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Hydrogen Peroxide, Its Measurement and Effect During Enzymatic Decoloring of Congo Red

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Abstract The color of Congo red hinders the spectrometric measurements of a concentration of hydrogen peroxide and enzyme activity (Horseradish peroxidase; HRP) during enzymatic decoloring of Congo red. In this study, a method was developed to measure peroxidase activity and hydrogen peroxide concentration in the presence of Congo red. The oxidation product of HRP/hydrogen peroxide and ABTS(2,2'-azino-bis-(3-ethylbenzotriazoline-6-sulfonic acid)) formed a dark green color. The spectrum of this product showed absorption bands at 420 nm and 734 nm. When compared with the Congo red spectrum, the absorption at 734 nm of this product did not overlap with Congo red, thus making the hydrogen peroxide measurement possible even in the presence of Congo red. Kinetic study of decoloring of Congo red performed by this method showed that the decoloring reaction followed the Michaelis-Menten kinetics. Pulse feeding of hydrogen peroxide, upon depletion, significantly increased the decoloring of Congo red. This result shows that this newly developed technique can monitor, predict, and improve the enzymatic decoloring process.

Key words: Congo red, H_2O_2, decoloring

Production of various types of dyes from industrial processes and release of dye-containing wastewater into natural ecosystems cause serious environmental pollution [8, 17]. Dyes, due to their calcitrant structure, are resistant to microbial biodegradation and generate severe problems [2, 15, 19].

Azo dyes, the most widely used dye in the textile industry, are reported to be resistant to biodegradation in aerobic conditions and to produce toxic carcinogenic aromatic amine anaerobically [2, 8, 11, 12, 16, 20, 21]. Although bleaching of azo dye by chlorine can remove the color of the dye, the toxicity of chlorine and its byproducts imposes a serious threat to the environment [3]. In order to overcome these problems, microbial or enzymatic decoloring of dye-wastewater has been developed [3, 4]. Lignin peroxidase which is produced from a lignin-degrading fungus, *Phanerochaete chrysosporium*, and horseradish peroxidase (HRP) which is produced from plants, are known to be involved in the decoloring of the dyes [3, 4]. Peroxidase and hydrogen peroxide generate free radicals which oxidize the double-bond of the dye, resulting in the loss of color [4]. Hydrogen peroxide is consumed during the reaction, and measuring its concentration can help predict the progress of the enzymatic decoloring process. Many methods have been developed to measure the hydrogen peroxide concentration in pharmaceutical, food, and environmental applications [6, 13, 14, 18]. Among these methods, an assay using peroxidase is widely used due to its simple procedure, and is widely applied to various samples. Among various peroxidases, horseradish peroxidase (HRP), a Fe^{2+}-containing peroxidase, has a high oxidation activity and is preferred for measurement of hydrogen peroxide. Previously, hydrogen peroxide consumption was determined by measuring the change of color at 555 nm which is due to the oxidation of 4-aminoantipyrine(4-AAP) in HRP/hydrogen peroxide. However, many dyes including Congo red and Orange II have their own color at 555 nm which interfere with the measurement of hydrogen peroxide during enzymatic decolorizations of dyes [7, 9, 16]. Therefore, alternative methods should be developed to measure hydrogen peroxide concentration in the presence of dyes such as Congo red.

This study was undertaken to develop a method to measure hydrogen peroxide concentration in the presence of Congo red. By using this technique, the effects of hydrogen peroxide and Congo red concentration on the decoloring rate were investigated by kinetic studies.

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Furthermore, conditions for the decoloring of Congo red by HRP/hydrogen peroxide were optimized.

Materials and Methods

Materials

Horseradish peroxidase (HRP) (Sigma, St. Louis, MO, U.S.A.), Congo red (Aldrich, St. Louis, MO, U.S.A.), ABTS (2,2'-azino-bis-(3-ethylbenzotriazoline-6-sulfonic acid)) (Sigma, St. Louis, MO, U.S.A.), and hydrogen peroxide (Junsei, Tokyo, Japan) were all reagent-grade. Their spectra were measured in 100 mM phosphate buffer (pH 7.0) by a UV-160A spectrophotometer (Shimadzu, Tokyo, Japan).

