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Activity, stability, and unfolding of reconstituted horseradish peroxidase with modified heme

Hai-Yan Song[a,b,c,d], Jian-Zhong Liu[a,b,c,*], Li-Ping Weng[a,b,c], Liang-Nian Ji[a,b,c]

[a] Key Laboratory of Gene Engineering of Ministry of Education, Zhongshan University, Guangzhou 510275, PR China
[b] Biotechnology Research Center, Zhongshan University, Guangzhou 510275, PR China
[c] State Key Laboratory of Biocontrol, Zhongshan University, Guangzhou 510275, PR China
[d] Department of Chemistry, South China Normal University, Guangzhou 510631, PR China

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ABSTRACT

Heme-propionates of horseradish peroxidase (HRP) were esterified by *p*-nitrophenol, phenol and *p*-methylphenol to change its electron character and to increase its hydrophobicity. These synthetic hemes were inserted apo-HRP to give a novel HRP, respectively. Of the three reconstituted HRPs, reconstituted HRP with *p*-nitrophenol-modified heme derivative had a larger initial rate, affinity, catalytic efficiency and substrate-binding efficiency than native HRP in aqueous buffer and some solvents. The reconstituted HRPs showed higher thermostability and tolerance of DMF because of the increase of the hydrophobicity of the active site. Changing the electron character of the aromatic moieties linked at each terminal of the two heme-propionates can control activity and stability of HRP. The initial rate, affinity, catalytic efficiency and substrate-binding efficiency increased with the increases of electron-withdrawing efficiency of substituents at 4-position of the phenolic used to synthesize the heme derivatives, contrariwise, the stability decreased. The modifications resulted in the increase in the temperature (T_m) at the midpoint of thermal denaturation and the decreases in both enthalpy and entropy change at T_m. The changes of catalytic properties and stabilities are related to the changes of the conformation of HRP. The modification changed the environment of heme and tryptophan, increased α-helix content of HRP. The present work demonstrates that enhancement of the hydrophobicity and the electron-withdrawing efficiency of heme improves the activity and stability of HRP.

1. Introduction

Horseradish peroxidase (HRP, EC 1.11.1.7) catalyzes the oxidation of aromatic compounds by hydrogen peroxide or alkyl hydroperoxide. The native enzyme consists of a single polypeptide chain with 308 amino acid residues, a heme prosthetic group and two Ca^{2+} ions maintaining enzyme conformation. It was widely applied in the synthesis of fine chemicals and polymer, and the removal of toxic phenolics from wastewater [1,2]. However, the oxidation of these hydrophobic aromatics by HRP is highly limited by the substrate partition between the protein active site and the bulk solvent. As the concentration of organic solvent increases the interaction between the hydrophobic substrate and the protein active site decreases, and then the biocatalytic activity is reduced. To our knowledge, there is no example of the use of HRP as catalyst in industrial organic synthesis. It is mainly due to low stability and low catalytic activity in organic solvents [3].

Over the years, several techniques have been developed to ameliorate this loss of catalytic function, including lyophilization in the presence of lyoprotectants and excipients such as KCl, crown ethers, cyclodextrins and molecular imprinters [4], the use of site-directed mutagenesis and directed evolution [5,6], or chemical modification [6,7]. Chemical modification has now reemerged as a powerful complementary approach to site-directed mutagenesis and directed evolution [6]. Chemical modification of a lysine residue on the protein surface is a useful method for the construction of a functional site, such as a substrate-binding site. Our previous papers reported that modification of lysine residues of HRP by phthalic anhydride [8–10], maleic anhydride and citraconic anhydride [11,12] improved HRP's stability and catalytic activity in water and organic solvents. Recently, some literatures demonstrated that reconstitution with chemically modified heme is another powerful method to convert myoglobin [13–16] into peroxidase. Because the aromatic moieties of the modified hemes formed a hydrophobic domain on the protein

* Corresponding author at: Biotechnology Research Center, Zhongshan University, Guangzhou 510275, PR China. Tel.: +86 20 84110115; fax: +86 20 84110115.
E-mail address: lssljz@mail.sysu.edu.cn (J.-Z. Liu).

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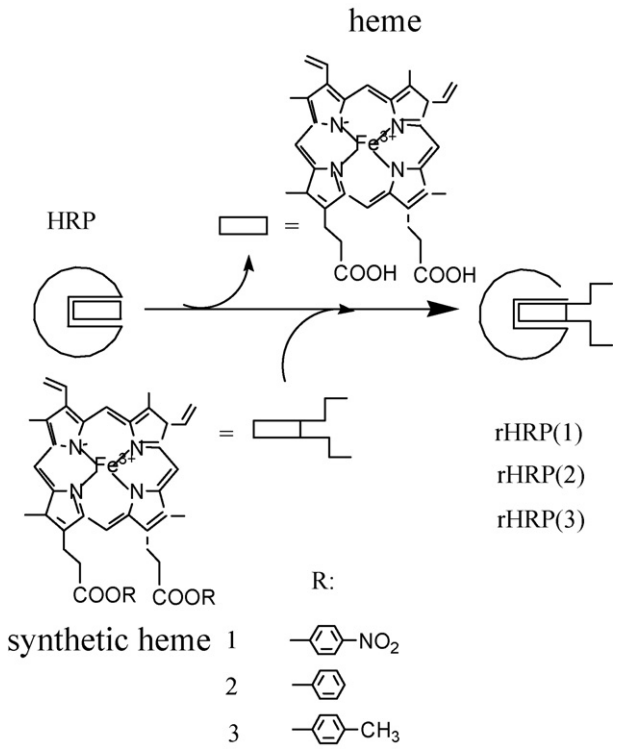

