0273-2289/96/5602—0129$07.75

Fluorometric Assay for Horseradish Peroxidase in Organic Media

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Received August 8, 1994; Accepted November 22, 1994

ABSTRACT

A fluorometric method for assaying the activity of horseradish peroxidase (HRP) in organic media has been developed. This method is designed on the basis of the disparity in the spectral properties of substrates and corresponding resultant polymers. It monitors the fluorescence quenching of substrate during enzymatic catalysis, and works efficiently in a number of organic media (such as dioxane–water mixture, acetone–water mixture, and alcohol–water mixture, and so forth) toward many substrates. This assay is simpler, more rapid, and more convenient compared with the commonly used HPLC method. It is qualitatively reproducible and can also be used for quantitative calculation of the substrate conversion.

Index Entries: Horseradish peroxidase; organic media; fluorescence; phenol; aromatic amine.

INTRODUCTION

Horseradish peroxidase (HRP) can catalyze the digestion of lignin and peroxidation of phenols and aromatic amines in the presence of H_2O_2 (*1*). Recently, the use of organic solvent as a reaction media has dramatically increased the diversity of enzymatic catalysis. The interest in organic enzymology, particularly in organic synthesis, is widespread (*2–4*). HRP has been applied to organic reaction media to catalyze the coupling of phenols and aromatic amines (*5–8*). The polymers show a wide potential

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application as substitutes for phenol-formaldehyde resins and other useful materials (*5–8*).

The methods of assaying the activity of HRP in aqueous solution for some special substrates have been developed previously by measuring the products absorbance spectrophotometerically (1). However, they are not suitable in organic media, because (*1*) the products might be coupled further in organic media to form a complex polymer (*6*), and the accuracy of the measurement will be affected; and (2) more and more substrates have been used as monomers in polymerization of phenols and aromatic amines (*5–10*). Thus, determining the activity of HRP for each substrate is necessary. Although the activity of HRP and the conversion of substrate can be determined by HPLC (*5,8*), it is not a ideal method for routine determination of HRP activity because of its complicated sample treatment and long time period. Therefore, it is important to develop a more rapid and simple method suitable for detecting the activity of HRP in organic media.

A flourescence assaying method for HRP in organic media is developed in the present work based on the disparity in the spectral properties of substrates and resultant polymers. It determines the activity of HRP by following the decrease in the fluorescence intensities of substrates during enzymatic catalysis. This assay is much more versatile, as efficient in many organic media for lots of substrates, and more simple and rapid compared with the HPLC method. The data obtained with this assay can also be used for quantitative calculation of substrate conversion according to the standard curves of the fluorescence intensities as a function of substrate concentrations.

MATERIALS AND METHODS

Preparation of Enzyme Solution

Horseradish peroxidase (EC 1.11.1.7) was purchased from Sigma Chemical Co. (St. Louis, MO) as a salt-free powder with an SA of 190 purpurogallin U/mg solid. It was dissolved in 0.01 mol/L phosphate buffer (pH 6.0) with the concentration of 0.071 mmol/L. It should be diluted before using.

Preparation of Substrate Solutions

All substrates, *p*-phenylphenol, *p*-cresol, 1-naphtol, and aniline were purchased from Aldrich Chemical Co. (Milwaukee, WI) and purified further. Each of them exhibited a single peak on HPLC. The solvents (dioxane, acetone, alcohol) and other chemicals of analytical grade were also available commercially, and used without further purification. Substrate solutions were prepared by dissolving each of them in the mixture of 70% pure organic solvent and 30% water (substituted by 0.01 mol/L, pH 6.00,

phosphate buffer) with concentration of 30 μmol/L. These solutions should be prepared freshly, because phenols and aromatic amines are susceptible to oxidation by air. Solution of H_2O_2 at a concentration of 0.03 mol/L was prepared by diluting 30% H_2O_2 aqueous solution into the mixture of organic solvent and water used above. It also should be prepared freshly because it is not stable.

Spectral Measurements

The fluorescence measurements were made on a HITACHI 850 fluorescence spectrophotometer equipped with 150 W Xenon lamp, and the UV absorption spectra were measured on a HITACHI 557 spectrophotometer.

