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Increased thermostability and phenol removal efficiency by chemical modified horseradish peroxidase

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Abstract

Horseradish peroxidase was modified by phthalic anhydride and glucosamine hydrochloride. The thermostabilities and removal efficiencies of phenolics by native and modified HRP were assayed. The chemical modification of horseradish peroxidase increased their thermostability (about 10- and 9-fold, respectively) and in turn also increased the removal efficiency of phenolics. The quantitative relationships between removal efficiency of phenol and reaction conditions were also investigated using modified enzyme. The optimum pH for phenol removal is 9.0 for both native and modified forms of the enzyme. Both modified enzyme could suffer from higher temperature than native enzyme in phenol removal reaction. The optimum molar ratio of hydrogen peroxide to phenol was 2.0. The phthalic anhydride modified enzyme required lower dose of enzyme than native horseradish peroxidase to obtain the same removal efficiency. Both modified horseradish peroxidase show greater affinity and specificity of phenol.

Keywords: Horseradish peroxidase; Chemical modification; Thermostability; Phenol removal

1. Introduction

Aromatic compounds, including phenols and anilines, are present at widely varying levels in the wastewater of a number of industries such as high temperature coal conversion, petroleum refining and the manufacture of plastics, resins, textiles, iron and steel. Many aromatics are toxic and are known or suspected to be carcinogens. Thus, the removal of such compounds from industrial effluents is of great importance. Current methods for removing phenolics from wastewater include microbial degradation, adsorption on activated carbon, chemical oxidation (using such agents hydrogen peroxide, chlorine dioxide and ozone), incineration, solvent extraction and irradiation [1]. The choice of system depends on economic and other factors.

Horseradish peroxidase (HRP, donor: hydrogen peroxide oxidoreductase, E.C. 1.11.1.7) catalyzes the oxidation of aqueous aromatic compounds by hydrogen peroxide, generating phenoxy radicals. These free radicals spontaneously form insoluble polymers which 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 [2]. Therefore, this approach is being studied as a method for the

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treatment of wastewaters containing aromatic compounds. Klibanov et al. first proposed a horseradish peroxidase method for the removal of toxic aromatics from wastewater [3]. One molecule of peroxidase can remove approximately 10^3 molecules of phenol [4]. Furthermore, two free radicals are generated for every molecule of peroxide consumed:

$$H_2O_2 + 2AH_2 \overset{HRP}{\rightarrow} 2AH + 2H_2O \quad (1)$$

This enzymatic approach has many advantages over conventional procedures [4,5] (such as solvent extraction and adsorption onto activated carbon) which are effective but which suffer from high cost, incomplete purification, formation of hazardous by-products and applicability to only a limited concentration range. Due to inactivation, large amounts of peroxidase are required to achieve a high degree of phenol removal. This has limited its use to date in industrial situation.

Enzyme stabilization has been achieved by several methods [6], including immobilization, site-directed mutagenesis, chemical modification and cross-linking. Chemical modification is a rapid and inexpensive method to stabilize enzyme [7]. The thermostability of HRP has been significantly enhanced by the modification with methoxypolyethylene glycols [8], succinimides [9,10]. Many small molecular compounds, such as acetic anhydride, citraconic anhydride, phthalic anhydride, succinimide, glucosamine, etc. have been used to modify enzyme [7,11]. Phthalic anhydride and glucosamine are cheaper than other compounds. Therefore, chemical modifications of HRP to increase its stability by phthalic anhydride and glucosamine hydrochloride were carried out. We have found that modification of HRP with phthalic anhydride and D-glucosamine improves HRP's thermostability and removal efficiency of phenolics.

2. Experimental

2.1. Enzyme and reagents

Horseradish peroxidase was purchased from Shanghai Lizhu Dong Feng Biotechnology Co. Ltd. and had a specific activity of 250 purpurogallin units mg^{-1}. Phthalic anhydride (PA, analytical grade) was obtained from Guangzhou Chemical Reagent Factory. Glucosamine hydrochloride (GA) was purchased from Shanghai Chemical Reagent Company. All other reagents were of analytic grade.

2.2. Chemical modifications

Chemical modification took place as follows: 0.15 ml 2 mmol l^{-1} phthalic anhydride in DMSO or 2 ml 1 mg ml^{-1} glucosamine hydrochloride in 0.01 mol l^{-1} phosphate buffer (pH 7.0) 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 dialyzed against 0.01 mol l^{-1} phosphate buffer (pH 7.0) at 4 °C to removal excess reagent.