Scheme 1. Reconstitution of HRPs with synthetic heme derivatives.

surface, the introduction of a hydrophobic benzene ring into two heme-propionate termini constructed a substrate-binding site in myoglobin, and thereby resulting in myoglobin having high activity of peroxidase [13–15]. However, all of these investigate focused on myoglobin and hemoglobin. Although Ryabov et al. reported that HRP reconstituted with ferrocene-modified heme could create a substrate-binding site and that the reactivity of the reconstituted HRP towards ABTS, which is commonly used as a substrate for peroxidase activity assay of HRP, dropped threefold compared with native HRP [17]. Adak and Banerjee reported that the reconstituted HRP with heme dimethyl ester had very low oxidative activity with guaiacol, iodide or thiocyanate in the presence of H_2O_2 [18]. And they thought that heme-propionates play a vital role in the oxidative and reductive reaction of HRP.

Thus, the hydrophobicity and electron character of heme prosthetic group of HRP were altered by chemical modification to evaluate their effects. The modification included the extraction of heme, esterification of hemin chloride, and the reconstitution of apo-HRP with synthetic heme (Scheme 1). The effects of this modification on the substrate affinity and catalytic activity were evaluated. This is the first report on enhancing the activity of HRP by modified-heme reconstitution method.

2. Materials and methods

2.1. Chemicals

HRP C was purchased from Shanghai Lizhu Dong Feng Biotechnology Co. Ltd. and had a specific activity of 250 purpurogallin units/mg and RZ = 3.0. Hemin chloride was obtained from Alfer Aesar. Ethyl-3-(-dimethylaminopropyl)carbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were obtained from Sigma Chemical. All other reagents were of analytic grade.

2.2. Synthesis of heme derivatives

Heme-propionates were esterified by *p*-nitrophenol, phenol or *p*-methylphenol using carbodiimide chemistry [17,19,20]. Fifty milligrams hemin chloride was mixed with 116 mg *p*-nitrophenol (86 mg phenol or 110 mg *p*-methylphenol) in 8 ml anhydrous dimethylformamide (DMF). 128 mg DCC for *p*-nitrophenol (65 mg DCC for phenol or *p*-methylphenol) was added and the solution was maintained under constant stirring at room temperature. For phenol or *p*-methylphenol, 38 mg DMAP were added. After 24 h, the solution was acidified with HCl until heme precipitation. The precipitate was washed with water and then 40 ml $CHCl_3$ was added. The organic solvents were evaporated off and dried under vacuum. The dried material was dissolved in a minimal amount of $CHCl_3$ and the products formed were separated by column chromatography on silica gel. The products were eluted with 100:3 CH_2Cl_2/MeOH mixtures. The first fuchsia eluate was collected. Finally, the eluate was evaporated off and dried under vacuum.

2.3. Extraction of heme from HRP

Apo-HRP was prepared using the acid methylethylketone method according Teale [21]. HRP (10 mg) was dissolved in 3 ml 0.1 M KCl and the pH of the ice-cold solution was adjusted to 1.9 by 0.1 M HCl. An equal volume of ice-cold methylethylketone was added to this mixture. The mixture was incubated for 10 s at 4 °C on a magnetic stirrer. The solution was kept at 0 °C and the lower aqueous layer containing apo-HRP was dialyzed against 0.01 M Tris/HCl buffer (pH 8.0) at 4 °C to remove the ketone and other low-molecular-weight impurities.

2.4. Reconstitution of apo-HRP with synthetic heme

Reconstitution of apo-HRP with synthetic heme derivative was carried out as described elsewhere [19]. The synthetic heme derivative was dissolved in 50 mM Tris/HCl buffer (pH 8.0) containing 2% DMF. The solution of apo-HRP was added dropwise with stirring to the solution of heme at 4 °C over 30 min. The molar ratio of synthetic heme derivative to apo-HRP was 5:1. The resulting solution was dialyzed against 0.01 M Tris/HCl buffer (pH 8.0) at 4 °C to remove the excess unbound heme and DMF. The dialyzed enzyme solution was ultrafiltrated by an ultrafilter retaining a molecular weight of 10 kDa and above. The concentrated enzyme solution was applied to a Sephadex G-75 column (1.6 cm × 80 cm) equilibrated with 50 mM Tris/HCl buffer (pH 8.0). The column was washed with the same buffer. The active fractions were pooled and then freeze-dried.

