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Kinetic Evidence for the Interactive Inhibition of Laccase from *Trametes versicolor* by pH and Chloride

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The interactive inhibitory effects of pH and chloride on the catalysis of laccase from *Trametes versicolor* were investigated by studying the alteration of inhibition characteristics of sodium chloride at different pHs for the oxidation of 2,2′-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid). At pH 3.0, the addition of sodium chloride (50 mM) brought about a 40-fold increase in K_m^{app} and a 4-fold decrease in V_{max}^{app}. As the pH increased to 7.0, the inhibitory effects of sodium chloride became significantly weakened. The mixed-inhibition mechanism was successfully used to quantitatively estimate the competitive and uncompetitive inhibition strengths by chloride at two different pHs (pH 3.0 and 6.0). At pH 3.0, the competitive inhibition constant, K_i, was 0.35 mM, whereas the uncompetitive inhibition constant, K_i', was 18.1 mM, indicating that the major cause of the laccase inhibition by chloride is due to the competitive inhibition step. At a higher pH of 6.0, where the inhibition of the laccase by hydroxide ions takes effect, the inhibition of the laccase by chloride diminished to a great extent, showing increased values of both the competitive inhibition constant (K_i = 23.7 mM) and uncompetitive inhibition constant (K_i' = 324 mM). These kinetic results evidenced that the hydroxide anion and chloride share a common mechanism to inhibit the laccase activity.

Keywords: Chloride, inhibition, laccase, pH effects, *T. versicolor*

Introduction

Laccases (benzenediol:oxygen oxidoreductase, E.C. 1.10.3.2) catalyze the oxidation of various phenolic compounds, aromatic amines, or nonphenolic compounds to the corresponding cationic radicals, by one electron using molecular oxygen, which is concomitantly reduced to water by four electrons. They are multicopper-containing extracellular enzymes of most of the white-rot fungi and involved in the *in vivo* function of the fungi to degrade lignin [10, 12, 21].

Until now, studies have been actively performed to elucidate the structures of laccases and the catalytic mechanisms of the enzymes for the oxidation of the substrates and the reduction of the molecular oxygen [3, 4, 13, 17, 19]. The active site of laccases is known to be conserved among various isoenzymes and contains four copper atoms in the 2^+ oxidation state, which are designated as T1 copper, T2 copper, and T3 coppers. The four one-electron oxidations of the substrates take place at the T1 copper site, followed by the subsequent transfer of the extracted electrons to the trinuclear T2/T3 copper site where an oxygen molecule is reduced to water by four electrons. Various laccases have been found from plants, insects, bacteria, and fungi. Among the sources of laccases, *Trametes* (which belongs to the white-rot fungi) are considered the major sources to produce laccases, which have been most comprehensively studied for their molecular properties and catalytic mechanisms [6, 18].

The catalytic capability of laccases to oxidize a variety of phenolic and nonphenolic organic compounds has been investigated for potential applications in a wide range of oxidative processes, including delignification in biopulping processes, dye or stain bleaching in the textile industry, bioremediation for effluent detoxification, and other processes [9, 11, 14–16, 20]. The enzymes have also been used to

synthesize various conducting polymers such as polyanilines and polypyrroles for the development of biosensors and biofuel cells [8].

For the efficient utilization of laccases in the various applications, the elucidation of the impact of environmental factors on the catalytic properties of laccases is essential. One of the most influential factors affecting the activity of laccases is solution pH. The optimum pH for the activity of laccases also depends on the types of sources [6]. For example, the optimal pH range is between 3 and 7 for fungal laccases and may increase to 9 for plant laccases. In general, the laccase activity decreases upon the increase of pH owing to the inhibition by the hydroxide anion, which is presumed to bind on the T2/T3 trinuclear site to interfere with the electron transfer from T1 to T2/T3 sites [5]. Most of the previous studies on the effects of pH on the catalysis of laccases, however, have been usually concerned with the apparent enzymatic activity at fixed concentrations of substrates. Such studies on the pH dependence of laccase activity under limited reaction conditions can result in an incorrect understanding of the pH effects on the intrinsic catalytic properties of the enzymes.

