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A Fluorometric Rate Assay of Peroxidase Using the Homovanillic Acid–*o*-Dianisidine–Hydrogen Peroxide System

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The oxidation of homovanillic acid by peroxidase in the presence of hydrogen peroxide is stimulated by various compounds. Among the compounds tested, *o*-dianisidine was found to stimulate the oxidation of homovanillic acid strongly. A rapid and highly sensitive method for the fluorometric rate assay of peroxidase using *o*-dianisidine as an oxidative stimulator in the homovanillic acid–hydrogen peroxide system was established. By this method, it is possible to determine peroxidase activity in the range of 0.5—18 mU/ml.

Keywords——homovanillic acid; *o*-dianisidine; hydrogen peroxide; peroxidase; fluorometric rate assay

Peroxidase catalyzes the oxidation of a wide variety of hydrogen-donating substrates in the presence of hydrogen peroxide. Among the many substrates, homovanillic acid is a fluorogenic substrate which is converted to highly fluorescent compound by oxidation with the peroxidase–hydrogen peroxide system, and it has been widely employed not only for the assay of peroxidase but also for the determination of hydrogen peroxide and compounds of clinical importance by means of coupling with an oxidative enzyme which serves to generate hydrogen peroxide.[1,2]

Yamazaki and Piette[3] reported that phenol, cresol, resorcinol, and aniline stimulate the oxidation of ascorbic acid and dihydroxyfumaric acid in the peroxidase–hydrogen peroxide system.

A compound that effectively stimulates the oxidation of homovanillic acid by the peroxidase–hydrogen peroxide system might be useful for developing a more sensitive and rapid assay of peroxidase. For this purpose, various compounds have been examined, and it was found that *o*-dianisidine is a strong stimulator which permits high sensitivity and rapidity in the determination of peroxidase activity.

Materials and Methods

Chemicals——Horseradish peroxidase (HRP), homovanillic acid (HVA), and *o*-dianisidine dihydrochloride (ODA) were purchased from Sigma Chemical Co., St. Louis. Orcinol was from Merck Co., Inc., West Germany. Pyrogallol, diphenylamine, and *N,N*-dimethylaniline were from Wako Pure Chemical Ltd., Japan. *p*-Dimethylaminophenol, *p*-aminodimethylaniline, and *o*-, *m*-, and *p*-toluidine were from Kanto Chemical Co., Japan. *o*-Anisidine, 4-aminoantipyrine, *o*-, *m*-, and *p*-phenylenediamine, guaiacol, 2,7-diaminofluorene, 3,4-diaminotoluene, and 1-amino-2-naphthol-4-sulfonic acid were purchased from Tokyo Kasei Co., Ltd., Japan.

Activity of HRP (about 200 purpurogelin units/mg) was standardized by means of the spectrophotometric method with guaiacol.[4] Hydrogen peroxide solution (Mitsubishi Gas Chemical, Japan) was also standardized by absorbance measurement at 240 nm.[5]

Procedure for Screening of Stimulators——Reaction mixture containing 2.5 ml of 0.1 M Tris-HCl buffer, pH 8.5, 0.1 ml of HRP (0.47 U/ml) solution, and 0.3 ml of 23 μM stimulator solution was placed in a quartz cell of the fluorometer, and incubated at 30 °C for 5 min. The reaction was started by adding 0.1 ml of 2.5 mg/ml HVA solution and 0.03 ml of 10 mM hydrogen peroxide solution. The rate of increase of fluoresence intensity (ΔF/min) was

continuously recorded for 1 to 5 min at an excitation wavelength of 315 nm and an emission wavelength of 425 nm.

Procedure for Fluorometric Rate Assay of Peroxidase——To 2.5 ml of 0.1 M Tris-HCl buffer, pH 8.5, was added 0.3 ml of 14.5 μg/ml ODA solution, 0.1 ml of 2.5 mg/ml HVA solution, followed by 0.1 ml of peroxidase (10 to 500 mU/ml), and then the mixture was incubated at 30 °C for 5 min. The reaction was started by the addition of 0.03 ml of 10 mM hydrogen peroxide solution and the change of fluorescence intensity, ΔF/ml, was recorded.

