Absorptiometric Measurement of Hydrogen Peroxide in Submicromolar Amounts

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N,N′-Diphenylethylenediamine (DPED) was found to be a useful hydrogen donor for the determination of hydrogen peroxide in the presence of peroxidase. Pigments formed by this reaction had a peak absorbance at 450 nm and had relatively high absorptivity; the measurable range of hydrogen peroxide was between 0.15 and 10 μM. The intra-assay and inter-assay precisions (coefficients of variation) of hydrogen peroxide determination were below 0.99 and 1.86%, respectively. The recovery was 97.2—102.8% ($n=10$).

Keywords hydrogen peroxide; *N,N′*-diphenylethylenediamine; spectrophotometry; peroxidase

Hydrogen peroxide in submicromolar amounts has been determined by spectrophotometry using various methods, which can be divided into three groups; oxidation of metal ions,[1,2] iodide ion,[3] and organic compounds.[4-10] Generally, the sensitivities of these methods are limited to concentrations of 10^{-5} M or more of hydrogen peroxide.

In clinical chemistry, enzymes are used in many methods for determination of hydrogen peroxide, either existing or produced by pre-reactions, because of their efficiency and specificity. Chromogens such as benzidine or its derivatives[4,5,7] have also been used in these enzymatic reactions, but their application is undesirable because of their carcinogenicity.[11-14] The more sensitive methods all present some problems, such as the requirement of special equipment, or complicated or time-consuming procedures.

We report here a new colorimetric reaction applicable to the determination of hydrogen peroxide. Studies on further applications, for example, the determination of peroxidase (POX), hemoglobin (Hb), and others, are proceeding.

Experimental

Chemicals, Reagents, and Apparatus *N,N′*-Diphenylethylenediamine dihydrochloride (DPED·2HCl) (Tokyo Kasei, Tokyo, Japan), POX from horseradish (Wako Pure Chemical Industries, Osaka, Japan), and 30% hydrogen peroxide solution (Mitsubishi Gas Chemical, Tokyo) were used. All other chemicals were of reagent grade.

The DPED solution (3.5 mM) was prepared by dissolving 100 mg of DPED·2HCl in 100 ml of water. This solution was prepared on the day of use. A standard solution of hydrogen peroxide for use in obtaining a calibration curve was prepared by diluting a 30% hydrogen peroxide aqueous solution, assayed by the potassium permanganate method, with water to give hydrogen peroxide concentrations of up to 10 μM.

Visible absorption spectra and absorbance were measured with a UV-240 spectrophotometer (Shimadzu, Kyoto, Japan) in 10 × 10 mm cells.

Assay Procedure To 3 ml of DPED solution in an amber test tube, 0.1 ml of POX solution (25 IU/ml) and 1.0 ml of the test or hydrogen peroxide standard solution were added successively. The mixture was incubated at 25°C for 15 min. The absorbance of the test or standard solution was measured at a wavelength of 450 nm, using water as a reference.

Results

Effect of Reagent Concentrations on the Extent of Color Reaction In the presence of hydrogen peroxide and POX, DPED is colored in aqueous solution (Fig. 1). Under the proposed conditions, several solvents for DPED, such as 0—100% acetic acid with or without 14% dimethylsulfoxide, were examined. Water was selected, because this coloring reaction gave a stable color with no precipitation.

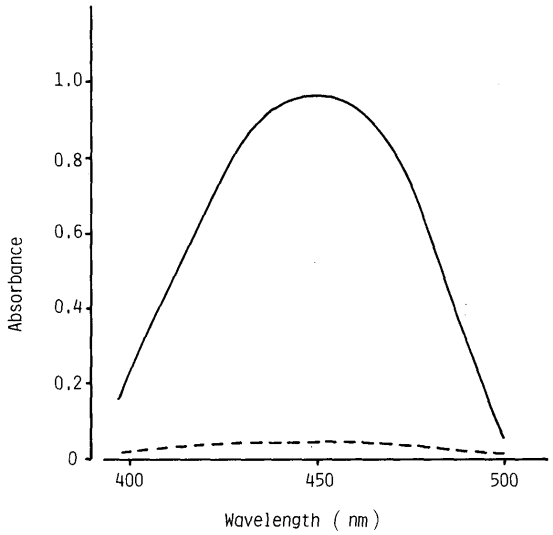

Fig. 1. Visible Absorption Spectra of Colored Products under the Proposed Conditions

Hydrogen peroxide concentrations in samples were 0 (- - - -) and 8.0 (——) μM.

