

Stabilizing and Suicide-Peroxide Protecting Effect of Ni²⁺ on Horseradish Peroxidase

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The present research discusses the structure stabilizing and protecting effects of Ni²⁺ against suicide-peroxide inactivation of horseradish peroxidase (HRP). Suicide inactivation of HRP by hydrogen peroxide (3 mM) was monitored by measuring change in the absorbance of the colored product (tetraguaiacol) of the catalytic reaction cycle at 470 nm. Progress curves of the catalytic reaction cycle were obtained at 27 °C, phosphate buffer (5 mM), pH 7.0. The corresponding kinetic parameters (*e.g.*, initial enzyme activity (α_0) and the apparent rate constant (k_i) of suicide inactivation of HRP by peroxide) were evaluated using a kinetic equation derived in this study. Comparative activatory and inhibitory effects of Ni²⁺ on the kinetics of suicide-peroxide inactivation of HRP are discussed.

Keywords: Horseradish Peroxidase, Suicide inactivation, Enzyme protection, Ni²⁺

INTRODUCTION

Horseradish peroxidase (HRP, E.C.1.11.1.7) catalyzes the oxidation of aromatic hydrogen donors by hydrogen peroxide through a two-electron transferring pathway [1-3]. Currently, industrial applications of peroxidase in chemistry and biotechnology are well developed. Peroxidase may be used in waste treatment in order to remove aromatic phenols and amines from aqueous solutions in the presence of hydrogen peroxide. In this treatment phenolic compounds are polymerized in the presence of hydrogen peroxide through a radical oxidation-reduction mechanism.

Suicide-peroxide inactivation, reactions between hydrogen peroxide and intermediates of the enzyme's catalytic cycle that reduce the sensitivity and efficiency of peroxidase [4-7], must

be considered, and various techniques for reducing the role of suicide inactivation must be applied. Among such methods, the use of polyethylene glycol (PEG, with various molecular weights) has been reported to increase the stability and lifetime of the enzyme [8-11].

Generally, it is believed that transition metal ions could coordinate to the active site donor residues leading to the activation of enzymes [12]. Conversely, such coordination may block substrate interaction causing inhibition [13]. Previously, we reported the effects of transition metal ions, including Mn²⁺, Co²⁺, Ni²⁺, and Cu²⁺, on the functional stability and kinetic parameters of HRP catalysis. It has been shown [14] that in the presence of low concentrations of metal ions activatory effects occurred, while at higher concentrations of metal ions inhibitory behaviors have been observed. An earlier study showed that the increased functional stability of HRP induced by an activatory

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concentration of Ni^{2+} was associated with an increase in its conformational stability [15]. Such an increase in enzyme activity and stability could be applied to improve industrially important processes such as phenol removal and wastewater treatment.

The present research investigates the protecting-effect of Ni^{2+} on the suicide-peroxide inactivation of HRP, including its increased catalytic activity and its reduction in the suicide inactivation rate.

EXPERIMENTAL

Materials

Horseshoe peroxidase type II, with a purity index of 2.30 ($R.Z = 2.3$), was obtained from Sigma Chemical Co., USA. (Guaiacol) *ortho*-methoxy phenol, nickel chloride ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$), ammonium sulfate, sodium phosphates, and hydrogen peroxide were obtained from Merck, Germany. All solutions were prepared in CO_2 -free deionized water (Barnstead Nanopure D4742, resistance = 18.3 $\text{M}\Omega$).

Methods

The concentration of peroxidase in the sample was determined spectrophotometrically ($\epsilon_{403} = 1.02 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$) [22]. According to our earlier procedure [15], each enzyme sample solution is stabilized with Ni^{2+} . Enzyme solutions of different concentrations were prepared by diluting the stock sample ($2.35 \times 10^{-5} \text{ M}$) with 5 mM phosphate buffer (pH 7.0) and 2 mM nickel chloride solution and stored for 24 h. The specific activity of the native enzyme was 230 U/mg.

A temperature controlled (water circulating thermobath, MLW 8) Shimadzu double beam spectrophotometer model 2101 PC was used to measure absorbance and record the electronic spectra at 27 °C. The specific activity of HRP was determined spectrophotometrically in a 1-min reaction time course (lag time = 7 s) using guaiacol as the hydrogen donor [17]. Progress curves of the reaction were obtained by following the change in absorbance of the product (tetraguaiacol) at 470 nm and by obtaining the maximum absorbance change upon the addition of 100 μl of fresh enzyme solution to the reaction mixture at the end of the process. The maximum change in absorbance (A) corresponds to the initial amount of guaiacol.

