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THE EFFECTS OF MEDIA PROPERTIES ON THE HORSERADISH PEROXIDASE-CATALYZED FLUOROGENIC REACTION

Q. G. Li,* J. G. Xu, X. Z. Huang and G. Z. Chen

Department of Chemistry, Xiamen University, Xiamen 361005, China

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Summary—The effects of media properties including buffers, acidity, solvents and surfactant on horseradish peroxidase-catalyzed fluorogenic reaction were investigated. The results showed that the so-called non-fluorescent hydrogen donors were in fact fluorescent. There existed an acid–base equilibrium in the fluorescent dimer product. For *p*-hydroxyphenylpropionic acid, a pK_a value of 8.0 for the product was obtained from its titration curve. The product fluorescence increased with higher pH, however, a longer time was needed to reach the reaction equilibrium due to the pH mismatch problem. Cationic micelles cetyltrimethylammonium bromide and cetyltrimethylammonium chloride could reduce the pH mismatch and offered a way to further increase the determination sensitivity. Finally, a micelle-enhanced flow-injection analysis of horseradish peroxidase is suggested.

Horseradish peroxidase (HRP)-catalyzed fluorogenic reactions have attracted much interest in recent years, mainly because of their wide applications in environmental and biochemical analyses. Fluorogenic hydrogen-donors exploited included dichlorofluorescein,[1] scopoletin[2] and *p*-substituted phenolic compounds (PSPCs). Due to their sensitivity, stability and low cost, PSPCs, especially *p*-hydroxyphenylpropionic acid (HPPA), have been regarded as the best choice.[3–5]

However, there is still much left to improve in the HPPA-based measurement. The preliminary study of Zaitsu *et al.*[6] showed that a basic media was suitable for HRP–HPPA–H_2O_2 reaction. However, HRP is more active in a neutral media.[7] More recently, Matsumoto *et al.*[8] reported that the alkalinization of the neutral solution after enzyme reaction resulted in a three- to five-fold increase in the product fluorescence. An extra alkalinization step, however, made it impossible to carry out reaction and fluorescence measurement in a single step.[9]

In order to find a way to overcome the pH mismatch and develop an easily automated single-step determination for HRP or H_2O_2, the effects of media including buffer, solvents and surfactant on the reaction and the product fluorescence were investigated. The results showed that the enhancing effects of some micelles offered a way to reduce the pH mismatch. Finally, a micelle-enhanced flow-injection analysis (FIA) of HRP was developed.

EXPERIMENTAL

Reagents

HRP (EC 1.11.1.7, RZ > 3.0, 250 IU/mg) was purchased from SABC, Beijing Co. HPPA(TCL-GR) and *p*-hydroxyphenylacetic acid (HPAA) (TCL-GR) were from Tokyo Kasei and homovanillic acid (HVA) (for biochemistry) was from Merck. Organic solvents, including methanol, absolute ethanol, iso-propanol, iso-butanol, iso-pentanol, acetonitrile, glycerol, formaldehyde reagent (>36% w/v), ethyl acetate, absolute ether and acetone were all analytical reagents purchased from Shanghai Chemical Co. Surfactant, including sodium dodecyl sulfate (SDS), sodium lauryl sulfate (SLS), Tween-20, Tween-80, Triton X-100, polyethylene glycol 400 (PEG-400), PEG-6000, cetylpyridine bromide (CPB), dodecyl triphenylphosphorus bromide (DTPB), cetyltrimethylammonium bromide (CTAB) and cetyltrimethylammonium chloride (CTAC), and β-cyclodextrin (β-CD) were all laboratory reagents purchased from Shanghai Chemical Co. H_2O_2 (30%, analytical reagent) was purchased from Shanghai Taopu Chemical Plant and used before its expiration

*Author to whom correspondence should be addressed.