Fluorometric Assays

All fluorometric assays were carried out at 25°C in a 1 x 1 cm quartz cell on HITACHI 850 fluorescence spectrophotometer. The excitation and emission wavelengths employed in the measurement were selected on the basis of the fluorescence properties of substrates. Usually the fluorometric assays were carried out at the maximal excitation and emission wavelengths of substrates. The quartz cuvet containing 2.89 mL, 30 μmol/L substrate solution and 10 μL HRP solution was placed in the sample holder. The reaction was started by the addition of 0.1 mL of 0.03 mol/L H_2O_2 solution, although no significant difference in rate was observed if the reaction was initiated by HRP. After the solution was mixed thoroughly, the decline of fluorescence intensity caused by enzymatic catalyzing was immediately recorded vs time for 3 min. From the slope of the plot, the relative activity of the enzyme was obtained. The activity of HRP in international unit and the conversion of substrate could be calculated according to the standard curve of the fluorescence intensity as a function of the substrate concentration.

RESULTS AND DISCUSSION

The object of this study is to develop a simple, rapid method suitable for assaying the activity of HRP in organic media toward different substrates and to overcome the drawbacks of HPLC method.

Spectral Properties of Substrates and Resultant Polymers

This method is set up on the basis of the difference in the spectral properties of the substrates and their corresponding polymers. Figure 1 shows the absorption spectra of the substrates and their corresponding polymers. It can be found that the absorption spectra of *p*-phenylphenol, *p*-cresol, 1-naphthol, and aniline almost coincide with those of their corresponding polymers, and the differences in the absorption values between

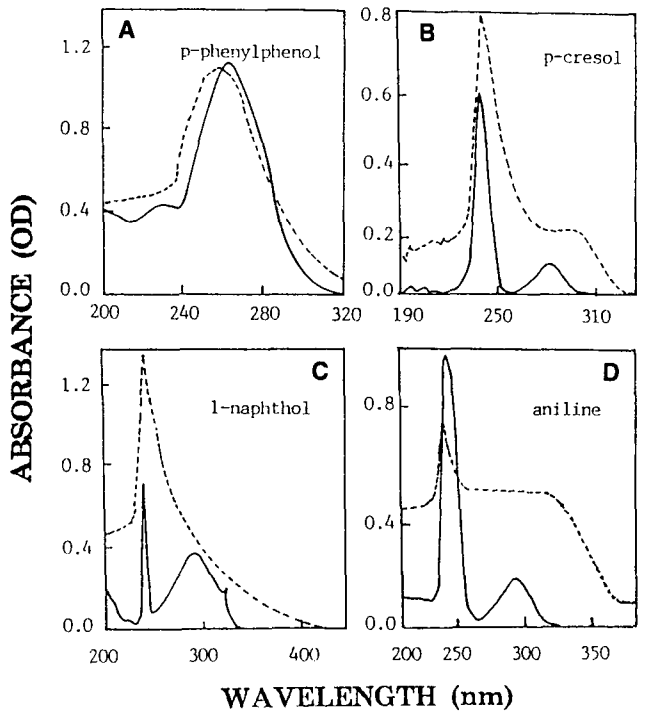

Fig. 1. UV absorption spectra of substrates (——) and their corresponding polymers (-----). Sample solutions were prepared by dissolving equal amounts of substrate and corresponding polymer in dioxane, with concentration of substrates being 100 μmol/L.

these substrates and their corresponding polymers are not significant. Moreover, the products generated by enzymatic catalysis in organic media are complex polymers with different molecular weights and structures (*6*). Thus, it will be difficult to measure the activity of HRP for these substrates in organic media by assaying the changes in the absorptions of substrates or resultant polymers.

The fluorescence emission spectra of various substrates and their corresponding polymers were investigated (Fig. 2). It is fortunate that the fluorescence spectra of the resultant polymers shift to long wavelength notably, and particularly the fluorescence intensities of the resultant polymers are so low compared with their corresponding substrates that they can be negligible. This result indicates that the fluorescence intensities of substrates will decrease during enzymatic catalysis. This fact can be observed by comparison of the fluorescence emission spectra of substrates before and after enzymatic catalyzing. Initially, the spectra of substrates with the concentration of 40 μmol/L in the mixture of 70% dioxane and 30% buffer were recorded. After the addition of HRP and H_2O_2 and incubation at 25°C for 2 h, the spectra of the substrates were recorded again. The comparison of the spectra is illustrated in Fig 3. A remarkable decrease in the fluorescence intensity of substrates can be observed. It suggests that the change of the substrates' fluorescence intensity can be empolyed as a

