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Kinetics of Horseradish Peroxidase-Catalyzed Nitration of Phenol in a Biphasic System

Mingming Kong†, Yang Zhang†, Qida Li, Runan Dong, and Haijun Gao*

School of Life Science, Beijing Institute of Technology, Beijing 100081, P.R. China

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**Corresponding author*
Phone: +86-10-6891-8396;
Fax: +86-10-6891-8397;
E-mail: hj_gao@bit.edu.cn

†These authors contributed equally to this work.

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The use of peroxidase in the nitration of phenols is gaining interest as compared with traditional chemical reactions. We investigated the kinetic characteristics of phenol nitration catalyzed by horseradish peroxidase (HRP) in an aqueous-organic biphasic system using *n*-butanol as the organic solvent and NO_2^- and H_2O_2 as substrates. The reaction rate was mainly controlled by the reaction kinetics in the aqueous phase when appropriate agitation was used to enhance mass transfer in the biphasic system. The initial velocity of the reaction increased with increasing HRP concentration. Additionally, an increase in the substrate concentrations of phenol (0–2 mM in organic phase) or H_2O_2 (0–0.1 mM in aqueous phase) enhanced the nitration efficiency catalyzed by HRP. In contrast, high concentrations of organic solvent decreased the kinetic parameter V_{max}/K_m. No inhibition of enzyme activity was observed when the concentrations of phenol and H_2O_2 were at or below 10 mM and 0.1 mM, respectively. On the basis of the peroxidase catalytic mechanism, a double-substrate ping-pong kinetic model was established. The kinetic parameters were $K_m^{H_2O_2} = 1.09$ mM, $K_m^{PhOH} = 9.45$ mM, and $V_{max} = 0.196$ mM/min. The proposed model was well fit to the data obtained from additional independent experiments under the suggested optimal synthesis conditions. The kinetic model developed in this paper lays a foundation for further comprehensive study of enzymatic nitration kinetics.

Keywords: Horseradish peroxidase, nitration, organic-aqueous biphasic system, kinetics, modeling

Introduction

Nitration is one of the most highly studied organic chemical reactions. Processes based on this reaction have become the most acceptable route for the manufacture of explosives, medicine, pesticides, and other industrial precursors [1]. Traditional aromatic nitration is performed in a "mixed acid pool" containing concentrated sulfuric acid and nitric acid, both of which are not environmentally friendly. With the advances in peroxidase-catalyzed nitration in vivo [2–4], Biochemistry researchers have paid more attention on enzymatic nitration in vitro, because nitration catalyzed by peroxidase offers significant advantages over conventional chemical reactions. Enzymatic nitration can be performed in the presence of $NaNO_2$ and H_2O_2 under mild conditions, effectively reducing energy consumption and the formation of by-products [5, 6]. Peroxidase comprises a group of enzymes that catalyze a variety of oxidation reactions, such as radical coupling [7], oxygen-atom insertion [8], halogenation [9], and nitration in the presence of nitrite [10]. Of the peroxidases, horseradish peroxidase (HRP) has been studied extensively [11–13].

Budde *et al.* [14] tested several peroxidases as catalysts for nitration of 4-hydroxy-3-methyl acetophenone and found that HRP can efficiently catalyze the nitration of phenols. Dai *et al.* [15] optimized the parameters of phenol nitration catalyzed by HRP and obtained yields of 4-nitrophenol and 2-nitrophenol of 14% and 12%, respectively, in the enzymatic-catalyzed nitration process.

Non-aqueous enzymatic catalysis is an active field of research; however, the processes constructed so far are rather limited [16]. One of the main reasons for the lack of

success is that many organic substrates are insoluble in aqueous media [17]. For overcoming this limitation, several researchers have contributed to the development of an aqueous-organic co-solvent system [14]. The high concentration of substances, such as substrates, products, or the solvent in the system, can markedly inhibit or inactivate the enzyme, leading to lower biocatalysis efficiency [18]. One possible solution is the use of an aqueous-organic biphasic system [16]. When the biphasic system is stirred or shaken appropriately, the enzyme-catalyzed reaction occurs in the aqueous phase and complies with the Michaelis-Menten kinetics exhibited in an aqueous system [19]. The biphasic system not only increases the solubility of hydrophobic substrates, but also minimizes inhibition of the biocatalyst by the organic substances, as the reaction occurs in the aqueous phase where the concentrations of substrates, products, and solvents are low.

