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A Spectrophotometric Method for the Determination of Glucose with Glucose Oxidase [E.C. 1.11.1.7] Using Titanium (IV)-4-(2'-pyridylazo)Resorcinol Reagent

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ABSTRAK

Satu campuran Ti(IV) dan 4-(2-piridilazo)resorsinol (Ti-PAR) didapati berguna untuk mengesan hidrogen peroksida dalam amaun yang sedikit secara spektrofotometri. Kompleks yang terbentuk menyerap pada 508 nm dan keserapan tersebut berkadar dengan kepekatan H_2O_2 yang ditambah. Reagen Ti-PAR ini telah digunakan di dalam pengasaian glukosa melalui pengkupelannya dengan enzim glukosa oksidase yang menghasilkan H_2O_2. Kaedah penentuan glukosa begini adalah cepat dan mudah dan tidak dipengaruh oleh bahan-bahan terturun.

ABSTRACT

A mixture of Ti(IV) and 4-(2-pyridylazo)resorcinol was found to be useful in the spectrophotometric determination of trace amounts of hydrogen peroxide. The absorbance of the complex formed at 508 nm was proportional to the concentration of hydrogen peroxide added. The reagent was applied to the assay of glucose through coupling with glucose oxidase which produces H_2O_2. This method of glucose determination was rapid, convenient and showed minimal interference from reducible substances.

Keywords: spectrophotometric determination, hydrogen peroxide, Ti-PAR reagent, glucose oxidase

INTRODUCTION

Matsubara and Takamura (1980) showed that a mixture of Ti(IV) and 4-(2'-pyridylazo)resorcinol (PAR) (denoted as Ti-PAR) can be used for the determination of trace amounts of hydrogen peroxide (H_2O_2). The usual spectrophotometric procedure for the determination of generated H_2O_2 (Claiborne and Fridovich 1979; Josephy *et al.* 1982) uses a chromogen in the presence of a peroxidase to yield a chromophore as shown below.

$$H_2O_2 + \text{Chromogen} \xrightarrow[\text{Peroxidase}]{} \text{Chromophore} + H_2O$$

Some foreign substances, however, can interfere with this determination. For example, serum which contains reducible substances such as bilirubin, ascorbic acid, uric acid, coenzyme-A and drug metabolites may influence the reaction either by competing with the chromogen for H_2O_2 or by reducing the chromophore. Analysis of H_2O_2 using Ti-PAR reagent, however, was not affected by the presence of reducible substances and its use in the colorimetric determination of trace amounts of H_2O_2 in biological fluids requires no peroxidase coupling step.

The present paper is concerned with the application of Ti-PAR reagent in the assay of H_2O_2 produced from the reaction of glucose oxidase on glucose.

MATERIALS AND METHODS

Materials

Ti(IV), PAR and 3,5,3',5'-tetramethylbenzidine (TMB) were obtained from Sigma Chemical Co. Glucose oxidase (GOD) and horseradish peroxidase were also purchased from Sigma. Hydrogen peroxide (30%) was obtained from Fisher Scientific. All chemicals used were of analytical reagent grade and were used without further purification.

The solutions of Ti(IV), 4-(2-pyridylazo)resorcinol (PAR) and Ti-PAR reagents were prepared as follows: The solution of Ti (IV) was prepared by dissolving titanium tetrachloride (24 ml) in 4M HCl (500 ml), and was standardized by titration with ethylenediaminetetra acetic acid (EDTA), followed by dilution with water to give 1 mM stock titanium (IV) solution. The solution of PAR was prepared by dissolving 22 mg of PAR in 5 ml of 0.2M sodium hydroxide and diluting to 100 ml with water to give a stock PAR solution (1 mM). The Ti-PAR reagent was prepared by mixing 40 ml of the stock Ti(IV) solution with 40 ml of the stock PAR solution. This solution was diluted with water to 100 ml.