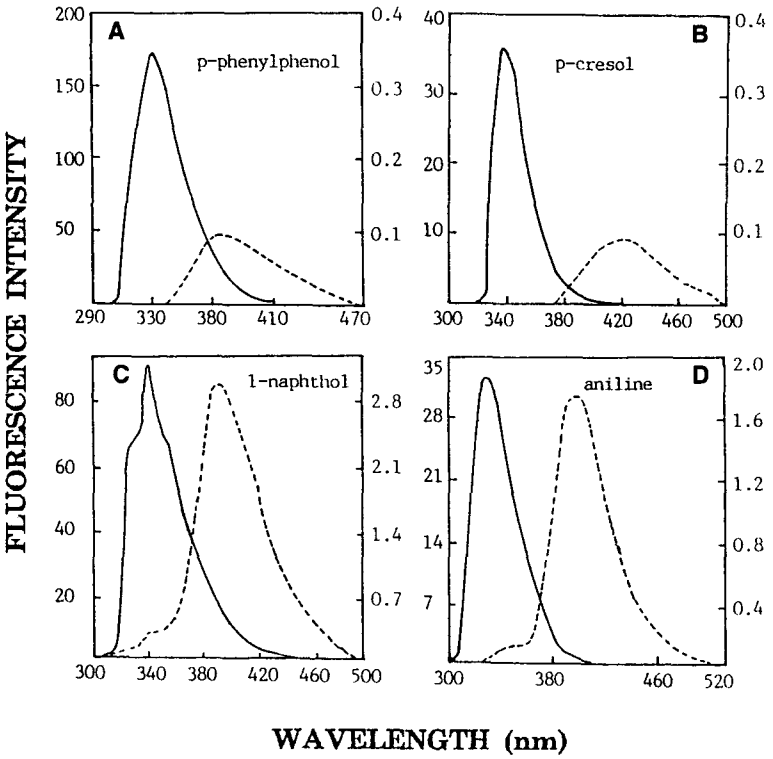

Fig. 2. Fluorescence spectra of substrates (——) and corresponding polymers (-----). The employed excitation wavelengths were (**A**) 279 nm, (**B**) 280 nm, (**C**) 300 nm, and (**D**) 270 nm. Preparation of the sample solutions was similar to that described in the legend of Fig. 1. The scale on the left axis is for substrates, and that on the right axis is for corresponding polymers.

criteria for assaying the activity of HRP for these substrates. Thus, the activity of HRP in the mixture of dioxane and water can be measured by following the fluorescence quenching of substrates caused by enzymatic catalysis.

Standard Curves

To calculate the conversion of the substrate and the activity of HRP in international units, it was necessary to make standard curves of fluorescence intensity a function of the substrate's concentration before performing the fluorometric assays. The standard curves were obtained by determining the fluorescence intensities of various substrate concentrations at their maximal excitation and emission wavelengths, and making the plot of the fluorescence intensity a function of substrate concentration (Fig. 4). This revealed that the fluorescence intensity values were proportional to the concentrations of substrates in the ranges tested. It substantiated that the changes in the fluorescence intensity could be regarded as a criteria in evaluating the activity of HRP for these substrates.