Assay of HRP Activity and Hydrogen Peroxide Concentration

HRP activity was determined by an increase in absorbance at 734 nm due to the oxidation product of ABTS. The reaction mixture contained 0.0225 μM HRP, 0.5 mM ABTS, and 80 μM hydrogen peroxide in 1 ml of 100 mM phosphate buffer (pH 7.0). One unit of enzyme was defined as the amount of enzyme required for an increase of 0.01 absorbance at 734 nm per min. Hydrogen peroxide concentration was measured from the standard curve, which related the hydrogen peroxide concentration with absorbance at 734 nm in 100 mM phosphate buffer (pH 7.0). The buffer contained 12.7 Unit/l HRP, 0.5 mM ABTS, and varying concentrations (from 0.8 μM to 880 μM) of hydrogen peroxide.

Decoloring of Congo Red by HRP/H_2O_2

Decoloring of Congo red was measured by the decrease of absorbance at 488 nm in 100 mM phosphate buffer (pH 7.0), containing 43 μM Congo red, 24.8 Unit/l of HRP, and 80 μM hydrogen peroxide. Kinetic constants of the decoloring reaction were determined in the 100 mM phosphate buffer (pH 7.0) that contained 24.8 Unit/l of HRP, by varying the concentration of Congo red (from 14.4 to 43.0 μM) and hydrogen peroxide (from 0.1 to 800 μM). The concentration of hydrogen peroxide during the decoloring reaction was determined by adding 0.5 ml of 100 mM phosphate buffer (pH 7.0) and 0.1 ml of ABTS (5 mM) to the 0.5 ml sample of the decoloring reaction. Also, HRP activity during the decoloring reaction was measured by adding 80 μM hydrogen peroxide to the sample.

Results and Discussion

H_2O_2 Measurement

Measurement the HRP activity and hydrogen peroxide by using ABTS in the presence of Congo red is difficult, if the oxidation product of ABTS has the same spectrum as Congo red. A spectrophotometrical analysis of each of the reactants was peformed. No absorption peak was observed above 400 nm in the tested reactants. Also, there were no changes in the spectra of ABTS and HRP, indicating that hydrogen peroxide was required for the reaction to occur (Fig. 1).

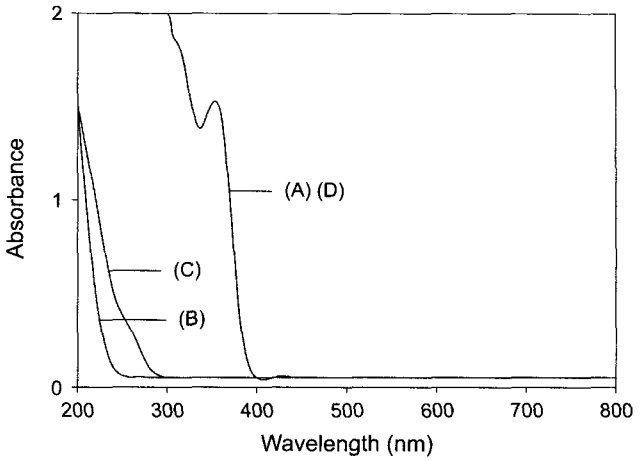

Fig. 1. Absorbtion spectra of reactants. (A) 0.5 mM ABTS, (B) 12.71 Unit/l HRP, (C) 80 μM H_2O_2, (D) ABTS (0.5 mM)+HRP (12.71 Unit/l).

