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STUDY AND ANALYTICAL APPLICATION OF RARE EARTH INHIBITION OF LACCASE

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Summary—The effect of metal ions on the laccase-catalyzed redox reaction of 5,6-dibromo-2,3-dicyanohydroquinone to 5,6-dibromo-2,3-dicyanosemiquinone was studied. The results show that rare earth ions strongly inhibited the reaction, while most of the common elements such as Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+}, *etc.* had no inhibition on the reaction. Further experimental results reveal that the inhibition of lanthanum (III) on laccase activity was of the comparative type and the degree of inhibition of La^{3+} on laccase activity was proportional to the concentration of La^{3+}. Based on this, a simple, rapid and new stopped-flow enzyme-catalyzed analytical kinetic method for determination of rare earth was proposed and applied to the determination of trace amounts of rare earth in water.

Enzyme-catalyzed analytical kinetic methods have been extensively used for substrate, enzyme, inhibitor and activator analysis and specificity inherent in most enzyme systems. However, a disadvantage of enzymes also exists due to their expense. To overcome this disadvantage, two methods are usually used: first, the enzyme is immobilized to some material so as to make use of the enzyme repeatedly;[1] on the other hand, the stopped-flow technique can be used to decrease the consumption of enzyme in the reaction.[2]

Laccase (EC1. 10. 3. 2)[15] is a multi copper-containing oxidase, it can catalyze the four-electron reduction of molecular oxygen by 5,6-dibromo-2,3-dicyanohydroquinone ($DDQH_2$) and the product is a semiquinone (DDSQ) radical anion, the process is shown as equation (1):

$$4DDQH_2 + 2O_2 \xrightarrow{\text{laccase}} \cdots \rightarrow 4DDSQ + 4H_2O \quad (1)$$

A mechanism of the laccase-catalyzed reaction with various substrates has been reported.[3–5] But studies of the effect of inorganic ions on the activity of laccase have been rare. In this paper, rare earth ions were found to be strong inhibitors of the enzyme-catalyzed reaction and further experimental results revealed the inhibition of lanthanum (III) on laccase was of the competitive type.

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Laccase has been used for the determination of catechols,[6] glucose,[7,8] and activities of other enzymes.[9–13] However, the determination of inhibitors for laccase has not been reported before; therefore, another purpose of this work is to establish a simple method for the determination of inhibitors of the laccase-catalyzed reaction. The stopped-flow technique was used and the method was applied to analytical chemistry.

EXPERIMENTAL

Apparatus

Spectrophotometric measurements were made with 1-cm light path silica cells in UV-240 spectrophotometer (Shimadzu, Japan) with a water bath thermostat controller to keep the temperature of reagents and reaction at $30 \pm 0.1°$.

Reagents

Laccase (EC1. 10. 3. 2) was prepared by extracting and purifying from Rhus Lacquer from Zhuxi, Hubei as the method proposed by B. Rainhammar;[14] the activity originally measured was 1.1 U/ml.

5,6-Dibromo-2,3-dicyanohydroquinone ($DDQH_2$) of $1.0 \times 10^{-2}M$ (obtained from Mr. Wang Guangfei) was prepared by dissolution of 33.7 mg of $DDQH_2$ in ethanol without water.

Rare earth standard solutions were prepared by dissolution of spectroscopic pure oxides with concentrated hydrochloridic acid to obtain 1 mg/ml stock solutions, the operating solutions could be prepared by dilution as required with doubly distilled water.

The HAc–NaAc buffer solution and other metal ions were prepared by usual methods.

The water used in all experiments was doubly distilled water.

Procedure

The HAc–NaAc (pH 4.50) buffer solution was preincubated at $30 \pm 0.1°$ for 30 min. Two solutions, A and B, were prepared to fill the two 3-ml tubes. Solution A contained 3 μl of laccase and the buffer solution to a final volume of 1.5 ml. Solution B contained 40 μl of $DDQH_2$, a certain volume of sample and the buffer solution to a final volume of 1.5 ml. After the 1.5 ml syringes had been filled with the corresponding solutions from the tubes, 1.5 ml of each solution was mixed quickly in the cell which was replaced in the spectrophotometer with the doubly distilled water as reference solution. The reaction was monitored at 389 nm by recording the decrease of the absorbance of substrate as a function of time while keeping the system at $30 \pm 0.1°$. The absorbance was printed every 5 sec and processed by linear regression with a microcomputer provided with a program for application of the initial rate method. The reaction rate was determined in 1 min after the solutions had been mixed for 1 min.