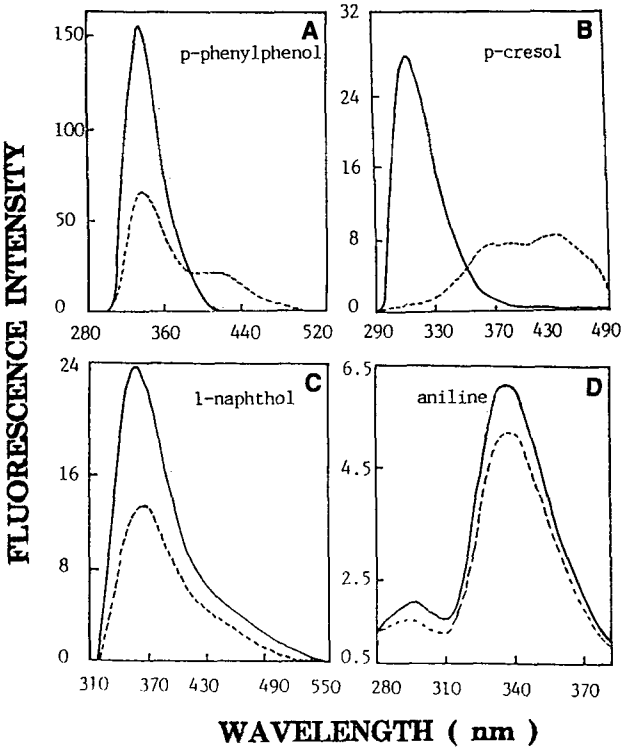

Fig. 3. Fluorescence spectra of substrates before (——) and after (-----) HRP catalyzing. The excitation wavelengths used were (**A**) 279 nm, (**B**) 280 nm, (**C**) 300 nm, and (**D**) 270 nm. The concentrations of substrates, HRP, and H202 were 40, 40, and 40 μmol/L, respectively. (For details of the experiment, *see text*).

Fluorometric Assays

The activity of HRP in the mixture of 70% dioxane and 30% phosphate buffer was measured by monitoring the decrease in the fluorescence intensity of substrate at its maximal excitation and emission wavelengths. The time-course of the fluorescence intensity change for HRP of various concentrations was recorded for 3 min (Fig. 5). It can be observed that the time-course curves show good linearity within 3 min. The relative reaction rate (represented by the fluorescence quenching rate) for HRP of each concentration was calculated from the slope of the time-course curves. The relationship between the relative reaction rates and concentrations of HRP, an evaluating criteria for this assay, is shown in Fig. 6. It can be observed that the relative reaction rate increases linearly with the concentration of HRP. This result confirms that this method is reliable in assaying the activity of HRP for these substrates in the mixture of dioxane and water.

This assay was also tested in the mixture of 70% acetone–30% water and in 70% alcohol–30% water with 1-naphthol and *p*-phenylphenol as substrate, respectively. The time-course curves of fluorescence intensity change and the relationship between the relative reaction rates and con-

