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Covalent bonding of chloroanilines to humic constituents: Pathways, kinetics, and stability

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ABSTRACT

Covalent coupling to natural humic constituents comprises an important transformation pathway for anilinic pollutants in the environment. We systematically investigated the reactions of chlorine substituted anilines with catechol and syringic acid in horseradish peroxidase (HRP) catalyzed systems. It was demonstrated that although nucleophilic addition was the mechanism of covalent bonding to both catechol and syringic acid, chloroanilines coupled to the 2 humic constituents via slightly different pathways. 1,4-addition and 1,2-addition are involved to catechol and syringic acid, respectively. 1,4-addition showed empirical 2nd order kinetics and this pathway seemed to be more permanent than 1,2-addition. Stability experiments demonstrated that cross-coupling products with syringic acid could be easily released in acidic conditions. However, cross-coupling with catechol was relatively stable at similar conditions. Thus, the environmental behavior and bioavailability of the coupling products should be carefully assessed.

1. Introduction

Cross-coupling with soil organic matter (SOM) to form non-extractable bound residue was widely observed and considered as an important sink of phenolic and anilinic pollutants in the environment (Barriuso et al., 2008; Bialk et al., 2007, 2005; Calderbank, 1989; Dankwardt and Hock, 2001; Thorn et al., 1996; Weber et al., 1996). The underlying reaction mechanisms were investigated in a number of studies (Gulkowska et al., 2012; Naidja et al., 1998; Tatsumi et al., 1994a; Thorn et al., 1996). It is revealed that nucleophilic addition and radical coupling are two principal mechanisms that result in the covalent incorporation of the organic pollutants into SOM (Bialk et al., 2005; Bollag, 1992b). These reactions occur naturally as part of humification processes, leading to the formation and growth of SOM from smaller building-block moieties (Bialk et al., 2007; Eriksson and Skyllberg, 2009; Naidja et al., 1998; Stone and Morgan, 1984). Nucleophilic addition usually proceeds in two steps, during which the phenolic moieties in humic constituents are first converted to quinones through humification reactions, which creates electron-poor carbon sites that are then susceptible to nucleophilic attack by anilinic/phenolic group in xenobiotics to form covalent adducts (Bialk et al., 2005). Radical coupling reactions also similarly comprise two stages (Park et al., 1999), the first of which involves oxidation of the substrates with phenolic/anilinic features to produce radical intermediates, which in the second stage chemically couple to form polymerized products. These reactions can be catalyzed by a variety of naturally-occurring extracellular enzymes, such as peroxidases and laccases, and by mineral oxides such as manganese dioxide (i.e. birnessite) (Bialk et al., 2007, 2005; Bollag, 1992a, 1992b).

The actual mechanism via which bound residues are formed is indeed dependent on the types of the pollutants (Park et al., 1999). For phenols, both nucleophilic addition and radical coupling could occur during the processes. However, direct oxidization of aniline by peroxidase/phenoxidase is substantially slower than the oxidation of phenols (Park et al., 1999). Anilinic compounds predominantly undergo nucleophilic addition processes to quinone intermediates generated from humic constituents (Simmons et al., 1989). Depending on the types of quinones and attached substituents on the ring, the addition of anilines could proceed via 1,2- and/or 1,4-pathways (Gulkowska et al., 2012; Thorn et al., 1996). Chloroanilines are typical anilinic

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pollutants and were extensively studied as models in the formation of bound residues (Adrian et al., 1989; Park et al., 1999; Simmons et al., 1987, 1989; Tatsumi et al., 1994a, 1994b, 1992; You et al., 1982). A number of research efforts were made to screen the specific humic constituents cross-coupling with chloroanilines thus facilitating their transformation (Park et al., 1999; Tatsumi et al., 1992). It was revealed that among a variety of model humic constituents, only catechol like and syringic acid like species were involved such process significantly. The former can be transformed to *ortho*-quinone intermediates upon enzymatic treatment and foster 1,4-addition subsequently to form anilinoquinones (Nonhebel and Walton, 1974). The later were transformed to *para*-quinone intermediates that induce 1,2-addition resulting in the formation of imines (Schiff base) (Bialk et al., 2005; Gulkowska et al., 2012). Similar reaction patterns were also found for other anilinic contaminants such as sulfonamides (Bialk et al., 2007). The details of these pathways were summarized by Gulkowska et al. in a recent publication (Gulkowska et al., 2012).

Chlorine substituted anilines are important building blocks and intermediates in the synthesis of pesticides, herbicides, drugs, and dyestuffs (Zhu et al., 2012). They enter the environment through incomplete treatment of industrial discharge and have been found ubiquitous. These compounds have significant toxic, carcinogenic, mutagenic and teratogenic effects. They are considered as important environmental pollutants and are subject to legislative control by the environmental protection agency of the United States and Europe (Hongsawat and Vangnai, 2011). It is reported that a major transformation pathway of the substituted anilines in environment is binding to soil humic substance (Park et al., 1999; Tatsumi et al., 1992). In this research, we intended to employ mass spectrometry (MS) with electron spray ionization (ESI) to study the cross-coupling between chlorine substituted aniline and selected humic constituents in horseradish peroxidase (HRP) catalyzed systems. Based on the understanding of the reaction mechanisms, we quantitatively examined the reaction kinetics of 1,2- and 1,4-addition, investigated the stability of the covalent bonds formed via each reaction pathway.

