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Inhibition of metal ions on *Cerrena* sp. laccase: Kinetic, decolorization and fluorescence studies

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ABSTRACT

Laccases (EC 1.10.3.2) have important industrial values in areas such as bioremediation, but they are often inactivated by heavy metal ions in real applications. In this report, laccase from a high laccase-producing *Cerrena* sp. HYB07 presented resistance to many metal ions except for Ag^{+}, Hg^{2+}, Li^{+} and Pb^{2+}, the inhibition of which on the laccase were all reversible. The presence of these four cations decreased Remazol Brilliant Blue R decolorization efficiencies catalyzed by the laccase, and the mediator 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) could restore the decolorization efficiency in spite of Hg^{2+} inhibition. Hg^{2+} had the lowest IC_{50} value (0.17 mM) on the laccase activity, followed by Ag^{+}, Li^{+} and Pb^{2+}. The inhibition type on laccase activity was competitive for Pb^{2+}, noncompetitive for Hg^{2+}, and mixed type for Ag^{+} and Li^{+}. The inhibition kinetic model of Hg^{2+} on laccase activity was established by using the kinetic method of substrate reaction. The microscopic forward inhibition rate constant (k_{+0}) of $Hg^{2+}$was 3.26×10^{-2}/s and the microscopic reverse inhibition rate constant (k_{-0}) was 1.36×10^{-3}/s, indicating the laccase would be completely inhibited when Hg^{2+} concentration was sufficiently high because k_{+0} was much larger than k_{-0}. Furthermore, Hg^{2+} reduced thermal and pH stability of the laccase. Fluorescence emission spectra demonstrated that Hg^{2+} had one binding site per laccase protein regardless of pH, and the binding was stronger at pH 6.0 than that at pH 5.0 or 9.0, in accordance with the lowest stability of the enzyme at pH 6.0 with Hg^{2+}. Dynamic simulation was performed to identify the binding sites of each ion on the Lac7 protein.

1. Introduction

Laccases (EC 1.10.3.2) are copper-containing oxidases that can catalyze oxidation of a wide range of phenolic and non-phenolic compounds, including various dyestuffs and environmental contaminants. With one-electron oxidation and radical formation, laccases catalyze oxidative coupling or bond cleavage of target compounds [1]. Since laccases have a wide substrate range and use only oxygen as the final electron receptor, they have widespread applications in various industries, such as wastewater treatment, biosensor development, food processing, biofuel production, organic synthesis and pulp biobleaching [2–5].

However, large-scale applications of laccases are limited by the economy and efficiency of the enzymes. Metal ions comprise a common group of enzyme activity modulators [6]; laccase activity is often impaired by metal ions present in wastewater or other environments/processes [7–10]. It is therefore a common practice to characterize the influences of metal ions on laccase activity, which vary with the ion and the enzyme [10–14]. For example, Mn^{2+} stimulates laccases from *Sporothrix carnis* [15] and papaya (*Carica papaya*) [16] but inhibits laccase from *Leptographium qinlingensis* [17]. Despite many reports describing effects of metal ions on laccase activity, the inhibition mechanism has seldom been studied, understanding of which could facilitate efficient laccase applications in the presence of metal ions [4].

Cerrena sp. HYB07 is an efficient laccase producer [18], and its laccase has shown potentials in bioremediation with high activity toward a broad range of environmental contaminants such as dyestuffs and antibiotics [19–22]. Lac7 is the predominant laccase isozyme of *Cerrena* sp. HYB07 [18,23]. Considering the metal cations in effluents may interfere with the efficiency of enzyme remediation [7,24], in this study, we investigated the effect of 15 metal ions on laccase activity and dye decolorization ability. The inhibition of four inhibitive metal ions on laccase activity, especially mercuric ions as the strongest inhibitor, was elucidated. Revealing the kinetic mechanism of metal ion inhibition on laccase provides a theoretical foundation for laccase applications.

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2. Materials and methods

2.1. Materials

Cerrena sp. HYB07 was used as the source of laccase. Fermentation of *Cerrena* sp. HYB07 preparation of enzyme were carried out as previously described [18], and the fermentation broth was harvested by paper filtration and centrifugation. Lac 7, a major laccase produced by HYB07, was purified from the fermentation broth with HiTrap DEAE column (GE Healthcare, UK) to electrophoretic homogeneity [18] and used. Guaiacol was purchased from Sinopharm Chemical Reagent Co., Ltd. All chemicals were of analytical grade. Metal ions Cu^{2+}, K^{+}, Li^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Zn^{2+} were in the form of sulfates, and Ag^{+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Ce^{3+}were in the form of nitrates.

2.2. Enzymatic assay methods

Enzyme activity was determined at 40 °C by following the increase in optical density at 465 nm ($\varepsilon = 1.21 \times 10^4$/M/cm) accompanying the oxidation of guaiacol. The reaction mixture contained 50 µL enzyme solution and 1 mM guaiacol in 50 mM sodium acetate-acetic acid buffer (pH 5.0). Absorption was recorded with a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, USA) for the first 60 s to measure the initial rate. One enzyme unit (U) is defined as the enzyme quantity required by optical density to increase by 0.01/min (ΔOD/min). Protein concentration was measured with an Easy II Protein Quantitative Kit (BCA) following the instructions of the manufacturer (TransGen, China).

