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Treatment of aqueous chlorophenol by phthalic anhydride-modified horseradish peroxidase

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Abstract

The reaction conditions of removal of chlorophenols from aqueous solution catalysed by phthalic anhydride (PA)-modified horseradish peroxidase were investigated. The optimal pH for chlorophenol removal decreased with increase in substituent number and it was not related to substituent position on aromatic ring. The optimum molar ratio of hydrogen peroxide to chlorophenol was 1.25. The effects of different substrates on phenolics removal were also investigated. The mixed phenolics were more easily removed. PA-modified horseradish peroxidase was more efficient in chlorophenol removal than native horseradish peroxidase either at low temperature or at high temperature. Aromatics with electron-withdrawing substituents (e.g. Cl) at the *p*-position favour removal over those with substituents at the *o*- or *m*-positions. The removal efficiency of 2,4-dichlorophenol, 2,4,6-trichlorophenol and pentachlorophenol was almost equal and it was all lower than that of 4-chlorophenol. The kinetic constants for chlorophenol oxidation with native and PA-modified horseradish peroxidase were also determined. The kinetic data also proved the trend of removal of different chlorophenols with native or PA-modified horseradish peroxidase.

Keywords: Phthalic anhydride-modified horseradish peroxidase; Removal of chlorophenols

1. Introduction

Chlorophenols are toxic phenolic compounds and are very often found in industrial effluents such as those generated by high-temperature coal conversion, petroleum refining and the manufacture of plastics, resins, textile, iron, steel and paper. Environmental legislation defines the maximum discharge limit in rivers as about $0.1\,mg\,l^{-1}$. However, the concentrations of chlorophenols found in effluents may vary from hundreds to thousands of milligram per liter and their degradation is usually very difficult [1]. Thus, efforts to develop new efficient methods to remove these compounds from wastewater become more and more important.

Current methods for removing phenolics from wastewater include microbial degradation [2,3], activated carbon adsorption [4,5], chemical oxidation [6,7], corona discharge [8], electrocatalytic oxidation [9] and enzymatic polymerization using peroxidase enzymes [10–12]. Of these, enzymatic polymerization

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offers the advantages of low process energy requirements and the low solubility of the polymerised product [13].

The enzyme-catalysed method to remove phenolics from wastewater was firstly proposed by Klibanov et al. [14] and has been continuously improved since then. Horseradish peroxidase (HRP) catalyses the oxidation of phenols in the presence of hydrogen peroxide generating phenoxy radicals. These free radicals spontaneously form insoluble polymers that can be removed from solution by sedimentation and filtration. The free radicals are non-specific and react with both good and poor peroxidase substrates. This has a practical value, since a wastewater system often contains a complex mixture of chemicals of varying susceptibility to peroxidase [15]. One molecule of peroxidase can remove approximately 1000 molecules of phenol [16]. Furthermore, two free radicals are generated for every molecule of peroxide consumed:

$$H_2O_2 + 2AH_2 \xrightarrow{HRP} 2^*AH + 2H_2O \quad (1)$$

where AH_2 is the substrate catalysed by HRP, such as phenol, chlorophenol and aniline, etc. $^*AH$ is the free radical formed from the enzymatic catalysis by HRP.

Many authors reported that peroxidase was used to remove chlorophenol from wastewater [2,13,17–20], but so far, detailed polymerization behaviours of chlorophenol removal have not been carried out. The reaction rule of chlorophenol removal has not also been investigated. Moreover, large amounts of enzyme are required because of enzyme inactivation, thus limiting its use to date in industrial situation. In our previous paper [21], the chemical modification of horseradish peroxidase by phthalic anhydride (PA) and glucosamine hydrochloride increased it's thermostability and in turn also increased the removal efficiency of phenol. Moreover, phthalic anhydride-modified HRP has greater thermostability and removal efficiency of phenol than glucosamine hydrochloride-modified HRP. In the literatures, only one paper reported chlorophenol removal by chemical-modified HRP [19]. They optimised the reaction conditions (such as reaction time, HRP concentration and H_2O_2 concentration, etc.) of enzymatic polymerization by ethylene glycol bis-succinimidyl sucinate or acetic acid *N*-hydroxysuccinimide eater-modified HRP. But the polymerization reaction rule of mono-, di-, tri- and penta-chlorophenol by chemical-modified HRP has not yet been investigated. Thus, removal of chlorophenols by phthalic anhydride-modified HRP was carried out in detail to understand its reaction rule.