RESULTS AND DISCUSSION

Effect of individual metal ion on laccase activity

Our initial work was aimed at finding a strong inhibitor for the laccase-catalyzed reaction and then establishing a trace analytical method for it, thus the experiments were carried out with very low concentrations of the individual metal ions. The degree of inhibition was given by the relation as below:

$$I(\%) = \frac{V_0 - V_i}{V_0} \times 100 \qquad (2)$$

where V_0 was the rate of the reaction under the same conditions with no inhibitor present and V_i is the rate when an inhibitor is present. The results of the effect of various ions on the laccase-catalyzed reaction is shown in Table 1. As can be seen, most of the common metal ions at low concentration hardly influence the catalysis activity of laccase, while rare earth ions such as Y^{3+}, Sc^{3+}, La^{3+}, Lu^{3+}, *etc.*, strongly inhibit the catalysis activity of laccase.

Absorption spectra and the type of inhibition of lanthanum (III) on laccase

Figure 1(a) showed a time sequence of the absorption spectra of the laccase-catalyzed reaction. The substrate ($DDQH_2$) absorbed strongly at 389 nm. In the absence of La^{3+}, as

Table 1. Effect of various metal ions on laccase

Metal Ion	Molar concn.	Inhibition (%)	Metal Ion	Molar concn.	Inhibition (%)
Be^{2+}	3.7×10^{-5}	0.04	La^{3+}	2.3×10^{-6}	54.41
	3.7×10^{-4}	0.20		2.3×10^{-5}	80.0
Ca^{2+}	8.3×10^{-6}	0	Y^{3+}	3.7×10^{-6}	40.02
	8.3×10^{-5}	0.10		3.7×10^{-5}	51.20
Ba^{2+}	2.4×10^{-6}	0.92	Sc^{3+}	7.3×10^{-6}	54.01
	2.4×10^{-5}	1.00		7.3×10^{-5}	70.23
Al^{3+}	5.0×10^{-5}	0.59	Lu^{3+}	1.9×10^{-6}	40.26
	5.0×10^{-4}	2.00		1.9×10^{-5}	59.36
Ga^{3+}	1.4×10^{-6}	1.21	Ti^{4+}	6.9×10^{-6}	0.27
	1.4×10^{-5}	2.00		6.9×10^{-5}	0.51
Pb^{2+}	1.6×10^{-6}	0	V^{5+}	6.5×10^{-6}	0.80
	1.6×10^{-5}	0.004		6.5×10^{-5}	1.00
Se^{4+}	4.2×10^{-6}	−0.34*	Cr^{6+}	6.3×10^{-6}	−1.18
	4.2×10^{-5}	−0.60		6.3×10^{-5}	−3.30
Cu^{2+}	5.2×10^{-6}	0.028	Mn^{2+}	6.0×10^{-6}	0.039
	5.2×10^{-5}	0.04		6.0×10^{-5}	0.2
Cd^{2+}	2.9×10^{-6}	0.71	Fe^{3+}	5.9×10^{-6}	0.92
	2.9×10^{-5}	0.90		5.9×10^{-5}	2.0
Zn^{2+}	5.0×10^{-6}	0.48	Co^{2+}	5.6×10^{-6}	1.38
	5.0×10^{-5}	0.60		5.6×10^{-5}	2.5
Hg^{2+}	1.6×10^{-6}	0.34	Ru^{3+}	1.0×10^{-6}	0.42
	1.6×10^{-5}	0.60		1.0×10^{-5}	2.11

*" − " stands for activative effect, it may be caused from the oxidation of the substrate by the metal ions.

