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Kinetic Study on Horseradish Peroxidase Interacting with Cyclodextrin

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Abstract: The catalyst reactivity of Horseradish peroxidase was enhanced in the presence of β-cyclodextrin. During this course, β- cyclodextrin played a role of stabilizing the intermediates of HRP. The results have been investigated using spectra and calculation.

Keywords: Horseradish peroxidase, β- cyclodextrin, compound, kinetics.

Horseradish peroxidase (HRP) catalyzes the oxidation of a wide variety of compounds in the presence of hydroperoxide. The products have been used as the important materials in extensive fields because of the conjuged bond. Many investigators have studied the mechanism of HRP. When native HRP reacts with hydroperoxide, compound I, which is two oxidizing equivalents of the ferric state of HRP, is formed. Compound I is subsequently reduced by one electron to form compound II. Reduction of compound II to native enzyme completes the catalytic cycle[1]. The ferriprotoporphyrin IX prosthetic group that is encapsulated in the proteinoid appendage is the active site of HRP. And the proteinoid appendage functions importantly as a hydrophobic environment. In recent years, research has been concentrated on the mimic of the active site and its environment of heme-containing proteins with porphyrin encapsulated in cyclodextrin and much progress had been achieved[2,3]. But the work had little effort on the real relationship between cyclodextrin and the proteinoid appendage[3]. In order to investigate the role of cyclodextrin, we report here the study of HRP interacting directly with cyclodextrin.

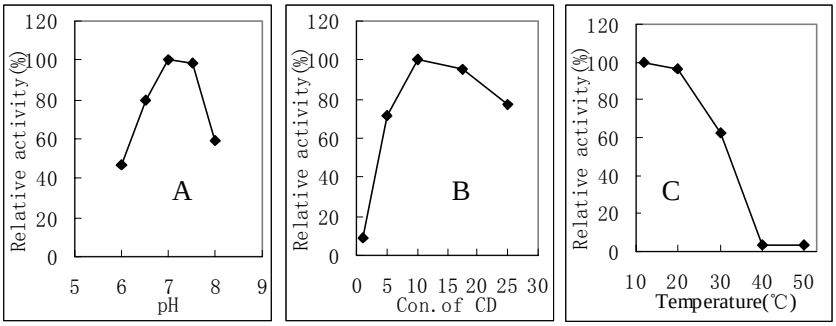
Experimental

Horseradish peroxidase (RZ>3.0) was purchased from Xibas Company. β-CD from Fluka was used without further purification. Other reagents were all analytical grade. Determination of HRP activity was performed on an UV-Vis Spectrophotometer (Shimadzu). And the activity was expressed as the absorbance change at 482 nm per minute per milligram protein. Kinetic measurement were carried out on a DX-17 MV Stopped-Flow Spectrofluorimeter (Applied Photophysics)

Results and discussion

HRP catalyses the polymerization of benzidine in the presence of hydrogen peroxide. **Figure 1** showed the effect of pH, concentration of β-CD and temperature on HRP activity in the solution containing β-CD. Compared with the buffer, the activity of HRP in solution with β-CD had a 3～5 times increase. And the optimum reaction condition had changed a lot. For example, the optimum pH value has risen from 5.9 in buffer to 7.0 in solution with β-CD, while the activity of HRP decreased continuously in solution containing β-CD with rising temperature and in buffer the optimum temperature is around 40℃. **Figure 1** also showed that the optimum concentration of β-CD was 0.01mol/L. Under the same conditions the two solutions including enzyme and substrates with and without β-CD were surveyed and we found in the system without β-CD, the

Figure 1 The relative activity of HRP in the solution with β-CD.
A: β -CD:0.01 mol/L HRP:0.0125μ mol/L H_2O_2 :1m mol/L benzidine:0.2m mol/L



B: PH:6.0 HRP:0.0125μ mol/L H_2O_2 :1m mol/L benzidine:0.2m mol/L
C: PH:6.0 β -CD:0.01 mol/L H_2O_2 :1m mol/L benzidine:0.2m mol/L

color changed immediately to deep blue after HRP was injected and then changed to deep brown. But in the other case converted to green immediately followed with yellow green and then brown. According to Wang[4], the intermediate compound I of HRP in the reaction cycle was green color, and its reaction rate was 10～100 times higher than compound II[5] . So we deduced that the reason why β-CD can accelerate the reaction was that β-CD stabilized the compound I for longer time. Whereas in buffer without β-CD, compound I was very unstable and the time for existence was very short which made the reaction rate slow. To demonstrate our conclusion, we carried out the rapid stopped-flow experiment subsequently. The results were listed in **Table 1**.