Results and Discussion

Screening of Stimulators

To examine the stimulatory effects of various compounds on the oxidation of HVA in the peroxidase–hydrogen peroxide system, each compound was added to the rate assay system for peroxidase activity described by Guilbault *et al.*[1] The ratio of molar concentration of HVA to stimulator was 200 : 1 and the rate of change of the fluorescence intensity was recorded.

As shown in Table I, the fluorescence intensity was evidently increased by the addition of diaminoderivatives in this reaction system. Among the various compounds tested, *o*-dianisidine was a very potent stimulator in this oxidative reaction, the rate of change of fluorescence intensity was 68 times higher than that of the control. The reaction product in the presence of ODA exhibited an excitation maximum wavelength of 315 nm and an emission maximum wavelength of 425 nm. These results are in close agreement with those for 2,2′-dihydroxy-3,3′-dimethoxybiphenyl-5,5′-diacetic acid, which is derived from HVA by oxidation with peroxidase in the presence of hydrogen peroxide. Therefore, it is concluded that ODA is a potent stimulator of the oxidation of HVA in the peroxidase–hydrogen peroxide system.

Optimum Conditions for the Rate Assay of Peroxidase Activity

To determine the optimum concentration of ODA, the reaction was performed with various amounts of ODA in the range of 0 to 25 μM.

As shown in Fig. 1, the increase of fluorescence intensity was proportional to the

TABLE I. Stimulatory Effects of Various Compounds on the Oxidation of Homovanillic Acid

Compounds	ΔF/min	Ratio
None	5.1	1.0
4-Aminoantipyrine	6.6	1.3
o-Anisidine	26.7	5.2
o-Toluidine	8.4	1.7
m-Toluidine	8.1	1.6
p-Toluidine	15.5	3.1
Diphenylamine	17.7	3.5
Dimethylaniline	6.0	1.2
p-Aminodimethylaniline	25.7	5.1
o-Phenylenediamine	157.8	32.1
m-Phenylenediamine	11.0	2.2
p-Phenylenediamine	21.3	4.2
3,4-Diaminotoluene	156.3	30.6
2,7-Diaminofluorene	132.1	25.9
o-Dianisidine (ODA)	248.8	68.4
3-Amino-2-naphthol-4-sulfonic acid	5.3	1.0
p-Methylaminophenol	22.4	4.4
Guaiacol	8.3	1.6
Orcinol	5.4	1.1
Pyrogallol	0	0

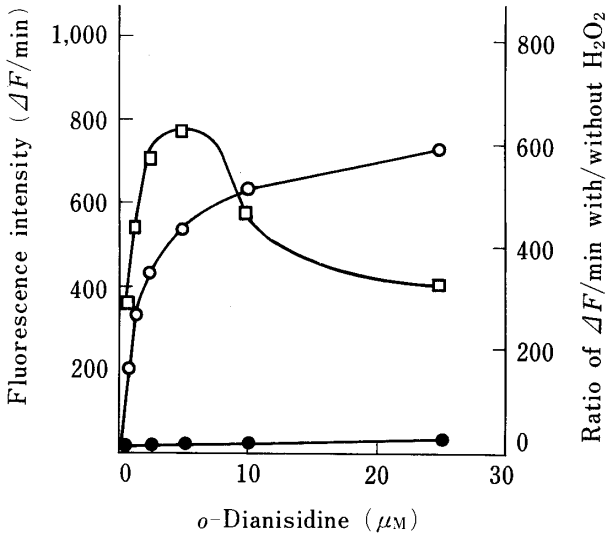

Fig. 1. Effect of *o*-Dianisidine Concentration on the Oxidation of Homovanillic Acid with Peroxidase and Hydrogen Peroxide

To 2.5 ml of 0.1 M Tris-HCl buffer, pH 8.5, was added 0.1 ml of 2.5 mg/ml of HVA and 0.1 ml of 0.47 U/ml HRP, and then 0.3 ml of various concentrations of ODA was mixed in. After the addition of 0.03 ml of 10 mM H_2O_2 (○) or H_2O (●) to the mixture, the rate of increase of fluorescence intensity was measured. (□); ratio of ΔF/min in the presence (○) and absence (●) of H_2O_2.