The percentage of phenol conversion or phenol removal (efficiency) was determined using the following relationships:

$$A_{\infty} \propto [\text{AH}]_0; [\text{AH}]_{\text{consumed}} = A_t/A_{\infty} \times [\text{AH}]_0$$

$$[\text{AH}]_t = [\text{AH}]_0 - [\text{AH}]_{\text{consumed}}$$

$$\% \text{Removal} = [\text{AH}]_{\text{consumed}}/[\text{AH}]_0 \times 100$$

where A_t and $[\text{AH}]_t$ are the absorbance and concentration, respectively, of AH at time t . At high concentrations of guaiacol, at which a high molecular weight polymer may be obtained, the concentration of the remaining guaiacol at each time is determined spectrophotometrically (510 nm) using 4-amino antipyrine. In order to stop the reaction at a specified time, 30 μl of 3 M sodium azide was added to 1 ml of reaction mixture. After filtration of the mixture, the amount of remaining guaiacol, and thus the removal efficiency, was determined. The concentration of hydrogen peroxide was determined ($\epsilon_{240} = 43.6 \text{ cm}^{-1} \text{ M}^{-1}$) as previously described [18].

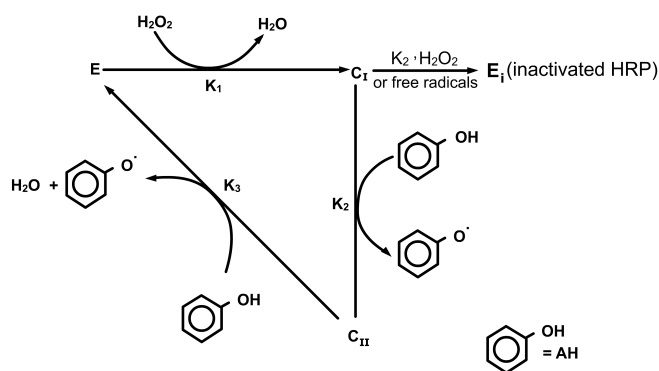
RESULTS AND DISCUSSION

Fundamentals of Suicide-Peroxide Inactivation of HRP

Scheme 1 shows the catalytic reaction cycle and the inactivation pathway for HRP. The two main reactions of the cycle are shown as reactions (I) and (II). Reaction (I) is the inactivation process and reaction (II) (oxidation of guaiacol by peroxide) is the enzyme-catalyzed reaction that is used to monitor the inactivation process. E_a , E_i , AH, and P denote the whole forms of the active enzyme (sum of E, C-I and C-II), inactivated enzyme, hydrogen donor (reductant-substrate), and the product (tetraguaiacol), respectively. The k_i and α denote the apparent rate constant of inactivation and the apparent rate constant of oxidation of the hydrogen donor, respectively.

In the catalytic reaction cycle, the active enzyme species are free active enzyme (E), C-I, and C-II. The rate constants of formation of C-I, C-II, and E_a are extremely large compared to the k_i value. Furthermore, k_i is about 100 times greater than k_2 and k_3 . Accordingly, all the active enzyme species are assumed to be in the form of C-I as the only species undergoing the inactivation process.

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Scheme 1



The following conditions are recommended for the investigation of the suicide inactivation through the simultaneous progress of the reactions (I) and (II):

- Higher concentration of peroxide with respect to the concentration of the hydrogen donor (AH), *i.e.* $[H_2O_2] \gg [AH]$.
- Selection and application of AH {(hydrogen donor) as a “benign substrate” (substrate which does not damage the enzyme)} concentration range, so that it does not lead to saturation of the enzyme by the substrate and coincides with the initial linear part of the Michaelis plot [19-21]. Therefore, the rate of reaction (I) is always proportional to the active enzyme concentration.

Our previous work [19] showed that the rates of consumption of the active enzyme (reaction (I)) and the phenolic compound (reaction (II)) could be written as:

$$-(dE_a)/dt = -d\alpha/dt = k_i [H_2O_2] \alpha \quad (1)$$

$$-(d[AH])/dt = [AH] = \times \alpha_0 e^{(-k_i \cdot t)} \quad (2)$$

Integration of Eq. (2) gives:

$$\alpha = \alpha_0 \times e^{(-k_i \cdot t)} \quad (3)$$

Substitution of α from Eq. (3) into Eq. (2) and the subsequent rearrangement and integration, can be used to obtain the integrated kinetic equation of the suicide inactivation process in which the AH and H₂O₂ substrates are considered to be the monitoring probe and the suicide inactivating substrate, respectively. Details of the kinetic model have been described previously [19].