To design an efficient reaction system and improve the transformation efficiency of enzymatic nitration, especially for further large-scale uses, it is critical to illustrate the mechanisms and kinetics of enzymatic nitration [20, 21]. HRP-catalyzed nitration of phenol could be represented by the following total Eq. (1), where one-electron oxidation occurs in the process (Fig. 1) [22, 23]. With coupling of phenoxy radicals and nitrogen dioxide generated in the process, the nitrophenol derivatives are produced with the nitro group in the *ortho* or *para* position of the phenol [24].

$$H_2O_2 + PhOH + NO_2^- \xrightarrow{HRP} O_2N - PhOH + H_2O \quad (1)$$

Although there have been several reports on the enzymatic nitration of phenols in vitro [15, 25], little is known about the nitration kinetics and kinetic models, especially for a biphasic system. In this paper, kinetic study on the enzymatic nitration of phenol was performed in a biphasic system. We investigated the effects of the biphasic system on enzymatic nitration, and proposed a ping-pong kinetic model based on the nitration mechanism. We evaluated the kinetic parameters and verified the predictive function of the proposed model under the suggested optimal synthesis conditions.

Materials and Methods

Chemicals and Reagents

HRP (300 U/mg) was obtained from MYM Biotechnology Co., Ltd (China). H_2O_2 (30% aqueous solution), sodium nitrite, and *n*-butanol were purchased from Beijing Chemical Reagent Co., Ltd (China). Phenol, 2-nitrophenol, and 4-nitrophenol were of GC-grade and were obtained from Aladdin Industrial Inc. (China). Methanol and acetonitrile for high-performance liquid chromatography (HPLC) were purchased from Fisher Scientific (China). Milli-q water was used throughout the study.

Enzymatic Nitration of Phenol

Enzymatic nitration of phenol was carried out in a 50 ml conical flask with a working volume of 12.5 ml. The reaction system contained two phases. Phosphate buffer (50 mM, pH 7.0) was used as the aqueous phase containing 100 mM $NaNO_2$, a certain amount of 30% H_2O_2, and HRP. *n*-Butanol was used as the organic phase containing phenol [14]. The flask was incubated at 25°C with agitation in a thermostated shaker provided by Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd (China). HRP (5 μg/ml in aqueous phase) was added to initiate the reaction. A volume of 0.5 ml of sample from the organic phase was taken out at different times for analysis.

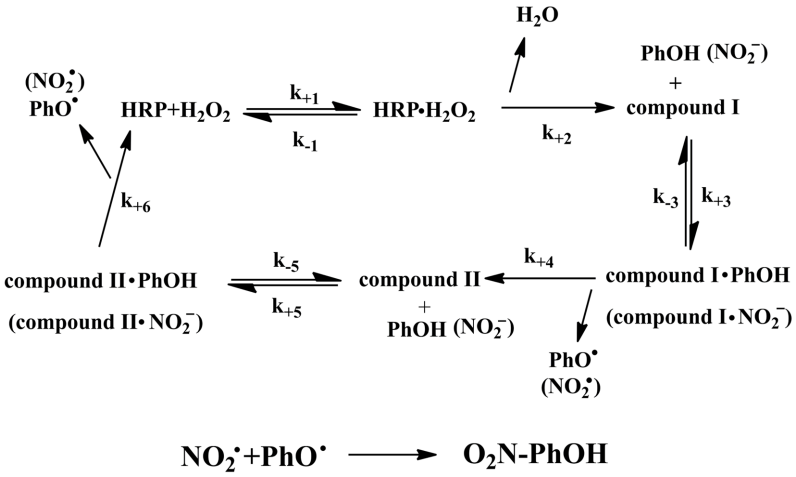

Fig. 1. The proposed ping-pong mechanism of horseradish peroxidase (HRP)-catalyzed nitration.

HPLC Analysis

The samples taken from the above systems were analyzed on a Shimadzu LC-15C HPLC instrument (Shimadzu, Japan) equipped with a SPD-15C detector (detection wavelength, 254 nm) [24]. The separation was performed on a Kromasil 100-5C18 column (250 × 4.6 mm) maintained at 55°C. The mobile phase was acetonitrile/water (containing 0.1% TFA; 40/60) mixture at 0.5 ml/min and the sample injection was 20 µl. Data were processed with the LC solution software. The concentrations of the substrate and products were estimated using calibration curves that was generated with standards obtained from Aladdin Industrial Inc.

Kinetic Analysis

The apparent steady-state reaction rates of enzymatic nitration were determined from the slope of the initial linear portions of the nitrated product concentration versus time plots. The apparent kinetic parameters were determined on the basis of the Michaelis equation: $v = V_{max} \times [S]/(K_m + [S])$, where v is the initial velocity, V_{max} is the maximal reaction velocity, $[S]$ is the concentration of substrate, and K_m is the Michaelis constant [20]. K_m and V_{max} were determined by the Lineweaver-Burk plot method. Our experiments showed that when excessive NO_2^- was used (at least 10 times the amount of phenol), its effect on nitration could be ignored and the enzymatic nitration could be considered as a double-substrate reaction where the reaction rate was a function of the concentrations of H_2O_2 and phenol. To further investigate the reaction mechanism, the standard reaction conditions were used to perform assays while altering the H_2O_2 concentrations at a fixed phenol concentration or vice versa.