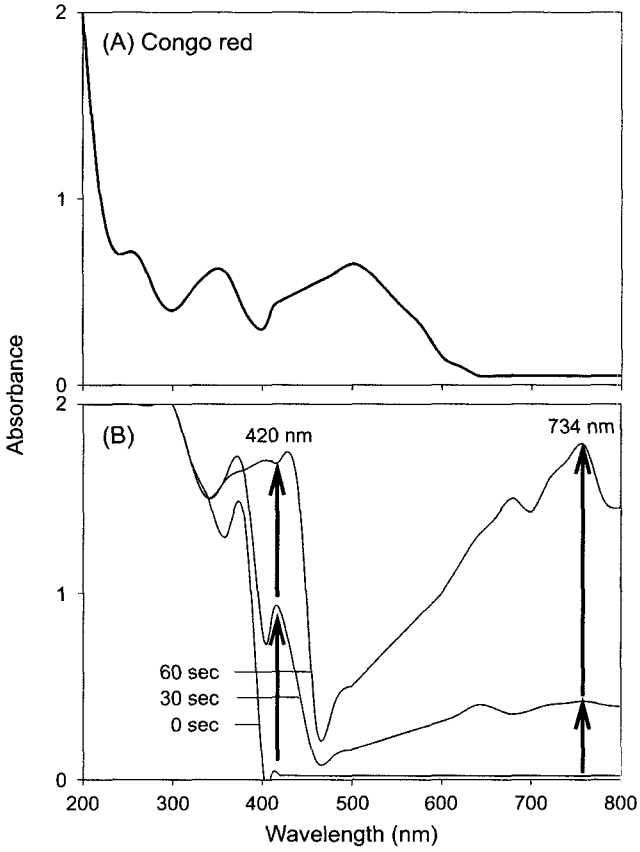

Fig. 2. Absorption spectra of (A) Congo red and (B) time-course changes of products after addition of H_2O_2 to HRP-ABTS. Arrows indicate the increases in absorbance at 420 nm and 734 nm.

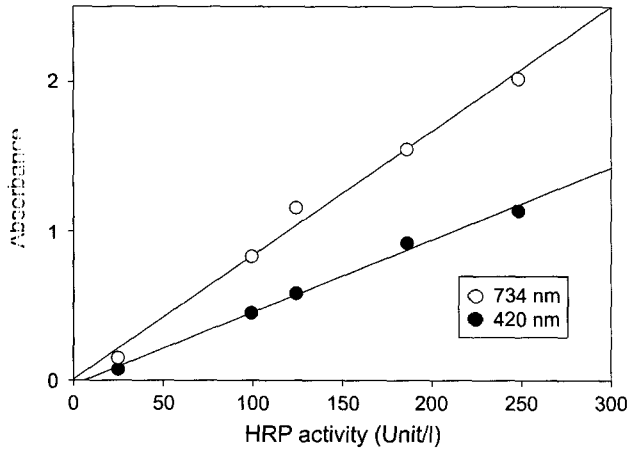

Fig. 3. Linear absorption of HRP-H_2O_2 product at 420 nm and 734 nm.

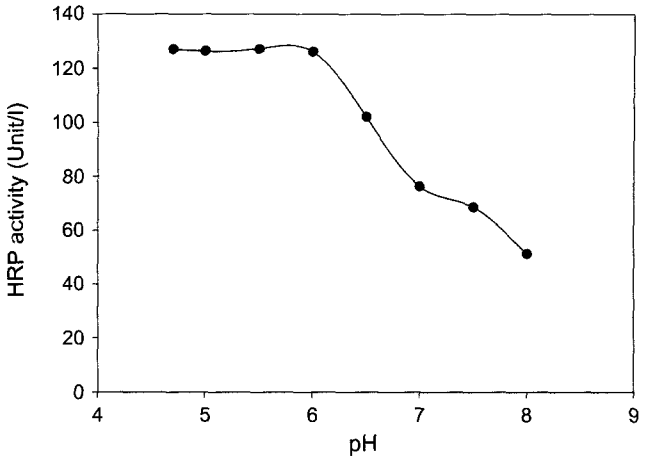

Fig. 5. Effect of pH on the HRP activity.

In order to investigate whether the spectra of the oxidation product of ABTS overlap that of Congo red, the spectrum of each reactant, such as HRP, ABTS, and hydrogen peroxide, was compared (Figs. 2A and 2B). When hydrogen peroxide was added to the ABTS/HRP, the oxidation product with two distinct absorption peaks appeared at 420 nm and 734 nm (Fig. 2B). The peak at 420 nm was due to the production of an ABTS cation radical. Peroxidase activity was previously determined by measuring absorbance at 420 nm [1, 10]. The absorption peak at 734 nm showed no overlapping with the spectrum of Congo red (Fig. 2A), therefore, this peak could be used for measuring the hydrogen peroxide concentration even in the presence of Congo red. The increase in the absorbance at 734 nm was compared with that at 420 nm without Congo red. A linear increase in absorbance was observed at both 420 nm and 734 nm at various HRP concentrations (Fig. 3).

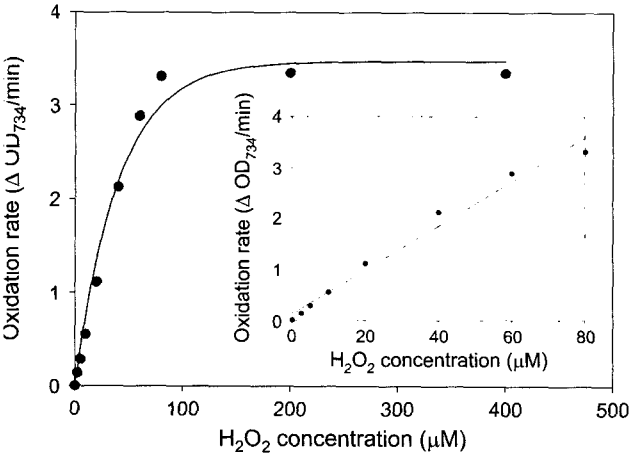

Fig. 4. Effect of H_2O_2 on the oxidation of ABTS by HRP. Oxidation rate was measured by the increase in the absorbance at 734 nm after 30 sec of oxidation.