The degree of modification was estimated by the method of Snyder and Sobocinski [12].

2.3. Thermostability assay

Native and modified HRP preparations were incubated in 0.01 mol l^{-1} phosphate buffer (pH 7.0) at 50 °C. Aliquots of each sample were withdrawn at different times and assayed for enzymatic activity. HRP activity was assayed using pyrogallol and hydrogen peroxide as substrates based on the Sigma Bulletin [1]. The assay mixture contained 1 ml 128 mmol l^{-1} pyrogallol in 0.1 mol l^{-1} phosphate buffer (pH 6.0) and 2 ml 12 mmol l^{-1} H_2O_2 in 0.1 mol l^{-1} phosphate buffer (pH 6.0). After addition of 10 μl HRP, changes of absorbance at 420 nm for 20 s was measured. One unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at above conditions.

HRP concentration was estimated from its Soret absorbance (molar extinction coefficient at 402 nm = 102 l $mmol^{-1}$ cm^{-1}) [13].

2.4. Phenol precipitation reactions

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 HRP enzyme. Aromatic substrate and enzyme were combined in phosphate buffer of the appropriate pH and then thermally equilibrated at corresponding temperature for 10 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 [$Al_2(SO_4)_3 \cdot 14H_2O$] to enhance colloidal particle coagulation and the pH was adjusted to approximately 6.3 using either HCl or NaOH to optimize floc formation. After 20 min, samples 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. The concentration of phenolics can be approximated using an extinction coefficient of 1400 l mol^{-1} cm^{-1} [1,14,15], but such estimates would be in error because of soluble products of the reaction (dimmers, trimers, etc.) which may have different extinction coefficients. Peroxide, HRP, catalase and alum did not interfere with absorbance measurements at this wavelength.

2.5. Determination of kinetic parameters

Kinetic parameters were assayed by colorimetric method [16]. The assay uses phenol, 4-aminoantipyrine (4-AAP) and H_2O_2 as color-generating substrates. With phenol, H_2O_2 and 4-AAP concentrations present in sufficient quantity, the rate of color generation at 510 nm is proportional to the rate of H_2O_2 consumption. The reaction mixture (3 ml) contained 2.62 mmol l^{-1} 4-aminoantipyrine (4-AAP), 1.0 mmol l^{-1} H_2O_2, 100 μl HRP (final HRP concentration was about 1.5 U ml^{-1}), 0–10 mmol l^{-1} phenol (dissolved in pH 7.0 0.01 mol l^{-1} phosphate buffer) and 0.1 mol l^{-1} phosphate buffer (pH 7.0). The volumes of buffer were selected based on phenol concentration. 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}.

3. Results and discussion

3.1. Thermostability

HRP treated with phthalic anhydride or glucosamine shows higher thermostability (Fig. 1). After single exponential fits of thermal inactivation data, the apparent half-lives of native HRP, phthalic anhydride modified-HRP and glucosamine modified-HRP were 0.46, 4.72 and 4.02 h, respectively, *k*-values were 0.926, 0.146 and 0.175, respectively. These values are for stability comparison only. They may not represent true half-lives since the thermal inactivation of HRP is not first-order [17]. The thermostability of

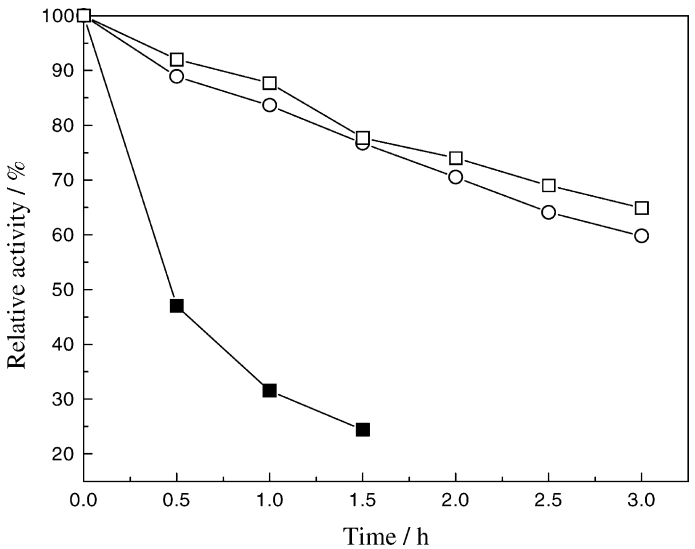

Fig. 1. Thermostability of native and modified HRP at 50 °C. Native HRP (■); HRP modified by phthalic anhydride (□); HRP modified by glucosamine (○).

