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Enthalpy change and mechanism of oxidation of *o*-phenylenediamine by hydrogen peroxide catalyzed by horseradish peroxidase

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Abstract

The hydrogen peroxide-oxidation of *o*-phenylenediamine (OPD) catalyzed by horseradish peroxidase (HRP) at 37 °C in 50 mM phosphate buffer (pH 7.0) was studied by calorimetry. The apparent molar reaction enthalpy with respect to OPD and hydrogen peroxide were $-447 \pm 8\,\mathrm{kJ\,mol^{-1}}$ and $-298 \pm 9\,\mathrm{kJ\,mol^{-1}}$, respectively. Oxidation of OPD by H_2O_2 catalyzed by HRP (1.25 nM) at pH 7.0 and 37 °C follows a ping–pong mechanism. The maximum rate V_{max} ($0.91 \pm 0.05\,\mu\mathrm{M\,s^{-1}}$), Michaelis constant for OPD $K_{m,S}$ ($51 \pm 3\,\mu\mathrm{M}$), Michaelis constant for hydrogen peroxide K_{m,H_2O_2} ($136 \pm 8\,\mu\mathrm{M}$), the catalytic constant k_{cat} ($364 \pm 18\,\mathrm{s^{-1}}$) and the second-order rate constants $k_{+1} = (2.7 \pm 0.3) \times 10^6\,\mathrm{M^{-1}\,s^{-1}}$ and $k_{+5} = (7.1 \pm 0.8) \times 10^6\,\mathrm{M^{-1}\,s^{-1}}$ were obtained by the initial rate method.

Keywords: Calorimetry; Horseradish peroxidase (HRP); Bisubstrate enzymatic reaction; Reaction enthalpy; Kinetics

1. Introduction

Peroxidases are ubiquitous heme-containing enzymes that catalyze the oxidation of a wide variety of organic and inorganic substances by hydrogen peroxide. Typical reducing substrates include aromatic phenols, phenolic acids, amines, indoles and sulphonates. Horseradish peroxidase (HRP EC 1.11.1.7) is an important heme-containing enzyme that has been studied for more than a century [1]. In recent years, new information has become available on the three-dimensional structure of the enzyme and its catalytic intermediates, mechanisms of catalysis and the function of specific amino acid residues [2–6]. The normal peroxidase cycle for HRP can be expressed by Scheme 1 [2], where E_I and E_{II} represent the oxidized states of HRP, and are called the enzyme-substrate compounds I and II, respectively, S is a reducing substrate and R is a free radical product.

In the reaction cycle of HRP, only steps I and III are rate-determining reactions [3]. The first step in the catalytic cycle is the oxidation of the Fe (III) resting enzyme by H_2O_2 to generate compound I, a high oxidation state intermediate comprising an Fe (IV) oxoferryl centre and a porphyrin-based cation radical. Generally, compound I is two oxidizing equivalents above the resting state. Step II is the first one-electron reduction step, in which a reducing substrate and compound I react to generate compound II, an Fe (IV) oxoferryl species that is one oxidizing equivalent above the resting state. In step III, the second one-electron reduction step returns compound II to the resting state of the enzyme. The rate-limiting step is the reduction of compound II to resting enzyme (k_4) [4,5].

Calorimetric methods provide thermodynamic and kinetic data on the reaction simultaneously without constraint on solvent, spectral, electrochemical and other properties of the reaction system [7–9]. In this paper, calorimetry has been used for studying the thermokinetics of HRP, a bisubstrate enzyme-catalyzed reaction, for the first time.

2. Materials and methods

HRP with a purity index $R_z \approx 3.0$ was purchased from Chinese Academy of Science, Shanghai institution of biochemistry and used without further purification. *o*-Phenylenediamine (OPD) and hydrogen peroxide (30%, v/v, solution) were of analytic grade. All the other chemical reagents used were of analytic grade. Doubly distilled water was used throughout the experiment. HRP stock solutions ($0.15\,\mathrm{mg\,mL^{-1}}$) were prepared by dissolving the enzyme in distilled water. OPD stock solu-

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$$E + H_2O_2 \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_I + H_2O \qquad \text{(Step I)}$$

$$E_I + S \xrightarrow{k_3} E_{II} + R \qquad \text{(Step II)}$$

$$E_{II} + S \xrightarrow{k_4} E + R + H_2O \qquad \text{(Step III)}$$

Scheme 1.