2. Experiments

2.1. Reagent

Horseradish peroxidase was purchased from Shanghai Lizhu Dong Feng Biotechnology Co. Ltd. and had a specific activity of 250 purpurogallin U mg^{-1}. Phthalic anhydride (analytical grade) was obtained from Guangzhou Chemical Reagent Factory. 2,4,6-Trichlorophenol (2,4,6-TCP) and 3-chlorophenol (3-CP) were obtained from Aldrich. All the other reagents were of analytic grade.

2.2. Chemical modification

Chemical modification by phthalic anhydride was based on our previous method [21]. A 0.15 ml, 2 mmol l^{-1} phthalic anhydride in DMSO and 2 ml 1 mg ml^{-1} HRP in 0.01 mol l^{-1} phosphate buffer (pH 7.0) were mixed. The reaction proceeded at 4 °C for 1 h and was then dialysed against 0.01 mol l^{-1} phosphate buffer (pH 7.0) at 4 °C to remove excess reagent.

2.3. Peroxidase activity assay

The enzyme activity was assayed by a colorimetric method [22]. A reaction mixture containing 0.1 mmol l^{-1} phosphate buffer (pH 7.0), 80.43 mmol l^{-1} phenol, 8.8×10^{-4} mmol l^{-1} hydrogen peroxide and 1.15 mmol l^{-1} 4-aminoantipyrin (4-AAP) in a total volume of 3.0 ml was incubated at 30 °C. The reaction was then started by adding 0.01 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 mmol of hydrogen peroxide per minute under the assay conditions.

2.4. Chlorophenol precipitation reaction

The removal efficiency is defined as the percentage of chlorophenol removed from solution under experimental conditions. Phenolic precipitation reactions were carried out in duplicate. The batch reactor consisted of a vial containing 5 ml of a mixture of aromatic compound, H_2O_2 and PA-HRP enzyme. Aromatic substrate and enzyme were combined in phosphate buffer of the appropriate pH and then thermally equilibrated at corresponding temperature for 15 min prior to the experiment. The reaction was initiated by adding H_2O_2. The reacting solution was stirred by a magnetic stirrer and terminated by the addition of a large dose of catalase (0.5 ml of 0.4 mg ml^{-1}). Each sample was treated with 0.2 ml of 4% alum [$Al_2(SO_4)\cdot 14H_2O$] to enhance colloidal particle coagulation and the pH was adjusted to approximately 6.3 using 1 M either HCl or NaOH to optimize floc formation. After 20 min, sample were centrifuged at 4000 × *g* for 20 min at room temperature. Residual aromatic compound in the clear supernatant was estimated by direct spectrophotometric measurement of absorbance at 275 nm for 2-chlorophenol (2-CP), 3-chlorophenol (3-CP), 4-chlorophenol (4-CP) and 2,4-dichlorophenol (2,4-DCP), 310 nm for 2,4,6-trichlorophenol and 300 nm for pentachlorophenol (PCP). The concentration of phenolics can be approximated using an extinction coefficient of 1400 l mol^{-1} cm^{-1} [19–21], but such estimates would be in error because of soluble products of the reaction (dimmers, trimmers, etc.) which may have different extinction coefficients. Peroxide, PA-HRP, catalase and alum did not interfere with absorbance measurements at this wavelength.

