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Inactivation of chloroperoxidase by arginine

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ABSTRACT

Inactivation of chloroperoxidase (CPO) from *Caldariomyces fumago* by arginine was investigated. It was found that the red native CPO solution was turned into a stable green species with a concomitant shift of the Soret band from 398 to 425 nm in the presence of arginine. The green CPO lost almost all of its catalytic activity, and this inactivation was irreversible.

Differential UV–vis spectrophotometry was used to examine the binding properties of arginine to CPO. The formation of CPO-arginine (1:1) complex was highly pH-dependent. Fluorescence investigation revealed the exposure degree of prosthetic group increased. Kinetic analysis indicated that CPO has both a high affinity and specificity to arginine.

This inactivation may be caused mainly by the binding of guanidinium group in arginine to the acid–base catalytic group Glu183 in CPO. The change of surrounding environment around heme induced by the interaction of heme propionates with arginine and the occupying of the sixth axial ligand position of heme iron by hydroxyl are also reasons bringing on this inactivation.

1. Introduction

Chloroperoxidase (CPO), a glycosylated hemoprotein secreted by the mold *Caldariomyces fumago*, is an enzyme of ecological and social significance [1,2]. It is thought to be the most versatile heme protein known in peroxidase super family, having extensive catalytic properties, such as halogenase-, peroxidase-, catalase and cytochrome P450-like activities [3–6]. However, due to high cost, instability at elevated temperatures, lower water solubility of many organic substrates of synthetic interest, and deactivation in high concentrations of hydrogen peroxide, industrial application of this enzyme has being hampered. To explore its huge potentials, many ongoing researches have focused on methods to improve the enzyme operational lifetime, storage stability and activity, such as increasing the initial enzymatic activity in reverse micellar systems [7], microencapsulating CPO as a recyclable catalyst [8], immobilizing CPO on some mesoporous or macromolecular materials [9–12]. Recent years, we have carried out some works about the effect of additives on CPO in our laboratory, and found some very efficient additives [13].

In this work, initially we wanted to continue the above-mentioned study in the search for more additives. However, when we came across arginine as the additive and evaluated it with MCD assay, we found that it had a drastically negative effect, which lead to an inactivation of CPO. Since our experimental finding shows contradiction with existing report, which suggested that arginine could enhance the catalytic activity of hemin which has a same active site with CPO [14], we decide to further our study in the reason for deactivation of CPO CPO. Though the inactivation mechanisms of typical peroxidases (e.g., horseradish peroxidase), especially in the presence of high concentration oxidants (i.e., hydrogen peroxide and t-butyl hydroperoxide) have been extensively investigated and are relatively well understood [15–17], very limited information regarding the inactivation of chloroperoxidase is available till now [18–20]. In this study, the interaction of arginine with CPO was investigated in detail, including the inactivation behavior of CPO, the measurements of apparent dissociation constant (K_d) and the number of arginine molecules bounding to each CPO molecule (h), as well as the determination of some kinetic characteristics parameters. Moreover, the inactivation mechanism was proposed based on the obtained data.

2. Materials and methods

2.1. Enzyme and chemicals

Chloroperoxidase was isolated from the growth medium of *C. fumago* according to the method established by Morris and Hager [21] with minor modifications, using acetone rather than ethanol in the solvent fractionation step. The enzyme had

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a specific activity of 7400 U/mL based on the standard Monochlorodimedon (MCD) assay (R_z = 1.41).

MCD was obtained from Fluka. Citrate, acetic acid, sodium citrate, arginine and hydrogen peroxide (30% in aqueous solution) were obtained from Xi'an Chemical Co. Ltd. All chemicals are of analytical grade unless otherwise indicated.

2.2. Enzyme activity assay

Enzyme activity was determined by MCD chlorination assay, according to the method reported previously [22]. Catalytic activity was always tested in pure phosphate buffer (0.1 M, pH = 2.75) containing 0.1 mM MCD, 20 mM KCl, and 2 mM H_2O_2. CPO activity was evaluated by the specific initial reaction rate ν (moles of MCD consumed per unit of time), which was calculated from the slope of changes in absorbance vs. time. Each data point of a set of results was obtained for three measurements and the discrepancy was below 5%.

2.3. Analytical methods

2.3.1. UV spectroscopy

UV–vis absorption spectra of CPO or green CPO were collected in a 1-mL quartz cuvette. 2.5 μM CPO was dissolved in pure 0.05 M citrate buffer (pH = 2–7) or in the presence of different concentration of arginine. Spectra were periodically recorded by a Shimadzu UV-1700 spectrophotometer. The concentration of CPO was determined by the absorbance at 398 nm (Soret band) using an extinction coefficient of 91,200 M^{-1} cm^{-1} [23].

2.3.2. Differential UV spectroscopy assay

The rich optical properties of CPO make it possible to characterize the nature of CPO–substrate interactions using these methods. Differential spectra were employed in this work to examine the binding properties of arginine to CPO because small changes caused by the binding process are difficult to measure directly. The apparent dissociation constant (K_d) can be calculated from the following expression [24,25]:

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_\infty} + \frac{K_d}{\Delta A_\infty} \times \frac{1}{S} \quad (1)$$

where ΔA is the difference between maximum and minimum absorption, [S] is the concentration of free substrate which is assumed to be equal to the initial substrate concentration, and ΔA_∞ is the absorbance change for the complete formation of the enzyme–substrate complexes.