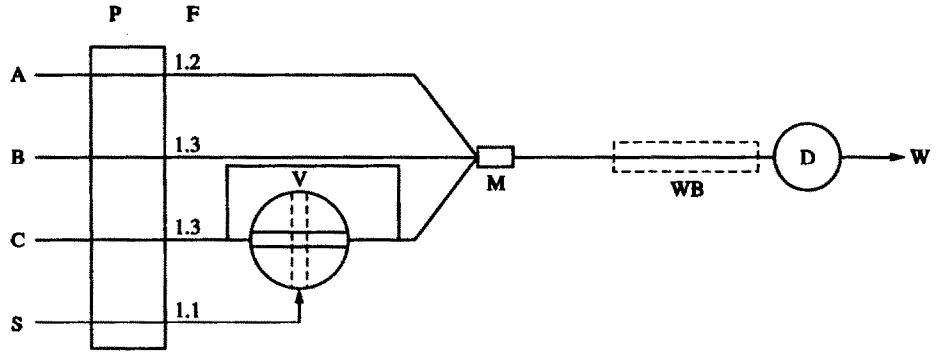

Fig. 1. Schematic illustration of FIA set up for HRP. A, CTAB + H_2O_2; B, HPPA; C, carrier; S, sample; P, pumps; F, flow rate (ml/min); V, valve; M, mixer; WB, water bath; D, detector; W, waste.

without standardization. Water was deionized twice and distilled once in a quartz bottle and was used to make all the solutions. All other reagents were of analytical grade commercially available.

Procedures

The fluorescence spectra of the equilibrium solution. Tris–HCl (pH 7.4) (2.5 ml, 0.1*M*) was added to a 1-cm quartz cell, followed by the addition of 50 μl of HRP (0.725 IU/ml), 200 μl of HPPA (7.5m*M*) and 100 μl of H_2O_2 (0.03%), and mixed immediately. The excitation and emission spectra were recorded after the fluorescence increase stopped (about 5 min later).

The effect of buffer on the reaction. (1) Type of the buffer on the reaction: 2.5 ml of buffer (pH 7.4), including 0.2*M* sodium phosphate, 0.1*M* potassium phosphate, 0.1*M* Tris–HCl, 0.2*M* sodium borate and 0.1*M* sodium citrate was added to the cell followed by HRP, HPPA and H_2O_2 as above. The kinetic curves (F-t) were recorded after $\lambda_{ex}/\lambda_{em}$ were selected. (2) The acid and concentration of the buffer on the reaction: 2.5 ml of Tris–HCl (0.05–2.5*M* Tris, pH 7–9) was added to the cell, followed by HRP, HPPA and H_2O_2 as above. *F–t* curves were recorded after $\lambda_{ex}/\lambda_{em}$ were selected. (3) The acidity of the buffer on the product fluorescence: the equilibrium solution was produced by the use of 0.1*M* of Tris–HCl (pH 7.4) as described, then 0–200 μl of NaOH (1.0*M*) or HCl (1.0*M*) was added, both excitation and emission spectra were recorded.

The effect of solvent on the product. To 1.5 ml of the equilibrium solution, 1.0 ml of solvent was added. Both the excitation and emission spectra were recorded.

The effect of surfactant. (1) On reaction: 2.5 ml of 0.1*M* Tris–HCl (pH 7.4) and 200 μl of surfactant solution (concentration listed in Table 2) was added to the cell, followed by HRP, HPPA and H_2O_2 as above, the *F–t* curve was recorded ($\lambda_{ex}/\lambda_{em}$ were selected for each surfactant). (2) On product: the equilibrium solution was produced as described, then 200 μl of the above surfactant solution was added, both excitation and emission spectra were recorded.

All the measurements were made on a Shimadzu RF-5000 Spectrofluorophotometer. The bandpass was 3 nm for both excitation and emission monochromators. The sensitivity was 'high'. The scan speed for wavelength was 17 nm/sec and there was a 0.1 sec time interval with end time 6.3 min. Temperature was 25 ± 0.5°C.