2.5. Peroxidase activity assay

The enzyme activity was assayed by colorimetric method [9]. A reaction mixture containing 10 mM phenol, 0.2 mM hydrogen peroxide and 2.4 mM 4-aminoantipyrin (4-AAP) in a total volume of 3.0 ml was incubated at 30 °C. All regents were dissolved in 0.1 M phosphate buffer (pH 7.0). The reaction was then started by adding 0.1 ml of diluted enzyme solution (about 2 μM), and the initial increase in absorbance was monitored at 510 nm during 1 min. The rate of reaction is proportional to the enzyme activity and is deduced from the rate of formation of the nonprecipitating product which absorbs light at a peak wavelength of 510 nm with a molar extinction coefficient of 7100 M^{-1} cm^{-1}. One unit of activity (U) is defined as the number of micromoles of peroxide utilized per minute under the assay conditions.

Native HRP concentration was estimated from its Soret absorbance (molar extinction coefficient at 402 nm = 102 mM^{-1} cm^{-1}) [9]. The reconstituted HRP concentrations were

determined by the pyridine hemochrome method [22]. These were 125 mM^{-1} cm^{-1} for rHRP(1), 100 mM^{-1} cm^{-1} for rHRP(2) and 104 mM^{-1} cm^{-1} for rHRP(3).

2.6. Kinetics

The steady-state kinetic experiments were performed using constant enzyme, 4-AAP and H_2O_2 concentration as the peroxidase activity assay, and varying the concentration of substrate under the same conditions of activity assay.

Formation and reduction of compound I, and reduction of compound II under pseudo-first-order condition, were monitored on a stopped-flow spectrophotometer (Model SX20, Applied Photophysics Ltd., UK) at 25 °C in 50 mM sodium phosphate buffer, pH 7.0. More than a 10-fold excess of H_2O_2 or phenol relative to the HRP concentration (2 μM) was utilized to ensure pseudo-first order kinetics. The elementary reaction rates of k_1, k_2 and k_3 were determined by the absorbance change at 395, 412, and 424 nm, respectively. The data were analyzed by fitting the absorbance versus time curves to exponential functions using a least-squares minimization program. The second order rate constant was determined from the slope of the plot of k_{obs} versus the H_2O_2 or phenol concentration.

2.7. Substrate binding

Apparent dissociation constants of phenol for HRP were determined by spectral titration on the Shimadzu UV2450 spectrophotometer at 25 °C as described elsewhere [23]. A 3.0 ml solution of HRP (about 3.3 μM) in 0.05 M phosphate buffer (pH 7.0) was added to the sample and reference cell, respectively, and then incubated for 5 min prior to spectrometric measurements. A few microlitters of concentrated phenol (30 mM) in 0.05 M phosphate buffer (pH 7.0) were added to the HRP solution in the sample cell and an equivalent volume of buffer to the enzyme solution in the reference cell. For titration studies, each solution was incubated at 25 °C for at least 5 min to reach equilibrium after phenol was added. And then the difference spectra of protein-substrate versus protein solutions were recorded. The obtained spectral changes caused by substrate addition were fitted to the following equation by a non-linear least-squares procedure:

$$\Delta A_{soret} = \frac{\Delta A_s[S]}{K_d + [S]}$$

where ΔA_{soret} and ΔA_s are the absorbance changes of Soret band at a given and saturating substrate concentration, respectively. [S] is the concentration of substrate.

2.8. Thermostability assay

Native and modified HRP preparations were incubated in 0.1 M phosphate buffer (pH 7.0) at 60 °C. Aliquots of each sample were withdrawn at different times and assayed for enzymatic activity under the above standard conditions.

2.9. Tolerance of organic solvent

DMF profiles of HRP samples were carried out at 60 °C with exposure times of 1 h. Reaction mixtures were set up with increasing percent volumes of organic solvent in 0.1 M phosphate buffer (pH 7.0) in 10% (v/v) increments. 100 μl were withdrawn from each reaction mixture and assayed for enzymatic activity under the above standard conditions.

2.10. Thermal unfolding

Thermal denaturations of native and reconstituted HRPs were monitored by heme absorption using Shimadzu UV2450 at pH 7.4 (0.1 M phosphate buffer). The temperature was raised from 25 to 95 °C in steps of 2 °C with an equilibration time of 2 min at each temperature. The reversibility of thermal denaturation was checked by cooling the denaturated sample and subsequent reheating. The refolding proteins showed an identical Soret peak to the native protein upon the refolding of the denaturational protein by cooling. It indicates heme was reincorporated to the original place. The reversibility usually exceeded 95%. The enzyme concentration was about 5 μM in 0.1 M phosphate buffer (pH 7.4).

The denaturation curves were plotted with the Soret absorbance of the enzyme against temperature. Following the approach of Santoro and Bolen [24], a non-linear least-squares analysis was used to fit the thermal denaturation curves to the equation:

$$y = \frac{y_N + m_N T + (y_U + m_U T)e^{(T-T_m)\Delta H_m/RT_m T}}{1 + e^{(T-T_m)\Delta H_m/RT_m T}} \quad (1)$$

where R is the universal gas constant (8.31 J mol^{-1} K^{-1}) and T is the absolute temperature. y is the observed Soret absorbance, and y_N and y_U are the intercepts and m_N and m_U the slopes of the pre- and posttransition baselines, T_m is the midpoint of the thermal unfolding curve, and ΔH_m is the enthalpy change for unfolding at T_m. Curve-fitting was performed using MicroCal Origin software (Version 5.0, MicroCal Software, Inc., Northampton, MA). The entropy change for unfolding at T_m, ΔS_m, was calculated using the following equation:

