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Transformation of triclosan by laccase catalyzed oxidation: The influence of humic acid-metal binding process☆

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ABSTRACT

Laccase is a widely present extracellular phenoloxidase excreted by fungi, bacteria, and high plants. It is able to catalyze one-electron oxidation of phenolic compounds into radical intermediates that can subsequently couple to each other via covalent bonds. These reactions are believed to play an important role in humification process and the transformation of contaminants containing phenolic functionalities in the environment. In this study, we investigated the kinetics of triclosan transformation catalyzed by laccase. It was found that the rate of triclosan oxidation was first order to the concentrations of both substrate and enzyme. Humic acid (HA) could inhibit the reaction by quenching the radical intermediate of triclosan generated by laccase oxidation. Such inhibition was more significant in the presence of divalent metal cations. This is because that binding to metal ions neutralized the negative charge of HA molecules, thus making them more accessible to laccase molecule that is also negatively charged. Therefore, it has greater chance to quench the radical intermediate that is very unstable and can only diffuse a limited distance after being released from the enzyme catalytic center. Based on these understandings, a reaction model was developed by integration of metal-HA binding equilibriums and kinetic equations. This model precisely predicted the transformation rate of triclosan in the presence of HA and divalent metal ions including Ca^{2+}, Mg^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, and Zn^{2+}. Overall, this work reveals important insights into laccase catalyzed oxidative coupling process.

1. Introduction

Catalyzed oxidative coupling reactions refer to a class of reactions that result in the polymerization of molecules having phenolic or anilinic features. These reactions are found to play a key role in natural humification processes, leading to the formation and growth of soil organic matter from smaller building-block moieties(Bollag, 1992a, 1992b; Huang et al., 2002; Huang and Weber, 2003, 2004; Piccolo et al., 2000). Heme peroxidases and laccase are the two groups of enzymes that are known to catalyze oxidative coupling reactions(Duran and Esposito, 2000; Torres et al., 2003). These enzymes are indigenously produced in extracellular forms by a large variety of fungi, bacteria, and plants, and are ubiquitous in soil/water matrices(Bollag, 1992a). Heme peroxidases comprise a large family of enzymes that contain iron protoporphyrin IX (heme) as their catalytic center and catalyze the oxidation of organic substrates in the presence of hydrogen peroxide as the ultimate electron acceptor(Dawson, 1988). All heme peroxidases share a common catalytic cycle in which the ferric resting enzyme is oxidized by two electrons of H_2O_2 to from an enzyme intermediate, referred to as Compound I. Compound I can oxidize one substrate molecule by one electron with a concomitant reduction of the enzyme to Compound II. Compound II can also oxidize one substrate by one electron and then return the enzyme to the resting state(Dunford, 1999). Laccase remarkably resembles peroxidases in terms of catalysis effects, but it has a completely different molecular structure and use oxygen as the electron acceptor. A laccase molecule contains four copper ions: one T1, one T2, and two T3 copper centers. The T2 and T3 copper centers form a trinuclear copper cluster site which is involved in the binding of oxygen during its reduction to water. The T1 copper center is involved in the oxidation of the substrate. A total of 4 substrate molecules are oxidized in a catalytic cycle(Torres et al., 2003).

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It is well recognized that the transformation of phenolic substrates in catalyzed oxidative coupling process comprises 2 steps. The first is a one-electron oxidation of the substrate by enzyme to form a phenoxyl radical. The second step is the coupling of the radicals, yielding dimers of the substrates(Anna K. Harding et al., 2006; Leonowicz et al., 2001; Lu et al., 2009). Because the dimers formed in such a pathway may retain the phenolic functionalities, they are still the substrates of the enzymes thus can be oxidized and coupled to each other again(Lu et al., 2009). In this way, catalyzed oxidation of phenolic compounds lead to the formation of polymerized products that are usually less soluble and bioavailable. Thus, it can be regarded as a detoxification process although the substrates are not degraded(Mao et al., 2010). Because of the wide presence of laccase and peroxidases in the environmental, catalyzed oxidative coupling process is believed to play an important role in governing the transformation and fate of certain organic contaminants in the environment(Bollag, 1992a). On the other hand, it can be manipulated in engineering systems to serve as an innovative scheme to address organic pollutants. A lot of efforts have been made in this direction(Dai et al., 2011; de Cazes et al., 2015; Gutierrez et al., 2007; Hou et al., 2014; Katuri et al., 2009; Savizi et al., 2012; Sun et al., 2016).