HRP was increased 9- and 10-fold by chemical modification using glucosamine and phthalic anhydride, respectively. No loss of HRP activity occurred during the modification reactions.

The modification degree of amino groups from HRP by phthalic anhydride was determined as about 52% of the native enzyme. According to the amino groups of HRP, this result showed that three of the six lysine ε-amino groups [18] from native enzyme were modified with phthalic anhydride. A few papers have reported various chemical modifications to enhance thermostability of HRP. Treatment of HRP using bis-succinimides and acetic acid *N*-hydroxysuccinimide increased 6- to 23-fold [9] and 5-fold [10] thermostability, respectively. They thought that these modification reagents reacted with HRP's lysine and that the neutralization of the amino group's positive charge during the modification reactions may account for the greater stabilization of HRP modified by these compounds [9,10]. Phthalic anhydride is the same lysine-specific reagent as bis-succinimides and acetic acid *N*-hydroxysuccinimide. Thus, we infer that the enhancement of thermostability of HRP results from the neutralization of lysine positive charge.

3.2. HRP-catalyzed removal of phenol

In order to examine the effect of pH on phenol removal, the precipitation reactions were performed at pH from 6.0 to 11.0. Fig. 2 shows the removal efficiencies at various pH. The optimum pH was 9.0 for native and modified HRP. These results are consistent with those of native HRP, ethylene glycol bis-succinimidyl succinate-modified HRP and acetic acid *N*-hydroxysuccinimide ester-modified HRP [1].

The effect of temperature on phenol removal of native and modified HRP was investigated. The results present in Fig. 3. Native HRP is susceptible to rapid inactivitation at temperature >60 °C, but modified HRP begin to rapid inactivitation >70 °C. Because the temperature of wastewaters containing aromatic compounds is higher, all further experiments were conducted at 70 °C.

Fig. 4 shows the effects of reaction times on phenol removal by native and modified HRP. Removal efficiencies of modified HRP were kept an approximate constant after 10 min, whereas that of native HRP was a constant after 15 min. Thus, the reactions of both modified HRPs were more rapid than that of native HRP. Both modified HRPs are more effi-

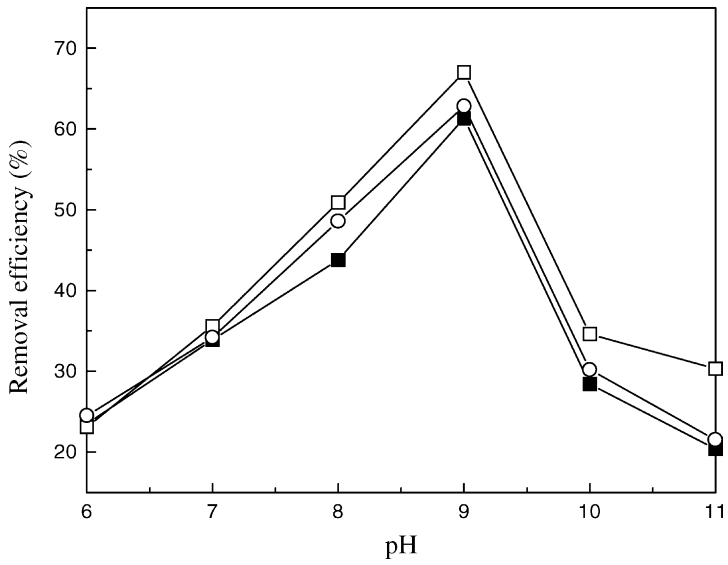

Fig. 2. The effect of pH on removal of phenol from aqueous solution by native and modified HRP. Native HRP (■); HRP modified by phthalic anhydride (□); HRP modified by glucosamine (○). Conditions: HRP concentration, 1.5 U ml^{-1}; initial phenol concentration, 0.8 mmol l^{-1}; H_2O_2 concentration, 1.0 mmol l^{-1}; reaction medium, 0.01 mol l^{-1} phosphate buffer or 0.1 mol l^{-1} borate buffer; reaction time, 20 min; temperature, 80 °C.

