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Enhanced dye decolorization efficiency by citraconic anhydride-modified horseradish peroxidase

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Abstract

Bromophenol blue and methyl orange removal capabilities of citraconic anhydride-modified horseradish peroxidase were compared with those of native horseradish peroxidase. Citraconic anhydride-modified horseradish peroxidase showed higher decolorization efficiencies for both dyes than native horseradish peroxidase. Upon the chemical modification, the decolorization efficiencies were increased by 1.8% and 12.4% for bromophenol blue and methyl orange, respectively. The quantitative relationships between decolorization efficiencies of dyes and reaction conditions were also investigated. Experimental data revealed that aqueous phase pH, reaction time, temperature, enzyme concentration and ratio of dye and H_2O_2 play a significant role on the dye degradation. Lower dose of citraconic anhydride-modified horseradish peroxidase was required than that of native enzyme for the decolorizations of both dyes to obtain the same decolorization efficiencies. Citraconic anhydride-modified HRP exhibited a good decolorization of dye over a wide range of dye concentration from 8 to 24 or 32 $\mu mol\,l^{-1}$ at 300 $\mu mol\,l^{-1}$ H_2O_2, which would match industrial expectations. Kinetic constants for two different dyes were also determined. Citraconic anhydride-modified horseradish peroxidase shows greater affinity and catalytic efficiency than native horseradish peroxidase for both dyes.

Keywords: Horseradish peroxidase; Dye decolorization; Citraconic anhydride modification; Bromophenol blue; Methyl orange

1. Introduction

Approximately 10,000 different dyes and pigments are produced annually worldwide and used extensively in the dye and printing industries [1]. It is estimated that about 10–14% of the total dye used in the dyeing process may be found in wastewater [2]. These dyes are considered to be recalcitrant, and toxic. They resist microbial biodegradation and are, therefore, not easily degraded in wastewater treatment plant [3]. Thus, treatment of dye is yet one of the challenging tasks in environmental field. Currently available methods such as chemical oxidation, reverse osmosis, adsorption, etc., suffer from disadvantages such as high cost, regeneration problem and secondary pollutant/sludge generation [4]. Recently, researchers have been focusing their attention to enzymatic treatment. Many peroxidases such as lignin peroxidase, manganese peroxidase, soybean peroxidase, horseradish peroxidase (HRP) and laccase, etc., were applied to decolorize and degrade dye in industrial effluents [1,4,4–7]. However, due to inactivation, large amounts of enzyme are required to achieve a high degree of decolorization, thus limiting its use in industrial situation. Immobilization of enzyme is one of the methods to overcome these limitations. Mohan et al. reported that acrylamide gel immobilized HRP showed effective performance compared to free HRP and alginate entrapped HRP [7]. Cheng et al. also reported that aluminum-pillared interlayered clay (Al-PILC) immobilized HRP could be applied over a broader range of pH from 4.5 to 9.3 for phenol removal and had better storage stability than free enzyme [8]. Chemical modification of HRP surface has been performed to improve its stability and catalytic efficiency. Our previous papers reported that modification of HRP by phthalic anhydride improved HRP's stability and catalytic activity both in aqueous buffer and some organic solvents [9–11]. O'Brien and Ó'Fágáin demonstrated that phthalic anhydride- and ethylene glycol-bis-(succine acid *N*-hydroxysuccinimide ester)-modified HRP showed slightly greater bleaching ability at 65 °C than native HRP for some of dyes [12]. However, to the best of our knowledge, no report has appeared on systematic evaluation of efficiency of modified

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HRP in degradation of dyes. We also reported that HRP-modified by citraconic anhydride (CA) had a greater thermostability both in aqueous buffer and organic solvents than native HRP [13]. Thus, in order to investigate the decolorization efficiencies of dye by CA-modified HRP, effects of parameters such as aqueous phase pH, temperature, H_2O_2, HRP and dye concentration on the dye decolorization efficiencies, were investigated, and then compared with those of native enzyme. The kinetic constants for selected dyes were also determined.

2. Materials and methods

2.1. Chemicals

Horseradish peroxidase was purchased from Shanghai Lizhu Dong Feng Biotechnology Co. Ltd. and had a specific activity of 250 purpurogallin units/mg and $RZ = A_{402}/A_{280} = 3.0$. Citraconic anhydride was purchased from Alfa Aesar. Methyl orange and bromophenol blue (analytical grade, the structure is shown in Scheme 1) were obtained from Shanghai Reagent Company. All other reagents were of analytic grade.