3. Results and discussion

3.1. Effect of pH on removal efficiency

The effect of pH on the removal efficiency of the substrate was examined in a broad pH range. The results are given in Fig. 1. The substrates with different structure had different optimum pH. Fig. 1 shows that the optimal pH was 9 for 2-CP, 3-CP, 4-CP and 2,4-DCP, and 6 for 2,4,6-TCP and 5 for PCP. The results indicate that the optimal pH for the removal of chlorophenols decreased with increase in substituent number and that it was not related to substituent position on aromatic ring. However, some authors reported different results. Dec and Bollay reported that optimal pH was 9 for 4-CP, 7 for 2,4-DCP, 6.3 for 2,4,5-TCP and 5 for PCP [23]. Klibanov et al. reported that the

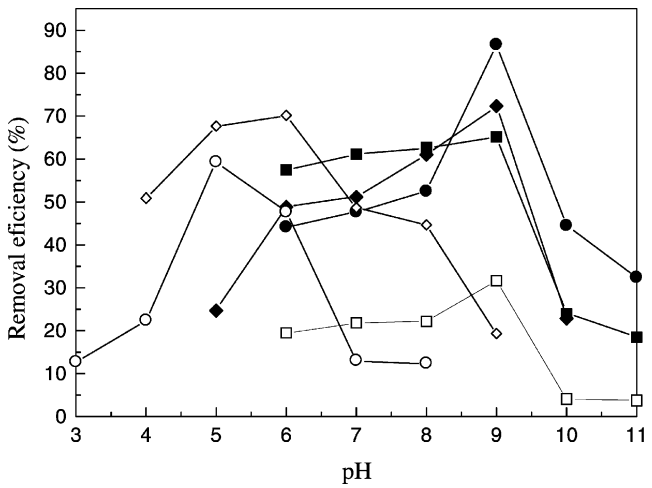

Fig. 1. The effect of pH on chlorophenol removal from aqueous solution by phthalic anhydride-modified HRP. (●) 4-CP; (■) 2-CP; (□) 3-CP; (◆) 2,4-DCP; (○) PCP; (◇) 2,4,6-TCP. Conditions: temperature, 80 °C; reaction time, 15 min; H_2O_2 concentration, 1.0 mmol l^{-1}; PA-HRP concentration, 1.5 U ml^{-1}; 2-CP, 4-CP and 2,4-DCP concentration, 0.8 mmol l^{-1}; 3-CP, 2,4,6-TCP and PCP concentration, 0.4 mmol l^{-1}.

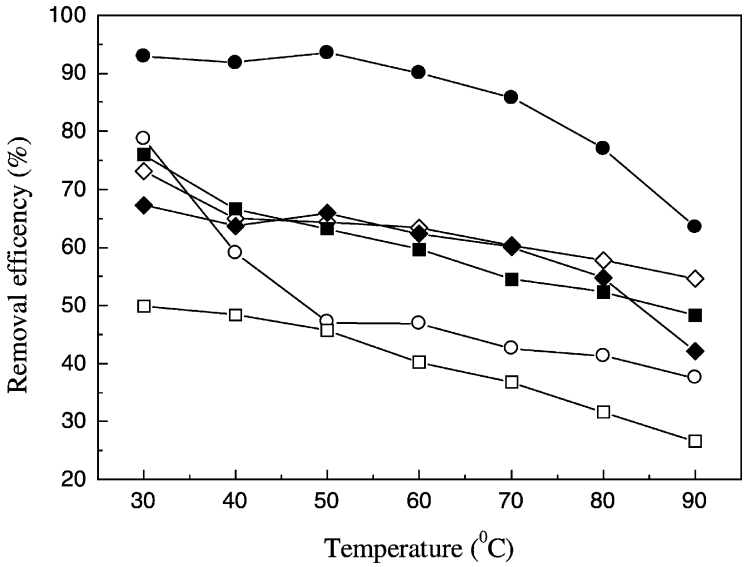

Fig. 2. The effect of temperature on chlorophenol removal from aqueous solution by phthalic anhydride-modified HRP. (●) 4-CP; (■) 2-CP; (□) 3-CP; (◆) 2,4-DCP; (○) PCP; (◇) 2,4,6-TCP. Conditions: corresponding optimal pH; reaction time, 15 min; H_2O_2 concentration, 0.5 mmol l^{-1}; PA-HRP concentration, 1.5 U ml^{-1}; CP concentration, 0.4 mmol l^{-1}.

optimal pH was 5.5 for 4-CP, and 7 for 2-CP and 3-CP [14].