In summary, according to the model, by monitoring the AH concentration in the catalytic reaction cycle, one can estimate the decrease in the level of the active forms of the enzyme (due to the suicide inactivation process). Hence, the rate of the consumption of the reducing substrate (AH) and the overall rate of the conversion of active enzyme (E_a) to the inactivated (E_i) form provides the means of obtaining the differential kinetic equation as well as the modified integrated kinetic relationship [19]:

$$[AH]_t = [AH]_0 \text{Exp} (\alpha_0/k_i) (e^{k_i \cdot t} - 1) \quad (4)$$

where α_0 , as the initial activity of the enzyme, is the value of α at time (t) = 0. $[AH]_t$ and $[AH]_0$ are the molar concentrations of AH at time t and t = 0, respectively. Equation (4) can be used for the determination (*via* non-linear regression) of α_0 and k_i by fitting the experimental data into it. EUREKA, a common computer software, was used for this purpose. Values of α_0 and k_i are given in Table 1.

The reductant substrate or hydrogen donor (phenolic compound) in the range of 0-8 mM is a benign substrate [19]. Indeed, the main inactivation effects are due to the hydrogen peroxide (above 3 mM). This poses a restriction on the above-mentioned model. This restriction is to keep the hydrogen

Table 1. Kinetic Parameters Obtained for Suicide Inactivation of Horseradish Peroxidase Induced by Ni²⁺

[Ni ²⁺] (mM)	k _i (min ⁻¹)	α ₀ (min ⁻¹)
0.0 (Native HRP)	(3.80 ± 0.08) × 10 ⁻⁶	(1.16 ± 0.09) × 10 ⁻⁵
2.0 (Activatory concentration)	(0.31 ± 0.01) × 10 ⁻⁶	(2.56 ± 0.06) × 10 ⁻⁵
8.0 (Inhibitory concentration)	(6.52 ± 0.02) × 10 ⁻⁵	(4.60 ± 0.03) × 10 ⁻⁵

peroxide concentration below the inactivatory range (0-1 mM). In our model, by using mass balance relations for the active peroxidase (reaction (I)) and phenolic compound (reaction (II)), the rate of inactivation of the enzyme [Eq. (1)] and also the rate of consumption of AH [Eq. (2)] was obtained. Meanwhile, the inactivatory effects of the suicide substrate (hydrogen peroxide) are taken into account [see Eq. (4)]. Another possible solution for reducing and/or preventing the suicidal effect of hydrogen peroxide is the stabilization and protection of the enzyme by suitable effectors like metal ions, which has been the aim of this study.

In recent years, the use of peroxidases to catalyze the removal of phenolic compounds from waste waters has been investigated by independent researchers [22-29], but in most models, suicide inactivation of the enzyme is not considered. In the process of homogeneous catalysis by HRP in the presence of high concentrations of hydrogen peroxide (> 3 mM), irreversible inactivation of the enzyme is of considerable importance [4,5].

It is known that metal ions coordinate to active site residues and activate enzymes [12] while, in some cases, coordination of metal ion results in inhibition [19]. In the previous work, the effects of some metal ions, including Mn^{2+} , Co^{2+} , Ni^{2+} , and Cu^{2+} , on the functional stability of HRP and the kinetics of enzyme catalysis were investigated using different concentrations of various metal ions [14]. This study showed that in the presence of low concentrations of metal ions (*e.g.*, 2 mM of Ni^{2+}) activatory effects is occurred. However, at higher concentrations of metal ions (*e.g.*, 8 mM of Ni^{2+}) an inhibitory behavior was observed appeared.

An earlier study showed that an increase in the functional stability of HRP induced by an activatory concentration of Ni^{2+} is associated with an increase in conformational stability of the enzyme (ΔG_{H_2O}) by 4.9 kJ mol⁻¹ [15]. For this reason, the enzyme used for phenol removal was incubated with an activatory concentration of Ni^{2+} (2 mM). Similar results were reported for the effect of PEG on HRP stability [8-12]. In such a process, peroxidase can be inactivated by several mechanisms:

- I) Adsorption of polymerized phenol on peroxidase resulting in reduced access of a substrate to the enzyme's active site [30-32].
- II) Irreversible reactions between the enzyme and phenyl or

phenoxy radicals formed by the one-electron oxidation of phenolic substrates during the catalytic cycle. This inactivation was thought to be caused by the bonding of the free radicals to the heme edge of the enzyme and disrupting the active active site [33,34].