$$\Delta H_m = T_m \Delta S_m \quad (2)$$

2.11. CD spectra

CD experiments were carried out using a Jasco J810 spectropolarimeter. CD in the UV region (200–300 nm) was monitored with a cell of 2 mm path length with enzyme concentration of about 2.5 μM in 50 mM Tris/HCl buffer (pH 8.0). CD spectra reported in the region were an average of three scans recorded at a scan speed of 250 nm/min, a slit width of 0.2 nm, a response time of 1 s and a resolution of 0.1 nm, corrected by subtracting the appropriate blank runs on HRP-free solutions. CD in the visible region was monitored using a cell of 2 mm path length with enzyme concentration of about 10 μM in 50 mM Tris/HCl buffer (pH 8.0). CD spectra reported in the region were an average of three scans recorded at a scan speed of 20 nm/min, a slit width of 15 nm, a response time of 2 s and a resolution of 0.5 nm, corrected by subtracting the appropriate blank runs on HRP-free solutions. The CD data were expressed in terms of mean residue ellipticity, [θ], in degree cm^2/dmol. The secondary structure percentage predictions were made using K2D software (http://www2.umdnj.edu/cdrw.jweb).

2.12. Fluorescence spectra

Fluorescence measurements were carried out using a Hitachi F4500 spectroflurimeter. The intrinsic tryptophan fluorescence on excitation at 295 nm was recorded for emission from 300 to 550 nm. The slit widths for both the excitation and the emission monochromators were set at 10 nm, the scan speed at 540 nm/min and the resolution at 1.0 nm. The enzyme concentration was about 1.8 μM in 50 mM Tris/HCl buffer (pH 8.0).

Table 1
Spectral properties of synthetic heme and HRP derivatives

Derivative	Soret (nm)	Visible (nm)		
Heme	389	540	510	641
Heme 1	386	538	510	
Heme 2	385	539	511	
Heme 3	385	539	511	
Native HRP	402		498	640
rHRP(1)	398		498	638
rHRP(2)	401		501	634
rHRP(3)	396		497	632

Table 2
Initial reaction rate of oxidation of phenol by native and modified HRP in 0.1 M phosphate buffer (PBS, pH 7.0) and organic solvent[a]

Enzyme	Initial rate ($1\ s^{-1}$)			
	PBS	10% ACN	10%DMF	10%DMSO
HRP	89.1 ± 2.7	58.4 ± 2.3	83.2 ± 2.5	89.8 ± 2.7
rHRP(1)	127.2 ± 3.9	91.1 ± 3.8	123.6 ± 4.9	126.7 ± 3.1
rHRP(2)	76.2 ± 2.8	53.7 ± 2.0	70.8 ± 3.1	88.9 ± 3.6
rHRP(3)	68.8 ± 3.2	49.6 ± 1.9	69.6 ± 1.4	72.9 ± 2.8

[a] *Conditions*: the concentration of phenol, hydrogen peroxide and enzyme was 5, 0.2 mM and 6 μM, respectively. Other conditions were the same as the activity assay.

3. Results and discussion

Heme-propionates were esterified by *p*-nitrophenol, phenol and *p*-methylphenol. The ESI-MS spectrum (LCQ DECA XP, Thremo of USA) of these synthetic heme derivatives gave the desired mass number of 858.3, 768.5 and 796.5 for heme 1, 2, and 3, respectively. The electronic spectra of the synthetic heme 1, 2 and 3 show the typical absorption features for high-spin Fe^{3+} species [25]: Soret band at 386 nm, Q bands at 511 and 539 nm, respectively (Table 1).

Apo-HRP was prepared according to an acidic methyl ethyl ketone procedure of Teale [21]. The UV–vis spectrum of the apoprotein shows that heme was removed from HRP because there was no absorbance at 403 nm. Apo-HRP was reconstituted with the synthetic heme derivative by usual reconstitution technique as shown in Scheme 1. The loading of the synthetic hemes into apo-HRP is strongly supported by UV/vis spectroscopy. Reconstitution of apo-HRP could be followed by the shift of the Soret band of free hemes in the buffer (about 385 nm) to 398 nm for rHRP(1), 401 nm for rHRP(2) or 396 nm for rHRP(3) (Table 1). It indicates that the synthetic heme derivative was located at the normal position in the heme cavity. Compared with native HRP, the Soret bands of these reconstituted HRPs shifted from 402 nm to a shorter wavelength. In order to check whether the synthetic hemes were reconstituted with the apo-HRP at the appropriate heme-binding site, the activities of the reconstituted HRPs were determined by adding heme, which will interact at the correct place to increase activity if the synthetic heme was not bound to the specific site. The reconstituted HRP did not show any further increase in activity after the addition of heme (data not shown). It indicates that heme cannot replace already bound synthetic heme. In other words, the synthetic heme has interacted with apo-HRP at the right place under the reconstituted conditions used.