Kinetic Model

The process of HRP-catalyzed nitration of phenol has been identified and involves the steps shown in Fig. 1 [26]. Referring to Fig. 1, a double-substrate ping-pong model was employed to describe the reaction mechanism. HRP combines with the first substrate to form the enzyme-substrate complex, and then releases the first product and produces the enzyme intermediate (compound I) before the second substrate binds to it.

The proposed model complies with the following assumptions:

(i) The reaction was performed isothermally (the experimental flask was placed in a thermostated shaker at 25°C).

(ii) External mass transfer limitations were ignored. Experiments performed at different stirring speeds showed that 165 rpm was sufficient to avert mechanical damage of HRP and avoid external mass transfer resistances. Neglecting external mass transfer limitations is a conventional practice when studying the kinetics of a heterogeneous system [27].

(iii) The concentration of phenol is much higher than that of the enzyme. This guarantees that the rate-determining step is determined by the enzymatic process.

(iv) The inhibition of enzyme by substrates and products could be ignored, and no enzyme inactivation occurred.

On the basis of the above assumptions, we have the differential equations for the intermediates shown in Fig. 1 as follows:

$$\frac{d[E]}{dt} = k_{-1}[E\cdot H_2O_2] - k_{+1}[E][H_2O_2] + k_{+6}[E_2\cdot PhOH] \quad (2)$$

$$\frac{d[E\cdot H_2O_2]}{dt} = k_{+1}[E][H_2O_2] - (k_{-1}+k_{+2})[E\cdot H_2O_2] \quad (3)$$

$$\frac{d[E_1]}{dt} = k_{+2}[E\cdot H_2O_2] + k_{-3}[E_1\cdot PhOH] - k_{+3}[E_1][PhOH] \quad (4)$$

$$\frac{d[E_1\cdot PhOH]}{dt} = -(k_{-3}+k_{+4})[E_1\cdot PhOH] + k_{+3}[E_1][PhOH] \quad (5)$$

$$\frac{d[E_2]}{dt} = k_{+4}[E_1\cdot PhOH] + k_{-5}[E_2\cdot PhOH] - k_{+5}[E_2][PhOH] \quad (6)$$

$$\frac{d[E_2\cdot PhOH]}{dt} = k_{+5}[E_2][PhOH] - (k_{-5}+k_{+6})[E_2\cdot PhOH] \quad (7)$$

where [E] is the concentration of free HRP, $[E\cdot H_2O_2]$ is the concentration of intermediate $E\cdot H_2O_2$, $[E_1]$ is the concentration of compound I, $[E_1\cdot PhOH]$ is the concentration of intermediate $E_1\cdot PhOH$, $[E_2]$ is the concentration of compound II, and $[E_2\cdot PhOH]$ is the concentration of intermediate $E_2\cdot PhOH$. k_{+1}, k_{-1}, k_{+2}, k_{+3}, k_{-3}, k_{+4}, k_{+5}, k_{-5}, and k_{+6} are the rate constants of the reactions.

The total enzyme loading in the reaction system is equal to the sum of all types of enzyme as shown in Eq. (8):

$$E_0 = [E] + [E\cdot H_2O_2] + [E_1] + [E_1\cdot PhOH] + [E_2] + [E_2\cdot PhOH] \quad (8)$$

It is assumed that the activity of the enzyme is kept constant throughout the assay, and the enzyme added to the system will be rapidly distributed in the reaction system and will achieve steady-state concentrations. Thus,

$$\frac{d[E]}{dt} = \frac{d[E\cdot H_2O_2]}{dt} = \frac{d[E_1]}{dt} = \frac{d[E_1\cdot PhOH]}{dt} = \frac{d[E_2]}{dt} = \frac{d[E_2\cdot PhOH]}{dt} = 0 \quad (9)$$