The DPED concentration of 3.5 mM in this solution gave almost maximum absorbance with hydrogen peroxide between 10.5 μM and 0.7 M. The optimal activity of POX was 25 IU/ml for hydrogen peroxide analysis (it was tested in the range of 0.25 to 250 IU/ml).

Effect of Reaction Time The color reaction of DPED was studied at 25°C, because this temperature was considered to be suitable for routine use, and the reaction proceeded easily. Under our conditions, the color developed within 15 min, and the absorbance of the colored solution was stable for at least 45 min (Fig. 2).

Accuracy and Precision A linear calibration curve was obtained between absorbance and hydrogen peroxide concentration in the range of 0.15—10 μM.

The accuracy was tested by adding hydrogen peroxide to water to give concentrations of up to 10 μM. The recovery was 97.2—102.8% ($n=10$).

Intra- and inter-assay precisions were evaluated in several test tubes at each concentration, and the results are shown in Table I.

Discussion

The chromogen, DPED, was employed to quantitate

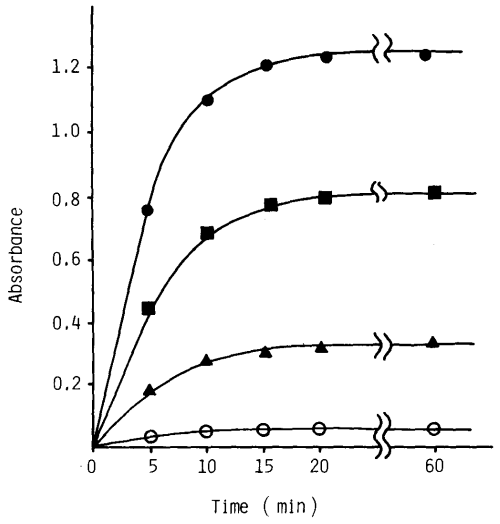

Fig. 2. Time Course of Coloring Reaction for Hydrogen Peroxide Analysis

Hydrogen peroxide concentrations in samples: 0 (○), 2.7 (▲), 6.4 (■), and 9.1 (●) μM.

TABLE I. Reproducibility and Precision of the Proposed Method for the Determination of Hydrogen Peroxide

	Hydrogen peroxide (μM)			C.V.[b] (%)
	Added	Measured		
		Mean	S.D.[a]	
Within-assay ($n=10$)	2.28	2.19	0.01	0.36
	5.02	4.92	0.02	0.41
	7.76	7.89	0.07	0.99
Between-assay ($n=10$)	5.02	5.18	0.09	1.86

a) Standard deviation. b) Coefficient of variation.

hydrogen peroxide in this method. The structure of this pigment, produced under the proposed conditions, is presumed to be the dimer of DPED formed by oxidative dimerization with hydrogen peroxide and POX with loss of 2 mol of water, because a similar reaction was reported with *o*-phenylenediamine.[15,16]

Generally, the sensitivity of most colorimetric methods is limited to more than 10 μM hydrogen peroxide, and for most luminescence methods it is less than 1 nM. The present colorimetric method for hydrogen peroxide analysis is very effective, because the molar absorptivity coefficient of the pigment at 450 nm under these conditions was 1.2×10^5 and the measurable range was 0.15—10 μM. This method is more sensitive than other submicro methods such as the leuco crystal violet (LCV)–POX method,[8] the LCV–Hb method,[10] and the 2,2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)–Hb method,[9] which can detect 10, 0.1, and 2 mM, respectively.

In the method presented, the measuring wavelength is 450 nm, so this method is more liable to interference from components in samples than the methods involving longer wavelengths. However, a high degree of accuracy is indicated by the good reproducibility. For example, the LCV–POX, the LCV–Hb, and the ABTS–Hb methods gave coefficients of variation (C. Vs.) of less than 2.0, 2.75, and 2.49%, respectively, whereas that of the present method was less than 1.86%. Good recoveries (97.2—102.8%) were obtained in the proposed method.

Therefore, the method presented is expected to be useful for determination of submicromolar amounts of hydrogen peroxide in various fields.

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