2.3. Effect of metal ions on laccase activity

Individual metal ions at 10 or 100 mM were added to the enzyme assay, and the residual activity was determined. Laccase activity without added metal ions was set as 100%. For selected metal ions, their concentrations leading to loss of 50% of laccase activity (IC_{50}) was evaluated.

2.4. Dye decolorization

Decolorization assays were carried out as in our previous study [21]. Each decolorization reaction (4 mL) contained 100 mg/L Remazol Brilliant Blue R (RBBR), 2 U/mL laccase and sodium acetate buffer (pH 5.0). When needed, individual metal ions were added. Decolorization was carried out at 25 °C according to our pervious study. The absorbance spectra and decolorization time courses of RBBR were recorded with Multiskan GO Spectrophotometer. Decolorization efficiency was calculated as previously described [19].

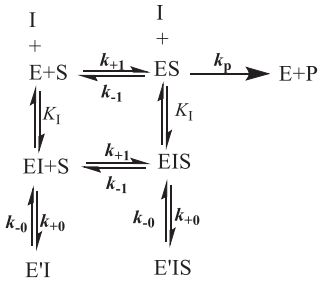
2.5. Determination of inhibition mechanism and type of selected metal ions on laccase

For determination of inhibition mechanism, different enzyme doses (6.7–20.2 µg/mL) were added to the enzyme assay system supplemented with individual metal ions, and enzyme activity was quantified. Concentration ranges of individual metal ions were as follows: 0–8 mM for $AgNO_3$, 0–0.6 mM for $Hg(NO_3)_2$, 0–100 mM for Li_2SO_4 and 0–150 mM for $Pb(NO_3)_2$.

To determine the effect of the inhibitive metal ions on the catalytic constants of laccase, the initial laccase catalytic oxidation rates (v) at various guaiacol concentrations (0.08, 0.12, 0.2, 0.4 and 1.0 mM) was measured in the presence of fixed concentrations of individual metal ions. The double-reciprocal plots of v *versus* substrate concentrations were drawn to determine the inhibition types. The values of K_I, inhibitory constants on free laccase, and K_{IS}, inhibitory constants on the substrate-enzyme complex, for each inhibitor were respectively obtained from the plots of the slopes and vertical intercepts of the Lineweaver–Burk curves *versus* concentrations of each metal cation.

2.6. Inhibition kinetics of Hg^{2+}on laccase

The progress-of-substrate-reaction method was used to study the inhibition kinetics of laccase by Hg^{2+} [25]. The substrate reaction progress curve was analyzed to obtain the reaction rate constants. The reversible reaction can be expressed as the following scheme:



where E, S, I and P denote the enzyme, substrate (guaiacol), inhibitor (Hg^{2+}) and product, respectively; ES, EI and ESI are the enzyme-substrate complex, enzyme-inhibitor complex and enzyme-substrate-inhibitor complex, respectively; E'I and E'IS are the inactive forms of enzyme and enzyme-substrate complex, respectively. k_{+1}, k_{-1} and k_p are the substrate reaction rate constants. k_{+0} and k_{-0} are the microscopic rate constants for forward and reverse inhibition on the enzyme, respectively. KI is the inhibition constant of Hg^{2+} as noncompetitive inhibitor. The enzyme concentration in this assay was 0.16 µM (8.9 µg/mL), meaning that $[S] >> [E_0]$, $[I] >> [E_0]$ and that the inhibition reactions are relatively slow compared with the setup of the steady-state of the enzymatic reaction [26]:

$$[E] = \frac{K_I K_m}{(K_I+[I])(K_m+[S])}[E_T]$$

$$[ES] = \frac{K_I[S]}{(K_I+[I])(K_m+[S])}[E_T]$$

$$[EI] = \frac{K_m[I]}{(K_I+[I])(K_m+[S])}[E_T]$$

$$[EIS] = \frac{[I][S]}{(K_I+[I])(K_m+[S])}[E_T]$$

where $[E_T] = [E] + [ES] + [EI] + [EIS]$ and $[E_T^*] = [E'I] + [E'IS]$. E_T denotes the active enzyme in all forms, E_T^* denotes all inactive enzymes, and K_m is the Michaeli–Menten constant. Since $[E_0] = [E_T] + [E_T^*]$, the rate of E_T decrease can be given as follows:

$$-\frac{d[E_T]}{dt} = \frac{d[E_T^*]}{dt}$$

$$= k_{+0}([EI] + [EIS]) - k_{-0}[E_T^*]$$

$$= A[E_T] - B[E_0]$$

$$\text{where } A = \frac{k_{+0}[I]}{KI + [I]} + k_{-0} \quad (1)$$

$$\text{and } B = k_{-0} \quad (2)$$

A and B are the apparent rate constants of inactivation and reactivation, respectively. The catalytic product formation can be written as

$$[P]_t = \frac{k_{-0}v}{A}t + \frac{(A-k_{-0})v}{A^2}(1-e^{-At}) \quad (3)$$