Therefore, Eqs. (2)–(7) may be simplified and solved to obtain the concentration of various forms of HRP:

$$[E] = \frac{k_{+6}[E_2\cdot PhOH]}{\left(\frac{k_{+1}k_{+2}}{k_{-1}+k_{+2}}\right)[H_2O_2]} \quad (10)$$

$$[E\cdot H_2O_2] = \frac{k_{+6}[E_2\cdot PhOH]}{k_{+2}} \quad (11)$$

$$[E_1] = \frac{(k_{-3}+k_{+4})k_{+6}[E_2\cdot PhOH]}{k_{+3}k_{+4}[PhOH]} \quad (12)$$

$$[E_1\cdot PhOH] = \frac{k_{+6}[E_2\cdot PhOH]}{k_{+4}} \quad (13)$$

$$[E_2] = \frac{(k_{-5}+k_{+6})[E_2 \cdot PhOH]}{k_{+5}[PhOH]} \quad (14)$$

In the enzymatic reaction, the reduction of E_2·PhOH is the rate-limiting step. The initial producing rate of nitrated products can be described as:

$$v = k_{+6}[E_2 \cdot PhOH] \quad (15)$$

Substituting Eqs. (10)–(14) into (8) and (15), we obtain the expression of v as follows:

$$v = \frac{[E_0]}{\frac{k_{-1}+k_{+2}}{k_{+1}k_{+2}} \times \frac{1}{[H_2O_2]} + \frac{k_{-3}+k_{+4}}{k_{+3}k_{+4}} \times \frac{1}{[PhOH]} + \frac{k_{-5}+k_{+6}}{k_{+5}k_{+6}} \times \frac{1}{[PhOH]} + \left(\frac{1}{k_{+2}} + \frac{1}{k_{+4}} + \frac{1}{k_{+6}}\right)} \quad (16)$$

The above equation could be rearranged and expressed as:

$$v = \frac{V_{max}[H_2O_2][PhOH]}{K_m^{PhOH}[H_2O_2] + K_m^{H_2O_2}[PhOH] + [H_2O_2][PhOH]} \quad (17)$$

where the constants V_{max}, K_m^{PhOH}, $K_m^{H_2O_2}$, and k_{cat} are defined by

$$V_{max} = k_{cat}[E_0] \quad (18)$$

$$K_m^{PhOH} = \frac{k_{cat}[(k_{-3}+k_{+4})k_{+5}k_{+6} + (k_{-5}+k_{+6})k_{+3}k_{+4}]}{k_{+3}k_{+4}k_{+5}k_{+6}} \quad (19)$$

$$K_m^{H_2O_2} = \frac{k_{cat}(k_{-1}+k_{+2})}{k_{+1}k_{+2}} \quad (20)$$

$$k_{cat} = \frac{k_{+2}k_{+6}k_{+4}}{k_{+2}k_{+6} + k_{+4}k_{+6} + k_{+2}k_{+4}} \quad (21)$$

where V_{max} is the maximal reaction velocity, $[H_2O_2]$ and [PhOH] are the concentrations of H_2O_2 and phenol, K_m^{PhOH} and $K_m^{H_2O_2}$ are the Michaelis constants of phenol and H_2O_2, respectively, and k_{cat} represents the turnover number of enzymatic nitration.

Results and Discussion

Effect of Organic Solvent Concentration

Enzymatic reactions conducted in mixtures of water and organic solvents are the focus of growing attention [28]. The partial replacement of water by an organic solvent leads to a high dissolved concentration of hydrophobic substrates, resulting in higher product yields. However, when an enzyme is placed in a non-aqueous medium, its native, aqueous-based structure and functions can be altered due to a number of factors [29, 30]. A theoretical kinetic model proposed by Lee and Kim [31] shows that the enzymatic reaction rate in non-aqueous media depends largely on the solubility of the substrates and enzyme hydration. Any variation in the structure or chemical nature of the enzyme upon hydration could change the constants of the kinetic constants [32]. The effect of phenol concentrations with varying concentrations of *n*-butanol on nitration efficiency was studied in this work. Both Michaelis-Menten and Lineweaver-Burk plots (Figs. 2A and 2B) were constructed to estimate the maximum reaction rate (V_{max}) and Michaelis constant (K_m) for each assay. The Michaelis-Menten plots clearly indicated that the phenol nitration rate increased as the phenol concentration increased. We determined the kinetic parameters by the double-reciprocal plots of $1/v$ versus $1/S$ and found that the apparent K_m