- III) Reactions between hydrogen peroxide and intermediates of the enzyme's catalytic cycle, referred to as 'suicide-peroxide inactivation' [33].

Figure 1 shows typical progress curves for the oxidation of guaiacol by hydrogen peroxide (0.3 mM) in the presence of the native HRP (lower curve) and Ni^{2+} -stabilized horseradish peroxidase (upper curve).

Figure 2 shows the inactivation progress curves at different concentrations of Ni^{2+} ion. The rate law of inactivation reaction is described by Eq. (4) from which, after fitting the experimental data into the equation, kinetic parameters including k_i and α_0 could be obtained.

Figure 2 shows that HRP in the presence of Ni^{2+} (lower curve) is more active than the native HRP (middle curve), and practically no suicidal effect of H_2O_2 was detected (see Table 1). In order to elucidate the metal ion effects, an additional experiment was performed using an inhibitory concentration of Ni^{2+} (8.0 mM). Results showed that, in the presence of

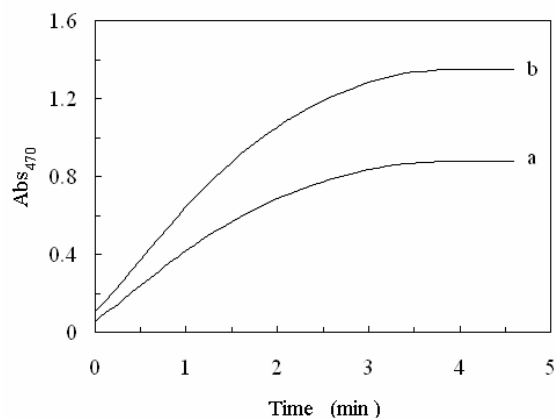


Fig. 1. Typical progress curves for oxidation of guaiacol (monitored spectrophotometrically at 470 nm) by hydrogen peroxide: (a) in the presence of native HRP, (b) in the presence HRP stabilized by Ni^{2+} (2 mM). Assay conditions: [HRP], 12 nM; [AH], 0.25 mM; [H_2O_2], 0.3 mM; Lag time, 7 s.

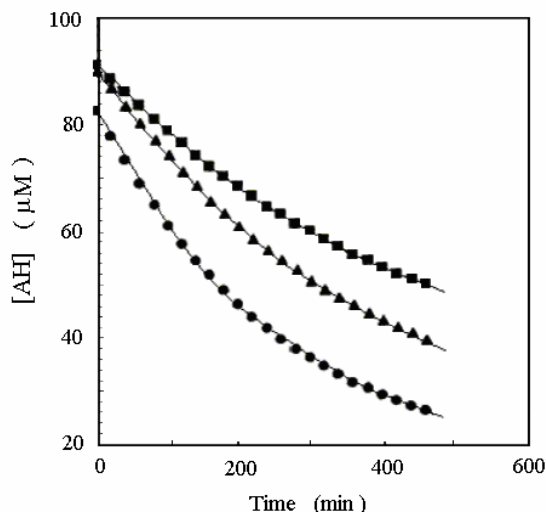


Fig. 2. Progress curves for suicide inactivation of HRP by hydrogen peroxide shown as plot of guaiacol concentration versus time of reaction: (■) HRP in presence of inhibitory concentration of Ni²⁺ ([Ni²⁺] = 8.0 mM), (▲) native enzyme (in the absence of Ni²⁺), (●) HRP in presence of activatory concentration of Ni²⁺ ([Ni²⁺] = 2.0 mM). Assay conditions: pH, 7.0 (phosphate buffer 5 mM); T, 27 °C; [HRP], 12.0 nM; [H₂O₂], 3 mM.

inhibited enzyme, the suicide inactivation process was stronger (see upper curve in Fig. 2). Comparative values of kinetic parameters are given in Table 1. In this Table, the low value of k_i (close to zero) together with an increased initial activity of HRP (α_0) in the presence of an activatory concentration of Ni²⁺ represent the suicide-peroxide protecting effect of the nickel ion on peroxidase. Indeed, the metal ion provides the simultaneous activation of HRP (two-fold in comparison with the native enzyme, see Table 1) and protection against the suicidal effect of hydrogen peroxide. On the other hand, the suicide inactivation effect is reinforced (increased value of k_i) and the initial activity of the enzyme decreased significantly in the presence of an inhibitory concentration of Ni²⁺ (2.5 times compared to native HRP). Consequently, in practical and operational cases, 2 mM Ni²⁺ can be used to stabilize HRP and to protect the suicidal effect of peroxide on the enzyme.

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