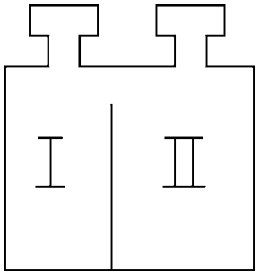


Fig. 1. The calorimeter cell.

tions (0.15 M) were prepared by dissolving OPD in distilled water. The H_2O_2 solutions (0.2 M) were prepared daily by an appropriate dilution of 30% H_2O_2 in distilled water and the concentrations were obtained by titration with potassium permanganate. The stock solutions were stored at 4 °C. The buffer was 50 mM sodium dihydrogenphosphate solution was adjusted to pH 7.0 by 0.1 mM sodium hydroxide with a pH meter (ORION Model 828 pH meter) at 37 °C. Reaction solutions were obtained by diluting the reagent stock solutions into buffer solution just before each experiment.

The heat of reaction was determined with an LKB-2107 batch calorimeter equipped with two glass vessels of about 7 mL total volume: one sample cell and one reference cell (see Fig. 1). Each vessel consisted of a chamber divided into two compartments (I and II, of about 2.5 and 4.5 mL, respectively) by an interior wall. The substrates and enzyme solutions were introduced into the two compartments separately.

In experiments, 2 mL H_2O_2 solution was put into the sample cell (I) and 4 mL of OPD solution with HRP (1.25 nM) was put into the sample cell (II). To avoid the influence of the heat effect of diluting and mixing, etc., the same concentrations and volumes of H_2O_2 solutions and OPD solutions without enzyme were added to the reference cell. When the calorimeter reached thermal equilibrium and a steady baseline was obtained, the reaction was initiated by rotating the calorimetric drum 360° clockwise and counter clockwise. The calorimetric signal was measured by a digital voltmeter (a UNI-TUT 70D model digital multi-purpose meter) that was part of a computerized recording system. Each of experiments was repeated twice.

3. Theory

3.1. Velocity equation of HRP reaction

The heat produced by a chemical reaction in a batch heat-conduction calorimeter is given by the Tian equation,

$$Q_t = \kappa a_t + \Lambda \Delta_t; \tag{1}$$

$$Q_\infty = \kappa A_\infty \tag{2}$$

where Q_t is the total heat produced to time t, Q_∞ is the total heat at time $t \to \infty$, a_t is the integral of the thermogenesis curve to time t, A_∞ is the total area enclosed by the thermogenesis curves, Δ_t is the height of the curve at time t, and κ and Λ are apparatus constants which can be obtained by an electrical energy calibration experiment as described in Ref. [10]. The apparent molar reaction enthalpy ($\Delta_r H_m$) can be obtained through Eq. (3):

$$\Delta_r H_m = \frac{Q_\infty}{n_s} = \pm\frac{\kappa A_\infty}{n_s} \tag{3}$$

where the negative value denotes an exothermic reaction and a positive value denotes an endothermic reaction and n_s is the number of moles of initial substrate. This equation only applies to single substrate enzymatic reactions or double substrate reactions where the stoichiometric coefficients of both substrates are equal. When substrates' stoichiometric coefficients are not equal, Eq. (3) changes to $\Delta_r H_{m,i} = \frac{Q_\infty}{n_{s,i}} = \pm\frac{\kappa A_\infty}{n_{s,i}}$, where i expresses substrate S_A or S_B in the double substrate enzymatic reactions, and $\Delta_r H_{m,i}$ is the apparent molar reaction enthalpy with respect to S_A or S_B.

Kinetics of the reaction was analyzed by initial rate method where product inhibition can be neglected. The initial rate of the reaction v_0 can be calculated from:

$$v_0 = -\frac{\Omega_0}{\Delta_r H_m V} \tag{4}$$

and

$$\Omega_t = \frac{\partial Q_t}{\mathrm{d}t} \tag{5}$$

in which V is the volume of the reaction system (always 6 mL in this study), Ω_t is the rate at time t and the initial rate Ω_0 is the slope of a linear-fit of Q_t versus t in the initial period of the reaction. A series data of v_0 at the different concentrations of OPD or H_2O_2 while the concentration of H_2O_2 or OPD is kept unchanged can be calculated from Eqs. (1), (4) and (5). The values of K_m for OPD and H_2O_2 and V_{max} of this enzyme-catalyzed reaction can be obtained through the double reciprocal plots of initial rate data both in the direction of OPD and in the direction of H_2O_2.