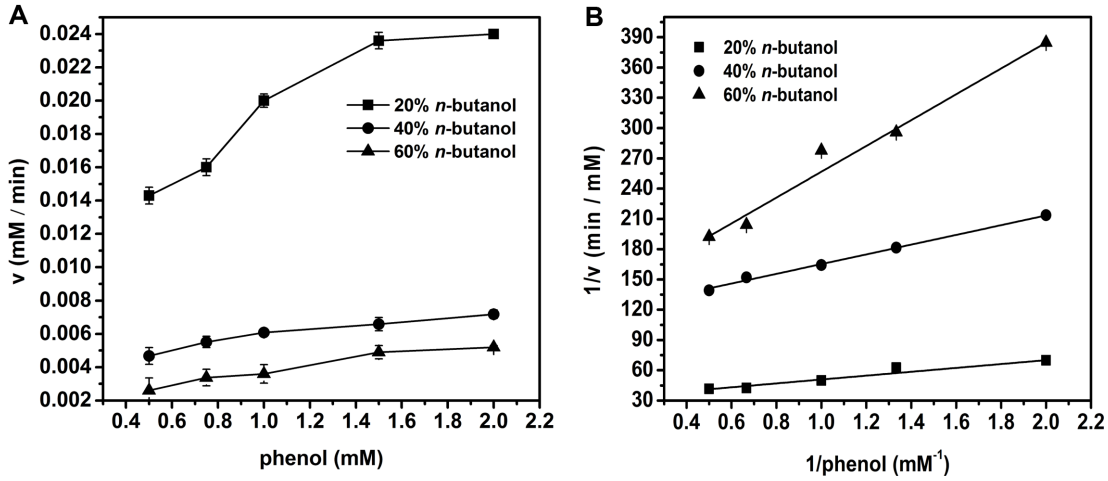

Fig. 2. Michaelis–Menten plot (**A**) and Lineweaver-Burk double-reciprocal plot (**B**) of the effect of concentrations of phenol on the initial velocity of horseradish peroxidase (HRP) in varying concentrations of *n*-butanol.
Reaction conditions: 0.05 mM H_2O_2, 100 mM sodium nitrite, 5 μg/ml HRP, pH 7, 25°C, 165 rpm.

largely depends on the concentration of organic solvent. Larger K_m and a regular decrease in V_m/K_m ratio were observed with increase of organic solvent concentration. These results demonstrated that an appropriate concentration of organic solvent is vital for the enzyme catalytic reaction, as a high concentration of *n*-butanol in the biphasic system lowered the HRP catalytic efficiency.

Effect of Mass Transfer and HRP Concentration

The aqueous-organic biphasic reaction medium is a heterogeneous system. The enzyme and hydrophobic substrates are mainly distributed in the aqueous phase and the organic phase, respectively [27], so the influence of mass transfer and diffusion on nitration must be considered. Here, we used mechanical agitation to evaluate the effect of mass transfer on enzymatic nitration. The reaction was performed at an agitation speed of 50–200 rpm. With the purpose of reaching a high concentration of substrate and enhancing product yields, we adopted 40% (v/v) *n*-butanol as the organic phase [24]. Fig. 3A depicts the effect of agitation speed on HRP-catalyzed nitration of phenol. As the speed increased, the reaction rate gradually increased until the speed reached 100 rpm. This indicated that the influence of mass transfer on the reaction could be ignored at speeds higher than 100 rpm. For the research of nitration kinetics, the agitation speed was set at 165 rpm.

The influence of different concentrations of HRP on nitration is shown in Fig. 3B. The initial velocity of the reaction increased with increasing HRP concentration. This suggested that the effect of mass transfer between two phases on the reaction was not obvious. Instead, the rate of enzymatic reaction was mainly controlled by the reaction kinetics in the aqueous phase. Based on these results, 5 μg/ml HRP was chosen as the appropriate dosage.

Effect of Substrate Concentrations

Substrate effects on nitration were investigated. The concentration of one substrate was maintained constant and the other reactant concentration was altered, and the initial reaction rates were plotted against the concentration of the variable substrate for each group. Fig. 4A shows that the nitration reaction rate significantly increased as the phenol concentration was increased from 0.5 to 2 mM, and then grew slowly. No enzyme inhibition of phenol was observed.

The relationship between the initial reaction rate and concentrations of H_2O_2 is shown in Fig. 4B. When the H_2O_2 concentration was lower than 0.1 mM, the reaction rate increased as the H_2O_2 concentration was increased from 0.02 to 0.1 mM. However, the reaction rate exhibited a downward trend if the concentration of H_2O_2 increased continually. This phenomenon indicated that a high concentration of H_2O_2 inhibits the enzyme in the reaction.

The role of H_2O_2 in enzyme activity and conformational stability has been reported [33, 34]. The exposure of HRP to a high concentration of H_2O_2 may result in irreversible inactivation. The molecular mechanism is quite complex, and the tendency of surface-exposed methionine to become easily oxidized is one of the main causes for the enzyme inactivation in the presence of H_2O_2 [35–37]. Even the chemical structure of the substrate or product(s) may also be influenced by H_2O_2, suggesting that the addition of