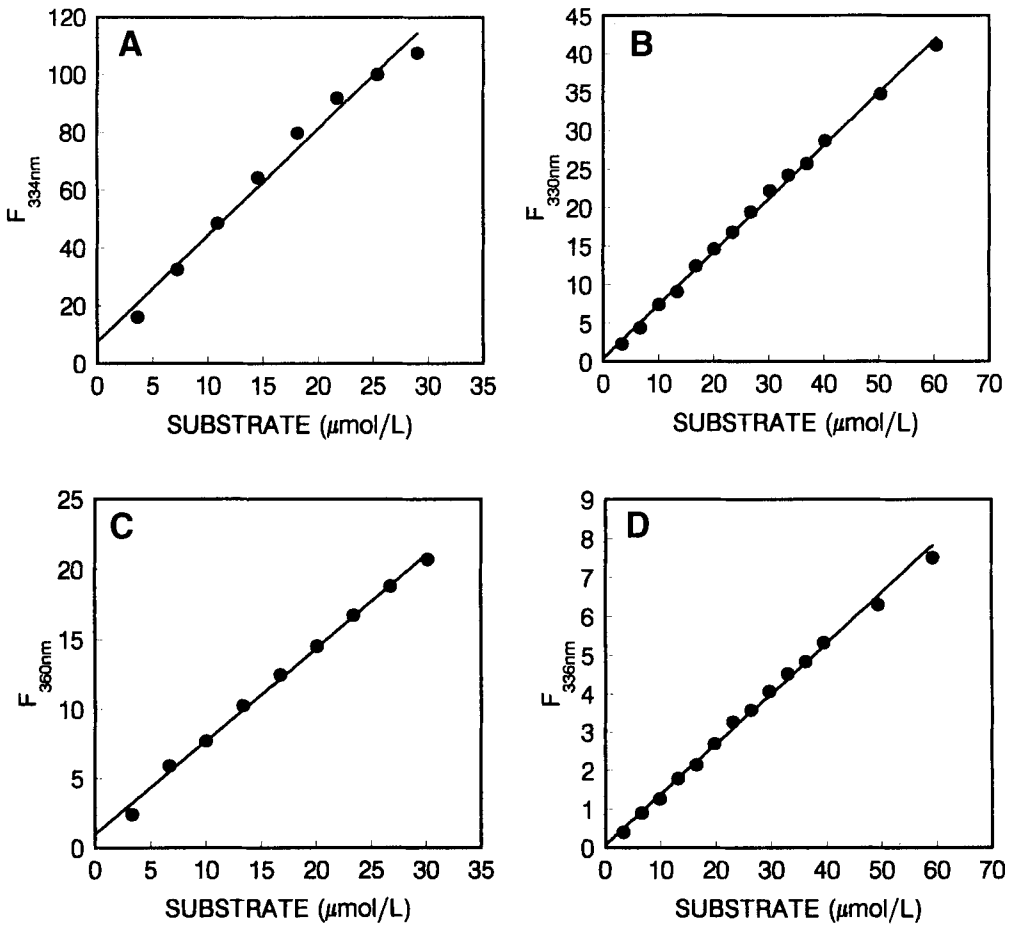

Fig. 4. Plots of the fluorescence intensities as a function of concentrations of substrates in the mixture of 70% dioxane and 30% 0.01 mol/L phosphate buffer (pH 6.0). The wavelengths of excitation and emission are the following: (**A**) *p*-phenylphenol, Ex 279 nm, Em 334 nm, (**B**) *p*-creasol, EX 280 nm, Em 330 nm, (**C**) 1-naphthol, Ex 300 nm, Em 360 nm, (**D**) aniline, Ex 270 nm, Em 336 nm. The linear regression equations calculated for lines are (**A**) $Y = 3.5106X + 11.95$, (**B**) $Y = 0.6899X + 0.3876$, (**C**) $Y = 0.6690X + 0.9600$, and (**D**) $Y = 0.1213X + 0.2582$.

centrations of HRP are shown in Figs. 7 and 8. It can be observed that the obtained results were similar to those in the dioxane–water mixture. These results demonstrate that this method can also work efficiently in these organic solvents. It implies that this method can be extended to apply in other water-miscible organic solvents.

Points for Attention

In the application of the fluorometric method, attention should be given to a number of considerations.