3.2. Bisubstrate enzyme kinetic mechanism

Bisubstrate enzyme-catalyzed reaction mechanisms can be divided into three types, the sequential ordered mechanism, the sequential random mechanism, and the ping–pong mechanism. The initial rate can be expressed by the following equations for sequential ordered and sequential random mechanisms [11], respectively:

$$v_i = \frac{1}{(K_A/V_{max}) \times (1/[S_A]) + (K_{iA}K_B/V_{max}) \times (1/[S_A] \times [S_B]) + (K_B/V_{max}) \times (1/[S_B]) + (1/V_{max})} \tag{6}$$

$$v_i = \frac{1}{(K_{iA}/V_{max}) \times (1/[S_A]) + (K_{iA}K_{iB}/V_{max}) \times (1/[S_A] \times [S_B]) + (K_{iB}/V_{max}) \times (1/[S_B]) + (1/V_{max})} \quad (7)$$

The initial rate of ping–pong mechanism can be expressed by Eq. (8):

$$v_i = \frac{1}{(K_A/V_{max}) \times (1/[S_A]) + (K_B/V_{max}) \times (1/[S_B]) + (1/V_{max})} \quad (8)$$

Where V_{max} is the maximum rate, K_A and K_B are the Michaelis constants for substrate S_A and S_B, the K_{iA} is the dissociation constant of the enzyme–S_A complex and K_{iB} is the dissociation constant of the enzyme–S_B complex. These parameters can be obtained from differences in slopes and intercepts of double reciprocal plots of initial rates versus the concentration of one substrate (S_A or S_B) while the concentration of the other substrate (S_B or S_A) is kept unchanged. The initial rate equations for sequential ordered and sequential random reaction mechanisms are very similar. Because there is no term containing the reciprocal concentrations of both substrates makes the formula of the ping–pong reaction mechanism much different.

Graphically, a double reciprocal plot for a bisubstrate sequential ordered or sequential random mechanism is a family of straight lines that intersect the vertical axis at different points. Furthermore, the family of straight lines of sequential ordered mechanism will intersect to the underside of the horizontal axis (see Fig. 2a) and the family of straight lines of sequential random mechanism will converge at a single point on the horizontal axis (see Fig. 2b) regardless whether S_A or S_B is the variable substrate. However, ping–pong mechanism results in a double reciprocal plot of a family of parallel lines rather than intersecting lines (see Fig. 2c) [11].