This indicates that hydrogen peroxide or HRP activity can be measured at 734 nm even when the sample contains dyes or colors such as Congo red, Orange II, lignin, or coal.

The concentration of hydrogen peroxide was varied to determine the range where the hydrogen peroxide was linear to the absorbance at 734 nm (Fig. 4). The rate of increase of the absorbance at 734 nm showed a linearity up to 80 μM hydrogen peroxide. Above 80 μM, no further increase in oxidation rate was observed, indicating that the effective detection range of the hydrogen peroxide was up to 80 μM by this method (Fig. 4). This is a similar range to that reported by Huang *et al.* [7], who demonstrated that a hydrogen peroxide concentration from 0 to 84 μM could be detected by β-CD-hemin.

The pH and temperature of the oxidation reaction were varied to find the effective range of pH and temperature so as to measure hydrogen peroxide at 734 nm. In order to determine the effect of pH on hydrogen peroxide by this method, the pH was varied from 4.5 to 8.0. As shown in Fig. 5, no significant change was observed in the pH range from 4.5 to 6.0. However, the detection efficiency of hydrogen peroxide decreased significantly at pHs above 6.5

The effects of temperature on HRP activity were investigated. HRP activity remained relatively constant between 25-35°C and decreased slowly as the temperature increased. The optimum pH and temperature of H_2O_2 measurements, therefore, were pH 4.5-6.0 and 20-35°C, respectively.

Characteristics of Congo Red Decoloring Reaction

The effects of hydrogen peroxide and HRP on Congo red decoloring were investigated. Fifty percent of added Congo red was degraded in 2 h. However, degradation of Congo red was not observed, if either HRP or hydrogen peroxide was not added, indicating the interaction of HRP and hydrogen peroxide in Congo red decoloring. The effects of two substrates, Congo red and hydrogen peroxide, on the

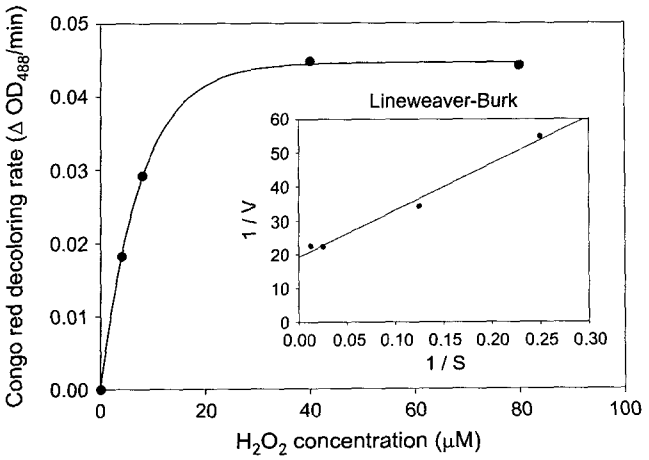

Fig. 6. Oxidation rate of Congo red as a function of H_2O_2 concentration.

Congo red decoloring rate were investigated by varying the concentration of one substrate while maintaining the other substrate at a constant. The effects of hydrogen peroxide on the decoloring rate showed a Michaelis-Menten type of kinetics. From the Lineweaver-Burk plot, the V'_{max} (apparent maximum reaction rate) and K'_m (apparent Michaelis constant) were determined to be 0.05 OD_{488}/min and 7.2 μM, respectively. Congo red showed similar effects on the decoloring rate (Fig 6). Increasing the Congo red concentration increased the decoloring rate. The V''_{max} and K''_m of Congo red were 0.14 OD_{488}/min and 91 μM, respectively (Fig. 7).

After 2 h of Congo red decoloring by HRP/ hydrogen peroxide, there was no more reaction observed. To elucidate the reason for the discontinuation of the reaction, HRP activity and hydrogen peroxide concentrations were determined by measuring the absorbance at 734 nm. As shown in Fig. 8, HRP activity remained constant.