A stock solution of TMB (1 mg/ml) was prepared by dissolving the compound in 1 M sodium acetate buffer, pH 4.5. A solution of H_2O_2 (0.1M) was prepared as follows: 30% H_2O_2 (5.5 ml) was diluted with water to 500 ml and standardized by titration with potassium permanganate.

Hydrogen Peroxide Determination

The oxidation of TMB by H_2O_2 through a peroxidase-coupled system, measured at 660 nm was used to quantify the presence of H_2O_2 (Josephy *et al.* 1982). Determination using Ti-PAR reagent was according to that described by Matsubara *et al.* (1985).

Determination of Glucose Oxidase Activity

The glucose oxidase activity was determined by two different methods and the results were compared. In the first method the reaction velocity was determined by an increase in absorbance at 660 nm resulting from the oxidation of TMB through a peroxidase-coupled system as described by Josephy *et al.*

(1982). One unit of enzyme activity (U) was defined as the amount of GOD which will oxidize 1.0 μmole of β-D-glucose to D-gluconic acid and H_2O_2 per min under the conditions used.

In the second method the H_2O_2 generated from the reaction of glucose oxidase and glucose was analysed using the Ti-PAR reagent. The effects of pH, temperature, reaction time, concentration of glucose oxidase and glucose used were studied in preliminary experiments in order to optimize the reaction conditions for the determination of H_2O_2 using Ti-PAR reagent. The following general conditions were used : To a mixture of 0.5 ml glucose solution (0.1mM in Tris buffer pH 7.0, 100 mM) and 0.4 ml Ti-PAR reagent previously equilibrated at 37°C was added 5 - 10 μl glucose oxidase (100 U/ml). The mixture was incubated for 15 min, cooled to room temperature and the absorbance measured at 508 nm. For the control assay, a solution containing the same components as above was prepared, except that heat-denatured GOD was added. All the measurements were made with reference to a reagent blank.

RESULTS

A comparison of the H_2O_2 determinations using Ti-PAR reagent with a peroxidase-coupled system is given in *Fig. 1.* The sensitivity of the determinations is comparable.

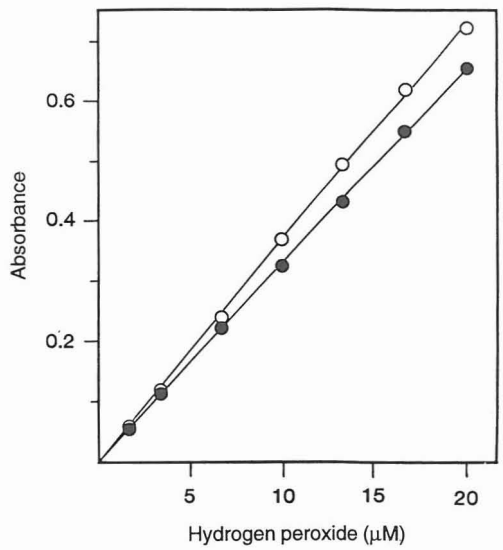

Fig. 1. Standard curve for H_2O_2 under standard assay conditions determined using the Ti-PAR system (absorbance at 508 nm, -●-) and the oxidation of TMB through a peroxidase-coupled system (absorbance at 660 nm, -○-). Each point is the mean of three determinations

In order to select the best conditions for the combined use of the Ti-PAR reagent and glucose oxidase in the determination of H_2O_2 and glucose, a careful consideration of the incubation time, pH and enzyme concentration

was made. The optimal pH for the glucose oxidase catalysed reaction was reported to be about 6.0 (Bright and Appleby 1969) while the optimal colour formation of the Ti-PAR reagent was between pH 7.6 and 12.0 (Matsubara *et al.* 1985). The effect of different levels of pH on the absorbance was thus investigated by examining the enzymatic and colour formation processes. The absorbance response was found to be fairly constant in the pH range of 6-7.5 (*Fig. 2*). Thus, to compromise on the optimal enzyme reaction and colour formation, the pH of the solution was maintained at 7.0 throughout all enzymatic and chromogenic reactions.