Table 4
Dissociation constants of native and modified HRP in 0.05 M phosphate buffer (pH 7.0) for phenol

Enzyme	K_d (mM)
HRP	9.2 ± 0.4
rHRP(1)	3.6 ± 0.2
rHRP(2)	10.3 ± 1.3
rHRP(3)	23.1 ± 0.5

In order to evaluate the reactivities of these modified HRPs, we monitored the oxidation of phenol in the presence of hydrogen peroxide. Table 2 shows the initial rate of oxidation of phenol by native and modified HRP in 0.1 M phosphate buffer (pH 7.0) and some organic solvents. Of the three reconstituted HRPs, only rHRP(1) had a larger initial rate than native HRP in aqueous buffer and some solvents. We can also find that the initial rate of native and reconstituted HRPs in organic solvents decreased as the hydrophobicity of organic solvent increased (log *P* of acetonitrile, DMF and DMSO is −0.33, −1.0 and −1.3, respectively). This is because the higher solvent hydrophobicity the weaker energy binding, and in consequence, lower enzymatic activity [26].

Steady-state kinetics was measured for the phenol oxidation by native and reconstituted HRPs in the presence of hydrogen peroxide at room temperature in 0.1 M phosphate buffer and some organic solvents. The oxidation follows Michaelis–Menten kinetics and the apparent kinetic parameters were shown in Table 3. Of the three rHRPs, rHRP(1) had the biggest affinity and catalytic efficiency (the value of k_{cat}/K_m) for phenol. These results suggest that phenol is easily accessible to the active site of rHRP(1). The dissociation constants of native and reconstituted HRPs were determined by the spectral titration approach and the data were given in Table 4. The replacement of native heme with synthetic heme 1 decreased the dissociation constant (K_d) by 2.5-fold, however, dissociation constants for rHRP(2) and rHRP(3) were increased.

From Tables 2–4, we can also find that the initial rate, affinity, catalytic efficiency and substrate-binding efficiency increased with the increases of electron-withdrawing efficiency of substituents at 4-position of the phenolic used to synthesize the heme derivatives. The same results were also obtained when the propionates of heme of hemoglobin were esterified with *p*-nitrophenol and *p*-aminophenol [19]. They thought that this biocatalytic behavior could be attributed to the electron character of the substituted group. When the carboxylic acids of heme are modified with an electron attractor, *p*-nitrophenol, electron density at the heme moiety is decreased, and the redox potential of hemoglobin is expected to be increase, increasing enzyme activity. On other hand, electron density is increased when the propionates of heme are esterified with an electron donor, *p*-methylphenol, and the electron transfer rate is expected to decrease, diminishing enzyme activity. Hayashi et al. reported that modification of the heme-propionates is effective for creating a binding site with specificity for a substrate [15]. The artificially created hydrophobic heme bearing a total of eight

Table 3
Kinetic parameters of native and modified HRP in 0.1 M phosphate buffer (pH 7.0) and 10% ACN

Enzyme	0.1 M phosphate buffer (pH 7.0)			10% acetonitrile		
	K_m (mM)	k_{cat} ($1\ min^{-1}$)	k_{cat}/K_m ($1\ mM^{-1}\ min^{-1}$)	K_m (mM)	k_{cat} ($1\ min^{-1}$)	k_{cat}/K_m ($1\ mM^{-1}\ min^{-1}$)
HRP	1.1	1.7×10^5	1.5×10^5	15.0	5.4×10^5	3.6×10^4
rHRP(1)	0.9	2.1×10^5	2.3×10^5	6.9	3.5×10^5	5.1×10^4
rHRP(2)	1.4	1.8×10^5	1.3×10^5	13.2	3.0×10^5	2.3×10^4
rHRP(3)	1.4	1.7×10^5	1.2×10^5	19.6	2.8×10^5	1.4×10^4

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

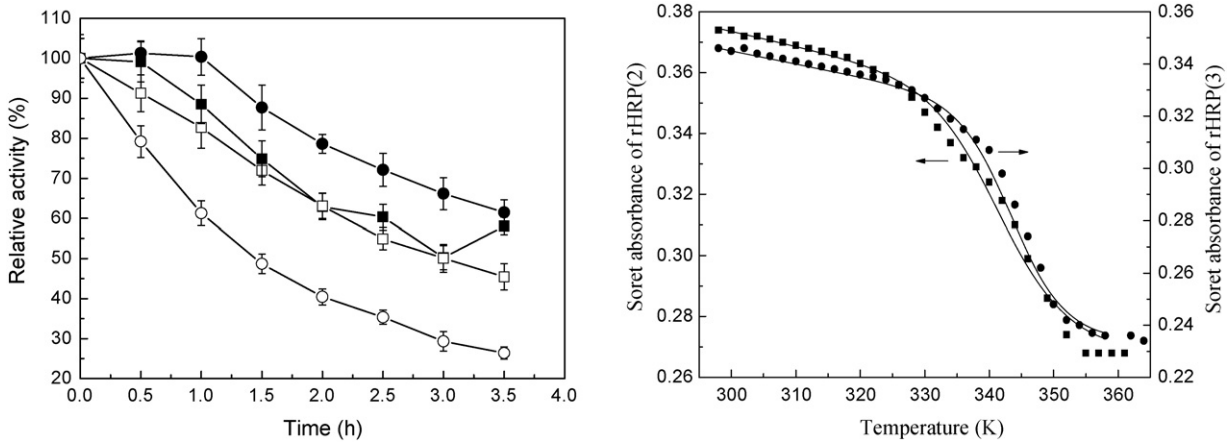

Fig. 1. Thermostability of native and reconstituted HRPs at 60 °C in aqueous buffer and DMF. Native HRP (○); rHRP(1) (□); rHRP(2) (■); rHRP(3) (●).