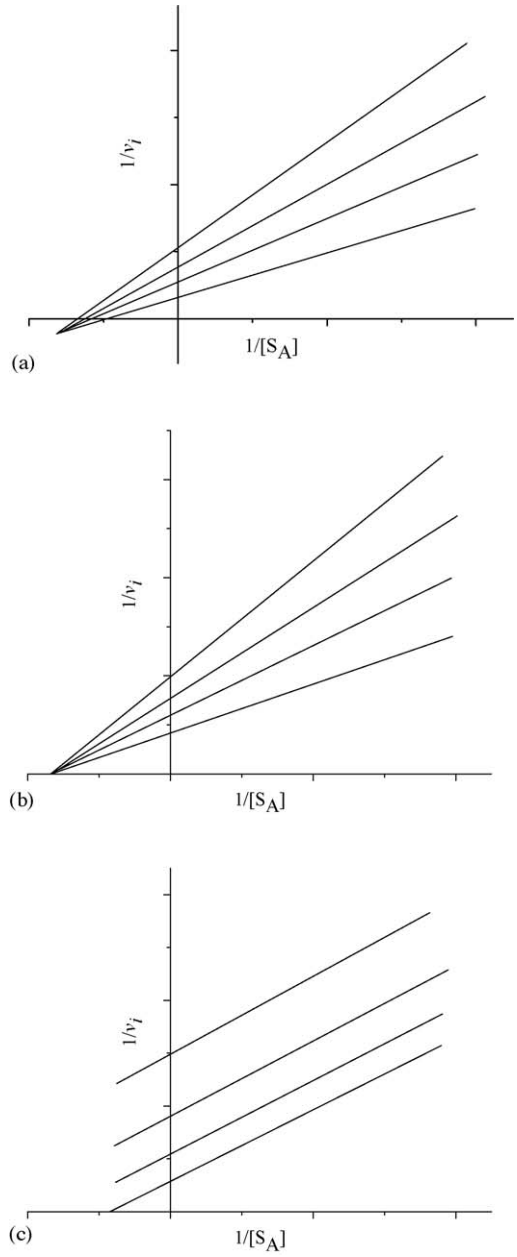


Fig. 2. Double reciprocal plots of initial rate of bisubstrate models (S_A is the variable substrate and S_B is the constant substrate). (a) A double reciprocal plot of sequential ordered mechanism. (b) A double reciprocal plot of sequential random mechanism. (c) A double reciprocal plot of ping–pong mechanism.

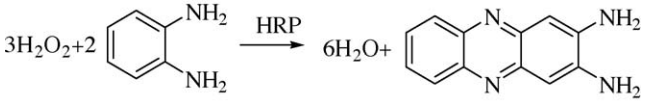
4. Results and discussion

4.1. The reaction enthalpy analysis

From Table 1, the apparent molar reaction enthalpy with respect of OPD ($\Delta_r H_{m,OPD}$) is 1.5 times of that of H_2O_2 ($\Delta_r H_{m,H_2O_2}$). This result shows that the stoichiometry is 3 mol H_2O_2/2 mol OPD. Scheme 2 expresses the overall reaction [12–14]. The apparent molar reaction enthalpy of producing 2,3-diaminophenazine in the peroxidase system is $-894 \pm 21\ kJ\ mol^{-1}$.

4.2. The reaction mechanism of this peroxidase reaction system

Fig. 3 shows a series of double reciprocal plots of initial exothermic rates of OPD oxidation as a function of the concentration of H_2O_2 with different concentrations of OPD, while Fig. 4 shows the double reciprocal plots of initial exothermic rates of OPD oxidation as a function of the concentration of OPD with different concentrations of H_2O_2. Compared with plots of three different types of kinetic mechanism (see Fig. 2), Figs. 3 and 4 indicate that the reaction of HRP-catalyzed oxidation of OPD follows ping–pong mechanism.



Scheme 2.

Table 1
The apparent molar reaction enthalpy of OPD and H_2O_2 for the oxidation of OPD by hydrogen peroxide catalyzed by HRP

n_{OPD} (μM)	$n_{H_2O_2}$ (μM)	Q_∞ (mJ)	$-\Delta_r H_m$ (kJ mol^{-1})
0.300	10.00	133.4	444.6
0.336	10.00	148.6	442.2
0.372	10.00	168.0	451.7
0.432	10.00	192.3	445.2
0.480	10.00	220.8	460.0
0.600	10.00	263.6	439.4
			447 ± 8
10.00	0.240	68.53	285.5
10.00	0.480	141.8	295.5
10.00	0.600	183.4	305.6
10.00	0.918	264.0	287.6
10.00	1.20	360.5	300.4
10.00	1.80	550.4	305.8
10.00	3.60	1107	307.6
			298 ± 9

Experimental conditions: $T = 37\,^\circ$C; 50 mM phosphate buffer; pH 7.0; [HRP] = 1.25 nM; $\kappa = 1.63$ mJ V^{-1} s^{-1}.

4.3. Kinetic data analysis

A conventional ping–pong (ordered two substrates, two products) mechanism was used for this reaction, assuming that the dissociation and transformation steps were the same as depicted in Scheme 3 [5], where E, E·H_2O_2, E_I, E_{II}, S, R, E_I·S and E_{II}·S represent HRP, the [HRP·H_2O_2] complex, compounds I and II, reducing substrate, free radical product, and the complexes of compounds I and II with S, respectively. From the Scheme 3 the total enzyme concentration, $[E]_0$ can be expressed as below:

$$[E]_0 = [E] + [E \cdot H_2O_2] + [E_I] + [E_I \cdot S] + [E_{II}] + [E_{II} \cdot S] \quad (9)$$