Micelle-enhanced FIA of HRP. FIA was made on a LZ-1000 FIA System (Zhaofa Auto. Anal. Inst., Shenyang). The schematic diagram is shown in Fig. 1. The following conditions were used with respect to the sensitivity and rapidness: CTAB + H_2O_2 was a buffered mixture of 10 ml containing 1.0 ml of CTAB (0.01*M*) and 500 μl of H_2O_2 (0.03%); HPPA was a buffered solution of 3.75 m*M*; the carrier was the buffer of 0.01*M* of Tris–HCl (pH 7.4). According to the flow rates as noted, the sample loading (the valve position shown in the dotted line in Fig. 1) was finished in 8 sec by pumping it into 20 μl of loop on the valve, extra sample returned to the sample container. The sample injection (the valve position is shown by the solid line in Fig. 1) was finished in 10 sec. The reaction was initiated in the mixer and continued in a PTFE tube (100 cm long, 1.0 mm i.d.) bathed in water of 37 ± 0.5°C. The fluorescence was detected with $\lambda_{ex}/\lambda_{em}$ = 300/405 nm.

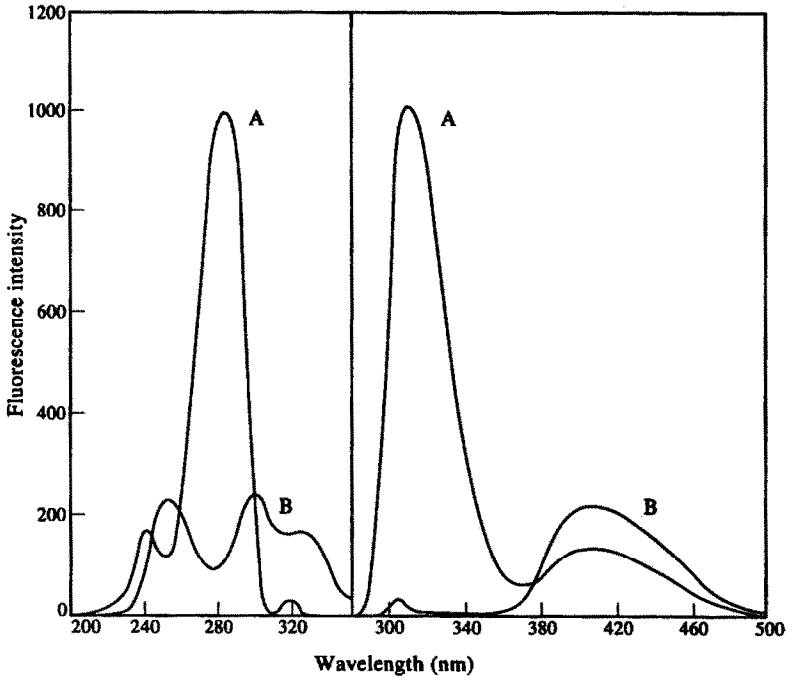

Fig. 2. Excitation and emission spectra of the equilibrium solution in 0.1*M* of Tris–HCl (pH 7.4). (A) HPPA, (B) product.

RESULTS AND DISCUSSION

Fluorescence spectra

The excitation and emission spectra of the equilibrium solution are shown in Fig. 2. A is the reactant HPPA, B is the dimer product. *F–t* curves also showed that the fluorescence of HPPA decreased during the reaction (data not shown). Similar results were observed when HVA and HPAA were used. For HPPA, $\lambda_{ex}/\lambda_{em}$ of reactant and product were 290/310 and 299/406 nm; for HPAA, 284/310 and 300/405 nm; for HVA, 270 300 and 315 430 nm.