Kinetics of laccase catalyzed oxidative coupling process has been extensively studied(Feng et al., 2013; Lu et al., 2009). One-electron oxidation of substrate by laccase is the rate-limiting step during its transformation. Overall, transformation of phenolic substrates is a second order reaction with first order to the concentrations of both substrate and enzyme as described by the follow equation(Lu et al., 2009):

$$\frac{dc_S}{dt} = -kc_S c_E \quad (1)$$

where c_S and c_E represent the concentrations of substrate and enzyme, respectively; k is second-order rate constant. Because laccase is relatively stable and its activity remains constant at environmental conditions, the removal of substrate apparently shows pseudo-first order kinetics (Eq. (2)).

$$\frac{dc_S}{dt} = -k_{obs} c_S \quad (2)$$

Although the kinetics of laccase catalyzed transformation of phenolic compounds has been well understood, such understandings are mostly gained at laboratory conditions. At real conditions, the reactions can be affected by various constituents encountered in the environmental matrices such as natural organic matter (NOM) and metal cations. In an earlier study, we examined the influence of humic acid (HA) on the transformation of halophenols in laccase catalyzed oxidative coupling process(Lu et al., 2015). It was demonstrated that the activity of laccase was not affected by HA but the removal of halophenols was significantly inhibited. Such a phenomenon can be well explained by considering HA acts as the inner filter of the phenolic radical intermediate formed upon the oxidation of the substrate by laccase. As such, the oxidation of substrate is reversed and the radical intermediate is reduced back to its original form. The reaction scheme is shown as:

$$S + \text{Laccase} \underset{+HA.k_{-1}}{\overset{k_1}{\rightleftharpoons}} S^* \xrightarrow{k_2} P$$

Assuming the reaction rate of intermediate reduction is proportional to HA concentration, equations for the reaction rates of the substrate (S), radical intermediate (S^*), and product (P) can be obtained. Assuming steady-state is attained with the concentration of the intermediate keeping constant, a numerical model can be developed and the relationship between the observed pseudo-first order rate constant (k_{obs}) and HA concentration can be established:

$$\frac{1}{k_{obs}} = \frac{k_{-1}}{k_1 k_2} c_{HA} + \frac{1}{k_1} \quad (3)$$

Eq. (3) can precisely describe the dependence of the pseudo-first order rate constant and HA concentration in laccase catalyzed oxidative coupling process and was validated in our earlier study(Lu et al., 2015). This reaction model was also successfully applied by Shi et al. in the study of laccase catalyzed transformation of antimicrobials chlorophenes(Shi et al., 2016).

This work is a continuation of our previous study. We systematically explored the reaction kinetics of triclosan, a widely used antibacterial and antifungal agent of great environmental concern due to its endocrine disrupting potential(Dann and Hontela, 2011), in laccase catalyzed oxidative coupling process in the presence of both HA and metal cations. It was revealed that metal cations such as Ca^{2+} and Mg^{2+} could facilitate the quenching of the phenoxyl radical intermediate by HA. By integration with HA-metal binding model, the dependence of reaction rate constant to HA and metal concentrations in the system can be analyzed quantitatively. This study established a comprehensive understanding of the laccase catalyzed oxidative process.

2. Material and methods

2.1. Chemicals

Laccase from *Trametes versicolor* and humic acid (HA) were obtained from Sigma-Aldrich (St. Louis, USA). Triclosan, NaCl, KCl, $CaCl_2$, $MgCl_2$, $BaCl_2$, $CoCl_2$, $CdCl_2$, $MnCl_2$, $ZnCl_2$, and 2,2-azinobis-3-ehtylbenzthiazolin-6-sulphonate (ABTS) were all purchased from Aladdin (Shanghai, China). HPLC grade methanol was purchased from Fisher Chemical. Other reagents were of analytical grade or better and used as received without additional purification. Laccase was dissolved in Milli-Q water (>18 MΩ/cm) generated by a Millipore Milli-Q system. Laccase stock solution was kept in refrigerator and assayed prior to use. HA stock solution was prepared in Milli-Q water and the total organic carbon (TOC) was analyzed using a Shimadzu 5050A TOC analyzer.

2.2. Enzyme assay

Laccase activity was determined by oxidation of 0.3 mM ABTS in citrate-phosphate buffer (pH 3.8) as described in our earlier study. One unit of laccase activity is defined as the amount of enzyme that causes a unit change per minute in absorbance at 420 nm in 3 mL of this solution in a 1-cm light path cuvette (Lu et al., 2015). A Varian Cary50 spectrophotometer was used to measure the absorbance.

2.3. Reaction setup

Transformation of triclosan in laccase catalyzed oxidative coupling process was performed in glass vials at 20 °C. Reaction solution (20 mL) was comprised of 3.5 μM triclosan and appropriate concentration of laccase. The pH of the solutions was maintained at 7.0 by 0.01 M phosphate buffer. Before laccase was dosed, the solution was vigorously stirred for 5 min to facilitate the saturation of dissolved oxygen. Laccase concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 unit/mL were tested. At every 20 min, 0.5 mL solution was withdrawn from each vial and mixed with 0.5 mL methanol immediately to deactivate laccase thus stopping the reaction. Negative controls with boiled laccase were also prepared.

The same reaction setup was used to explore the influence of HA