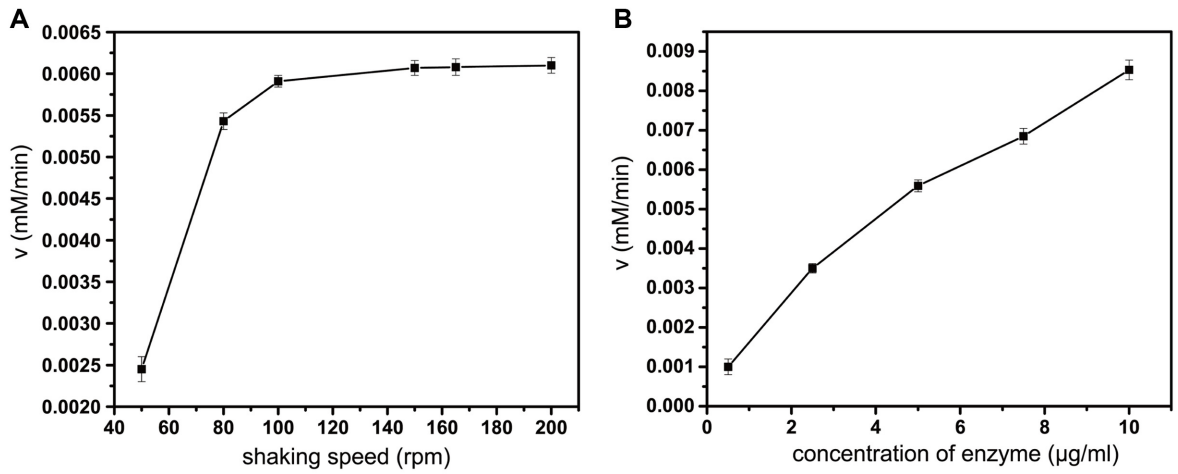

Fig. 3. Effect of agitation speed (**A**) and enzyme concentration (**B**) on horseradish peroxidase (HRP)-catalyzed nitration of phenol. Reaction conditions: 1 mM phenol, 40% (v/v) *n*-butanol, 0.05 mM H_2O_2, 100 mM sodium nitrite, 25°C, 5 μg/ml HRP (**A**) or 165 rpm (**B**).