HPPA, HPAA and HVA have long been considered as non-fluorescent substances.[3,9,10] This misunderstanding may be attributed to several factors: first, the original work of Guilbault *et al.*[10] was done on an Amico fluoromicrophotometer filter instrument, the filters used may have filtered the emission spectra of the substrate; second, the strong basic media used as suggested by Matsumoto *et al.*[8] greatly inhibited the substrate fluorescence as noticed in our experiments. According to their molecular structure, all the PSPCs should exhibit fluorescence under some condition. In fact, HPPA and HPAA have been recorded as fluorescent substances.[11]

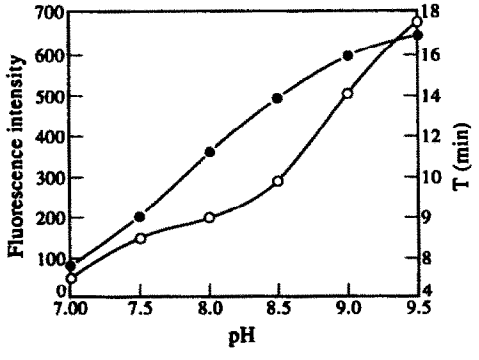

Fig. 3. The effect of acidity on the fluorescence intensity of the product (●) and the time to reach reaction equilibrium (○).

The effect of buffer

The effects of different types of buffers were studied by recording the fluorescence increase in 30 sec from *F–t* curves. It was concluded that the Tris–HCl buffer gave the biggest fluorescence increase, and was selected for the reaction. The concentration study of Tris–HCl buffer showed that 0.1*M* gave the biggest reaction rate. The effect of acidity on the reaction equilibrium is shown in Fig. 3. The product fluorescence was stronger at higher pH, but a longer time was needed to reach the equilibrium. These results showed an apparent pH mismatch problem. HRP is more active at neutral pH, and the reaction proceeded faster than in a basic media. Without noticing the pH mismatch,

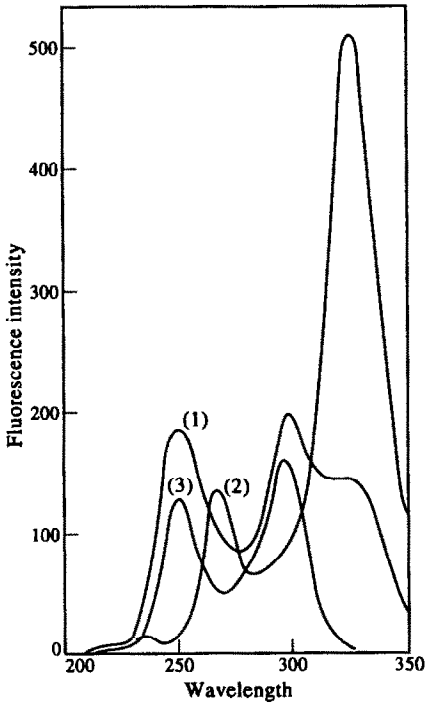

Fig. 4. Excitation spectra (λ_{em} = 406 nm) of the product in (1) 0.1*M* of Tris–HCl buffer (pH 7.4), (2) (1) + 300 μl NaOH (1.0*M*), (3) (1) + 100 μl HCl (1.0*M*).

Zaitsu *et al.*[6] adopted pH 8.5 to determine HRP and H_2O_2. This pH was apparently a trade-off between the fluorescence intensity and the enzyme activity.

Figure 4 shows the excitation spectra of the product in three different acidity (pH values were not measured in the strong acid and base). We could see that 325 nm peak was of the basic product, 299 nm peak was the acid, and the base was more florescent than the acid. The identification of these peaks explained the spectra changes observed by Matsumoto *et al.*[8] The acid–base equilibrium in the product is clearly shown in the titration curve of the basic product in Fig. 5. A pK_a value of 8.0 of the product was obtained according to the point of inflection of the curve. A slight decrease that appeared at high pH was attributed to dilution effect with continuous addition of 1.0*M* of NaOH. The decrease between pH 2 and 5 was thought to be another potential inflection point corresponding to pK_a of the excited DHPPA.