Another influencing factor of the laccase activity is the presence of halide ions. Various laccases have been reported to be inhibited by halides, thus hampering their applications in the decolorization and detoxification of dye effluents containing high concentrations of halide ions [5, 22, 23]. Xu [22, 23] reported the I_{50} values of halide ions such as F^-, Cl^-, and Br^-, where I_{50} was defined as the concentration of inhibitors to decrease the enzyme activity by 50%. In these studies, however, mechanistic analyses for the inhibition of the laccases by the halides have not been performed. Enaud *et al*. [5] reported that chloride ion exerts both competitive and weaker uncompetitive inhibitory effects on the activity of laccase. They also proposed that the inhibition of laccase by chloride is similar to that by hydroxide ion, by inhibiting the electron transfer from the T1 site to T2/T3 trinuclear sites. However, the interactive inhibitory roles of pH and chloride have not been kinetically explored until now. The present study reports the first kinetic evidence that pH strongly affects the inhibitory strength of chloride for the laccase-catalyzed oxidation of 2,2′-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).

Materials and Methods

Laccase from *Trametes versicolor* was purchased from Sigma (Sigma 53739) and used without further purification. The enzyme content of the product is 10 wt% as informed by the supplier. The laccase sample was previously reported to show a single band on Native PAGE (polyacrylamide gel electrophoresis) with the approximate molecular mass of 66 kDa [1]. The purity of the laccase was also confirmed by sodium dodecyl sulfate (SDS)-PAGE, as shown in Fig. 1. The molecular mass of the major component of the denatured laccase sample on SDS-PAGE was estimated to be about 66.4 kDa, which is almost the same as previously reported [1].

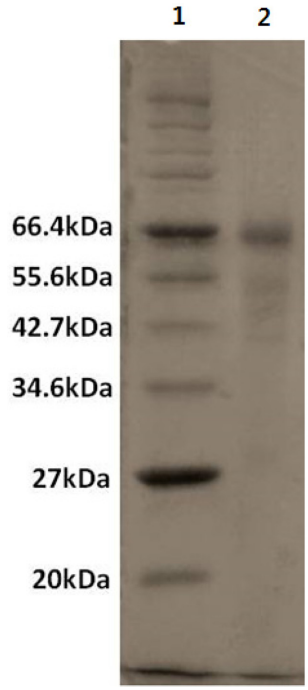

Fig. 1. SDS-polyacrylamide gel electrophoresis of laccase (Sigma 53739) on 12% polyacrylamide gel.
Proteins were stained with Coomassie brilliant blue R-250. Lanes 1 and 2 are the molecular weight standards and the laccase sample, respectively.

Laccase-catalyzed oxidation of ABTS was performed on a spectrophotometer as follows. A 1 ml citrate-phosphate buffer (20 mM, pH 2.6~7.0) containing 0.05 μg of laccase component and different concentrations of ABTS (10 μM~6 mM) was placed in a 1 cm quartz spectrophotometer cuvette. The formation of the oxidation products of ABTS was measured by monitoring the increase in absorbance at 420 nm for 1 min at 25°C. The initial rates of the activity were obtained from the asymptotic linear lines near time 0 on the absorbance versus time curves. All the aqueous buffers were saturated with air by air bubbling prior to the reaction. An extinction coefficient of 36,000 M^{-1} cm^{-1} at 420 nm was employed for the oxidation product of ABTS [7]. The two apparent kinetic parameters, V_{max}^{app} and K_m^{app}, were determined by linear fitting of the experimental data to the Lineweaver-Burk plots.