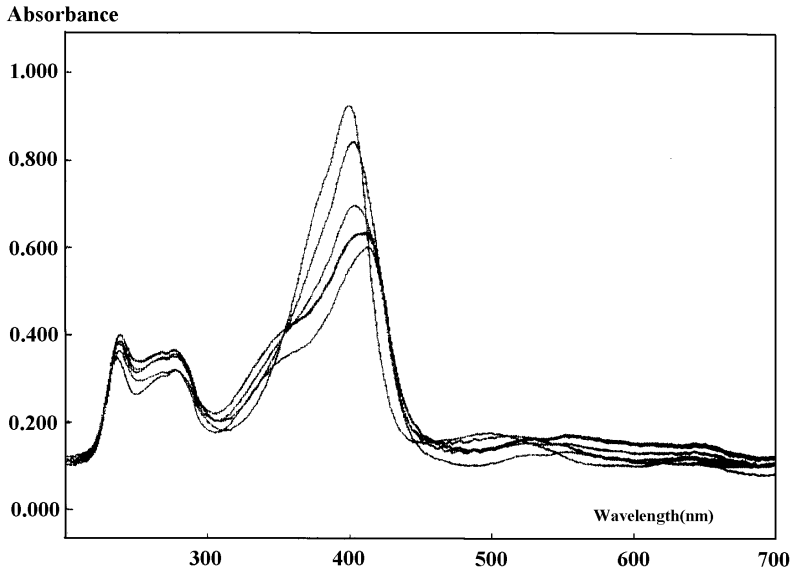
Table 1 The pseudo-first-order rate constants (k/s^{-1}) at 395nm and 411nm

Systems	k (395nm)	k(411nm)
Buffer	163	303
Buffer (β -CD:HRP=250:1)	223	16.3

It showed that the pesudo-first-order rate constant k_{395nm} was smaller than k_{411nm} in buffer while in solution with β -CD the result was on the contrary. According to Johan *et al*[5] , the rate of compound I reacted with substrate is 10～1000 times higher than that of

compound II. We considered that β-CD may play a role of stabling the intermediate compound I. This was in good agreement with the result of steady kinetics. In this experiment encapsulation of benzidine by β-CD was not detected, therefore the possibility for the acceleration of β-CD was that β-CD interacted with HRP. **Figure 2** illustrates the UV-Vis spectra of HRP with various concentrations of β-CD. It showed that the λ_{max} shifted toward longer wavelength and the absorbance decreased continuously. The interloculum of β-CD had a diameter of 780pm, and the crystal structure of HRP-C, the major isoenzyme, was recently resolved to 215pm resolution [6]. The possibility was that the encapsulated compounds of HRP with β-CD were formed in certain proportion, and some residues of HRP interacted with β-CD occurred which changed the polarity around the active site of HRP continuously.

Figure 2 The UV-Vis curves of HRP with diverse concentrations of β-CD
HRP: 25μmol/L β-Cyclodextrin: 2.5, 3.75, 6.25, 7.5,12.5, 25mmol/L



A method of calculation was used to treat the UV-Vis spectra. By postulating the proportion of compound formed with host and guest was 1:1, the coordination can be expressed by equation [7] (1):

$$H + G \longleftrightarrow G \cdot H \qquad (1)$$

Because the concentration of β-CD was much higher than that of HRP, in other word, $[H]_0 >> [G]_0$, the result was expressed as:

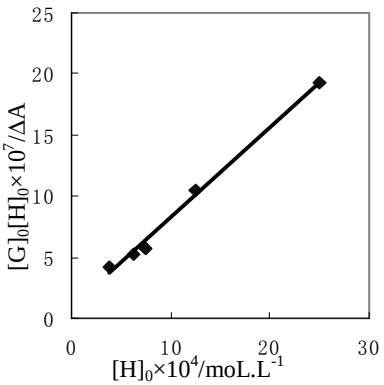
$$[G]_0[H]_0/\Delta A = 1/K_S\Delta\varepsilon + [H]_0/\Delta\varepsilon \qquad (2)$$

where $[G]_0$, $[H]_0$, $\Delta\varepsilon$ and ΔA are the total concentration of enzyme, concentration of β-CD, difference of mole absorbance factor of HRP before and after host-guest compound formed and varieties of absorbance after β-CD was added , respectively. A diagram plotted with $[G]_0[H]_0/\Delta A$ *versus* $[H]_0$ was showed in **Figure 3**. It can be seen

that when β-CD and HRP were in different proportions of 150:1, 250:1, 300:1, 500:1 and 1000:1，$[G]_0[H]_0/\Delta A$ *versus* $[H]_0$ gives a linear relationship. Therefore in this area of concentration ratio, the compound formed by β-CD and HRP was in a proportion of 1:1.

By means of the above-mentioned analysis we suggested that the activity of HRP interacted with β-CD was higher than its activity in buffer through analysis. The possibility was that the composites in proportion of 1:1 was formed in which the conformation of HRP may have changed and the changed conformation is more beneficial for the reaction rate than that in buffer, so the reaction rate of benzidine catalyzed by HRP increased. In this course β-CD provided a hydrophilic environment.

Figure 3 The plot of $[G]_0[H]_0/\Delta A$ Values *vs.* $[H]_0$ for the system of HRP and β-CD in buffer.



HRP: 25μmol/L　β-Cyclodextrin: 3.75,6.25, 7.5, 12.5, 25mmol/L　pH=7

Acknowledgment

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