3.2. Effect of reaction temperature on removal efficiency

To examine the effect of temperature on chlorophenol removal, the polymerization and precipitation reactions were performed at optimal pH and temperatures from 30 to 90 °C under the same conditions. The results are presented in Fig. 2. The removal efficiencies decreased with an increase in reaction temperature. That is in agreement with that of *Coprinus cinereus* peroxidase [10] and HRP [24]. It may be due to the lower solubility of the polymer at low temperature; that is, precipitation occurred without adsorption of enzyme on the polymers, resulting in extending catalyst lifetime at low temperature [10]. Another reason may be the lower concentration of free radicals, which reduce the enzyme inactivation. From Fig. 2, we can find that the removal efficiencies of 4-CP and 2,4-DCP kept near a constant at temperatures ranging from 30 to 60 °C and that the removal efficiencies of PCP decreased sharply above 30 °C. It may be because the polymerization reaction of different chlorophenols formed different polymers and the extent of inactivation of polymers on enzyme was also different.

3.3. Effect of reaction time on removal efficiency

Fig. 3 shows the effects of reaction time on chlorophenols removal by PA-HRP. Removal efficiencies of 2-CP, 3-CP, 2,4-DCP and PCP were kept an approximate constant after 15 min. The polymerization of 4-CP was more rapid than that of above chlorophenols and that of 2,4,6-TCP was slower than that of above chlorophenols. In our previous paper, removal efficiencies of phenol by PA-HRP were kept an approximate constant after 10 min, whereas that by native HRP was a constant after 15 min [21]. Miland et al. reported a similar result in 4-CP removal by native HRP and ethylene glycol bis-succinimide ester or acetic acid *N*-hydroxysuccinimide ester-modified HRP [19]. After 15 min, the removal reaction is 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). Base on these results, the reaction time was selected as 15 min in all the further experiments.

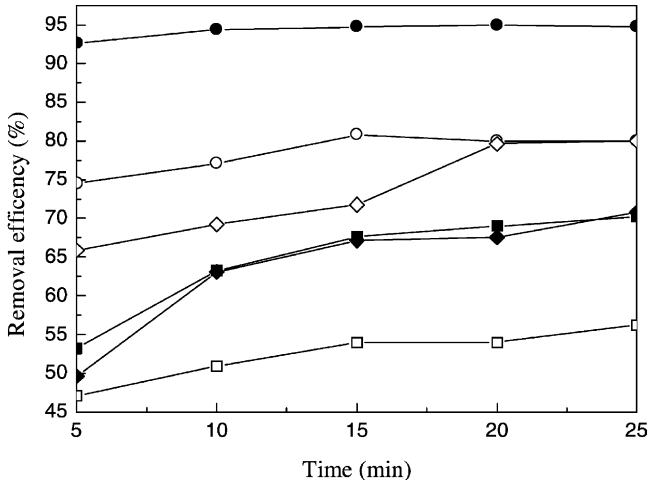

Fig. 3. The effect of reaction time on chlorophenol removal from aqueous solution by phthalic anhydride-modified HRP. (●) 4-CP; (■) 2-CP; (□) 3-CP; (◆) 2,4-DCP; (○) PCP; (◇) 2,4,6-TCP. Conditions: corresponding optimal pH; temperature, 30 °C; H_2O_2 concentration, 0.5 mmol l^{-1}; PA-HRP concentration, 1.5 U ml^{-1}; CP concentration, 0.4 mmol l^{-1}.