2.2. Chemical modification

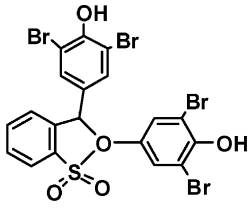
Chemical modification was based on our previous method [10,14]. Twenty-five microlitres 50% (v/v) citraconic anhydride in 0.1 mol l^{-1} phosphate buffer (pH 7.4) and 2 ml 1 mg ml^{-1} HRP in 0.1 mol l^{-1} phosphate buffer (pH 7.4) were mixed. The reaction proceeded at 4 °C for 1 h and was then dialyzed against 0.1 mol l^{-1} phosphate buffer (pH 7.4) at 4 °C to removal excess reagent.

The degree of modification was about 50% estimated by the method of Snyder and Sobocinski [14].

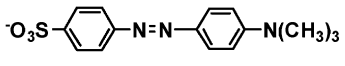
2.3. Peroxidase activity assay

The enzyme activity was assayed by colorimetric method [11]. Reaction mixture containing 10 mmol l^{-1} phenol, 0.2 mmol l^{-1} hydrogen peroxide and 2.4 mmol l^{-1} 4-aminoantipyrin (4-AAP) in a total volume of 3.0 ml was incubated at 30 °C. All reagents were dissolved in 0.05 mol l^{-1} phosphate buffer (pH 7.0). The reaction was then started by adding 0.1 ml of diluted enzyme solution, and the initial increase in absorbance was monitored at 510 nm during 1 min. Under such conditions, the rate of formation of colored product which absorbs light at a peak wavelength of 510 nm was calculated using a molar extinction coefficient of 7100 l mol^{-1} cm^{-1}. One unit of peroxidase activity was defined as the amount of the enzyme consuming 1 μmol of hydrogen peroxide per minute under the assay conditions.

HRP concentration was estimated from its Soret absorbance (molar extinction coefficient at 402 nm = 102 l mol^{-1} cm^{-1}) [11].



Bromophenol blue



Methyl orange

Scheme 1. The structure of bromophenol blue and methyl orange.

2.4. Dye assay

Quantitative estimation of the dye in the aqueous phase was carried out by colorimetry. A solution of 0.24 mol l^{-1} of the dye was scanned over a wavelength range of 200–700 nm by using Shimadzu UV2450. λ_{max} and absorbance at λ_{max} were determined. λ_{max} for bromophenol blue and methyl orange is 592 and 462 nm, respectively. And then the molar extinction coefficient was calculated (31.09 and 26.81 l $mmol^{-1}$ cm^{-1} for bromophenol blue and methyl orange, respectively).

2.5. Dye degradation

The dye was first dissolved in 50 mmol l^{-1} citrate buffer (pH 5.0). The assay medium was 50 mmol l^{-1} citrate buffer. Typically, 0.1 ml of diluted enzyme solution and 30 μl of 30 mmol l^{-1} H_2O_2 were used in 3 ml of reaction mixture for the assay. The decrease in absorbance of the dye solution at the respective λ_{max} was monitored. Series of experiments were performed by varying the process parameters such as aqueous phase pH, reaction temperature, reaction time, enzyme concentration and dye concentration. The conditions are detailed in the legends to figures.

2.6. Kinetic studies

The kinetic experiments were performed using constant enzyme and H_2O_2 concentration as the dye degradation and varying the concentration of substrate under the optimum conditions for dye degradation.

The values given in the paper represent the mean of three independent sets of experiments with S.D. of less than 5%.

3. Results and discussion

Enzyme has an optimum pH range at which its activity is maximum. The efficiency of dye removal by CA-modified HRP (CA-HRP) at various pH values of the reaction mixture is depicted in Fig. 1. The similar result was obtained in the dye degradation using native HRP (data not shown). From Fig. 1, we can find that the decolorization efficiency of bromophenol blue was highest at pH 4.0–5.0. The bell-shaped curve with a defined pH optimum was also obtained in HRP and phthalic anhydride-modified HRP catalyzed removal of phenol [8,9]. The reason is that bromophenol blue and phenol are also phenolic compound. However, the optimum pH for bromophenol blue was different from that for phenol.