carboxylate groups at the terminal of two propionate side chains was incorporated into apomyoglobin to obtain a new reconstituted myoglobin with a unique binding domain structure [13]. The resultant reconstituted myoglobin showed higher peroxidase activity than native myoglobin. HRP reconstituted using heme with a modified (by aminomethylferrocene) propionic acid residue also showed more effective binding for hydrophobic substrate and higher reactivity towards water-soluble ferrocenes, however, its reactivity towards ABTS dropped threefold compared with native HRP [17]. However, Adak and Banerjee reported that heme-propionates play a vital role in the oxidative and reductive reactions of HRP and that the reconstituted HRP containing protoheme dimethyl ester oxidized neither guaiacol (aromatic electron donor) nor iodide or thiocyanate (inorganic donor) [18]. Thus, we speculate that the enhancement of catalytic activity of rHRP(1) may result from having a more hydrophobic and the change in the electronic state of the heme. In general, the reduction of compound II with a donor substrate is usually the rate-limiting step in peroxidase catalysis [27]. When the carboxylic acids of heme are modified with an electron attractor, *p*-nitrophenol, electron density at the heme moiety is decreased, and then attracting the electron of substrate, thus increasing the reduction rate of compound II. In order to testify the point, we determined the rate of compound I formation (k_1), compound I reduction (k_2) and compound II reduction (k_3) of different HRPs with phenol as a reductant by stopped-flow pre-steady-state kinetic measurements. The reduction rates of compound II (k_3) of native HRP, rHRP(1) and rHRP(2) were 3.5×10^5, 8.5×10^5 and $9.1 \times 10^4\ M^{-1}\ s^{-1}$, respectively. It indicates that the modification of carboxylic acids of heme with *p*-nitrophenol enhanced the reduction rates of compound II, and then increasing its catalytic efficiency.

The thermostabilities of the reconstituted HRPs were compared with native HRP in aqueous buffer and organic solvent (Fig. 1). The reconstituted HRPs showed higher thermostabilities and tolerances of DMF. After the exposure for 3.5 h at 60 °C in aqueous buffer, native HRP retained only about 26% activity. However, the reconstituted HRPs retained up to 45% activity. After the treatment of 40% DMF for 1 h at 60 °C, native HRP retained only about 13% activity. However, the reconstituted HRPs retained up to 43% activity. This biocatalytic behavior could be attributed to the hydrophobicity increase of active site. Our previous paper also reported that the enhancement of hydrophobicity of HRP by modification with phthalic anhydride resulted in higher tolerance of some organic solvents [9]. Torres et al. also reported that reconstituted hemoglobins using modified heme with *p*-nitrophenol and *p*-aminophenol showed a higher activity in acetonitrile [19]. From Fig. 1, we can also find that the stability of reconstituted HRPs increased with the increases of electron-donating efficiency of substituents at 4-position of the phenols used to synthesize the heme derivatives. This behavior could be attributed to the electron character of the substituted group. When heme-propionates are esterified by an electron donor (*p*-methylphenol), the electron density at the heme moiety is increased, and neutralizing positive charges of the enzyme molecular, then forming tighter binding of structural calcium ions, thereby

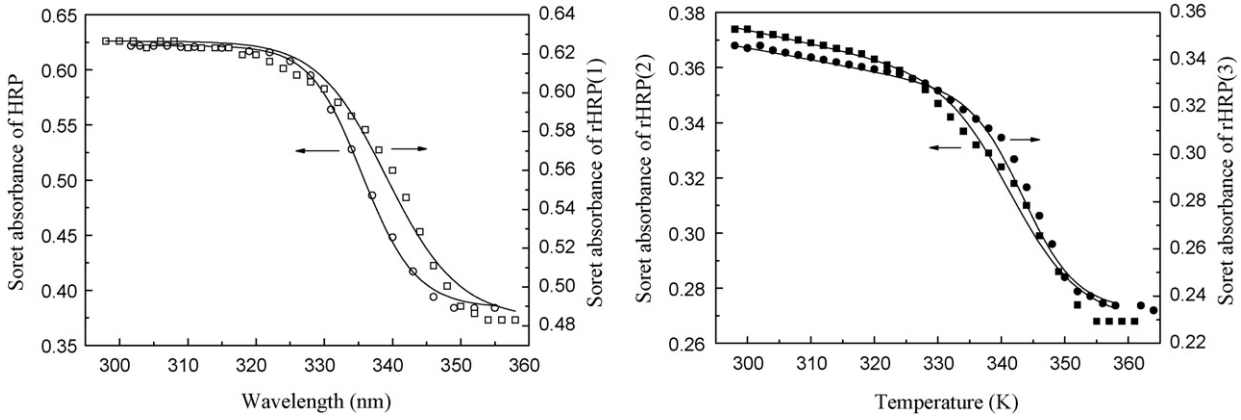

Fig. 2. Change in Soret absorbance of native HRP and reconstituted HRPs. Native HRP (○); rHRP(1) (□); rHRP(2) (■); rHRP(3) (●). The continuous lines are the best-fit curves based on Eq. (1).

