stirrer. Aliquots of the solution inside the tubing were removed for phenanthrene analysis every 24 h for 168 h. It was found that a 4-day period was enough for the equilibrium for phenanthrene-LHA binding. To study the binding capabilities of phenanthrene to dissolved HS or DOM, the same experiment was carried out, except using AHA, SRFA, WFA and DOM.

Phenanthrene desorbing from the HS and DOM were also determined by equilibrium dialysis. The dialysis tubing contained 3 mL of the mixture of 15 mg L^{-1} of HS or DOM spiked with the phenanthrene at equilibrium concentration. The tubing was placed in 17 mL of fresh 200 mM sodium acetate buffer pH 5.0 in 22 mL test tubes. The samples were shaken at 200 rpm on the shaker. Then, free phenanthrene in the tubes was extracted and analyzed by GC-FID at 48 h. This 48 h was the time to allow phenanthrene desorbed from AHA (Vacca *et al*., 2005).

Analysis of phenanthrene: The samples were extracted by hexane (1:2 hexane/sample volume) and dewatered with sodium sulfate. Then, phenanthrene concentrations were analyzed by GC-FID. The GC equipped with HP 5 MS column (30 m×0.25 mm id×0.25 µm) and set the condition of carrier, helium 33 cm sec^{-1} constant flow; oven, 100°C for 0 min, 100-200°C at 6°C min^{-1} for 2 min, 200-250°C at 50°C min^{-1} for 0 min; injector, 5 µL splitless 250°C, retention time, 12.3 min. Percent recovery by this procedure was 86-103%.

RESULTS AND DISCUSSION

Reaction of crude fungal ligninolytic enzyme with dissolved HS and DOM: The objective of this section was to prove whether HS and DOM could act as a substrate for ligninolytic enzyme. We found the increase in 465 nm absorption upon the incubation period. The 465 nm absorbance could imply the heterocyclic, aromatic, carboxylic and monoester functional groups of HS (Olk, 2006) and is useful in dissolved HS quantification (Mazzuoli *et al*., 2003). Therefore, dissolved HS and DOM could react with the enzyme. Another objective in this section was to test whether dissolved HS and DOM deactivated the enzyme, the activity of laccase in the mixtures of dissolved HS and DOM was analyzed by catechol assay. Laccase was the most predominant among the ligninolytic group induced from *Agrocybe* sp. CU 43 (Chupungars *et al*., 2009). It was found that laccase activity was active during the experimental period for all types and concentrations (0-40 mg L^{-1}) as shown in Fig. 1a-e. Therefore, we could disprove hypothesis that dissolved HS and DOM could inactivate the enzyme.

Reaction of ligninolytic enzyme with phenanthrene and dissolved HS or DOM mixtures: The reaction of phenanthrene with 6 U of ligninolytic enzyme showed Michaelis-Menten kinetics in the substrate range of 0-20 mg L^{-1}, with a K_m value of 0.20±0.04 mM and a V_{max} of 0.01±0.00 µM min^{-1}. The samples with 10 and 15 mg L^{-1} of HS were converted in terms of dissolved organic carbon (DOC) concentration (DOC concentration (mg L^{-1}) = DOM concentration (mg L^{-1}) x % organic carbon/total mass of HS) so that K_m and V_{max} of HS and DOM could be compared. K_m and V_{max} of the enzyme for phenanthrene with dissolved HS or DOM were shown in Table 2. The addition of dissolved HS and DOM did decrease phenanthrene degradation by the enzyme. Our results also indicated that dissolved HS and DOM showed the inhibitory effect, which was influenced differently dependent on the types of dissolved HS and DOM. Enzyme-inhibitor constants (K_i) were shown in Table 2.

Sorption of phenanthrene to dissolved HS or DOM Phenanthrene sorption was experimented using equilibrium dialysis method. After 96 h equilibrium time for allowing phenanthrene diffusion across dialysis membrane, phenanthrene-HS complex was collected. Phenanthrene concentration (free and HS-bound phenanthrene) per mg of HS inside the dialysis tubing was determined. It was found that phenanthrene partition coefficient (K_p) to HS was SRFA (0.46±0.00) < WFA (0.48±0.00)<AHA (0.64±0.04)<LHA (0.69±0.01) < DOM (0.71±0.02). The group of aquatic fulvic acids (WFA, SRFA) bound less strongly than that of terrestrial humic acids (AHA, LHA) and DOM. Desorption experiment showed very low concentration (<0.05 mg L^{-1}) of phenanthrene that could desorb out from the bound compound.