However, the decolorization efficiency of methyl orange decreased with the increase of pH value of the reaction mix-

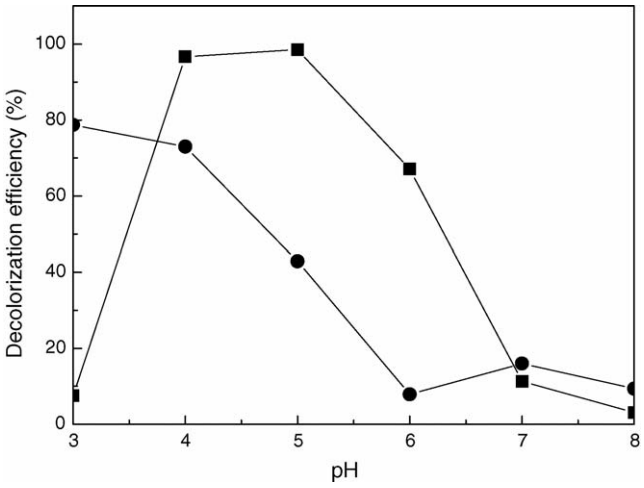


Fig. 1. Effect of aqueous phase pH of the reaction mixture on dye removal by CA-modified HRP. Bromophenol blue (■); methyl orange (●). Conditions: temperature, 30 °C; reaction time, 5 min; dye concentration, 8 μmol l^{-1}; H_2O_2 concentration, 0.3 mmol l^{-1}; enzyme concentration, 0.15 U ml^{-1}.

ture. That was to say, a conventional bell-shaped curve with a defined pH optimum could not be obtained within the pH range tested. This result is similar to that of Deveci et al. for azo dye decolorization using laccase [4] and Bhunia et al. for Remazol dye decolorization using HRP [5]. Bhunia et al. thought that this behavior could be attributed to the inhibition of Remazol blue to HRP at pH values of greater than 6.0 [5]. To approve this view, we conducted an inhibition assay with CA-modified HRP using the standard activity assay. The inhibition assay was performed in the presence of 5.0 mg l^{-1} bromophenol blue or 2.5 mg l^{-1} methyl orange in the reaction mixture of activity assay. Control experiments were performed in the absence of both dyes. The inhibition efficiencies were about 55% and 85% for bromophenol blue and methyl orange, respectively. This indicates that both dyes showed a strong inhibition at higher pH values.

Thus, we selected pH 5.0 for bromophenol blue degradation and pH 3.0 for methyl orange degradation in the further experiments.

To examine the effect of temperature on dye removal, the degradation reactions were performed at optimal pH and temperatures from 30 to 80 °C by keeping all the other experimental conditions constant (see the legends). The results are presented in Fig. 2. The decolorization efficiency decreased with an increase in reaction temperature above 40 °C for methyl orange or 50 °C for bromophenol blue. That is in agreement with that of phenol removal using *Coprinus cinereus* peroxidase [15] and HRP [9,10]. It may be due to the lower solubility of the formed polymer at low temperature; that is, precipitation occurred without adsorption of enzyme on the polymers resulting in extending catalyst lifetime at low temperature [15]. Another reason may be the lower concentration of free radicals, which reduce the enzyme inactivation and the coupling of free radicals. It was reported that when laccases from various fungi were used for colour removal, the optimal temperature was 50–60 °C [4,16–18]. Greco et al. also reported that when peroxidase purified from *Geotririchum candidium* was used for colour removal of Remazol Brilliant Blue R, the activity decreased at temperatures above 30 °C, e.g. 90% decrease at 50 °C [19]. Therefore, considering of the cost of temperature control, 30 °C was selected in the further experiments.

Fig. 3 shows the effects of reaction time on dye degradation by native and modified HRP. It is evident from the figure that 5 min of the reaction time is sufficient for the dye degradation. However, Mohan et al. reported that 45 min of reaction time was required for HRP catalyzed Acid Black 10 BX degradation [7]. After 5 min, the removal reaction followed by a very slow removal process. This slowdown can be attributed to the simultaneous decrease in the concentration of all the reacting species (phenol, HRP and H_2O_2). Subsequent experiments were conducted for 5 min of reaction time.