Table 5
Thermodynamic parameters for thermal denaturation of native and reconstituted HRPs

Enzyme	T_m (°C)	ΔH_m (kJ/mol)	ΔS_m (J/mol/K)	$T_{m,HRP}\Delta S_m$ (kJ/mol)	ΔG (kJ/mol)
HRP	62.7 ± 0.1	242 ± 6	721 ± 19	242	0
rHRP(1)	66.4 ± 0.4	185 ± 10	545 ± 30	183	2
rHRP(2)	69.4 ± 0.4	179 ± 10	523 ± 30	176	3
rHRP(3)	71.9 ± 0.2	262 ± 10	760 ± 28	255	7

$\Delta G = T_m\Delta S_m - T_{m,HRP}\Delta S_m = \Delta H_m - T_{m,HRP}\Delta S_m$.

Table 6
The percentage of secondary structure elements of native and reconstituted HRPs, estimated from CD spectra using K2D software (http://www2.umdnj.edu/cdrw.jweb)

Enzyme	α-Helix	β-Structure	Random coil
HRP	37	13	49
rHRP(1)	50	10	40
rHRP(2)	41	13	45
rHRP(3)	61	7	32

The values represent the mean of three independent sets of experiment with S.D. of <10%.

stabilizing the modified HRP as in the case of charge neutralization of the amino groups of α-amylase by citraconic anhydride [28], of HRP by acetic acid *N*-hydroxysuccinimide ester [29], ethylene glycol bis-succinimidyl succinate [30], phthalic anhydride [9,10] and maleic anhydride and citraconic anhydride [11]. On the other hand, when heme-propionates are modified an electron attractor, *p*-nitrophenol, electron density at the heme moiety is decreased, and strengthening the electrostatic repulsion between structural calcium ions and active site. The interest results are the activations of 10–30% DMF on reconstituted HRPs. In our previous paper, we also reported the activation of methanol, acetonitrile on phthalic anhydride-modified-HRP [9] and that of THF on maleic anhydride and citraconic anhydride-modified-HRP [11]. Khmelnitsky et al. have reported numerous examples of enzyme activation by moderate concentrations (10–30%) of solvents [31].

The temperature dependence of the Soret absorbance is shown Fig. 2. For native HRP and reconstituted HRPs, a typical two-state transition was observed. Based on two-state model, the denaturation curves were analyzed using Eq. (1) to obtain T_m and ΔH_m (Table 5). Thus, ΔS_m was calculated from Eq. (2). From Table 5, it is clear that T_m at pH 7.4 was increased from 62.7 to 66.4 °C (for rHRP(1)), 69.4 °C (for rHRP(2)) and 71.9 °C (for rHRP(3)) upon modifications. These results also indicate that the thermal stability of reconstituted HRPs was enhanced. Table 5 also shows that the thermostabilization of HRP after modification is mostly accompanied by a decrease in ΔH_m and ΔS_m, as in the case of our previously reported maleic anhydride and citraconic anhydride-modified-HRP [11]. The reduction in both the enthalpies and entropies upon modification is an example of enthalpy–entropy compensation. The free energies contributed from the entropy at the T_m of HRP ($T_{m,HRP}\Delta S_m$) and The changes of the free energies contributed from the entropy (ΔG) were calculated (Table 5). This says that at 62.7 °C, rHRP(1), rHRP(2) and rHRP(3) are 2, 3 and 7 kJ/mol more stable than HRP, respectively. Because enthalpies and entropies upon modification became less positive, unfoldings were less favored.

CD spectra in UV and UV–vis regions provide information on the structure of protein and prosthetic heme of HRP [31]. Fig. 3 shows CD of native and reconstituted HRPs in water. Native and reconstituted HRPs have similar CD spectra with negative bands at 208 and 220 nm, which agreed with the previous results [11,32]. The reconstituted HRPs showed stronger CD bands at 208 and 220 nm than native HRP (Fig. 3A), indicating an increase of α-helix content. The percentages of secondary structure elements calculated using K2D software are summarized in Table 6. The α-helix content were increased from 37% of native HRP to 50% of rHRP(1), 41% of rHRP(2) and 61% of rHRP(3). It indicates that the modification of heme-propionate increases compactness of the secondary structure of HRP, and then enhances thermostability and tolerance of DMF [33,34]. When heme CD spectra were recorded (Fig. 3B), the reconstituted HRPs also showed significant Soret CD bands, as shown by native HRP. The intensity of the Soret CD band of rHRP(1) was almost identical to that of native HRP, however, rHRP(2) and rHRP(3) had weaker the Soret CD signal than native HRP. The large decrease in the Soret CD suggests a loss in interaction of heme with the apoprotein [34,35]. This may be the reason that rHRP(1) showed higher catalytic efficiencies and the other rHRPs lower than native HRP. Akita et al. reported the same trend between the activity and the intensity of the Soret CD [35]. Adak and Banerjee also reported similar results [18]. The reconstituted HRP with heme dimethyl ester had similar CD bands in the far-UV region to native HRP and not any Soret CD band, and then resulting in having very low activity of oxidation of guaiacol [18]. These results indicate that these modifications resulted in the increase in α-helix content and the change of the environment of heme.