3.4. Optimisation of enzyme dose on removal efficiency

Chlorophenol removal at different levels of PA-HRP is illustrated in Fig. 4. The removal efficiency increased with increase in the concentration of peroxidase. Nearly constant removal efficiencies were observed above 1.0 U ml^{-1} for 4-CP, 2,4-DCP, 2,4,6-TCP and PCP, and 1.25 U ml^{-1} for 2-CP and 3-CP. Miland et al. reported that the removal efficiencies of 4-CP by native and modified HRP became nearly constant when the dose of enzyme was above 1.2 U ml^{-1} [19]. The number of substrate molecule catalysed by each molecule of peroxidase can be

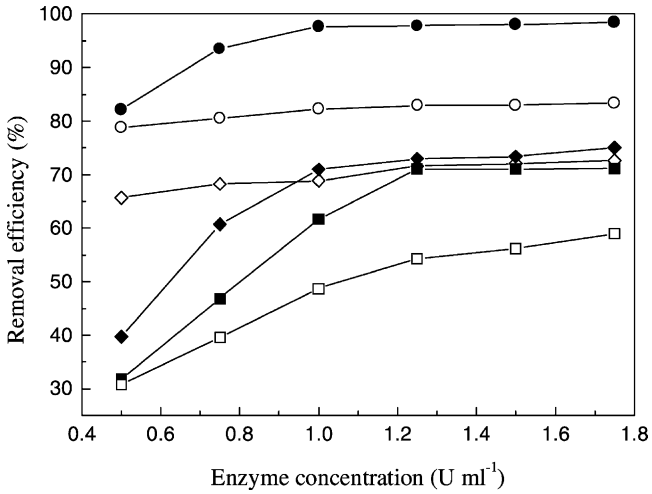

Fig. 4. The effect of enzyme concentration on chlorophenol removal from aqueous solution by phthalic anhydride-modified HRP. (●) 4-CP; (■) 2-CP; (□) 3-CP; (◆) 2,4-DCP; (○) PCP; (◇) 2,4,6-TCP. Conditions: corresponding optimal pH; temperature, 30 °C; reaction time, 15 min; H_2O_2 concentration, 0.5 mmol l^{-1}; CP concentration, 0.4 mmol l^{-1}.

increased by keeping an initially low concentration of enzyme in the system, i.e. <1.0 or 1.25 U ml^{-1}. At higher enzyme concentration, i.e. >1.0 or 1.25 U ml^{-1}, it is decreased and this represents a decrease in catalytic efficiency. On the other hand, the interaction between enzyme molecules is strengthened with increase in enzyme dose and it can form the steric hindrance which blocks substrates into catalytic site. In addition, it is clear that the dose of PA-HRP required for 4-CP, 2,4-DCP, 2,4,6-TCP and PCP removal was less than that for 2-CP and 3-CP removal.

3.5. Effect of the amount of hydrogen peroxide on removal efficiency

One must limit the addition of H_2O_2, as excess of H_2O_2 would inhibit HRP catalytic ability [25]. Experiments involving a range of H_2O_2 concentrations were conducted to determine the effect of the amount of peroxide on the removal of chlorophenol by PA-HRP. The results are shown in Fig. 5. For initial H_2O_2 concentrations ranging from 0 to 0.5 mmol l^{-1} (i.e. H_2O_2/chlorophenol = 1.25), the removal efficiencies of chlorophenol increased proportionally. Therefore, when the H_2O_2/chlorophenol ratio is lower than 1.25, the quantity of peroxide directly controlled the removal efficiency. The H_2O_2/chlorophenol ratio is higher than the theoretical stoichiometry of 0.5 for HRP [26] and that of 1.0 reported in many previous studies [10,12,19,24]. Zhang and Nicell reported that H_2O_2/PCP was about 0.5 in the treatment of aqueous PCP by HRP [12]. The H_2O_2/4-CP ratio of 1.0 was optimal for 4-CP removal with HRP [19]. It is also lower than that of Klibanov et al. report (2.0) [16] and our previous report (2.0) [21]. In our previous paper, the H_2O_2/phenol ratio of 2.0 was beneficial for phenol removal by native and phthalic anhydride HRP [21]. The deviation may be that the products of the catalytic process of different substrate are different and formation of larger polymers required more peroxide [16,24,26].