In general, removal of aromatic compound is dependent on the amount of catalyst added since the catalyst has a finite lifetime and also the conversion is found to be dependent on the reaction time. There is an optimal relationship between the concentration of enzyme and substrate for achieving maximum activity. Decolorization efficiencies of bromophenol blue and methyl orange at different HRP doses are illustrated in Fig. 4. The decolorization efficiencies increased with the increase in the concentration of enzyme from 0.078 to 0.15 U ml^{-1}. However, subsequent increase in enzyme dose up to 0.198 U ml^{-1} had a significantly low impact on the dye degradation. The reason may be each peroxidase molecular catalyses fewer reactions under higher enzyme concentration, and decreasing the catalytic effi-

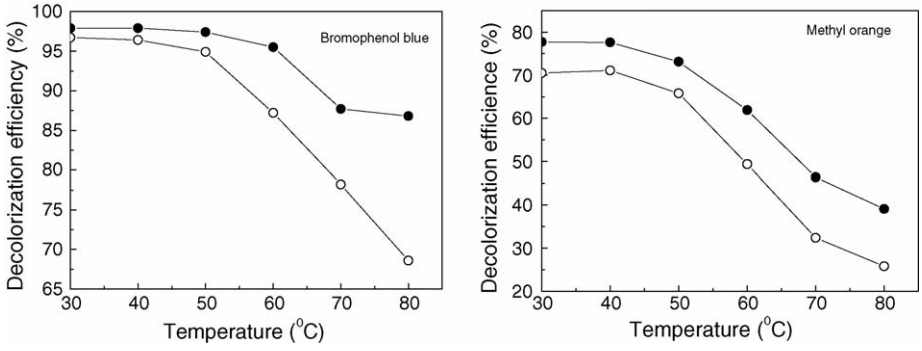


Fig. 2. Effect of reaction temperature on dye removal by native HRP (◯) and CA-modified HRP (●). Conditions: corresponding optimal pH; reaction time, 5 min; dye concentration, 8 μmol l^{-1}; H_2O_2 concentration, 0.3 mmol l^{-1}; enzyme concentration, 0.15 U ml^{-1}.

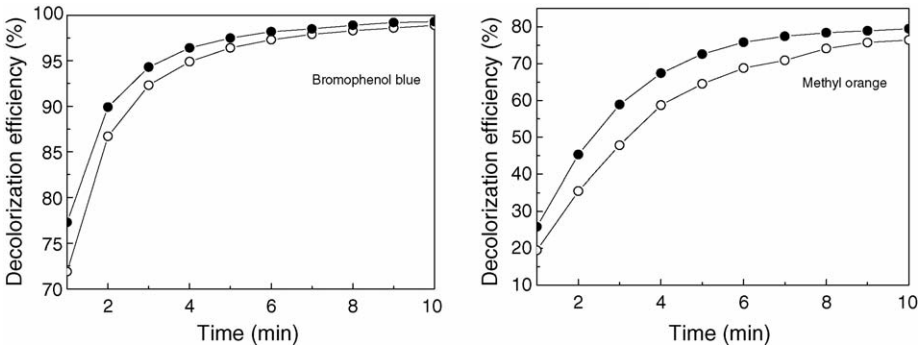


Fig. 3. Effect of reaction time on dye removal by native HRP (◯) and CA-modified HRP (●). Conditions: corresponding optimal pH; temperature, 30 °C; dye concentration, 8 μmol l^{-1}; H_2O_2 concentration, 0.3 mmol l^{-1}; enzyme concentration, 0.15 U ml^{-1}.

ciency. The results indicate that the enzyme dose of 0.15 U ml^{-1} was enough to the removal of 8 μmol l^{-1} of both dyes (about 2.6 mg l^{-1} of methyl orange and 5.4 mg l^{-1} of bromophenol blue) after 5 min. However, 0.22 U ml^{-1} immobilized HRP was required for 2.0 mg l^{-1} for acid azo dye (Acid Black 10 BX) removal after 45 min [7].

The price of HRP was very high. The cost of enzyme had always been the bottleneck of application of enzymatic process on the treatment of wastewater. Thus, one could increase the reaction time to offset the reduction in decolorization efficiency at low enzyme concentration.

From Fig. 4, we can also find that lower dose of CA-HRP was required than that of native HRP for the decolorization of both dyes when the decolorization efficiencies were the same.