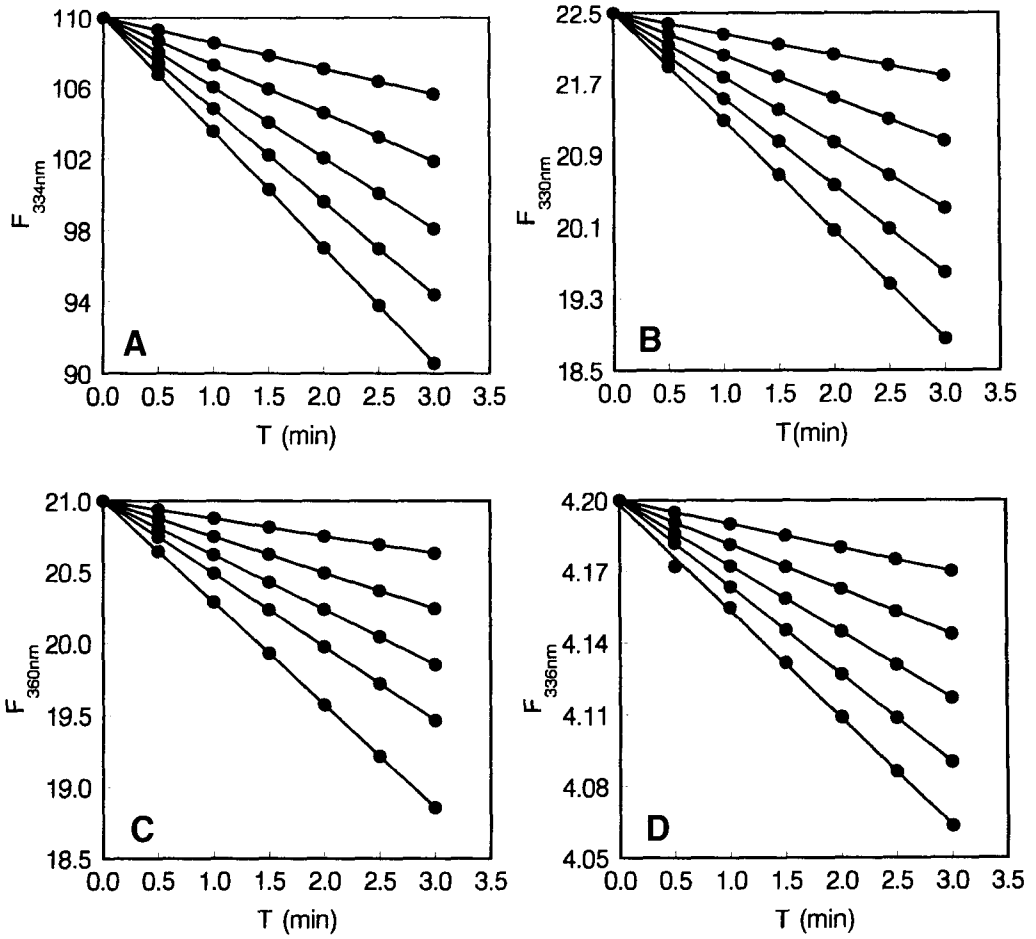

Fig. 5. The time-course of the fluorescence intensity changes of substrates in dioxane solvent for various concentrations of HRP. All measurements were carried out at 25°C with 30 μmol/L substrates in the mixture of 70% dioxane and 30% 0.01 mol/L phosphate buffer (pH 6.0). The wavelengths of excitation and emission used were consist with that described in Fig. 4. The concentrations of HRP (μmol/L) for the curves (from high to low) in the figure are the following: (**A**) *p*-phenylphenol, 0.016, 0.032, 0.048, 0.064, 0.08; (**B**) *p*-presol, 0.16, 0.32, 0.48, 0.64, 0.80; (**C**) 1-naphtol, 1.8, 3.6, 5.4, 7.2, 9.0; and (**D**) aniline, 1.8, 3.6, 5.4, 7.2, 9.0.

1. The used concentration of substrate should not exceed the linear range shown in the standard curve (Fig. 4); otherwise, the accuracy of this assay might be affected;
2. The concentration of H_2O_2 should not be added over 0.02 mol/L in the reaction system to avoid the inhibition of excessive H_2O_2 (5);
3. In order to obtain a reproducible constant rate of fluorescence quenching, the reaction solution should be mixed thoroughly; and