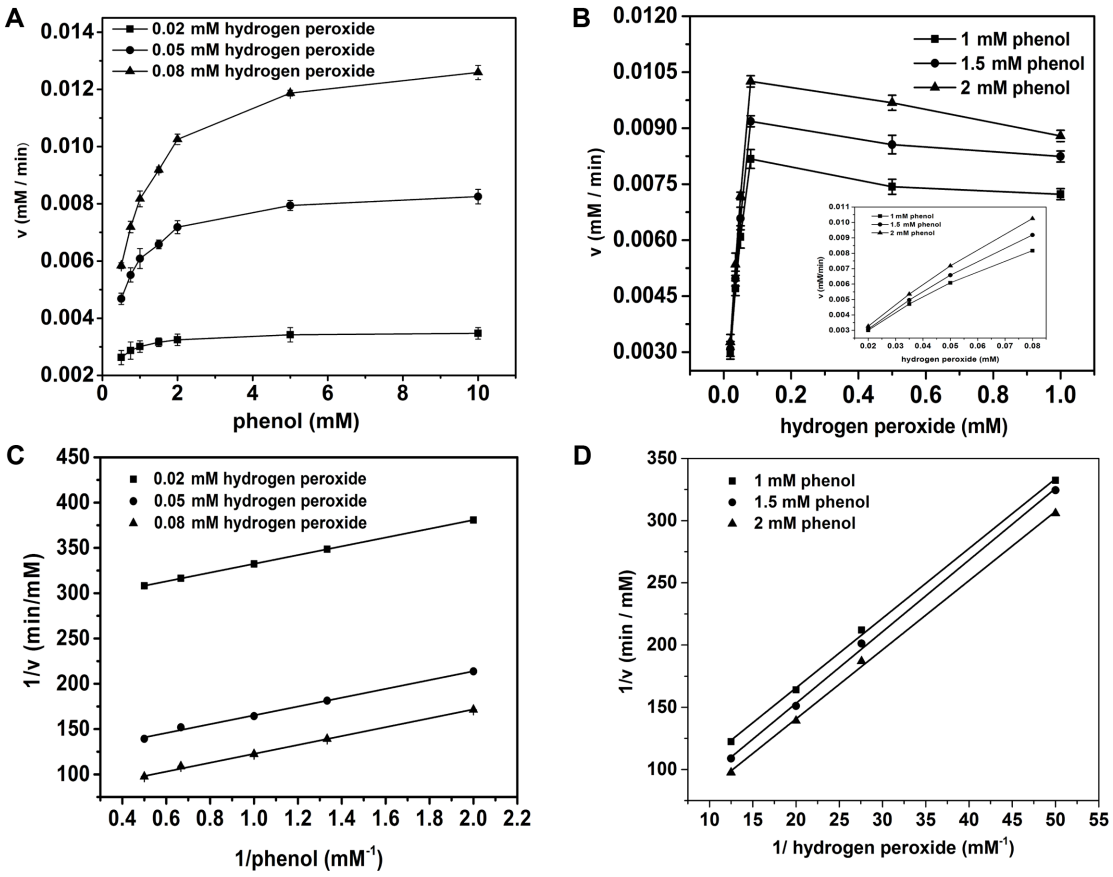

Fig. 4. Kinetic diagram of horseradish peroxidase (HRP)-catalyzed nitration.
(**A** and **B**) The velocity (v) of the reaction was measured under standard reaction conditions with varying concentrations of phenol and fixed concentrations of H_2O_2, or vice versa. (**C** and **D**) Double-reciprocal plots of the activity of HRP with the concentration of one substrate (H_2O_2 or phenol) kept constant and the other varied. The reaction conditions: 40% (v/v) *n*-butanol, 100 mM sodium nitrite, 5 μg/ml HRP, pH 7, 25°C, 165 rpm.

substrate to the reaction system must be done with caution [38]. Many studies attempted to redesign the enzyme using site-directed mutagenesis [39] or directed evolution techniques to reduce enzyme inactivation by H_2O_2 [40, 41], but these processes are difficult and have had limited success [42]. On the laboratory-scale, enzyme stability could be substantially improved when addition of H_2O_2 was controlled to maintain it at a low level of concentration [43, 44]. Another technique to reduce enzyme inactivation by high concentration of H_2O_2 is in situ production; for example, exploiting the process of oxidizing glucose by glucose oxidase to produce H_2O_2 [25].

Kinetic Model and the Parameters

To further understand the catalytic process of HRP, we investigated the qualitative aspects of the reaction and a series of tests was performed (Fig. 4). The process of HRP-catalyzed nitration using phenol as the substrate was well depicted by typical Michaelis–Menten curves (Fig. 4A). As noted above, high H_2O_2 concentrations inhibited the HRP-catalyzed nitration. However, typical Michaelis–Menten curves were recognized when H_2O_2 concentrations was confined in a proper range (Fig. 4B). This indicated that the initial reaction rate depends on the substrate concentration. Then, we applied the Lineweaver-Burk plot method to examine the kinetic behavior of the catalytic reaction of HRP. As shown in Figs. 4C and 4D, generation of the parallel data lines, which are double-reciprocal plots of the initial reaction rate versus the concentration of phenol or H_2O_2, implies a feature of the ping-pong mechanism. Next, a model (written as Eq. (17)) was developed to describe the kinetics of enzymatic nitration based on the ping-pong mechanism. The catalytic parameters of Eq. (17) determined by using the double-reciprocal of the Michaelis–Menten equation are as follows: $K_m^{H_2O_2} = 1.09 \times 10^{-3}$ mol/l, $K_m^{PhOH} = 9.45 \times 10^{-3}$ mol/l, and $V_{max} = 0.196$ mM/min. The equation

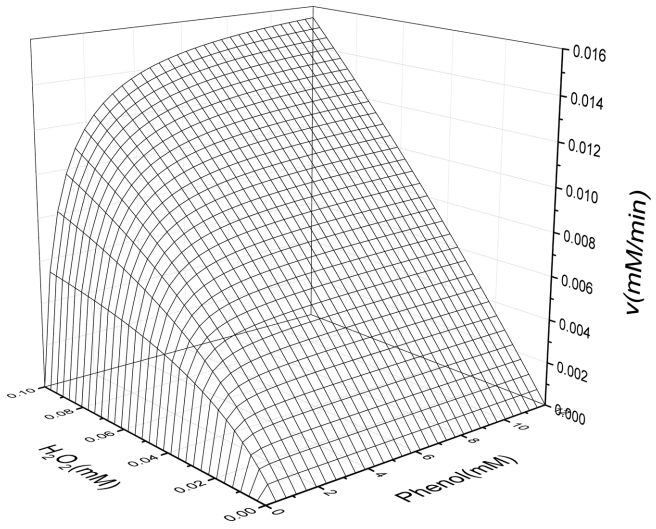

Fig. 5. Illustration of the obtained ping-pong model.
Dependence of the initial reaction rate on the concentrations of phenol and H_2O_2.