Concentration of substrate present in the aqueous phase has significant influence on any enzymatic reaction. In the process of HRP catalyzed reaction, dye and H_2O_2 act as substrates. H_2O_2 activates the enzymatic action of peroxidase radical. It contributes in the catalytic cycle of peroxidase, to oxidize the enzyme to form an enzymatic intermediate, which accepts the aromatic compound to carry out its oxidation to a free radical form. However, excess of H_2O_2 leads to an inactivation of HRP. Enzyme immobilization or in situ generation of H_2O_2 was successively applied to overcome the problem [20,21]. Thus, the ratio of dye and H_2O_2 has significant influence on the reaction rate. In our previous paper, the ratio of H_2O_2 and phenol of 2.0 was beneficial for phenol removal by native and phthalic anhydride-modified HRP [9]. However, for chlorophenol removal by native and phthalic anhydride-modified HRP, the optimal ratio of H_2O_2 and phenol was 1.25 [10]. Cheng et al. reported that ratio of H_2O_2 and phenol was 1.5 for phenol removal using immobilized HRP [8]. Zhang and Nicell reported that ratio of H_2O_2 and pentachlorophenol was about 0.5 in the treatment of aqueous pentachlorophenol [22]. The ratio of H_2O_2 and 4-chlorophenol of 1.0 was optimal for 4-chlorophenol removal by HRP. From these literatures, it is clear that the ratio of H_2O_2 and phenolics is different for different phenolics removal by HRP. Thus, we investigated the effect of ratio of H_2O_2 and dye on dye degradation by changing dye concentration. The results are shown in Fig. 5. The decolorization efficiencies increased with the increase of dye concentration when dye concentration was smaller than 8 μmol l^{-1}, then kept a constant up to dye concentration of 24 or 32 μmol l^{-1} and finally decreased. Thus, the optimal ratio of H_2O_2 and dye was about 18 for both dyes by native and CA-modified HRP. This result is similar to that of Ferreira et al. [23]. Ferreira et al. reported that the oxidation of methylene blue by *Phanerochaete chrysosporium* lignin peroxidase occurred via stepwise *N*-demethylation followed by aromatic ring cleavage to result in a decolorization level of about 85% by means of changing methylene blue:H_2O_2 from 1:1 to 1:10 [23]. They also found that the optimal ratio of methylene blue:H_2O_2 was 1:40 for methylene blue decolorization by HRP [24]. A few researchers thought formation of larger polymers required more peroxide [25–27]. However, the optimal ratio of H_2O_2 and acid azo dye was about 0.16 for acid black 10 BX decolorization by HRP [7].

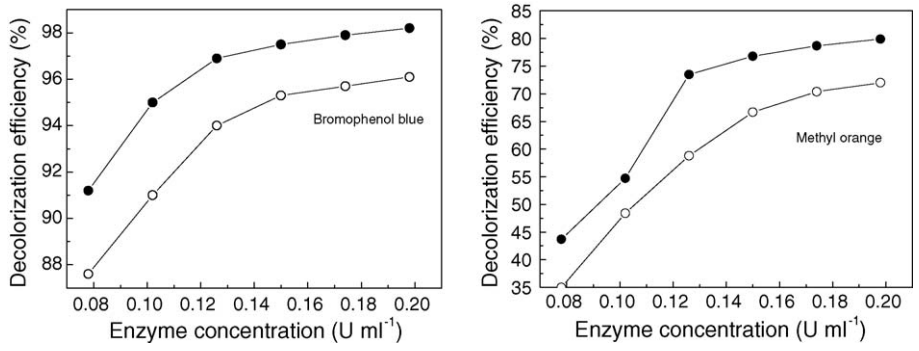


Fig. 4. Effect of enzyme concentration on dye removal by native HRP (◯) and CA-modified HRP (●). Conditions: corresponding optimal pH; temperature, 30 °C; reaction time, 5 min; H_2O_2 concentration, 0.3 mmol l^{-1}; enzyme concentration, 0.15 U ml^{-1}; dye concentration, 8 μmol l^{-1}.