Results and Discussion

For the initial assessment of the combined inhibitory effects of chloride and pH, the oxidation of ABTS by laccase was performed without chloride or in the presence of 50 mM chloride in the pH range of 2.6 ~ 7.0 and then the apparent kinetic parameters, V_{max}^{app} and K_m^{app}, were calculated and compared (in Fig. 2). In the absence of chloride in the reaction mixtures, V_{max}^{app} decreased continuously from 17.5 μM/min to 0.07 μM/min as the pH increased from 2.6 to 7.0. The K_m^{app}, however, increased steadily up to pH 5.0 and then decreased smoothly to pH 7.0. Upon the addition of 50 mM chloride, which is within the range of I_{50} for laccases [20], V_{max}^{app} was reduced up to 3.5-fold in acidic solutions. As the pH increased to 7, the inhibition of V_{max}^{app} by chloride was diminished and became negligible at pHs 6.0 and 7.0. The inhibitory effect of chloride on K_m^{app} was most pronounced in acidic solutions. At pH 2.6, K_m^{app} increased more than 100-fold. Similar to the case of V_{max}^{app}, the inhibitory effect of chloride on K_m^{app} was weakened significantly as the pH increased.

Two pHs (pH 3 and 6) were selected for further analyses of the combined inhibitory effects of chloride and pH on the kinetics of the laccase. In Figs. 3 and 4, the Michaelis-Menten plots and Lineweaver-Burk double reciprocal linear plots for the oxidation of ABTS are shown at pH 3.0 and 6.0, respectively. The concentration of sodium chloride was varied from 0 to 50 mM. The Michaelis-Menten plots revealed that the increased chloride concentration decreased the maximum oxidation rate of ABTS pronouncedly at pH 3.0, but affected little at pH 6.0. The Lineweaver-Burk plots clearly show that the mixed-inhibition mechanism shown in Fig. 5 can describe the inhibition of the laccase by chloride at both pHs.

According to the mixed-inhibition scheme shown in Fig. 5, the oxidation rate of ABTS (v) is represented by the

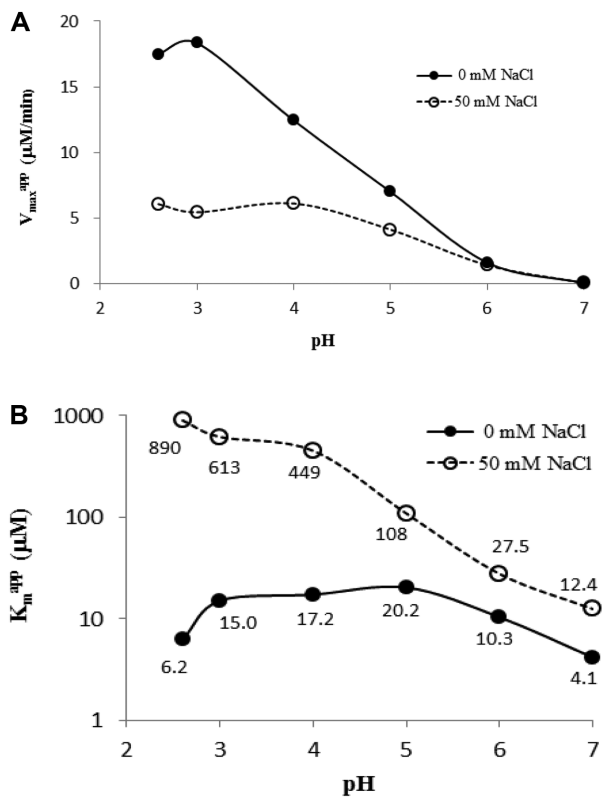

Fig. 2. The pH dependences of the two apparent kinetic parameters, V_{max}^{app} (**A**) and K_m^{app} (**B**), of *Trametes versicolor* laccase for the oxidation of ABTS in the absence or in the presence of 50 mM NaCl.

Numbers are the K_m^{app} (μM) values at each different pH.