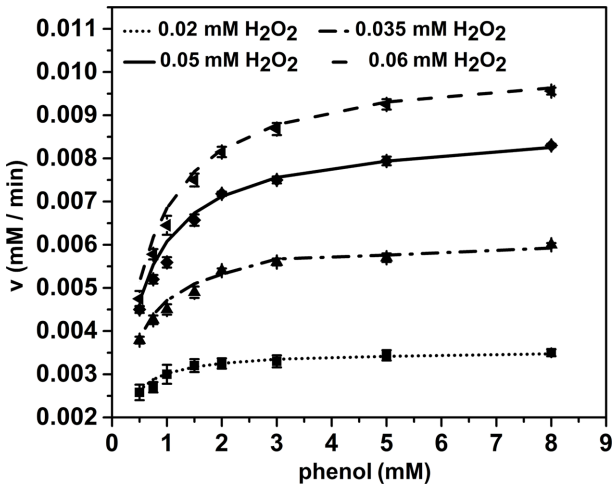

Fig. 6. Experimental and calculated initial rates with a constant H_2O_2 concentration.
The symbols and lines represent the experimental data and model-simulated data, respectively.

could be expressed as

$$v = \frac{0.196[H_2O_2][PhOH]}{9.45[H_2O_2] + 1.09[PhOH] + [H_2O_2][PhOH]} \quad (22)$$

As noted above, HRP-catalyzed nitration follows a ping-pong kinetic mechanism. The initial step in the process is the binding of H_2O_2 to HRP to form the active intermediate compound I with the concomitant release of H_2O. Next, PhOH binds to intermediate compound I and forms the active intermediate compound II with the concomitant release of PhO$^\bullet$. Compound II can continue to bind to PhOH, forming PhO$^\bullet$. Finally, the enzyme is regenerated. In the process of the reaction, both enzyme intermediates (compound I and compound II) can also react with nitrite, generating free diffusible radicals of $NO_2^\bullet$. Coupling of PhO$^\bullet$ and $NO_2^\bullet$ gives the nitrophenol derivatives. Studies have shown that $NO_2^\bullet$ is also able to generate PhO$^\bullet$ from PhOH and then give rise to nitrophenol, but the former nitration path is faster [26, 45–48]. When a high concentration of nitrite is used, the steps containing $NO_2^\bullet$ can also be assumed as a very fast reaction, and the whole reaction can be described as a double-substrate (H_2O_2 and phenol) ping-pong mechanism. The graphic illustration of the obtained ping-pong model is depicted in Fig. 5.

Application of the Kinetic Model

The performance of this prediction model was assessed by additional independent experiments performed according to the suggested reaction conditions of initial buffer pH 7.0, reaction temperature 25°C, 40% (v/v) *n*-butanol as organic phase, agitation speed of 165 rpm, and 5 μg/ml HRP. Comparisons of the experimental and calculated results are shown in Fig. 6. Overall, the model fits the data well, with a relative error that is under 8%, which confirms that the ping-pong model without enzyme inhibition by the substrates that is presented in this work is appropriate to illuminate the kinetic behavior of HRP-catalyzed nitration of phenol.

Many studies have shown that enzymatic nitration is accompanied by side reactions [6, 26]. The PhO$^\bullet$ produced can yield dimeric products during the process of peroxidase catalytic reaction. However, the yield of these dimers decreases significantly as $NO_2^\bullet$ increases. This demonstrates that a relatively high concentration of nitrite can not only accelerate reaction, but inhibit the formation of by-products. The investigation of the kinetics of enzymatic nitration suggests that effective enzymatic nitration depends upon the interaction of the HRP with nitrite, H_2O_2, and phenol at proper concentrations.

Based on the comparison of the experimental and calculated data, we can conclude that the proposed kinetic model in this study is a reasonable representation of the HRP-catalyzed nitration process and can be used for reaction simulations. Additionally, the developed kinetic model provides a foundation for further comprehensive study of enzymatic nitration kinetics.

In this paper, we investigated the kinetics of HRP-catalyzed nitration of phenol in an organic-aqueous biphasic

system. The kinetic characteristics of HRP-catalyzed nitration largely depend on mass transfer between two phases and the concentrations of organic solvent, enzyme, and substrates. The initial rate of the reaction increases with increasing HRP concentration. Moreover, the increase of substrate concentrations, such as phenol (0–2 mM) or H_2O_2 (0–0.1 mM), enhances nitration efficiency catalyzed by HRP. In contrast, the increase of organic solvent (*n*-butanol) provided a regular decrease in V_{max}/K_m for the process. No inhibition was observed when the concentrations of phenol and H_2O_2 were 10 mM or lower (in the organic phase) and 0.1 mM (in the aqueous phase), respectively. The effect of mass transfer on nitration can be ignored when using an appropriate agitation speed. Based on the peroxidase catalytic mechanism and experimental results, a double-substrate ping-pong kinetic model was established. The kinetic parameters were $K_m^{H_2O_2} = 1.09$ mM, $K_m^{PhOH} = 9.45$ mM, and $V_{max} = 0.196$ mM/min. Even though the kinetic model has some limitations, it allows a fast prediction of transformation after the operating conditions are constrained.

Acknowledgments

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