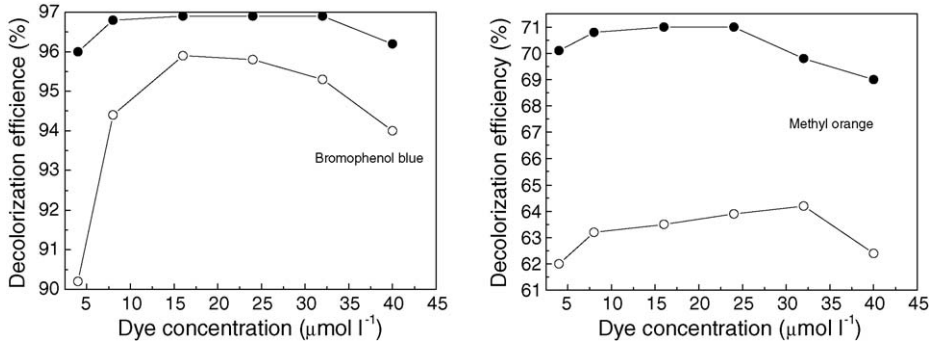


Fig. 5. Effect of dye concentration on dye removal by native HRP (◯) and CA-modified HRP (●). Conditions: corresponding optimal pH; temperature, 30 °C; reaction time, 5 min; dye concentration, 8 U ml^{-1}; enzyme concentration, 0.15 U ml^{-1}; H_2O_2 concentration, 0.3 mmol l^{-1}.

Table 1
The apparent kinetic parameters of dye degradation by native and modified HRP at infinite dye concentration and 0.3 mmol l^{-1} H_2O_2 at 30 °C

Dye	Native HRP			CA-HRP		
	K_m (μmol l^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (l μmol^{-1} min^{-1})	K_m (μmol l^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (l μmol^{-1} min^{-1})
Bromophenol blue	25.95	9.25×10^3	3.56×10^2	14.15	1.10×10^4	7.77×10^2
Methyl orange	37.39	4.23×10^3	1.13×10^2	30.26	6.59×10^3	2.18×10^2

From Fig. 5, we can also find that CA-modified HRP had a broader range of dye concentration from 8 to 24 or 32 μmol l^{-1} at the same amount of H_2O_2 (300 μmol l^{-1}) than native HRP. It indicates that CA-modified HRP exhibited a good decolorization of dye over a wide range of dye concentration than native HRP, which was benefit to the removal of the real industrial wastewater.

We measured the initial rates of dye degradation at various concentration of the dye but at a fixed concentration of H_2O_2 (0.3 mmol l^{-1}) and the enzyme at pH 5.0 for bromophenol blue or pH 3.0 for methyl orange. The plot of initial rate versus dye concentration follows a hyperbolic pattern as expected Michaelis–Menten kinetics (data not shown). The apparent kinetic parameters are reported in Table 1. As shown, the chemical modification increased the catalytic constant (k_{cat}) and decreased the values of the apparent Michaelis constant (K_m) for both dyes. Thus, the catalytic efficiencies (k_{cat}/K_m) of CA-HRP for both dyes were higher than that of native HRP. From Figs. 2–5, we can also find that CA-modified HRP showed higher decolorization efficiencies of both bromophenol blue and methyl orange than native HRP. The decolorization efficiency of bromophenol blue increased to about 97.8% from 96.1%, and that of methyl orange increased to about 74.5% from 66.3% after this modification. The trend is consistent with the results of kinetic studies (Table 1).

Table 1 also shows that the affinity, the turnover number and the catalytic efficiencies (k_{cat}/K_m) for bromophenol blue were greater than that for methyl orange. It indicates that bromophenol blue is more easily removed than methyl orange. From all figures, it is also observed that the decolorization efficiency of bromophenol blue was higher than that of methyl orange. This behavior could be attributed to the different mechanisms of degradation of both dyes. Methyl orange is an azo dye; however, bromophenol blue is a sulphonephthalein dye, which contains two hydroxyl groups. Kim et al. reported that the azo bond was broken, and then produced sulfanilic acid, aromatic amine and other intermediate products when azo dye was degraded using HRP [21]. Moreover, the catalytic rate of phenol is higher than that of aniline using HRP [28].

In our previous paper [13], we reported that CA modification of HRP neutralized lysine charges and then formed tighter binding of a structural calcium ions, resulting in a greater stability. CD studies proved that the improvement of stability is related to side chain reorientations of aromatics upon CA modification. Thus, we can infer that these structural changes of HRP causing by CA modification also was beneficial for dye decolorization.

4. Conclusion

Chemical modification of HRP by citraconic anhydride increased the decolorization efficiencies of bromophenol blue by 1.8% and methyl orange by 12.4%. Lower dose of CA-HRP was required than that of native HRP for the decolorization of both dyes when the decolorization efficiencies were the same. CA-modified HRP exhibited a good decolorization of dye over a wide range of dye concentration, which could be expected in industry. The decolorization efficiency of bromophenol blue by native and modified HRP was higher than that of methyl orange. Kinetic studies proved that citraconic anhydride-modified horseradish peroxidase shows greater affinity and catalytic efficiency than native horseradish peroxidase for both dyes.

Acknowledgments

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