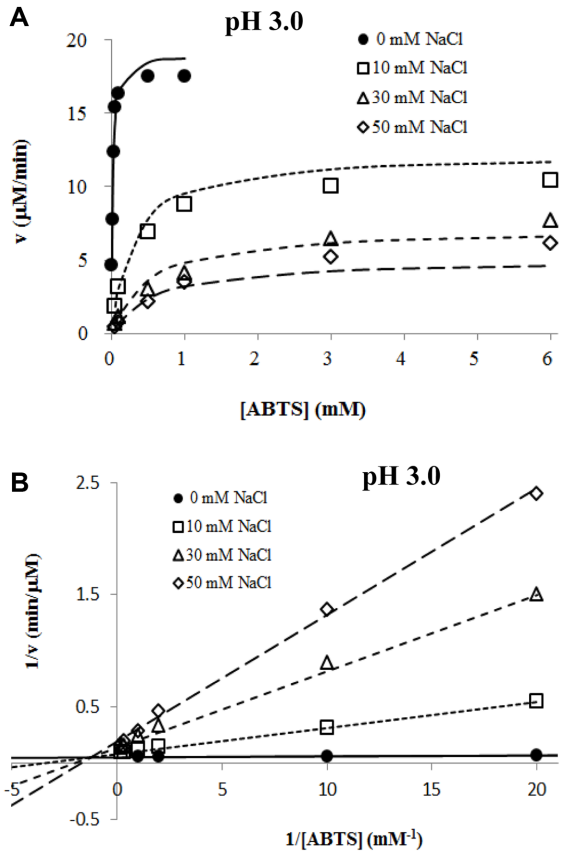

Fig. 3. Michaelis-Menten plots (**A**) and the corresponding Lineweaver-Burk plots (**B**) of the activity of laccase from *Trametes versicolor* at pH 3.0.

Lines were drawn from Eq. (1) with V_{max} = 19 μM/min, K_m = 15 μM, K_i = 0.35 mM, and K_i' = 18.1 mM.

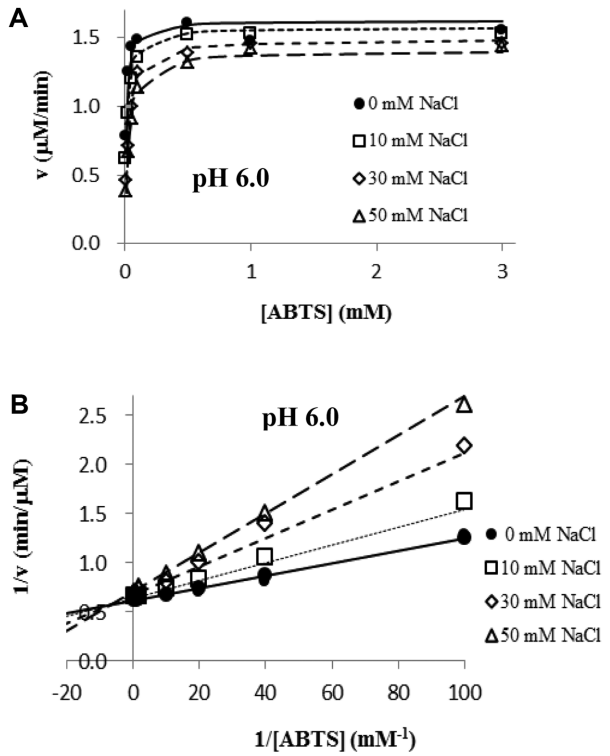

Fig. 4. Michaelis-Menten plots (**A**) and the corresponding Lineweaver-Burk plots (**B**) of the activity of laccase from *Trametes versicolor* at pH 6.0.

Lines were drawn from Eq. (1) with V_{max} = 1.63 μM/min, K_m = 10.3 μM, K_i = 23.7 mM, and K_i' = 324 mM.

following equation:

$$v = \frac{V_{max}[S]}{[S](1 + [I]/K_i') + K_m(1 + [I]/K_i)} \quad (1)$$

where V_{max} and K_m are the kinetic parameters in the absence of the inhibitor, chloride [2]. The two inhibition constants, K_i and K_i', in Eq. (1) are competitive and uncompetitive inhibition constants, respectively. From Eq. (1), the following linear equations between the apparent kinetic parameters and the inhibitor concentration are derived.

