A Fluorometric Determination of Peroxidase Using o-Phenylenediamine as Hydrogen Donor.

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SUMMARY A sensitive fluorometric determination of peroxidase activity using o-phenylenediamine as substrate is described.

Upon oxidation with peroxidase and hydrogen peroxide, o-phenylenediamine is converted to the 2,3-diaminophenazine which has an excitation maximum at 410 nm and an emission maximum at 550 nm in neutral solution. Under the optimum conditions, peroxidase was possible to determine in the range of 0.62 to 31.23 µU/ml.

Introduction

Peroxidase (EC 1.11.1.7) can be determined by the decrease of the hydrogen peroxide or the hydrogen donor and by the increase of the oxidized compound is employed, and many different substrates have been used.

o-Phenylenediamine, one of the typical hydrogen donor, is converted to red brown compound, 2,3-diaminophenazine by oxidation with peroxidase and hydrogen peroxide. The oxidized compound of o-phenylenediamine exhibited the sharp absorption maximum at 492 nm in strong acid solution. In the previous report, the optimum conditions for colorimetric determination of peroxidase using o-phenylenediamine as substrate were examined.

On the other hand, the o-phenylenediamine oxidized compound is the highly fluorescent compound, which has an excitation maximum at 410 nm and an emission maximum at 550 nm. In this study, therefore, the fluorometric determination of peroxidase utilized the optimum conditions for colorimetric method was investigated.

Materials and Methods

Chemicals and Reagents

Horseradish peroxidase (HRP, Type IV, 300 purpurrogallin units/mg, Sigma Chemical Co., st. Louis Missouri) was dissolved in 0.1 M phosphate buffer, pH 5.0 and the activity of peroxidase was standardized by the spectrophotometric method using guaiacol. o-Phenylenediamine

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(o-PD, Sigma Chemical Co.) was further purified by sublimation method and dissolved in 0.1M phosphate buffer, pH 5.0 to 3 mM in final concentration. Hydrogen peroxide solution (10 mM) was prepared by diluting a 30% stock solution (Mitsubishi Gas Chemical, Japan) with distilled water.

Assay Method for peroxidase

The assay method is performed as colorimetric method described in the previous report except 5% Na$_2$SO$_3$ solution was used in place of 1 N HCl and the fluorescence intensity was measured. Namely, 0.25 ml of peroxidase solution was added to 1.5 ml of 3 mM o-PD solution, and then preincubated at 37°C for 5 min. The reaction was started by adding 0.25 ml of 10 mM H$_2$O$_2$. After 30 min. incubate at 37°C, the reaction was stopped by adding 1.0 ml of 5% Na$_2$SO$_3$ solution. The fluorescence intensity was measured with an excitation wavelength at 410 nm and an emission wavelength at 550 nm.

Results and Discussion

An excitation and an emission spectrum of the HRP-o-PD-Hydrogen peroxide reaction product were determined in various pH solutions. As shown in Fig. 1, an excitation and an emission maximum in fluorescence spectra were shown at the wavelength of 410 nm and 550 nm, respectively, and a high fluorescence intensity was observed in pH 8.0. From those results, 5% Na$_2$SO$_3$ solution was employed for stopping of the reaction and for achieving in pH of the solution to about 8.0.

The fluorescence intensity developed by use 5% Na$_2$SO$_3$ for stop of the reaction was completely stable at room temperature.

![Fig. 1 Excitation and emission spectra of o-PD oxidized compound in various pH solutions.](image1)

![Fig. 2 Calibration curve for peroxidase by fluorometric method.](image2)

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A linear relationship exists between the fluorescence intensity against the concentration of HRP as shown in Fig. 2. The lower limit for determination of HRP activity was 0.62 μU/ml, and the sensitivity in this fluorometric method was about 10 times higher than that of colorimetric method described in the previous report. The coefficients of variation for this fluorometric method were 3.04, 5.47, and 4.29% (n=8 each) for 6.25, 12.50, and 31.25 μU/ml of HRP, respectively.

As described in the previous report, the absorption spectra of the o-PD oxidized compound exhibited the sharp absorption maximum at 492 nm in the strong acid solution, and the absorption maximum was shifted to short wavelength in the neutral solution and remained constantly at 420 nm in the range of pH 6.0 to 8.0, though the absorbance at absorption maximum was decreased. Accordingly, the peroxidase activity was possible to determined by the measurement of absorbance at 420 nm in the same experimental conditions of fluorometric method. Figure 3 showed that the calibration curve by the proposed colorimetric method was linear from 6.2 to 125.0 μU/ml of HRP, though a low sensitive than the colorimetric method described in the previous report. The coefficients of variation for this colorimetric method were 6.62, 3.25, and 3.97% (n=5 each) for 6.25, 31.25, and 62.50 μU/ml of HRP, respectively.

The peroxidase assy method described in the present paper is especially available in both the colorimetric and fluorometric method with the reactive solution of the same experimental conditions, and permitted to determine peroxidase in the wide range of 0.62 to 125.0 μU/ml.

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