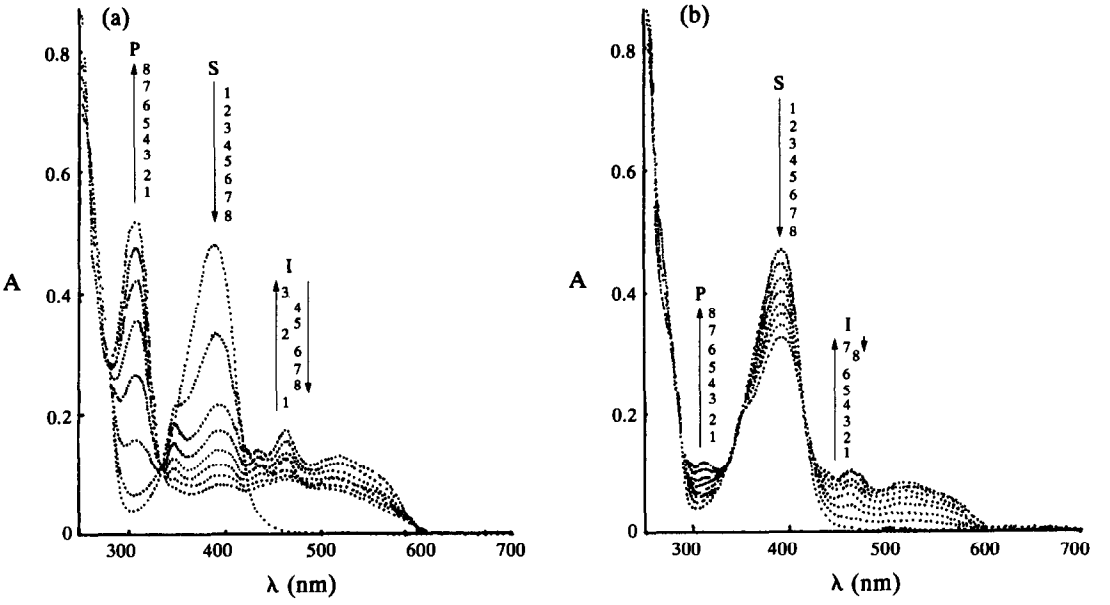

Fig. 1. Time sequence of the absorption spectra of the laccase-catalyzed reaction (a) in the absence of inhibitor and (b) in the presence of 0.1 ppm of lanthanum (III) in 0.2*M* HAc–NaAc (pH 4.50) buffer solution at 30 ± 0.1°. laccase: 3 μl, $DDQH_2$ concentration: $1.33 \times 10^{-4}M$, spectra scan speed: 2000 nm/min, time between scans: 3 min, "↑↓" indicates the changing direction of absorption curve, S: substrate, I: intermediate, P: product, 1, 2, 3, 4, 5, 6, 7 and 8 are reaction curve numbers.

the reaction was initiated, the absorption peak of substrate decreased quickly from no. 1 to no. 8; the wide absorption band (between 440 and 600 nm) of intermediate increased quickly from no. 1, through no. 2, to the top curve no. 3 at first, and then decreased slowly from no. 3 to no. 8. There was an isosbestic point at 420 nm between the substrate peak and the intermediate absorption band during the reaction from no. 1 to no. 3; the sharp absorption peak (at 310 nm) of product increased quickly from no. 1 to no. 8, there was an isosbestic point at 338 nm between the product peak and the substrate peak. In the presence of La^{3+}, the reaction process and the peak shape were not altered, but the rate of the reaction was much slower than the case in the absence of La^{3+}, as shown in Fig. 1(b).

To reveal the inhibition mechanism of lanthanum (III) on laccase, further experiments were run with various concentration of substrate[s]: 1.67×10^{-4}, 2.00×10^{-4}, 2.33×10^{-4}, 2.67×10^{-4}, 3.00×10^{-4}, $3.33 \times 10^{-4}M$, respectively, the same experiments were done with addition of 0.2, 0.5, 0.8 μg of La^{3+}, and the initial reaction rate (v) were obtained from each reaction curve. By the method of Lineweaver-Burke, the plots of $1/v$ *vs.* $1/[s]$ were shown in Fig. 2, the Michaelis-Menten constant K_m and the maximum reaction rate V_{max} were obtained from the plots, the results were shown in Table 2.