$$1/V_{max}^{app} = (1+ [I]/ K_i')/V_{max} \quad (2)$$

$$K_m^{app} / V_{max}^{app} = (1+ [I]/ K_i) K_m/V_{max} \quad (3)$$

The two inhibition constants, K_i and K_i', can be determined from the linear Eqs. (2) and (3). As shown in Fig. 6 for the apparent kinetic parameters *vs.* [NaCl] at pH 3.0, the chloride affected K_m^{app} more significantly than V_{max}^{app}. As [NaCl] increased from 0 to 50 mM, V_{max}^{app} decreased 4-fold,

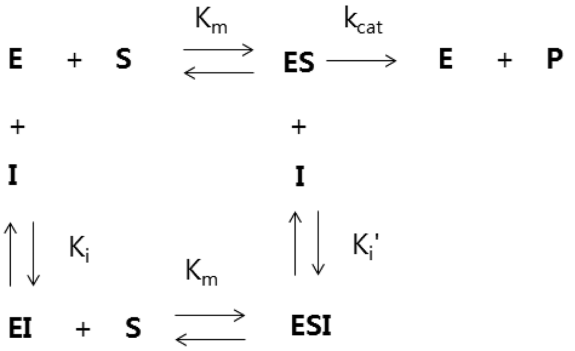

Fig. 5. Schematic mechanism for the mixed inhibition of laccase by chloride.

Table 1. Effects of ionic strength on the kinetic parameters for the oxidation of ABTS by laccase from *Trametes versicolor*.

pH	Buffer	V_{max}^{app} (μM/min)	K_m^{app} (μM)	V_{max}^{app} / K_m^{app} (min^{-1})
3	100 mM	18.3	16.3	1.12
3	20 mM	19.0	15.0	1.27
6	100 mM	1.60	9.5	0.17
6	20 mM	1.63	10.3	0.16

Buffers were citrate-phosphate buffers.

while K_m^{app} increased almost 40-fold. The values of K_i and K_i' were estimated to be 0.35 mM and 18.1 mM, respectively, indicating that the inhibition of the laccase activity by chloride is mainly due to the competitive inhibition component. Enaud *et al*. [5] also reported that the competitive inhibition is dominant over the uncompetitive inhibition for another laccase purified from *T. versicolor*. However, the effects of pH change on the inhibition mechanism of the laccases by NaCl have not been reported previously.

As shown in Fig. 7, when the solution pH was increased from 3.0 to 6.0, the effects of chloride on the kinetic parameters were altered: the increase in the chloride concentration slightly reduced V_{max}^{app} but increased K_m^{app} only about 3-fold. Comparing the results at pH 6.0 with those at pH 3.0, it becomes obvious that the inhibitory effects of chloride is weakened to a great extent as pH is increased. At pH 6.0, the two inhibition constants calculated from Eqs. (2) and (3) increased significantly (K_i = 23.7 mM, K_i' = 324 mM) indicating that the inhibitory strength of NaCl decreased.

As listed in Table 1, the effects of increased concentration of the buffers from 20 mM to 100 mM on the two apparent kinetic parameters at pH 3.0 and 6.0 are negligible, indicating