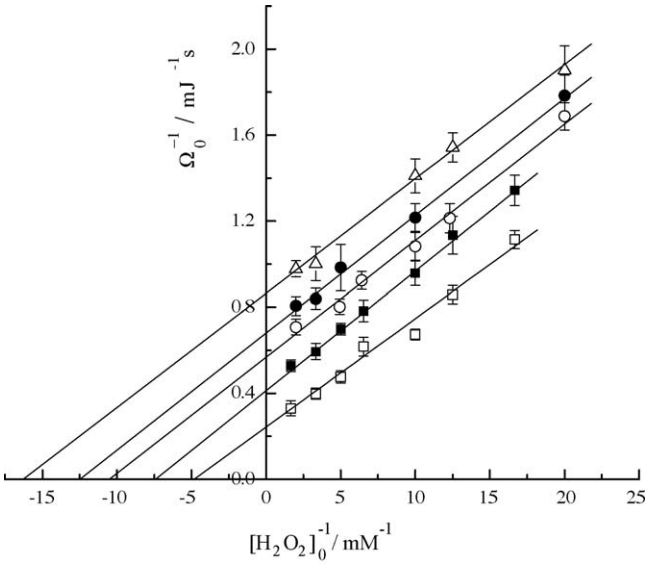


Fig. 3. Inverse plot of 1/initial exothermic rate vs. $1/[H_2O_2]_0$. Reaction mixture contained 1.25 nM HRP in 50 mM phosphate buffer, pH 7.0, 37 °C. OPD concentrations of 0.05 (△), 0.08 (●), 0.1 (◯), 1.68 (■) and 5.0 mM (□) were used.

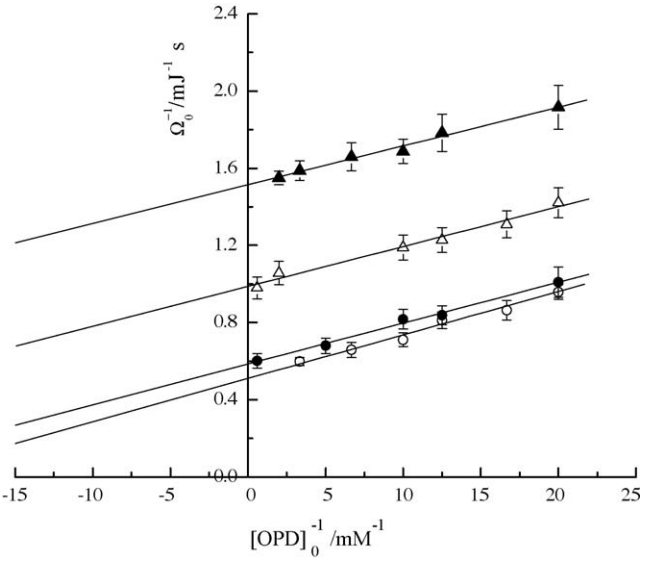


Fig. 4. Inverse plot of 1/initial exothermic rate vs. $1/[OPD]_0$. Reaction mixture contained 1.25 nM HRP in 50 mM phosphate buffer, pH 7.0, 37 °C. H_2O_2 concentrations of 0.05 (▲), 0.1 (△), 0.3 (●) and 0.5 mM (◯) were used.

The concentration of each enzyme species can be written as:

$$\frac{d[E]}{dt} = k_{-1}[E \cdot H_2O_2] - k_{+1}[E][H_2O_2]_0 + k_{+6}[E_{II} \cdot S] \quad (10)$$

$$\frac{d[E \cdot H_2O_2]}{dt} = k_{+1}[E][H_2O_2]_0 - (k_{-1} + k_{+2})[E \cdot H_2O_2] \quad (11)$$

$$\frac{d[E_I]}{dt} = k_{+2}[E \cdot H_2O_2] + k_{-3}[E_I \cdot S] - k_{-3}[E_I][S]_0 \quad (12)$$