The effect of solvent

The basic product (λ_{ex} = 325 nm) fluorescence decreased from water, methanol, ethanol to iso-propanol (Table 1) while the acidic product (λ_{ex2}) increased. Additionally, the excitation wavelength of the latter exhibited a slight red shift. These phenomena agreed with the solvent polarity and the hydrogen bond effect. The solvent polarity decreased from water to iso-propanol. Lower solvent polarity would make the acid dissociation of the solute more difficult, and lower down the relative concentration of the basic form, leading to the basic product fluorescence decrease. The hydrogen bond formed between the solvent and the fluorophore promoted the non-fluorescent inner conversion from the first excited singlet state to the ground state, and caused a fluorescence decrease.[12] From water to iso-propanol, the hydrogen bond became increasingly weak and thus the fluorescence of the acid product was increasingly strong. The red shift of the acid can be also attributed to the hydrogen bond, which, when weak, would facilitate the charge transfer from the electron donor group OH to benzene ring, widen the conjugated bond, promote the excitation of the molecule, and finally lead to red shift.

Acetone had a strong quenching effect on the product fluorescence. Experimental results proved that the most significant quenching appeared at the wavelength of 280 nm, which corresponded to the biggest absorption of acetone. Thus the quenching effect was due to the absorption of acetone.

The effect of surfactant

Table 2 shows the effect of several surfactant and β-CD on the initial reaction rates ($\lambda_{ex}/\lambda_{em}$ were not shown) defined as the fluorescence increase in 1 min and the excitation spectra. Under the experimental conditions, the final concentration of the surfactant was above the

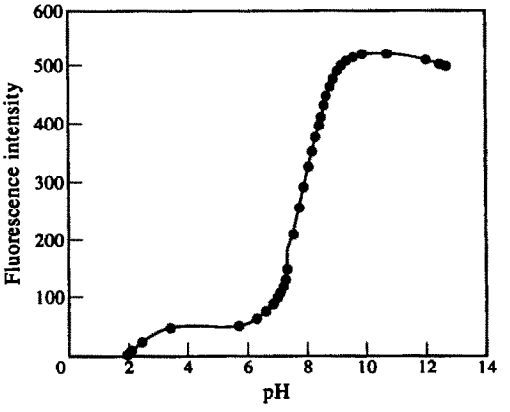

Fig. 5. Fluorimetric titration curve of the basic product. ($\lambda_{ex}/\lambda_{em}$ = 325/406 nm). Different acidity was obtained by the addition of different volume of NaOH (1.0*M*) and HCl (1.0*M*).

Table 1. The effects of solvents on the excitation spectra of the product

Solvent	λ_{ex1}‡	F1‡	λ_{ex2}‡	F2‡	F(λ325 nm)‡
Water	250.8	373	299.0	389	312
Methanol	252.0	494	301.8	490	115
Ethanol	253.0	542	302.0	573	102
iso-Propanol	253.4	612	303.0	610	85
iso-Butanol*	251.8	595	301.0	835	285
iso-Pentanol*	251.8	453	300.8	555	110
Acetonitrile	251.8	478	301.9	557	110
Glycerol*	252.5	380	301.8	485	288
Formaldehyde	251.8	348	299.0	394	14
Ethyl acetate†	254.0	287	299.0	380	20
Ether	251.0	253	300.2	310	10
Acetone	—	0	—	0	10

*Heterogeneous solution.

†Heterogeneous solution, $\lambda_{em} = 400$ nm.

‡λ_{ex1}, λ_{ex2} and λ_{325} nm are three peaks of the excitation spectra; $F1$, $F2$ and F are intensities of these peaks, respectively.

critical micelle concentration (cmc). It is shown that only the cationic micelles exhibited significant effects. CTAB and CTAC increased the reaction rate and the product fluorescence; DTPB and CPB, on the other hand, decreased both.

The most important property of micelles is their ability to solubilize within their distinct structured regions, compounds which are insoluble or sparingly soluble in water, allowing their location into or at the surface of the aggregate.[13] Moreover, ionic micelles can provide charged structures, where attractive or repulsive interaction with ionic solute may be present. We propose that the charge interactions between the basic product and the micelle gave rise to the above effects. The interactions not only increased the fluorescence by offering a non-aqueous environment to the basic product molecules, but also accelerate the reaction rate by promoting the acidic dissociation of the product. This point of view was supported by the observations that the influence of CTAB and CTAC on the excitation spectra was similar to that of excess NaOH and that the presence of CTAB micelle increased the conversion rate of HPPA during the reaction (data not shown).