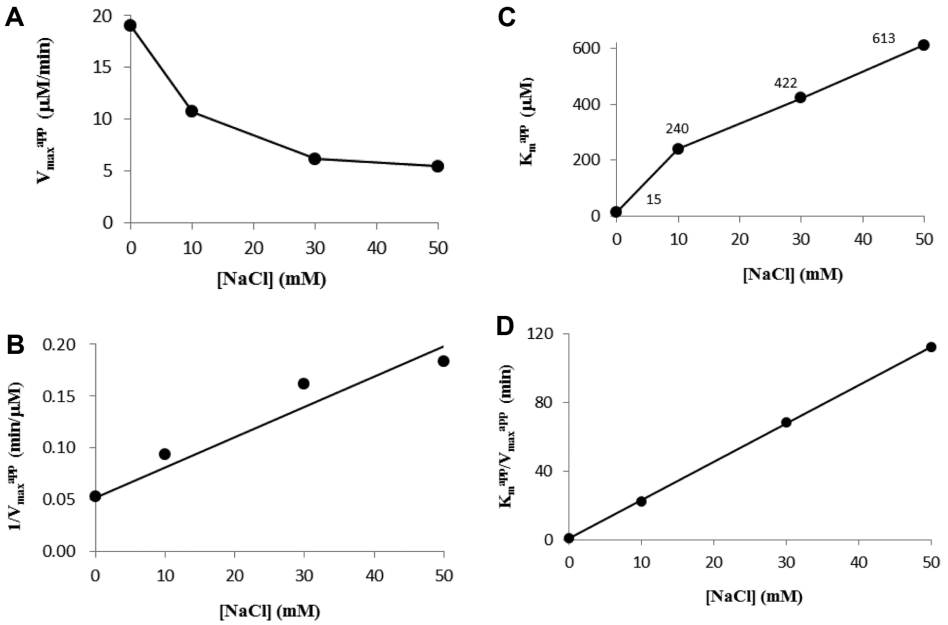

Fig. 6. Correlations of the kinetic parameters to NaCl concentration for the oxidation of ABTS by *Trametes versicolor* laccase at pH 3.0.

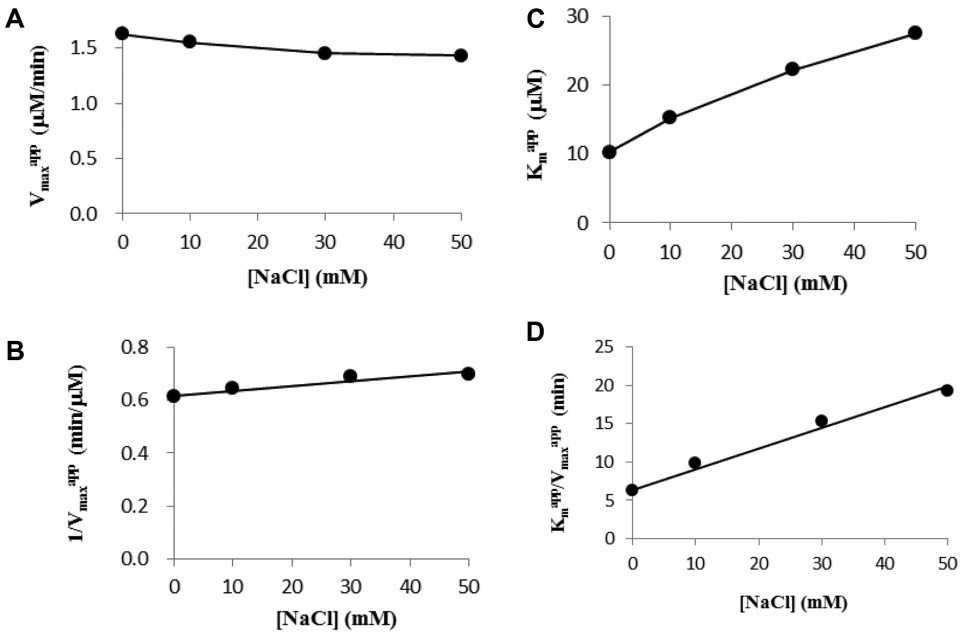

Fig. 7. Correlations of the kinetic parameters to NaCl concentration for the oxidation of ABTS by *Trametes versicolor* laccase at pH 6.0.

that the variation of the kinetic parameters upon the addition of NaCl are not due to the increased ionic strength.

In conclusion, two inhibitory factors for laccases, hydroxide anion and halide, were found to interact so that the inhibition strength by chloride is significantly weakened at higher pH. The competitive and uncompetitive inhibition constants were significantly increased at higher pH, suggesting that the inhibitions of laccase by the hydroxide anion and halide share a common mechanism. Therefore, when laccases are to be used for applications in the

presence of halide ions, an adjustment of the pH could alleviate the inhibition of the enzymes by the halides.

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