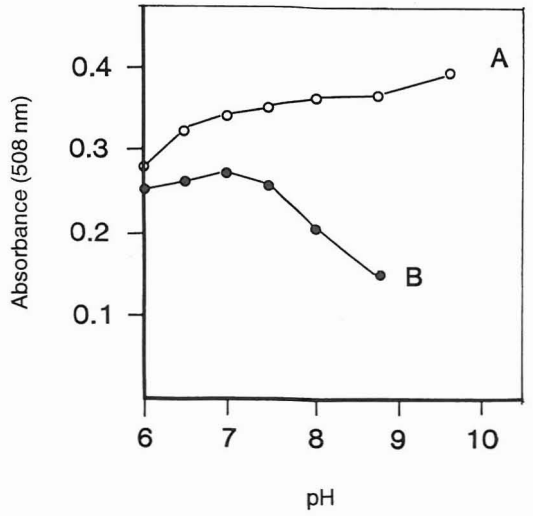

Fig. 2. Effects of pH on the absorbance of the Ti-PAR-H_2O_2 system (A) and the Ti-PAR reagent combined with glucose oxidase system (B) under standard assay conditions

In order to determine the appropriate glucose oxidase concentration, the effect of enzyme activity on the absorbance was examined. Relatively constant absorbance values were obtained for GOD activity values above 0.5 U after incubation for 15 min at 37°C with glucose (*Fig. 3*).

A plot of absorbance against glucose concentration in a GOD-coupled Ti-PAR system (GOD = 0.5 U/ml solution) gave a linear relationship (*Fig. 4*). The method may thus be applicable for the determination of glucose, especially in the lower concentration range of up to about 20 μM.

The effects of certain foreign substances, such as the anticoagulant EDTA and ascorbic acid, added to the test solution were also examined. Addition of ascorbic acid (10 mg/l) and EDTA (200 mg/l) (results not shown) had no significant effects on the glucose assay (*Fig. 4A*). On the other hand, the presence of ascorbic acid in the peroxidase-TMB system significantly reduced the absorbance (*Fig. 4B*).

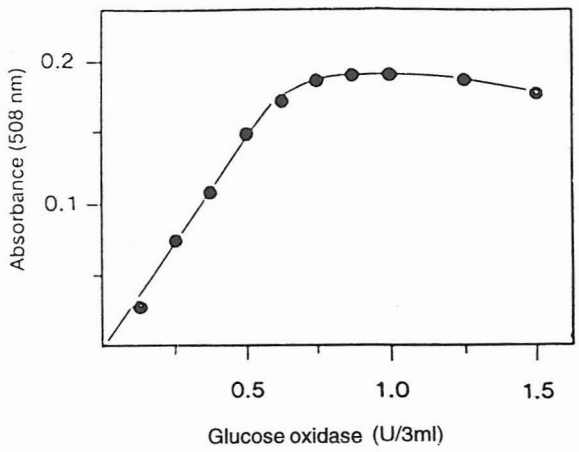

Fig. 3. Effects of glucose oxidase concentration on the absorbance under standard assay conditions. Each point is the mean of two determinations