Our study is unique and different from previous study because it is the first to compare and contrast the role of HS and DOM through the measurement of kinetic parameters, K_m, V_{max}, K_i, K_p as a function of HS and DOM concentrations and types. From our results, the hypothesis of DOM and HS could deactivated the enzyme

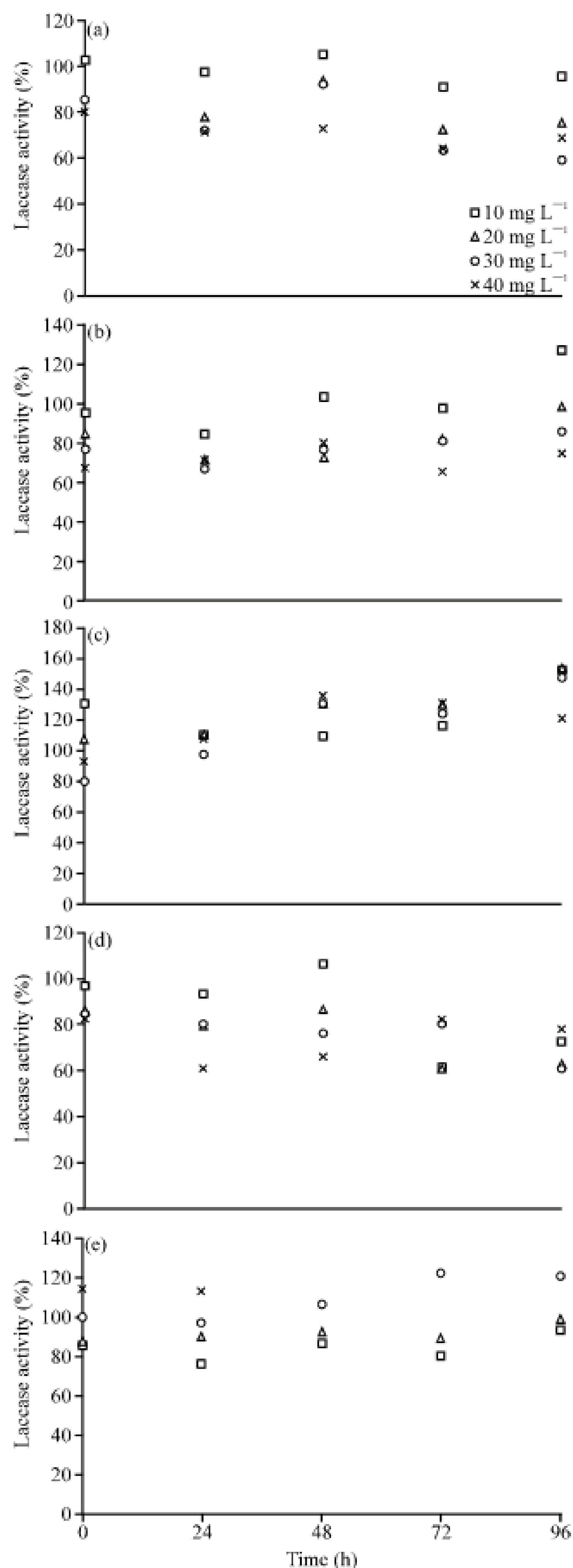

Fig. 1: Percent laccase activity for each HS and DOM. Activity of laccase in oxidation of catechol without HS and DOM is 100%. Symbols represent HS and DOM concentration. (a) AHA, (b) LHA, (c) SRFA, (d) WFA and (e) DOM

Table 2: K_m, V_{max} and K_i of ligninolytic enzyme for phenanthrene with and without HS and DOM addition

HS	DOC conc. (mg L^{-1})*	K_m (mM)	V_{max} (μM min^{-1})	K_i (μg mL^{-1})**
Control (Phenanthrene)	-	0.20±0.04	0.010±0.00	-
AHA	6.53	0.43±0.02	0.010±0.00	5.64±0.05
	9.80	0.55±0.00	0.010±0.00	
LHA	6.38	0.22±0.03	0.005±0.001	4.25±0.59
	9.57	0.23±0.02	0.003±0.000	
SRFA	5.30	0.36±0.03	0.010±0.00	7.10±0.66
	7.95	0.41±0.27	0.010±0.00	
WFA	5.36	0.41±0.07	0.010±0.00	4.76±0.48
	7.95	0.56±0.09	0.010±0.00	
DOM	10	0.08±0.01	0.002±0.000	3.11±0.86
	15	0.09±0.02	0.002±0.000	