2. Material and methods

2.1. Reagents

All reagents were ACS grade or better. 2-Chloroaniline (CA), 2,4-dichloroaniline (DCA), catechol, syringic acid, gallic acid, salicylic acid, syringaldehyde, 4-hydroxylbenzoic acid, and resorcinol were all obtained from Aladdin (Shanghai, China). Stock solutions at the concentration of 1 mM for each of the chemicals were prepared and stored at 4 °C. HRP was purchased from Sigma–Aldrich (St. Louis, USA). 1.0 g/L HRP stock solution was prepared in deionized (DI) water and stored at 4 °C. Its activity was determined photometrically as described in the early study (Bhunia et al., 2001).

2.2. Coupling reactions

Experiments were performed to assess chloroaniline removal by HRP catalyzed reactions in the presence of various humic constituents. The reactions were carried out in flasks as batch reactors under room temperature. Each reactor contained a 50 mL reaction medium prepared in 10 mM citrate–phosphate buffer solution (pH 7). The reaction solution initially contained 100 μM CA or DCA, an individual humic constituent with predetermined concentration, and 0.15 unit/mL HRP. 500 μM H_2O_2 was added to each reactor as the last component to initiate the reaction. After predetermined incubation time, 0.5 mL reaction solution was sampled and mixed with 0.5 mL methanol immediately to denature the enzyme thus quenching the reaction. The residual CA or DCA was quantified using a Hitachi L-2000 HPLC equipped with a photo-diode array (PDA) detector. A C18 reverse phase column (Hitachi LaChrom, 5 μM × 250 × 4.6 mm) was used for separation. An isocratic elution consisting of 50% methanol and 50% water (1% acetic acid) at a flow-rate of 1.0 mL/min was used as mobile phase. Controls in the absence of humic constituents were also prepared. Three replicate experiments were performed for each reaction condition.

2.3. Characterization of reaction products

To characterize the reaction products, each reaction solution after 2 h incubation was loaded onto a C18 solid-phase extraction (SPE) cartridge (Restec, 6 mL, 500 mg) preconditioned with 5 mL methanol at a flow rate of 10 mL/min. After loading, the SPE cartridge was eluted with 5 mL methanol and the elution was collected and reconstituted to 3 mL with methanol. The samples thus obtained were analyzed using an Agilent G6410B Triple Quad Mass spectrometer with an electron spray ionization source. The ionization source was operated in positive mode (ESI+) and the mass analyzer was run at scanning mode from m/z 100 to 1000. Nitrogen was used as desolvation gas and maintained at a flow rate of 10 L/min. The desolvation temperature was set at 350 °C. The fragmentor was set at 90 V.

2.4. Stability of the coupling products

The same reaction setup and conditions described above were used in the study of the stability of the coupling products formed in the presence of catechol and syringic acid. Total volume of the reaction media was 150 mL. Initial concentrations of the aniline and humic constituent were both 100 μM. After 2 h incubation, when HRP catalyzed reactions were determined to be completely ceased in pre-experiments, appropriate amount of H_2SO_4 was added to the solutions to decrease the pH to 4.0 and 2.0, respectively. Immediate after acidification, 10 mL sample was taken and extracted with SPE. The remaining solutions were incubated in water bath at 37 °C. After 2, 4, 6, 8, 12, and 24 h, 10 mL sample was withdrawn from each solution and extracted. The contents of chloroanilines in the samples were quantified with HPLC. The residual chloroanilines in controls without acidification were also analyzed.

2.5. Kinetic assay

A similar setup as described above using glass test tubes as the reactors and following a scheme used in earlier studies (Mao et al., 2009). Each reactor contained 5 mL reaction medium prepared in citrate–phosphate (10 mM, pH 7), comprising various initial concentrations of CA and a humic constituent, 0.1 unit/mL HRP, and 500 μM H_2O_2. Following the initiation of the reaction by the addition of H_2O_2, the reactor was hand shaken and allowed to react for 3 min prior to the termination of the reaction by the addition of equal volume methanol. The residual pollutants and humic constituents were quantified using HPLC. Three replicate reactors were prepared and tested for each reaction condition along with a blank reactor that was prepared at otherwise the same condition except with HRP absent.

3. Results and discussion

3.1. Transformation of CA and DCA in HRP catalyzed reactions

Anilinic compounds are substrates of peroxidases and can be directly oxidized in HRP catalyzed systems. However, anilines are apparently less reactive than their phenolic analogs. The transformation of CA and DCA in HRP system was rather limited. We investigated their removal in the presence of an array of humic constituents with different structures. The data are presented in Fig. 1. In accordance with the findings of other researchers (Park

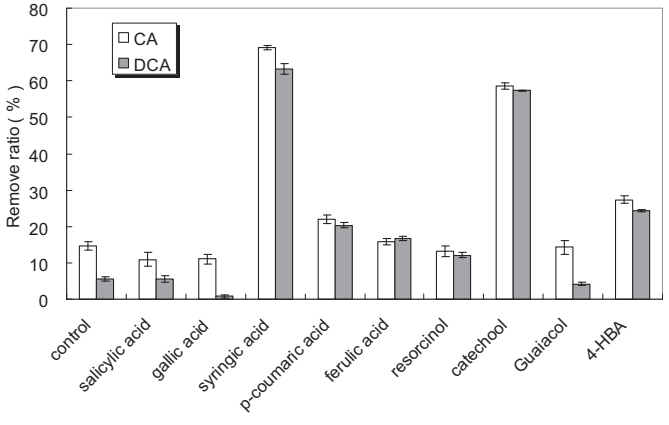

Fig. 1. Removal of chloroaniline by HRP catalyzed reactions in the presence of various model humic constituents.