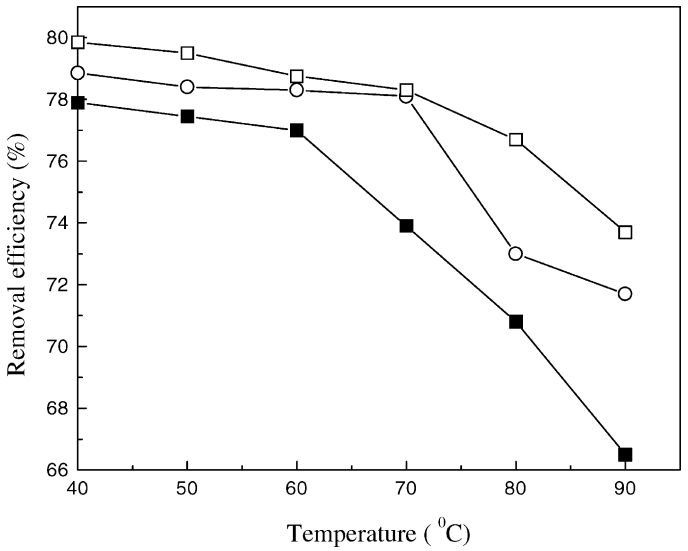

Fig. 3. The effect of temperature on removal of phenol from aqueous solution by native and modified HRP. Native HRP (■); HRP modified by phthalic anhydride (□); HRP modified by glucosamine (○). Conditions: HRP concentration, 1.5 U ml^{-1}; initial phenol concentration, 0.8 mmol l^{-1}; H_2O_2 concentration, 1.0 mmol l^{-1}; reaction medium, 0.1 mol l^{-1} borate buffer (pH 9.0); reaction time, 15 min.

cient in removing phenol at 70 °C than native HRP. Removal efficiencies of phthalic anhydride-modified HRP were higher than that of glucosamine-modified HRP. 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

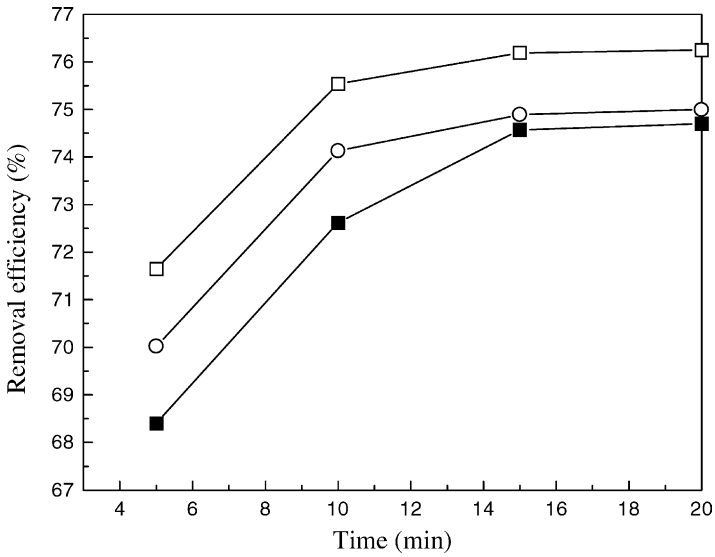

Fig. 4. The effect of reaction time on removal of phenol from aqueous solution by native and modified HRP. Native HRP (■); HRP modified by phthalic anhydride (□); HRP modified by glucosamine (○). Conditions: HRP concentration, 1.5 U ml^{-1}; initial phenol concentration, 0.8 mmol l^{-1}; H_2O_2 concentration, 1.0 mmol l^{-1}; reaction medium, 0.1 mol l^{-1} borate buffer (pH 9.0); reaction time, 15 min; temperature, 70 °C.