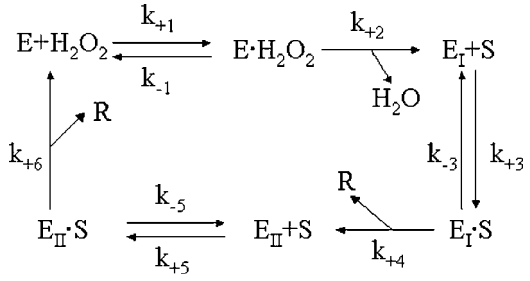
$$\frac{d[E_I \cdot S]}{dt} = -(k_{-3} + k_{+4})[E_I \cdot S] + k_{+3}[E_I][S]_0 \quad (13)$$

$$\frac{d[E_{II}]}{dt} = k_{+4}[E_I \cdot S] + k_{-5}[E_{II} \cdot S] - k_{+5}[E_{II}][S] \quad (14)$$

$$\frac{d[E_{II} \cdot S]}{dt} = k_{+5}[E_{II}][S]_0 - (k_{-5} + k_{+6})[E_{II} \cdot S] \quad (15)$$

Since the initial quantity of native enzyme will be rapidly distributed among different oxidation states and will achieve steady-state concentration. Thus,

$$\frac{d[E]}{dt} = \frac{d[E \cdot H_2O_2]}{dt} = \frac{d[E_I]}{dt} = \frac{d[E_I \cdot S]}{dt} = \frac{d[E_{II}]}{dt} = \frac{d[E_{II} \cdot S]}{dt} = 0 \quad (16)$$



Scheme 3.

Therefore, Eqs. (10)–(15) can be simplified and the concentration of each enzyme species can be expressed by following equations:

$$[E] = \frac{k_{+6}[E_{II} \cdot S]}{(k_{+1}k_{+2}/k_{-1} + k_{+2})[H_2O_2]_0} \quad (17)$$

$$[E \cdot H_2O_2] = \frac{k_{+6}[E_{II} \cdot S]}{k_{+2}} \quad (18)$$

$$[E_I] = \frac{(k_{-3} + k_{+4})k_{+6}[E_{II} \cdot S]}{k_{+3}k_{+4}[S]_0} \quad (19)$$

$$[E_I \cdot S] = \frac{k_{+6}[E_{II} \cdot S]}{k_{+4}} \quad (20)$$

$$[E_{II}] = \frac{(k_{-5} + k_{+6})[E_{II} \cdot S]}{k_{+5}[S]_0} \quad (21)$$

In the cycle of this enzymatic reaction the reduction of enzyme-substrate compound II E_{II}·S is rate-limiting step, so the initial rate of radical production v_0 can be shown as below:

$$v_0 = 2k_{+6}[E_{II} \cdot S] \quad (22)$$

Substitution of Eqs. (17)–(21) into Eqs. (9) and (22), the initial rate of radical production v_0 can be derived:

$$v_0 = \frac{2[E]_0}{\frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}} \times \frac{1}{[H_2O_2]_0} + \frac{k_{-3}+k_{+4}}{k_{+3}k_{+4}} \times \frac{1}{[S]_0} + \frac{k_{-5}+k_{+6}}{k_{+5}k_{+6}} \times \frac{1}{[S]_0} + \left(\frac{1}{k_{+2}} + \frac{1}{k_{+4}} + \frac{1}{k_{+6}}\right)} \quad (23)$$

It has been assumed that the free enzyme is limiting in order to derive the rate of radical product formation. Assuming that the initial concentrations of hydrogen peroxide and S (reducing substrate), ($[H_2O_2]_0$ and $[S]_0$), are much higher than that of the free enzyme (HRP), ($[E]_0$). Furthermore, in the mechanism shown in Scheme 3 we assume the following relationships [2–4]:

$$k_{+2} \gg k_{-1},$$

$$k_{+4} \gg k_{-3},$$

$$k_{+6} \gg k_{-5},$$

$$k_{+3} \gg k_{+5},$$

$$k_{+4} \gg k_{+2}, k_{+6}$$

Eq. (23) is simplified as:

$$v_0 = \frac{2[E]_0}{\frac{1}{k_{+1}} \times \frac{1}{[H_2O_2]_0} + \frac{1}{k_{+5}} \times \frac{1}{[S]_0} + \frac{k_{+2}+k_{+6}}{k_{+2}k_{+6}}} \quad (24)$$

