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STUDIES OF THE REACTION BETWEEN LACCASE AND HYDROQUINONE BY MICROCALORIMETRY *

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ABSTRACT

The Michaelis constant and the total molar enthalpy of the reaction between the *Rhus vernicifera* laccase and hydroquinone have been determined at 298.15 K by microcalorimetry to be $K_m = 0.21$ M and $\Delta_r H_m = -70.28 \pm 0.13$ kJ mol^{-1}, respectively.

The laccase activity at different laccase concentrations was calculated.

INTRODUCTION

At the present time several methods are available for kinetic studies of the reaction between laccase and different substrates, but no study of the reaction of *Rhus vernicifera* laccase by microcalorimetry has been reported.

The oxidase laccase belongs to a very small group of enzymes, including cytochrome *c* oxidase, ceruloplasmin and ascorbate oxidase, which are known to reduce O_2 and H_2O and to contain Cu. Like ceruloplasmin and ascorbate oxidase, laccase contains three different types of bound Cu which may be distinguished by light and EPR spectroscopy. Another feature common to the three enzymes is a poor selectivity for oxidizable substrate. It seems reasonable to believe that investigation of the mechanism of the laccase reaction may give clues to the function of Cu. By anaerobic reduction of the enzyme and subsequent re-oxidation by O_2 it has been shown that a Cu^{2+}–Cu^{+} cycle is likely to be a part of the catalytic mechanism [1].

In the present report the measurement of the properties of the laccase-catalyzed reaction by the heat-conduction calorimetric technique are described. The reaction investigated is a laccase-catalyzed oxidation reaction of

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hydroquinone (H_2Q) under anaerobic conditions and in the presence of phosphate buffer at pH 7.0

$$\text{L-Cu}^{2+} + \text{H}_2\text{Q} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{L-Cu}^{+}\text{–H}_2\text{Q} \xrightarrow{k_2} \text{L-Cu}^{+} + \text{SQ} \quad (1)$$

where SQ is semiquinone

EXPERIMENTAL

Reagents

Doubly distilled water was used throughout. Analytical grade sodium orthophosphate (dimetallic) and sodium orthophosphate (monometallic) were used for the preparation of the buffer solution. The pH of the solution was adjusted to 7.0 by means of the pH meter, mixing slowly both 0.1 mol l^{-1} phosphate solutions.

The solid laccase which was extracted from China lacquer of *Rhus vernicifera* was obtained from the Institute of Resource Chemistry of Wuhan University and was purified. A laccase solution of 1.0 mg ml^{-1} was prepared by dissolving the solid laccase in the buffer solution and the stock solution was stored in a refrigerator.

A 0.2 mol l^{-1} substrate solution was made by dissolution of analytical grade hydroquinone in buffer solution. In order to prevent oxidation of the hydroquinone by dissolved oxygen, purified oxygen-free nitrogen was passed through the substrate solution which was then stored in the refrigerator.

All solutions were freshly prepared before each set of experiments.

Instrumentation

The heat of reaction of laccase and hydroquinone was determined at 298.15 K using a LKB-2107 batch microcalorimeter system. One of the main components of the instrument consists of two separate calorimeter cells (Fig.

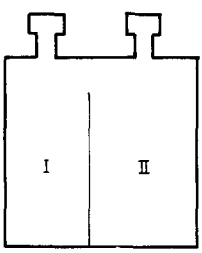

Fig. 1. The calorimeter cell.

1), one of which is the reaction cell and the other a reference cell, each cell being divided into two parts.

4 ml of the substrate solution and 2 ml of laccase solution, already separately diluted to the required concentration by buffer solution, were placed in the compartments II and I, respectively. In order to avoid the influence of the heat of mixing on the results of the measurement, the contents and their quantities in both cells were made as identical as possible except that laccase was not added to compartment I of the reference cell. When the microcalorimetry system had been equilibrated and a steady baseline obtained on the recorder, the reaction run was initiated by starting rotation of the calorimeter so as to mix the laccase and substrate solutions. The heat generated in the reaction process was recorded on a chart in the form of the recorder response; the heat capacity of the microcalorimetry system was calibrated by electrical energy.

RESULTS AND DISCUSSION

Determination of the overall molar reaction enthalpy

In order to determine the enzyme activity, the overall molar reaction enthalpy must first be assigned. Accordingly, substrate-limiting experiments at 298.15 K were made in which the laccase-catalyzed reaction of hydroquinone was allowed to go to completion. The results of the determination under different substrate concentrations and anaerobic conditions are given in Table 1. The uncertainty is "uncertainty intervals" defined as the final overall standard deviation of the mean.

Determination of enzyme activity

The "working equation" for the enthalpimetric determination of enzyme activity (EA) with pseudo-zero-order kinetics prevailing is given by [2]

$$EA = -\Delta_r H_m^{-1} q_t t^{-1} \quad (2)$$

TABLE 1

Overall molar reaction enthalpies of laccase with hydroquinone

H_2Q ($\times 10^{-6}$ mol)	$-Q_{tot}$ ($\times 10^{-3}$ J)	$-\Delta_r H_m$ (kJ mol^{-1})
0.25	17.47	69.88
0.30	21.05	70.17
0.40	28.25	70.62
0.50	35.12	70.24
0.60	42.28	70.47

Mean $\Delta_r H_m = -70.28 \pm 0.13$ kJ mol^{-1}

where $q_t t^{-1}$ is the overall rate of heat evolution from the reaction and $\Delta_r H_m$ is the overall molar enthalpy of the reaction given in Table 1. The minus sign represents an exothermic reaction. In the determination of enzyme activity, the substrate concentration should greatly exceed the Michaelis constant, K_m, e.g. 10 K_m. Under this condition, the Michaelis–Menten equation