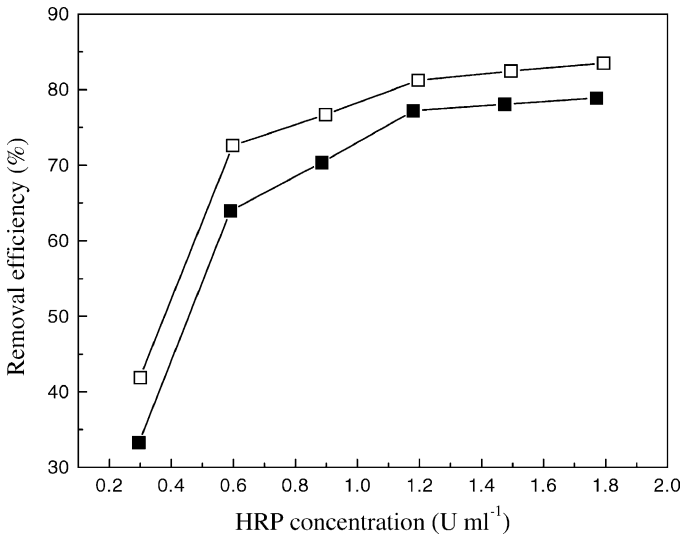

Fig. 5. The effect of HRP concentration on removal of phenol from aqueous solution by native HRP (■) and phthalic anhydride modified-HRP (□). Conditions: initial phenol concentration, 0.8 mmol l^{-1}; H_2O_2 concentration, 1.0 mmol l^{-1}; reaction medium, 0.1 mol l^{-1} borate buffer (pH 9.0); reaction time, 15 min; temperature, 70 °C.

time of all further experiments was selected as 15 min.

Phenol removal at different levels of native HRP and phthalic anhydride-modified HRP preparation is illustrated in Fig. 5. The removal efficiency increased with the increase in the concentration of peroxidase (0–1.0 U ml^{-1}). Near-constant removal efficiencies were observed >1.2 U ml^{-1}. Miland et al. reported that the removal efficiencies of 4-chlorophenol by native and modified HRP became near constant when

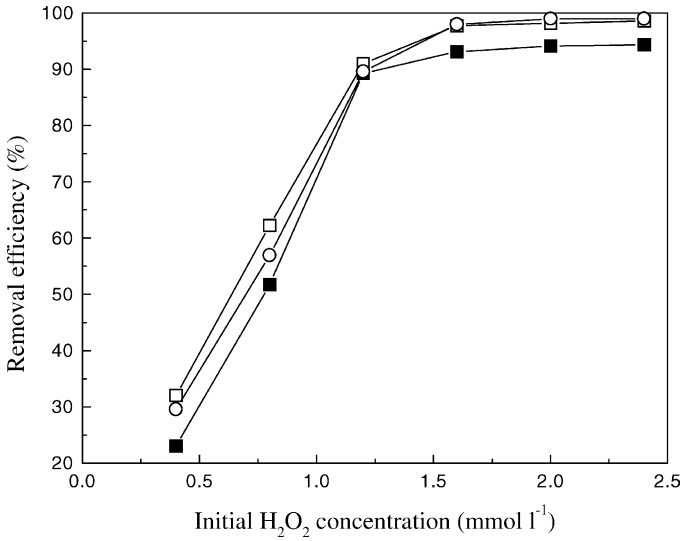

Fig. 6. The effect of H_2O_2 concentration on removal of phenol from aqueous solution by native and modified HRP. Native HRP (■); HRP modified by phthalic anhydride (□); HRP modified by glucosamine (○). Conditions: HRP concentration, 1.5 U ml^{-1}; initial phenol concentration, 0.8 mmol l^{-1}; reaction medium, 0.1 mol l^{-1} borate buffer (pH 9.0); reaction time, 15 min; temperature, 70 °C.

the dose of enzyme was >1.2 U ml^{-1} [1]. The number of reactions catalyzed by peroxidase can be increased by keeping an initially low concentration of enzyme in the system, i.e. <1.0 U ml^{-1} [1,13]. At higher enzyme concentrations, i.e. >1.0 U ml^{-1}, each peroxidase molecule catalyses fewer reactions and this represents a decrease in catalytic efficiency. In this case, the phenoxy radicals may find an enzyme's active site more rapidly but as the reaction proceeds and the concentration of aromatic decreases, free radical polymerization becomes much more difficult [19]. Thus, they thought that one could increase the time of treatment to offset the reduction in removal efficiency at low enzyme concentrations [1,14]. When the removal efficiency reached about 75 %, the dose of phthalic anhydride-modified HRP and native HRP was required about 0.76 and 1.08 U ml^{-1}, respectively. Thus, lower dose of phthalic anhydride-modified HRP was required than that of native HRP for phenol removal when the removal efficiencies were the same.