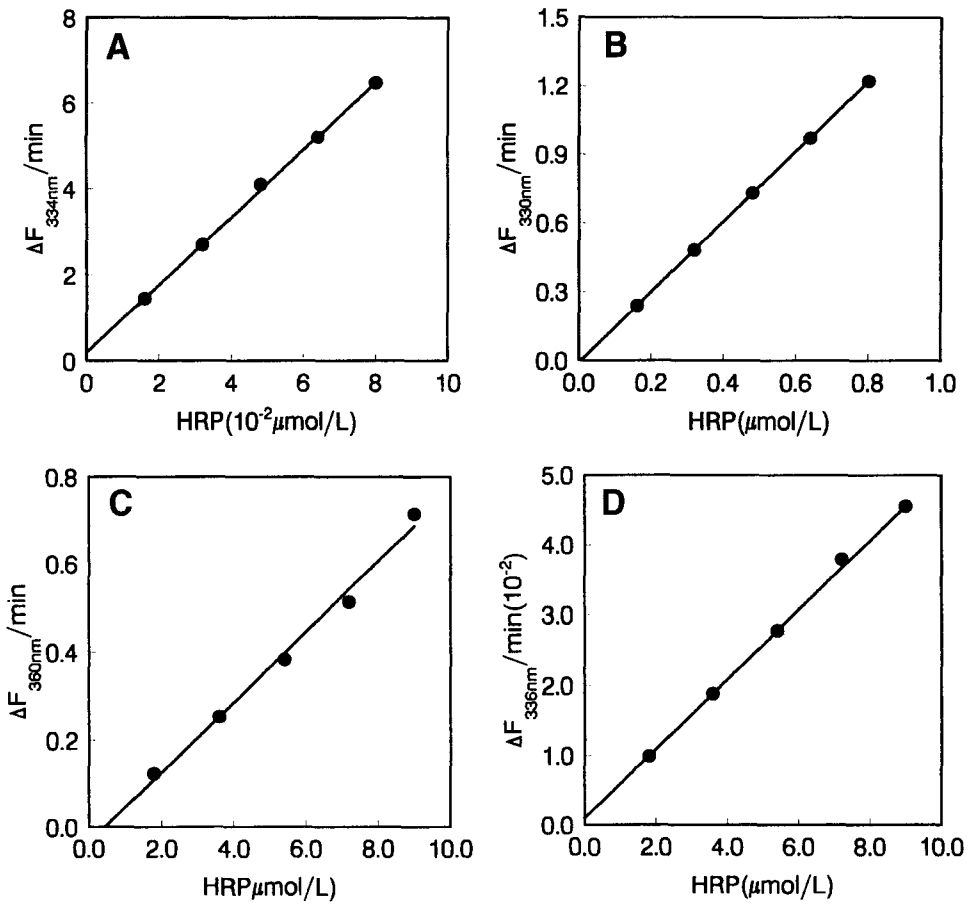

Fig. 6. Relationship between the relative reaction rates and the concentrations of HRP in 70% dioxane-30% water mixture. The linear regression equations calculated for the lines are (**A**) *p*-phenylphenol, $Y = 7.867X + 0.193$, (**B**) *p*-cresol, $Y = 0.152X - 0.00557$, (**C**) 1-naphthol, $Y = 0.0072X - 0.006$, and (**D**) aniline, $Y = 0.0004945X + 0.001$

4. The effect of the intrinsic fluorescence of HRP on the accuracy of this assay is considered in the present work. Since the fluorescence intensity of HRP at the usual concentration cannot be compared to that of substrate, it is assumed to be negligible. If HRP of high concentration is used in special cases, the appropriate adjustments should be made to basal fluorescence generated separately by HRP so that the interference can be eliminated.

Advantages of This Assay

The method presented in this work offers the possibility of determining the activity of HRP in organic media, which cannot be successfully achieved by the methods developed in aqueous buffer. Since this method

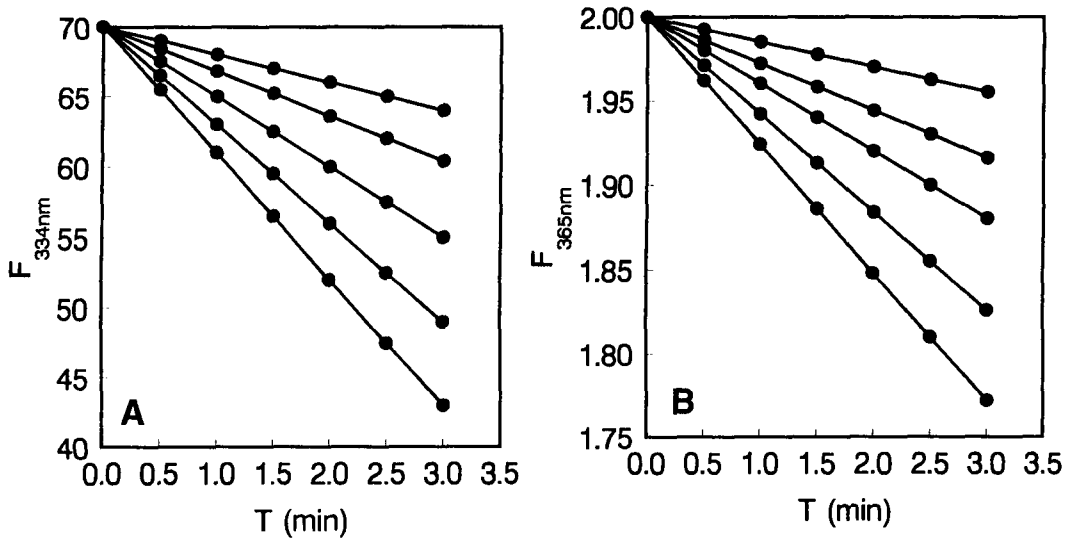

Fig. 7. The time-course of the substrate's fluorescence intensity change for various concentrations of HRP in acetone and alcohol. (**A**) In the mixture of 70% alcohol and 30% 0.01 mol/L, pH 6.0, phosphate buffer with 30 μmol/L *p*-phenylphenol as substrate; Ex 279 nm, Em 334 nm; concentrations of HRP (10^{-2} μmol/L) for the curves (from high to low) 0.48, 0.95, 1.43, 1.90, 2.38; (**B**) in the mixture of 70% acetone and 30% 0.01 mol/L, pH 6.0 phosphate buffer with 30 mol/L 1-naphthol as substrate, Ex 320 nm, Em 365 nm; concentrations of HRP (μmol/L) for the curves (from high to low) 0.57, 0.9, 1.4, 1.9, 2.4, 2.5.