The inhibition effect of H_2O_2 was obviously observed in this study for chlorophenols (Fig. 5). Under our experiment conditions, the optimum concentration is 0.5 mmol l^{-1}.

3.6. Effect of different substrate on removal efficiency

Table 1 shows that the easy-to-remove chlorophenol (i.e. 2-CP) have a certain positive effect on the difficult-to-remove chlorophenols (i.e. 3-CP). The mixed phenolics were more easily removed than their corresponding single phenolic. The reason may be

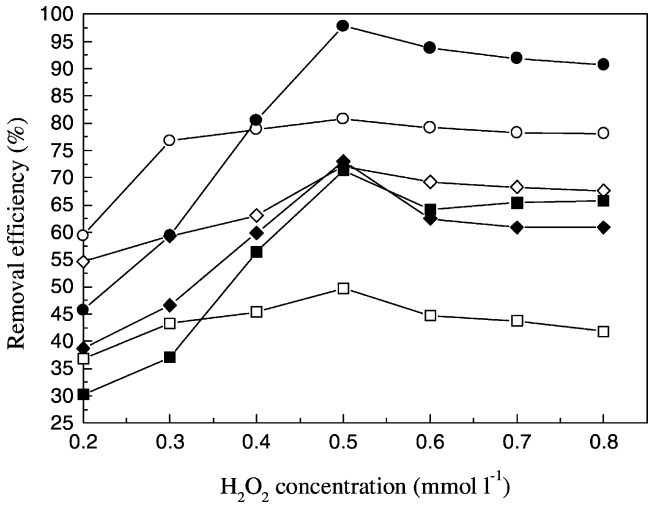

Fig. 5. The effect of H_2O_2 concentration on chlorophenol removal from aqueous solution by phthalic anhydride-modified HRP. (●) 4-CP; (■) 2-CP; (□) 3-CP; (◆) 2,4-DCP; (○) PCP; (◇) 2,4,6-TCP. Conditions: corresponding optimal pH; temperature, 30 °C; reaction time, 15 min; CP concentration, 0.4 mmol l^{-1}.

Table 1
Effect of different phenolics on the removal efficiency

Substrate	Removal efficiency (%)
0.4 mM 2-CP	67.1
0.4 mM 3-CP	51.1
0.4 mM 4-CP	100
0.4 mM 2,4-DCP	72.6
0.4 mM phenol	67.0
0.2 mM 2-CP + 0.2 mM 3-CP	79.5
0.2 mM 3-CP + 0.2 mM 4-CP	86.7
0.2 mM 3-CP + 0.2 mM 2,4-DCP	76.6
0.2 mM phenol + 0.2 mM 2-CP	80.7
0.2 mM phenol + 0.2 mM 3-CP	75.5
0.2 mM phenol + 0.2 mM 4-CP	95.4
0.2 mM phenol + 0.2 mM 2,4-DCP	94.2

that the phenolics that are difficult to remove were adsorbed on the polymer surface resulting from the phenolics that are easy to remove and then they were removed together.

3.7. The removal efficiency by native and modified HRP

Polymerization of chlorophenols by native and modified HRP was conducted under optimal conditions. Table 2 shows the results of HRP-catalysed oxidation of several different chlorophenols by native and modified HRP. PA-HRP was more efficient in chlorophenol removal than native HRP either at low temperature or at high temperature. Because pathalic anhydride is lysine-specific reagent, the stability of HRP resulting from the neutralisation of lysine position charge was enhanced after chemical modification with pathalic anhydride [21]. Therefore, the removal efficiency of PA-HRP was higher than that of native HRP.