One must limit the addition of H_2O_2, as an excess would inhibit HRP's catalytic ability [20]. Experiments involving a range of H_2O_2 concentrations were conducted to determine the effect of the amount of peroxide on the removal of phenol by native and modified HRP. The results are shown in Fig. 6. For initial H_2O_2 concentrations ranging from 0 to 1.2 mmol l^{-1} (i.e. H_2O_2/phenol = 1.5), the removal efficiencies of phenol increased proportionally. Therefore, when the H_2O_2/phenol ratio is <1.5, the quantity of peroxide exerted direct control on the removal efficiency. When initial H_2O_2concentration was 1.6 mmol l^{-1}, the removal efficiencies of phenol by native and modified HRP reached a constant value (>95%). This result indicate that almost two equivalent of H_2O_2 to phenol was required. It is consistent with that of Klibanov et al. [4]. The H_2O_2/phenol ratio is higher than the theoretical stoichiometry of 0.5 for peroxidase [21] and that of 1.0 reported in many previous studies [1,5,21–23]. The deviation may be because the products of the catalytic process are polymers larger than dimmers [4,5,21].

Table 1
Removal of aromatic compounds by native and modified HRP at 70 °C after 15 min

Substrate	pH	Native HRP	PA-HRP	GA-HRP
Phenol	9.0	70.37	77.63	74.19
Catechol	10	84.6	86.2	86.5
Hydroquinone	10	53.39	61.91	57.14
Cresol	10	92.9	93.1	95.3
Pyrogallic acid	10	92.7	94.8	93.5
Phloroglucinol	10	81.2	81.8	82.3
2-Methoxyphenol	10	21.9	35.4	34.4
1-Naphthol	10	96.7	96.4	96.4
2-Naphthol	10	97.8	98.4	98.1
3-Aminophenol	10	49.4	57.4	57.2
4-Aminophenol	10	52.7	56.5	55.7
2-Chlorophenol	10	80.7	83.4	84.0
4-Chlorophenol	10	100	100	100
2,4-Dichlorophenol	10	77.8	81.3	79.8
Pentachlorophenol	10	17.73	22.21	17.41

PA-HRP: phthalic anhydride modified-HRP; GA-HRP: glucosamine hydrochloride modified-HRP. Conditions: HRP concentration: 1.5 U ml^{-1}; phenolics concentration: 0.8 mmol l^{-1}; H_2O_2 concentration: 1.0 mmol l^{-1}; temperature, 70 °C; reaction time, 15 min; pH: set up as the table.

Clearance tests in batch systems have been performed for a variety of phenolic substrates (Table 1). The reaction conditions used in this survey were not optimized for near or complete removal of phenolics but were standardized to determine the relative susceptibilities of these compounds to oxidation by native and modified HRP. In some cases, removal efficiencies were very high (cresol, pyrogallic acid, 1-naphthol, 2-naphthol and 4-chlorophenol) whereas 2-methoxyphenol and pentachlorophenol were less prone to oxidation. Preliminary experiments indicated that complete removal of such substrates could be attained by prolonged exposure to an HRP/H_2O_2

Table 2
The apparent kinetic parameters of removal of phenol by native and modified HRP at infinite phenol concentration and 1.0 mmol l^{-1} H_2O_2

Enzyme	V_{max} (mmol min^{-1} l^{-1})	K_m (mmol l^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (l mmol^{-1} min^{-1})
Native HRP	3.70	12.99	8.71×10^5	6.70×10^4
PA-HRP	2.94	8.16	6.42×10^5	7.86×10^4
GA-HRP	3.69	8.33	8.68×10^5	1.04×10^5

PA-HRP: phthalic anhydride modified-HRP; GA-HRP: glucosamine hydrochloride modified-HRP.

system. For comparative purposes, the reaction time was limited to 15 min. For most substrates, removal efficiencies of both modified HRPs were significant higher than that of native HRP.

From Table 2, we can find that K_m of both modified HRPs was lower than native HRP and that k_{cat}/K_m of both modified HRP was greater than native HRP. These results indicate that the chemical modification of HRP increased the affinity and specificity of phenol.

4. Conclusions

Chemical modification of HRP by phthalic anhydride and glucosamine hydrochloride increased their thermostability (about 10- and 9-fold, respectively) and in turn also increased the removal efficiency of phenolics. The optimum molar ratio of hydrogen peroxide to phenol was 2.0. The phthalic anhydride modified enzyme required lower dose of enzyme than native horseradish peroxidase to obtain the same removal efficiency. Both modified HRPs show greater affinity and specificity of phenol.

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