Eq. (24) can be rearranged as follows:

$$v_0 = \frac{V_{max}[H_2O_2]_0[S]_0}{K_{m,S}[H_2O_2]_0 + K_{m,H_2O_2}[S]_0 + [H_2O_2]_0[S]_0} \quad (25)$$

where:

$$V_{max} = 2k_{cat}[E]_0 \quad (26)$$

$$K_{m,S} = \frac{k_{cat}}{k_{+5}} \quad (27)$$

$$K_{m,H_2O_2} = \frac{k_{cat}}{k_{+1}} \quad (28)$$

and:

$$k_{cat} = \frac{k_{+2}k_{+6}}{(k_{+2} + k_{+6})} \quad (29)$$

The V_{max} is maximum steady-state rate, $K_{m,S}$ is Michaelis constant of HRP toward OPD, and K_{m,H_2O_2} is Michaelis constant of HRP toward H_2O_2. k_{+1} is the second-order rate constant for the oxidation of HRP by H_2O_2 and k_{+5} is the second-order rate constant for the reduction of compound II by OPD. The catalytic constant k_{cat} is a function of two first-order constants (k_{+2}, k_{+6}), the heterolytic cleavage of the O–O bond of hydrogen peroxide (k_{+2}), and the electron transfer from the reducing substrate to compound II (k_{+6}).

The reciprocal plot of $1/v_0$ versus $1/[S]_0$ has the following expression:

$$\frac{1}{v_0} = \frac{K_{m,S}}{V_{max}}\frac{1}{[S]_0} + \frac{K_{m,H_2O_2}}{V_{max}}\frac{1}{[H_2O_2]_0} + \frac{1}{V_{max}} \quad (30)$$

The double reciprocal plot is a family of parallel lines and each of the lines intersects the vertical intercept. The y-axis intercept of the primary plots of $1/v_0$ versus $1/[H_2O_2]_0$ has the following expression:

$$y\text{-axis} = \frac{K_{m,H_2O_2}}{V_{max}}\frac{1}{[H_2O_2]_0} + \frac{1}{V_{max}} \quad (31)$$

The kinetic constants V_{max}, $K_{m,S}$, and K_{m,H_2O_2} for the reaction of HRP with reducing substrate can be calculated from primary and secondary plots of $1/v_0$ versus $1/[S]_0$ (Eq. (25)), and k_{cat}, k_{+1} and k_{+5} can be calculated from V_{max}, $K_{m,S}$, and K_{m,H_2O_2} using Eqs. (26)–(28).

From Eq. (4) it is known that the initial reaction rate is proportional to the initial exothermic rate. And Eqs. (30) and (31) can be rewritten as:

$$\frac{1}{\Omega_0} = \frac{K_{m,S}}{\Omega_{max}}\frac{1}{[S]_0} + \frac{K_{m,H_2O_2}}{\Omega_{max}}\frac{1}{[H_2O_2]_0} + \frac{1}{\Omega_{max}} \quad (32)$$

$$y\text{-axis} = \frac{K_{m,H_2O_2}}{\Omega_{max}}\frac{1}{[H_2O_2]_0} + \frac{1}{\Omega_{max}} \quad (33)$$

Fig. 4 shows the double reciprocal plots of Ω_0 versus $[OPD]_0$ at different concentrations of H_2O_2 and Fig. 5 shows the y-axis intercepts of all lines in Fig. 4 versus $1/[H_2O_2]_0$. From the intercept and slope of Fig. 5, the maximum rate and Michaelis constant of H_2O_2 of the reaction can be obtained. Michaelis constant of OPD, $K_{m,S}$, can be calculated from the slopes of the family of lines in Fig. 4. The maximum exothermic rate Ω_{max} for the reaction is $2.44 \pm 0.08\,mJ\,s^{-1}$ in 50 mM phosphate buffer, at pH 7.0, 37 °C. The maximum rate is calculated by Eq. (4) with $\Delta_r H_{m,OPD} = -447 \pm 8\,kJ\,mol^{-1}$. The values of the kinetic