*DOC conc. (mg L^{-1}) = DOM conc. (mg L^{-1})×% organic carbon/Total mass of HS (g). **AHA, SRFA, WFA used competitive inhibition model. LHA used linear mix inhibition model

could be invalidated. However, DOM and HS could act as substrate for the ligninolytic enzymes. This finding was expected because the complex structures of DOM and HS containing functional groups that can be substrates for ligninolytic enzymes (Kirk *et al*., 1992). The increase in 465 nm absorption was found after incubation of DOM, HS and enzymes. Yaropolov *et al*. (1994) explained that during lignin degradation by laccase two reactions, polymerization and depolymerization, can occur depending on initial molecular weight distribution in lignin preparations: polymer degradation with formation of low molecular weight products or condensation to form high molecular weight fractions indicative of existence of condensation-depolymerization equilibrium. In lignin degradation, ligninolytic enzyme is responsible for its demethylation, cleavage of C_α-C_β and alkyl-aryl bonds of phenolic substructures and side-chain elimination. Condensation occurs due to spontaneous polymerization of free radicals, formed as a result of oxidation of hydroxyl groups in the presence of molecular oxygen (Reinhammer, 1984; Thurston, 1994).

Inhibitory effect of HS and DOM was expected because functional groups of HS and DOM can be a substrate for oxidoreductive enzyme (Kirk *et al*., 1992). The inhibitory effect of HS was in accord to the results of Gianfreda and Bollag (1994) and Zavarzina *et al*. (2004). They reported a linear relationship between the organic matter content and its inhibitory effect on activities of laccase. The inhibitory properties were shown for other enzymes incubated with HS such as lignin peroxidase (Wondrack *et al*., 1989), pronase, trypsin and carboxypeptidase.

Phenanthrene sorption to dissolved HS and DOM could sequester phenanthrene and prevent it from being degraded by ligninolytic enzyme. Nature and extent of HS could be important in controlling the association of HS