Special attentions should be paid to DTPB and CPB, where the quenching effect was proposed to be due to the aromatic rings of the surfactant molecules. Shi *et al.*[12] in a systematic study on a variety of surfactant effects on the fluorescence, pointed out that only those surfactants in which the charge-bearing groups did not approximate to the conjugated bonds might exhibit enhancing effects. They indicated that the aromatic rings often had a lower energy level than the excited fluorescent molecule and an excitation energy transfer could take place from the product molecules to the aromatic rings of the surfactant, thus causing fluorescence quenching.

Table 2. The effects of surfactant on the reaction rates and the excitation spectrum of the product

Surfactant	Reaction rate (min^{-1})	$F(\lambda_{299}\ nm)$	$F(\lambda_{325}\ nm)$
Water	1.0	340	160
SDS ($2 \times 10^{-2}M$)	0.9	335	155
SLS ($1 \times 10^{-2}M$)	0.8	330	152
Tween-20 (0.5%)	1.0	320	140
Tween-80 (0.136%)	1.0	310	130
Triton X-100 (0.2%)*	1.1	—	—
β-CD ($1 \times 10^{-2}M$)	1.1	320	110
PEG-400 (0.8%)	0.7	330	140
PEG-6000 (0.8%)	1.1	338	158
CPB ($1 \times 10^{-3}M$)	0.0	0	0
DTPB ($1 \times 10^{-2}M$)	0.1	66	30
CTABV ($1 \times 10^{-2}M$)	2.6	370	387
CTAC ($1 \times 10^{-2}M$)	2.4	363	370

*The surfactant has strong fluorescence background at $\lambda_{em} = 406$ nm when excited at $\lambda_{ex} = 245$ and 289 nm.

The enhancing effects of CTAB and CTAC had analytical potentials for both HRP and H_2O_2 with some advantages. First, it could be applied to increase the product fluorescence in a strong basic media and resulted in a higher determination sensitivity than Matsumoto's method. Second and more important, the increase of the reaction rate in a neutral media can be used to develop a sensitive single-step determination, *e.g.* FIA of HRP. The latter strongly suggested that CTAB micelle actually offered a way to reduce the pH mismatch of the reaction.

FIA of HRP

Experimental conditions, *i.e.* concentrations of CTAB, HPPA and H_2O_2 were optimized using a orthogonal design method regardless of the interaction of the factors.[14] Different volume of CTAB ($1.0 \times 10^{-3} M$) and H_2O_2 (0.03%) were mixed and diluted with buffer to a final volume of 10 ml. Different volume of HPPA (75 mM) was diluted with buffer to a final volume of 25 ml. HRP (7.25×10^{-2} U/ml) was used as the sample. The optimal condition selected was: 1.0 ml of CTAB, 500 μl of H_2O_2 and 12.5 ml of HPPA.

The calibration curve for F–log(c) was linear from 0.029 to 290 mg/l., the linear regression equation was $y = 509.1 + 375.0x$ (six data points), the relative standard deviations of 10 data points was 2.1%, the correlation coefficient was 0.993. Though the presence of CTAB obviously increased the fluorescence in the FIA of HRP, the sensitivity obtained was still less than that of the results of Zaitsu *et al.* These may be ascribed to several factors, *e.g.* the adoption of FIA, long storage duration of the enzyme solution and the necessity of further optimization of the experimental conditions.

Above all, the fluorescent product of HRP–HPPA–H_2O_2 fluorogenic reaction was sensitive to the medial properties, and this helped to clarify the reaction of its own and offer ways to facilitate its progress. From the point of view of analytical interest, the enhancing effect of micelle not only improved the sensitivity, but also extended the use of this reaction in fast and automated determination.

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