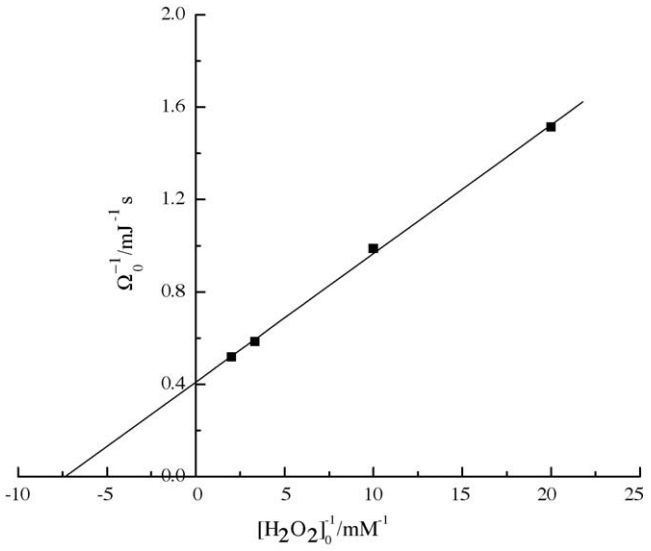


Fig. 5. Replot of the *y*-axis intercept of the primary plots of $1/\Omega_0$ vs. $1/[H_2O_2]_0$.

constants (V_{max}, $K_{m,S}$, and K_{m,H_2O_2}) at 50 mM phosphate buffer, pH 7.0, 37 °C and 1.25 nM HRP have been determined as follows: $V_{max} = 0.91 \pm 0.05\ \mu M\ s^{-1}$; $K_{m,S} = 51 \pm 3\ \mu M$; $K_{m,H_2O_2} = 136 \pm 8\ \mu M$ and by inserting these into Eqs. (26)–(28), $k_{cat} = 364 \pm 18\ s^{-1}$; $k_{+1} = (2.7 \pm 0.3) \times 10^6\ M^{-1}\ s^{-1}$ and $k_{+5} = (7.1 \pm 0.8) \times 10^6\ M^{-1}\ s^{-1}$. Since k_{cat} [$k_{cat} = k_{+2}k_{+6}/(k_{+2} + k_{+6})$] is a function of two first-order rate constants, k_{+2} and k_{+6}. The values of k_{+2} and k_{+6} could not be obtained in this experiment.

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References

[1] C. Veitch Nigel, Phytochemistry 65 (2004) 249–259.
[2] H.B. Dunford, in: J. Everse, K.E. Everse, M.B. Grisham (Eds.), Peroxidases in Chemistry and Biology, vol. 2, CRC Press, Boca Raton, Florida, 1991, pp. 1–24.
[3] B. Chance, A.C. Maehly, in: S.P. Colowick, N.O. Kaplan (Eds.), Methods in Enzymolgy, vol. II, Academic Press, New York, 1955, pp. 764–775.
[4] T.S. Andrew, A.S. Stephen, N.F.T. Roger, F.B. Julian, R.C.B. Robert, Eur. J. Biochem. 207 (1992) 507–519.
[5] J.N. Rodríguez-López, M.A. Gilabert, J. Tudela, R.N.F. Thorneley, F. García-Cánovas, Biochemistry 39 (2000) 13201–13209.
[6] Banci Lucia, J. Biotechnol. 53 (1997) 253–263.
[7] Utzig.E. Neat, J. Therm. Anal. Calorim. 54 (1998) 391–397.
[8] Y. Liu, C.N. Yan, T.Z. Wang, R.M. Zhao, S.S. Qu, P. Chen, Thermochim. Acta 333 (1999) 103–108.
[9] X.Y. Xie, X. Li, Z.Y. Wang, C.X. Wang, Thermochim. Acta 414 (2004) 19–23.
[10] Z.Y. Wang, C.X. Wang, S.S. Qu, Thermochim. Acta 360 (2000) 141–146.
[11] Kenneth B. Taylor, Enzyme Kinetics and Mechanisms, Kluwer Academic Publishers, 2002, pp. 49–61.
[12] P. Griess, Ueber ein neues phenylendiamin, J. Prakt. Chem. 3 (1871) 143–144.
[13] E. Knoevangel, J. Prakt. Chem. 89 (1914) 25–26.
[14] J.T. Peter, P.C. Victor, W. Dave, Anal. Biochem. 165 (1987) 230–233.