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

where v is the initial rate of reaction and [S] is the substrate concentration, can be simplified to $v = V_{max}$ and the rate of reaction, represented by the rate of heat evolution, is proportional to the concentration of the enzyme. For increasing substrate concentration, however, we encountered a difficulty because of the limited solubility of hydroquinone in the buffer solution, and we therefore had to reduce the amount of enzyme participating in the reaction. A test for the required amount of hydroquinone was made by changing the amount of hydroquinone added to the cell but leaving the laccase concentration in the cell unchanged. Experiments showed that, when the lacasse concentration in the cell is equal to 0.0417 mg ml^{-1} and the hydroquinone concentration exceeds 6.7×10^{-5} mol ml^{-1}, the enzyme activity is almost invariant. This means that, under these conditions, the active sites on the lacasse molecule are fully "saturated" by substrate. Hence, in the determination of lacasse activity, we took a constant substrate concentration of 6.7×10^{-5} mol ml^{-1} in all the experiments and the lacasse concentrations were varied but were not greater than 0.0417 mg ml^{-1}.

The results of the measurement of the activities at different laccase concentrations are given in Table 2. It can be seen from Table 2 that the observed laccase activities increased linearly with increasing amounts of enzyme (H_2Q concentration constant). Thus it was concluded that the condition necessary for the utilization of the zero-order form of the Michaelis–Menten equation had been satisfied.

TABLE 2

Laccase activities

No.	H_2Q (mol cm^{-3} in cell)	Laccase (mg cm^{-3} in cell)	EA ($\times 10^{-6}$ mol min^{-1})
1	6.7×10^{-5}	0.0167	0.024
2	6.7×10^{-5}	0.0250	0.039
3	6.7×10^{-5}	0.0333	0.043
4	6.7×10^{-5}	0.0417	0.068

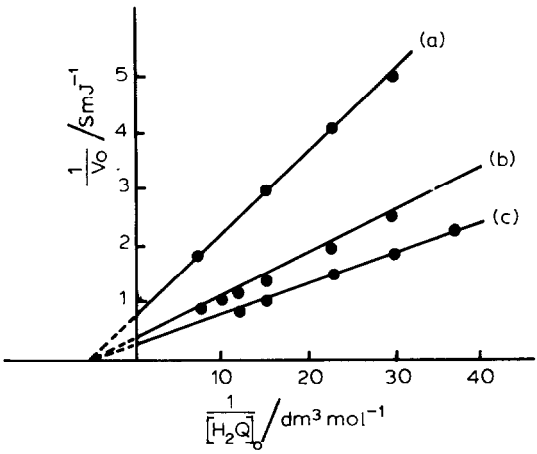

Fig. 2. Lineweaver–Burk plot of $1/V_0$ against $1/[H_2Q]_0$ at enzyme concentrations of (a) 4.17×10^{-3} mg cm^{-3}, (b) 8.33×10^{-3} mg cm^{-3}, and (c) 12.50×10^{-3} mg cm^{-3}.

Determination of the Michaelis constant

The Michaelis constant, K_m, for laccase-catalyzed reaction with hydroquinone was obtained in terms of a plot of $1/V_0$ versus $1/[S]_0$ according to the equation [3]

$$\frac{1}{V_0} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S]_0} \tag{3}$$

TABLE 3

Michaelis constant, K_m, for the reaction of laccase with hydroquinone

Laccase (mg cm^{-3} in cell)	$[H_2Q]_0$ (mol dm^{-3} in cell)	V_0 (mJ s^{-1})	K_m (mol dm^{-3})
4.17×10^{-3}	0.1333	0.56	
4.17×10^{-3}	0.0667	0.34	0.20
4.17×10^{-3}	0.0433	0.24	
4.17×10^{-3}	0.0333	0.20	
8.33×10^{-3}	0.1333	1.11	
8.33×10^{-3}	0.1000	0.95	
8.33×10^{-3}	0.0833	0.87	0.22
8.33×10^{-3}	0.0667	0.74	
8.33×10^{-3}	0.0433	0.51	
8.33×10^{-3}	0.0333	0.39	
12.50×10^{-3}	0.0833	1.19	
12.50×10^{-3}	0.0667	0.99	
12.50×10^{-3}	0.0433	0.69	0.22
12.50×10^{-3}	0.0333	0.55	
12.50×10^{-3}	0.0267	0.45	

Mean $K_m = 0.21$ mol dm^{-3}.

This is a linear equation called the Lineweaver–Burk reciprocal plot in which V_0 is the initial rate of the reaction (represented by mJ s^{-1} in the enthalpimetric determination) and $[S]_0$ is the substrate concentration before the start of the reaction. The maximum rate, V_{max}, at very large substrate concentrations and the Michaelis constant, K_m, can be calculated from the intercept of $1/V_0$ and the slope K_m/V_{max}, respectively. Figure 2 shows a plot of $1/V_0$ versus $1/[S]_0$ at different enzyme concentrations with the relative data in Table 2. It can be seen from Fig. 2 that the curves are linear and intersect the abscissa. The slope is proportional to the reciprocal of the enzyme concentration. The results calculated for constant K_m are given in Table 3. The mean value of K_m (0.21 M) is in excellent agreement with the published value (0.20 M) [4].

REFERENCES

1 T. Nakamura, Biochim. Biophys. Acta, 30 (1958) 44.
2 J.K. Grime, Analytical Solution Calorimetry, Wiley-Interscience, New York, 1985, p. 319.
3 J.K. Grime, Analytical Solution Calorimetry, Wiley-Interscience, New York, 1985, p. 359.
4 L.C. Petersen and H. Degn, Biochim. Biophys. Acta, 526 (1978) 85.