The stoichiometry of substrate binding to CPO can be estimated from the logarithmic form of the Hill equation shown below:

$$\log\left[\frac{\Delta A}{\Delta A_\infty - \Delta A}\right] = h \log[S] + \log[K_d] \quad (2)$$

where ΔA_∞ and K_d are calculated from Eq. (1). A plot of $\log[\Delta A/(\Delta A_\infty - \Delta A)]$ vs. $\log[S]$ gives a straight line with the slope h as the number of substrate molecules bound to each CPO molecule.

2.3.3. Fluorescence spectroscopy

1.5 μM CPO was dissolved in pure 0.05 M citrate buffer (pH = 7) or in the presence of different concentration of arginine at room temperature. The CPO samples were excited at 287 nm and fluorescence spectra were registered from 310 to 330 nm. Intensity was recorded for absorbance of the solutions with and without the arginine after 1 min of reaction.

2.4. Measurements of kinetic parameters

Kinetic studies were performed in a 1-mL quartz cuvette filled with enzyme and arginine solutions both prepared in buffer. The enzymatic reaction was started by adding different concentrations of arginine. The reaction follows Michaelis–Menten kinetics. Kinetic parameters K_m and V_{max} values were determined by measuring the initial rates. k_{cat} value, catalytic efficiencies k_{cat}/K_m was obtained from Lineweaver–Burk plots.

3. Results and discussion

3.1. Inactivation of CPO by arginine

Fig. 1 shows the UV–vis absorption spectra of the native and arginine-interaction forms of chloroperoxidase. The spectrum of native CPO exhibits a Soret maximum at 398 nm with additional peaks at 515 and 547 nm. The Soret band is characteristic π–π* electronic absorption spectrum of the porphyrin ring (line a). This peak position changed gradually with the increase of arginine amount and finally shifted to 425 nm at pH = 7 (line b). In the meantime, the red native CPO solution turned into a green one, accompanying the loss of its catalytic activity measured by MCD assay. This green species was very stable, and no color change was found after 72 h observations.

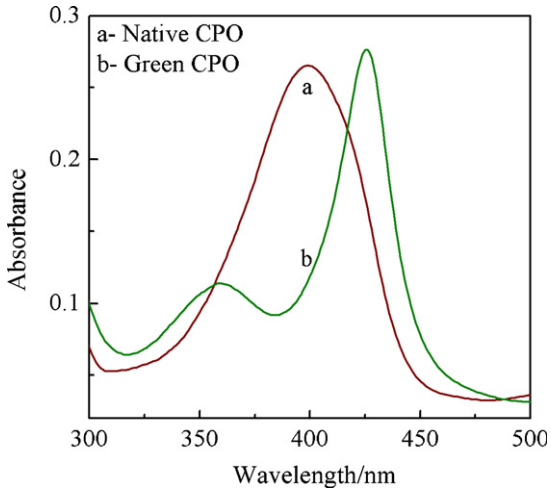

Fig. 1. UV–vis absorption spectra of the native ferric CPO (line a) and CPO–arginine complex (line b).

3.2. Fluorescence analysis

Fig. 2 shows that the intensity of the maximal emission peak at 322 nm of CPO began to increase when the arginine was increased to 1.5 mM, and then kept this increasing continuously. Fluorescence intensity can indicate the exposure degree of prosthetic group of enzyme molecules in the surrounding environment. The increase in intensity revealed the exposure degree of prosthetic group increased. In addition, the interaction between arginine and CPO would also make α-helix loosed. The looseness of α-helix caused the surface fluorescent chromophore of CPO molecules exposed further to the environment.

3.3. Effect of pH on CPO–arginine complex

The stability of CPO was greatly influenced by pH. Because native CPO is stable even at quite low pH, the effect of pH on the formation of CPO–arginine complexes was investigated in a wide pH range. It was found that the formation of the

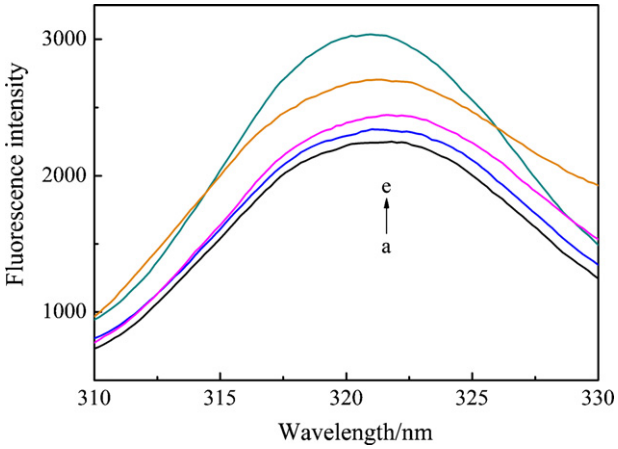

Fig. 2. Effect of the concentration of arginine on the fluorescence spectra of CPO in 0.05 M citrate buffer, pH = 7 at room temperature; the concentration of arginine: (a) 0 mM, (b) 1.5 mM, (c) 5 mM, (d) 10 mM, and (e) 25 mM.