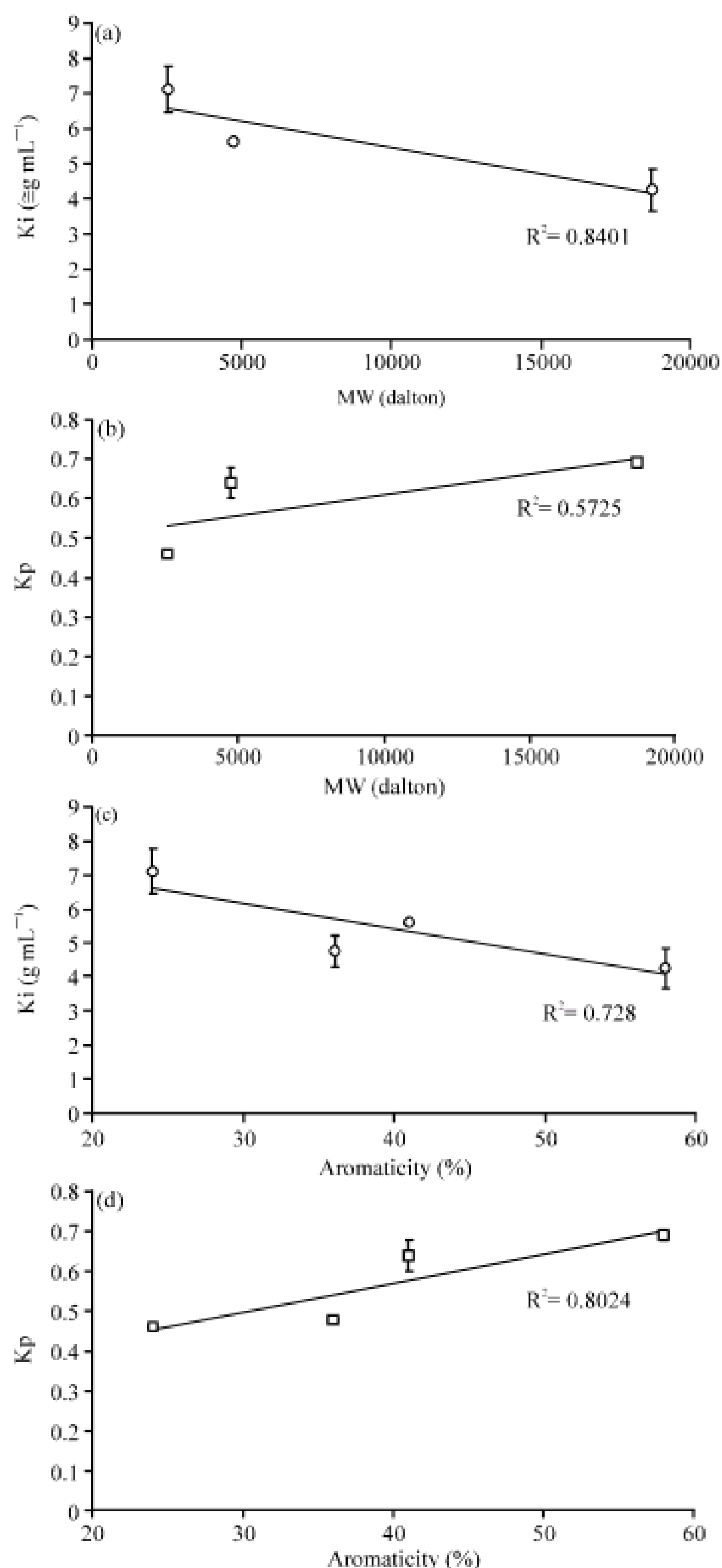

Fig. 2: Relationship between kinetic and thermodynamic parameters and dissolved HS and DOM properties: (a) K_i and MW, (b) K_p and MW, (c) K_i and (d) aromaticity (%), K_p and aromaticity (%)

and phenanthrene, affecting enzymatic degradation rate. Clapp *et al*. (1997) noted that the origin of HS played a key role in binding and suggested that the extent of coiling of HA polymer strands is an important factor in their complex formation with nonionic organics.

Since our results suggested that dissolved HS and DOM could act as another substrate and sequester phenanthrene, chemical properties of dissolved HS and DOM should associate with experiment kinetic parameters. The inhibitory effect (K_i) of dissolved HS and DOM accorded well with both molecular weight ($R^2 = 0.84$) and % aromaticity ($R^2 = 0.73$) (Fig. 2a, c). Since, aromatic functional groups of HS and DOM have been known to be substrate of oxidoreductive enzyme (Kirk *et al*., 1992), the high relationship between K_i and % aromaticity was expected. 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 *et al*. (2004) suggested that MW of HS would not affect inhibitory effect as significant as those hydrophobic properties.

The strong relationship between % aromaticity ($R^2 = 0.80$, Fig. 2d) and the degree of phenanthrene binding supports sequestration mechanism. The high correlation with % aromaticity indicates a specific interaction mechanism involving aromatic functional groups, e.g., pi-pi interactions. The same trend was also reported for Chin *et al*. (1997), Uhle *et al*. (1999), Perminova *et al*. (1999) and Gadad *et al*. (2007). It was possible that polarizability of HS is increased for the more aromatic HS (Gauthier *et al*., 1987; Chin, 1997). An increase in the polarizability of the HS could cause an increase in van der waals interactions between phenanthrene and HS. However, poor relationship between binding coefficient and molecular weight were found ($r^2 = 0.57$, Fig. 2b). The lack of correlation with weight averaged MW suggests that non-specific mechanisms such as hydrophobic interactions are not as important as specific interactions such as pi-pi bonding between phenanthrene and dissolved HS. MacCarthy and Rice (1990) explained a weak correlation between binding coefficient and molecular weight of HS that it might be due to heterogeneity and complex structure of HS.

CONCLUSION

We would like to underline that dissolved HS and DOM which are complex macromolecules and predominant in environment must be taken into account in bioremediation strategies. We proposed the model of which sequestration as well as inhibitory effect of dissolved HS and DOM could decrease degradation rate of the aromatic pollutant by oxidoreductive enzyme. The intrinsic chemical properties, especially % aromaticity and

molecular weight, of dissolved HS and DOM suggested as important parameters for the degradation rate.

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