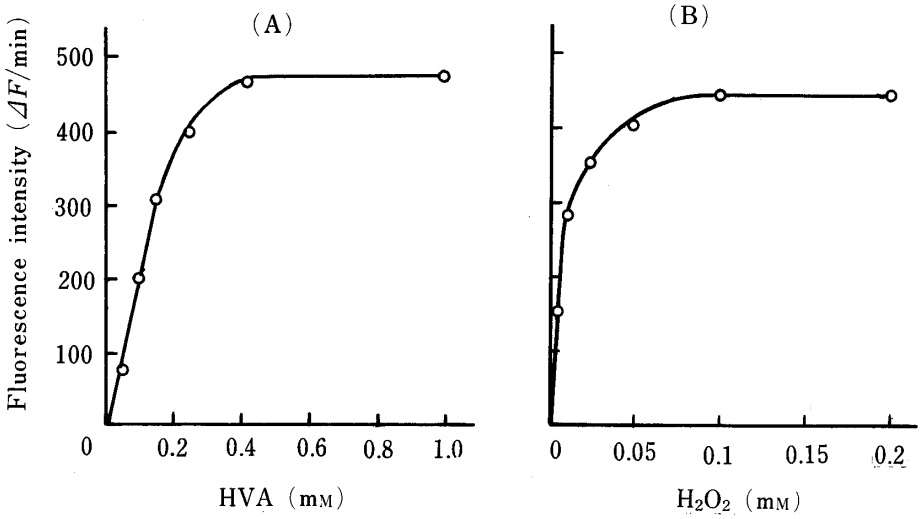

Fig. 2. Effects of Homovanillic Acid (A) and Hydrogen Peroxide (B) Concentrations

The conditions were as described in Materials and Methods except for the use of various concentration of HVA (A) or H_2O_2 (B).

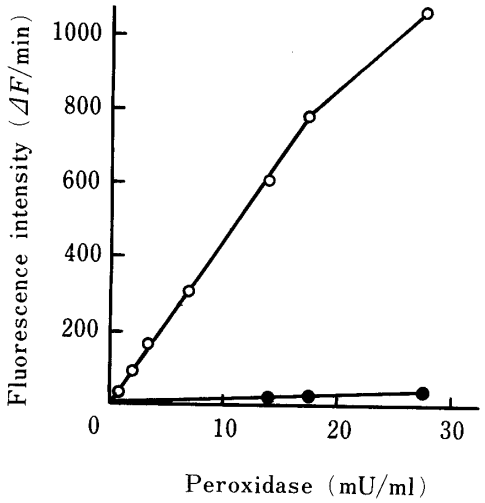

Fig. 3. Calibration Curves for Peroxidase

—○—: present method. —●—: Guilbault method.

concentration of ODA, though the background fluorescence (which seems to be produced by autoxidation of HVA) was also increased. The rate of oxidation of HVA with ODA relative to that of autoxidation was maximum at about 4.35 μM ODA. Therefore, 4.35 μM ODA (0.3 ml of 14.5 μg/ml ODA solution in 3.03 ml) was used in all determinations of peroxidase activity. The optimum concentrations of HVA and hydrogen peroxide were also examined and the results are shown in Fig. 2. From these results, 0.45 mM HVA (0.1 ml of 2.5 mg/ml HVA) and 0.1 mM hydrogen peroxide (0.03 ml of 10 mM hydrogen peroxide) were used in all experiments.

Calibration Curve for the Determination of Peroxidase Activity

A calibration curve for the determination of peroxidase activity was prepared according to the method described in Materials and Methods. As shown in Fig. 3, the plot passed through the origin and was linear over the range of 0.5 to 18.0 mU/ml of HRP. The sensitivity of the present method is about 40 times higher than that of the Guilbault method. The coefficients of variation were 1.41, 1.66, and 3.32% ($n=10$ each) for 17.2, 6.9, and 2.3 mU/ml of HRP, respectively.

Recently, Zaitsu and Ohkura[6] reported that 3-(*p*-hydroxyphenyl)propionic acid (HPPA) is a highly effective fluorogenic substrate for the sensitive, rapid, and precise assay of HRP activity. However, the sensitivity of the present method comparable to that of the HPPA method. Thus, the present method should be useful for the vary rapid and sensitive determination of peroxidase activity.

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