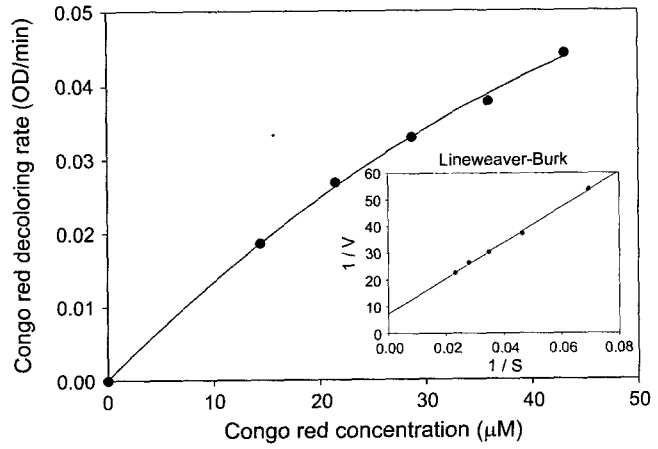

Fig. 7. Oxidation rate of Congo red as a function of Congo red concentration.

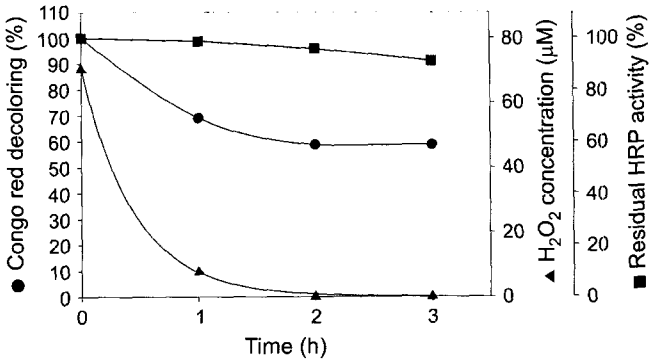

Fig. 8. Time course of Congo red decoloring by HRP.

Discontinuation of the decoloring reaction was due to complete consumption of hydrogen peroxide. However, excessively high hydrogen peroxide concentration over 80 μM caused the inactivation of the enzyme, resulting in a decrease in the decoloring rate (data not shown). The enzyme, which was recovered by ultrafiltration of the enzyme solution, was inactive, indicating that the enzyme had been inactivated by hydrogen peroxide (data not shown). Therefore, Michaelis-Menten kinetics, as described in Fig. 6, was effective within the hydrogen peroxide concentration of 80 μM. Supplying a low concentration of hydrogen peroxide by pulse was attempted, so as to decolorize the Congo red without discontinuation. As shown in Fig. 9, the pulsewise feeding of hydrogen peroxide every 2 h decolorized the Congo red at a constant rate, achieving 33% conversion in 8 h.

Conclusions

In this study, a method was developed to determine hydrogen peroxide and HRP concentrations in the presence

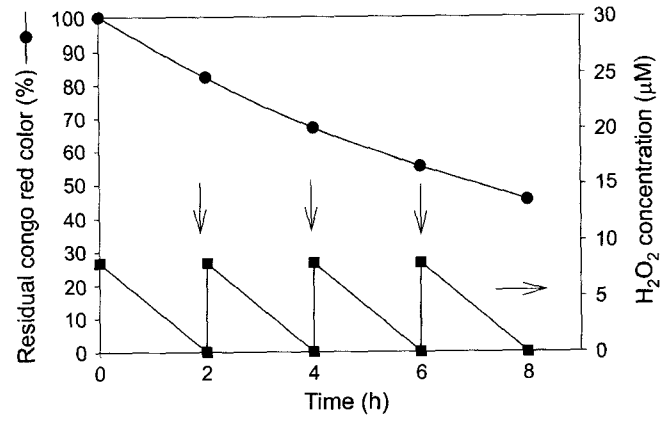

Fig. 9. Effect of H_2O_2 pulse feeding on Congo red decoloring. Arrows indicate the point of addition of 80 μM of H_2O_2.

of Congo red dye. By using this assay method, the decoloring kinetics of Congo red was investigated. Also determined were the Michaelis-Menten kinetic constants describing the decoloring rate of Congo red and hydrogen peroxide.

By measuring the hydrogen peroxide concentration during Congo red decoloring, it was found that hydrogen peroxide was a limiting substrate that resulted in the discontinuation of the reaction. Pulsewise feeding of the hydrogen peroxide increased the decoloring reaction.

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