On the other hand, for mono-chlorophenol, the greatest transformation was observed for phenols substituted in the position 4, followed by those in the positions 2 and 3. This observation is in agreement with the results of many previous studies [18,19,27,28]. Aromatics with electro-donating substituents at the *m*-position favour removal over those with substituents at the *o*- or *p*-positions. The opposite applies for electron-withdrawing groups (e.g. Cl) [14]. From Table 2, we can also find that the removal efficiency of 2,4-DCP, 2,4,6-TCP and PCP was almost equal and it was all lower than that of 4-CP. But some authors reported different results. Wright and Nicell reported that the removal efficiency of 2,4-CP was higher than that of 4-CP with soybean peroxidase [18]. Ikehata and Nicell reported that monochlorophenols were more easily removed than

Table 2
Comparison of the removal efficiency by native and phthalic anhydride-modified HRP

Temperature (°C)	Substrate	2-CP	4-CP	2,4-DCP	3-CP	2,4,6-TCP	PCP
30	HRP	62.3	89.9	67.5	43.8	69.6	66.2
	PA-HRP	71.0	97.9	73.0	49.8	71.6	77.8
80	HRP	54.1	79.4	57.3	31.7	58.7	50.8
	PA-HRP	61.2	86.8	61.3	34.4	62.3	59.3

H_2O_2: 0.5 mmol l^{-1}; CP: 0.4 mmol l^{-1}; enzyme: 1.25 U ml^{-1}; reaction time: 15 min.

Table 3
The apparent kinetic parameters of removal of chlorophenols by native and modified HRP at infinite phenol concentration and 1.0 mmol l^{-1} H_2O_2 at 30 °C

Substrate	Native HRP			PA-HRP		
	K_m (mmol l^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (l $mmol^{-1}$ min^{-1})	K_m (mmol l^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (l $mmol^{-1}$ min^{-1})
3-CP	3.70	4.70×10^5	1.27×10^5	3.38	6.42×10^5	1.90×10^5
2-CP	3.05	5.83×10^5	1.91×10^5	3.21	2.23×10^6	6.95×10^5
4-CP	2.72	1.45×10^6	5.34×10^5	2.53	2.88×10^6	1.14×10^6
2,4-DCP	3.58	1.02×10^6	2.86×10^5	3.33	1.43×10^6	4.30×10^5
2,4,6-TCP	3.36	1.07×10^6	3.20×10^5	3.33	1.64×10^6	4.92×10^5

2,4-DCP from aqueous solution with tyrosinase [28]. The deviation was correlated to enzyme used in the reaction.

Kinetic constants for chlorophenol oxidation with native HRP and PA-HRP are reported in Table 3. As shown, the chemical modification increased the catalytic constant (k_{cat}) and decreased the substrate affinity (K_m). Thus, the catalytic efficiency (k_{cat}/K_m) of PA-HRP was higher than that of native HRP. From Table 3, we can also find that K_m of 4-CP was lowest and k_{cat}/K_m of 4-CP was greatest. It indicates that of all the chlorophenols, 4-CP was most easily removed by HRP or PA-HRP.

4. Conclusion

The substrates with different structures have different optimum pH in the polymerization of chlorophenol by phthalic anhydride-modified HRP. The optimal pH for chlorophenols removal decreased with increase in substituent number and it was not related to substituent position on aromatic ring. The dose of PA-HRP required for 4-CP, 2,4-DCP, 2,4,6-TCP and PCP removal was less than that for 2-CP and 3-CP removal. When the H_2O_2/chlorophenol ratio is lower than 1.25, the quantity of peroxide directly determined the removal efficiency. H_2O_2 had inhibition for chlorophenols removal above concentration of 0.5 mmol l^{-1}. The mixed phenolics were more easily removed. PA-HRP was more efficient in chlorophenol removal than native HRP either at low temperature or at high temperature. The removal efficiency of 4-CP was greatest in the three monochlorophenols and that of 2,4-DCP, 2,4,6-TCP and PCP was almost equal and it was all lower than that of 4-CP.

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