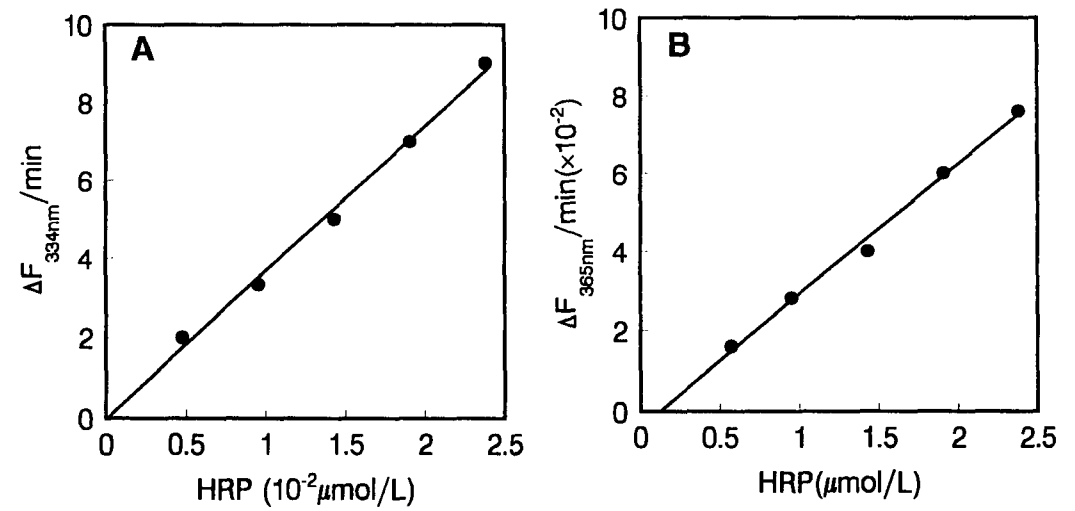

Fig. 8. Relationship between the relative reaction rates and the concentrations of HRP (**A**) In alcohol and (**B**) in acetone. The linear regression equations for the lines are (**A**) $Y = 3.709X - 0.006$, (**B**) $Y = 3.329X - 0.421$.

measures the activity of HRP by following the fluorescence quenching of substrates, the interferences of by-products can be avoided. Therefore, it will show a degree of accuracy. More importantly, this method represents the feature of versatility as working efficiently in many organic media and for a number of substrates.

This method overcomes some drawbacks of HPLC, which was a commonly used method for measuring the activity of HRP in organic media. The initial rate of HRP catalyzing can be easily determined by this method. In contrast, it will be difficult for HPLC. Particularly, it is very simple and rapid compared to HPLC, which requires complicated sample treatment and long time periods.

CONCLUSION

This fluorometric assays is a versatile method for determining the activity of HRP in organic solvents. In addition to the several model substrates and solvents used in the present work, the activity of HRP for other substrates in other organic solvents can also be assayed by this method, if the fluorescence properties of the substrates fulfill the requirement that the fluorescence emission spectra of the substrates and their resultant polymers are not overlapped, or the fluorescence intensities of the resultant polymers are so low that it can be negligible.

REFERENCES

1. Saunders, B. C., Holmes-sedle, A. G., and Stark, B. P. (1964), *Peroxidase*, Butterworth, Washington.
2. Dordick, J. S. (1992), *Biotechnol. Prog.* **8,** 256–267.
3. Dordick, J. S. (1987), *Enzyme Microb. Technol.* **11,** 194–211.
4. Klibanov, A. M. (1986), *Chemtechnology* **16,** 354–359.
5. Dordick, J. S., Marletta, M. A., and Klibanov, A. M. (1987), *Biotechnol. Bioeng.* **30,** 31–36.
6. Akkara, J. A., Senecal, K. J., and Kaplan, D. L. (1991), *J. Polym. Sci. Polym. Chem.* **29,** 1561–1574.
7. Pokora, A. R. and Cyrus, W. L. (1987), US Patent 4,647,952.
8. Rao, A. M., John, V. T., Gonzalez, R. D., Akkara, J. A., and Kaplan, D. L. (1993), *Biotechnol. Bioeng.* **41,** 531–540.
9. Blinkovski, A. M. and Dordick, J. S. (1993), *J. Polym. Sci. Polym. Chem.* **31,** 1839–1846.
10. Popp, J. L., Kirk, T. K., and Dordick, J. S. (1991), *Enzyme Microb. Technol.* **13,** 964–968.