The fluorescence emission excited at 295 nm arises solely from the only one tryptophan residue (Trp 117) of HRP [36]. Thus, the

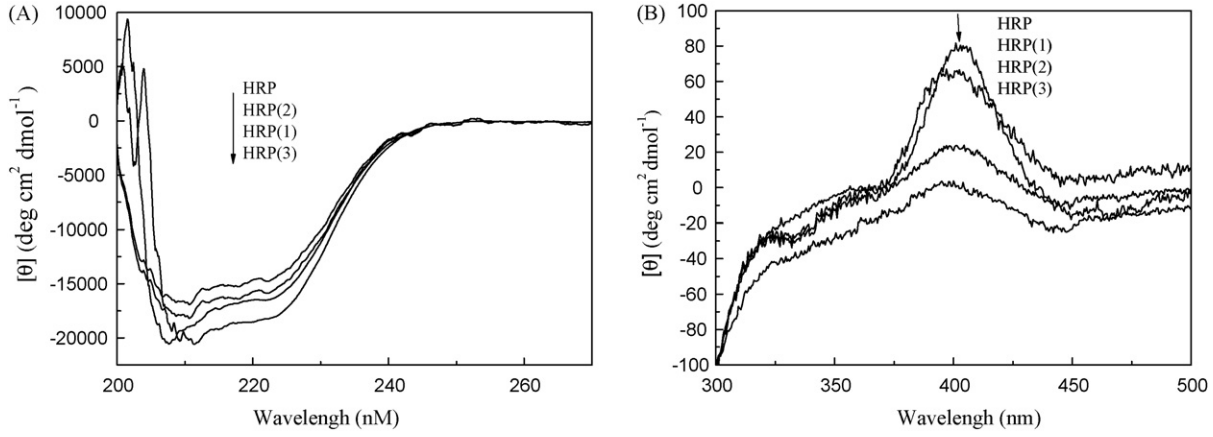

Fig. 3. CD spectra of native and reconstituted HRPs in 50 mM Tris/HCl buffer (pH 8.0). (A) CD in the UV region. The concentration of native HRP, rHRP(1), rHRP(2) and rHRP(3) was 1.8, 2.7, 2.1 and 3.0 μM, respectively. (B) CD in the visible region. The concentration of native HRP, rHRP(1), rHRP(2) and rHRP(3) was 10.8, 8.8, 11.0 and 10.6 μM, respectively.

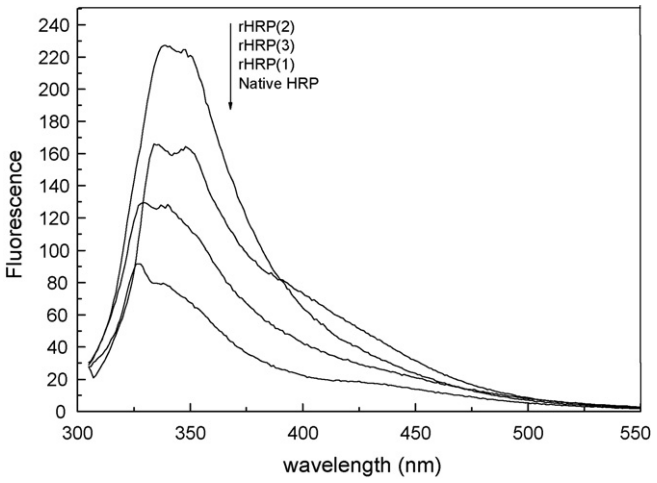

Fig. 4. Fluorescence spectra of native and reconstituted HRPs in 50 mM Tris/HCl buffer (pH 8.0).

tryptophan fluorescence was used to probe the structural change of reconstituted HRPs compared to native HRP. Fig. 4 shows that the tryptophan fluorescence emission red shifted from 327 nm of native HRP to 329 nm of rHRP(1), 334 nm of rHRP(3) and 339 nm of rHRP(2), respectively. It indicates that the tryptophan residue of the reconstituted HRPs more exposed and located in a more polar environment [9,37,38]. From Fig. 4, we can also find that the intensity of the trytophan fluorescence emission increased after modifications because of a change in the relative orientation or distance between the heme and the tryptophan residue leading to a decrease in the efficiency of energy transfer [37]. This is also denotes that the distance between the heme and the tryptophan residue increased [38]. Another explanation may be these modifications introduced a bulky benzene ring structure into HRP.

4. Conclusions

Changing the electron character of the aromatic moieties linked at each terminal of the two heme-propionates can control binding efficiency for substrate, catalytic efficiency and stability. The initial rate, affinity, catalytic efficiency and substrate-binding efficiency increased with the increases of electron-withdrawing efficiency of substituents at 4-position of the phenolic used to synthesize the heme derivatives, contrariwise, the stability decreased. However, we must further study on the trend using more synthetic heme derivatives. We have engineered a new HRP by modification of heme-propionates to enhance HRP activity. The above results demonstrate that the replacement of the native heme with a chemically modified hydrophobic one having an electron-withdrawing group is useful for improving the activity and stability of HRP. The changes of catalytic properties and stabilities are related to the changes of the conformation of HRP. The modification changed the environment of heme and tryptophan, increased α-helix content of HRP.

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