The results from Fig. 2 and Table 2 show that the K_m value increased with larger amounts of inhibitor, while the maximum reaction rate was kept the same. This revealed that the inhibition

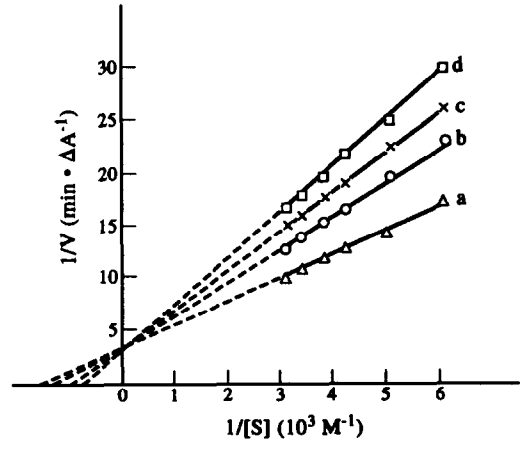

Fig. 2. Lineweaver-Burke plots for inhibition in the presence of various amounts of La^{3+}: a 0, b 0.2, c 0.5, d 0.8 μg.

Table 2. K_m and V_{max} in the presence of different amounts of La^{3+}

Added La^{3+} (μg)	0	0.20	0.50	0.80
K_m ($10^{-4}M$)	6.25	8.69	10.63	13.33
V_{max} (ΔA/min)	0.278	0.278	0.278	0.278

Table 3. Determination of rare earth and test of addition and recovery in water

Sample No.	Measured (μg)	Added (μg)	Measured (μg)	Recovery (%)
1	0.003	0.30	0.298	98.3
2	0.004	0.30	0.349	115.0
3	0.002	0.30	0.375	124.3
4	0.002	0.50	0.503	100.2
5	0.004	0.50	0.450	89.2
6	0.002	0.50	0.503	100.2

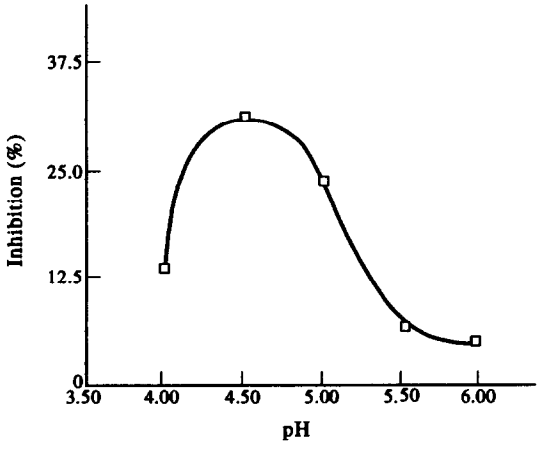

Fig. 3. Effect of pH on the degree of inhibition of La^{3+} on laccase activity.

of lanthanum (III) on laccase activity was of the competitive type. The relation between the degree of inhibition and the inhibitor concentration is linear at low substrate and inhibitor concentration.

Stopped-flow enzyme-catalyzed kinetic determination of rare earth

Optimization of variables. The optimal pH of the determination was selected according to the maximum degree of inhibition at various pH of HAc–NaAc buffer solution, as shown in Fig. 3, A pH 4.50 buffer media was chosen as optimal pH. The optimal volume of substrate was chosen at 40 μl as the similar method.

The temperature was chosen at $30 \pm 0.1°$ and the volume of laccase was 3 μl for experimental convenience.

Analytical characteristics of the method. It was found that the degree of inhibition of lanthanum (III) on the laccase-catalyzed reaction was linear with the concentration of inhibitor between 0.033 and 0.200 ppm, the regression equation was: $I(\%) = (213.1 \pm 3.2)C_{La^{3+}} + (0.653 \pm 0.099)$ ($C_{La^{3+}}$ was concentration of La^{3+}, unit is μg/ml) with a standard error of 0.232 and a detection limit of 0.033 ppm, relative standard deviation was 5.3%, which was obtained from 11 times runs in parallel in the presence of 0.5 μg of La^{3+}.

Application for determination of rare earth. Sample was prepared by concentrating 500 ml water to 25 ml. A 5-μl sample was taken to run the determination of rare earth and test of the addition and recovery of rare earth. Lanthanum (III) was chosen as the standard rare earth ion. The results were shown in Table 3.

CONCLUSIONS

The study carried out in this work indicates that (1) the stopped-flow technique is a very useful sampling tool for a study of enzyme-catalyzed reactions due to speed of analysis and small consumption of catalyst; (2) rare earth ions are found to be strong inhibitors for the laccase-catalyzed reaction while most of the common ions are not; (3) inhibition of lanthanum (III) on laccase activity was of the competitive type; (4) the inhibition of rare earth ions on laccase are applied to the determination of trace amounts of rare earth ions in water with good results.

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