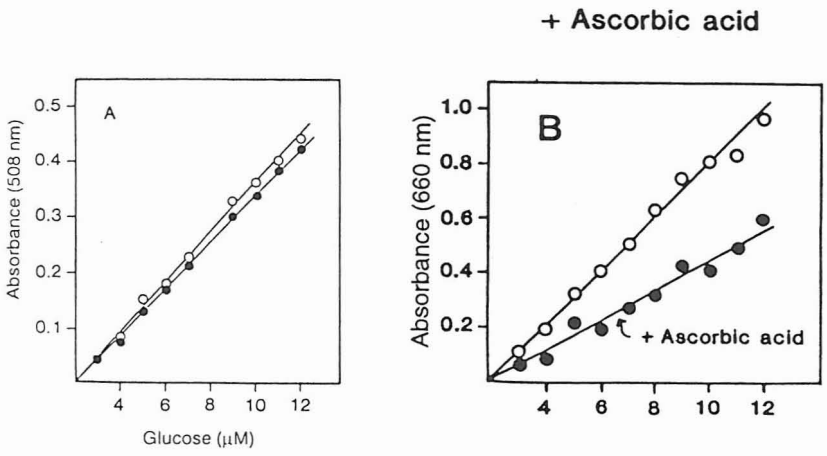

Fig. 4. Standard curve for glucose under standard assay conditions using Ti-PAR reagent (A) and peroxidase-coupled system (B) in the presence and absence of ascorbic acid. Each point is the mean of three determinations

In addition, the GOD-coupled Ti-PAR method was also applied to the measurement of glucose in solutions which were spiked with known amounts of glucose. The determinations were compared with the peroxidase-coupled method (*Fig. 5*). A good correlation was observed for the parameters under analysis (r=0.878).

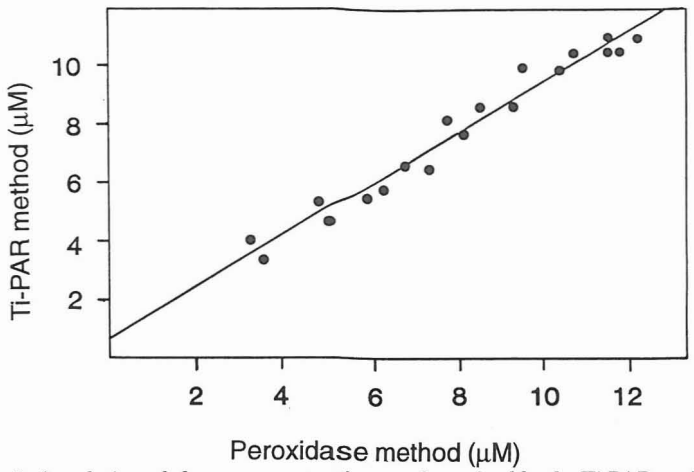

Fig. 5. Correlation of glucose concentrations as determined by the Ti-PAR method with that determined by the peroxidase-coupled method

DISCUSSION

It was previously reported that the Ti-PAR reagent could be used to detect trace amounts of hydrogen peroxide (Matsubara *et al.* 1983). Thus Ti-PAR reagent was used in this experiment for the determination of free hydrogen peroxide by glucose oxidase reaction. The method was rapid, convenient and reproducible compared to using a chromogen in the presence of peroxidase.

As reported previously (Matsubara and Takamura 1980) and confirmed in the present work, the absorbance spectrum of the Ti-PAR-H_2O_2 complex shows a peak in the pH range between 7.6 and 12.0. The absorbance at 508 nm was proportional to the concentration of added H_2O_2, ranging from 0.5 to 30 μM (*Fig. 1*) with a molar absorption coefficient (ε) of about 3.6×10^4 M^{-1} cm^{-1}. Constant absorption values were obtained within a few minutes following the addition of hydrogen peroxide to the Ti-PAR reagent and remained unchanged over 24 h at room temperature. Interference which was caused by the presence of excess Ti-PAR can be minimized by incubation at 37°C for a few minutes.

The results obtained in this research show that the Ti-PAR reagent can be used to assay free hydrogen peroxide and glucose and has an advantage over other methods. It does not require the use of peroxidase in the determination. The presence of peroxidase is known to prompt the oxidation of chromogens and other reducible substances. Thus, the Ti-PAR method is not affected by the presence of reducible substances, such as ascorbic acid. The sensitivity of the Ti-PAR reagent is also quite high. These findings and those reported by Matsubara *et al.* (1983) strongly support the use of this reagent for the colorimetric determination of trace amounts of hydrogen peroxide in biological fluid.

The Ti-PAR reagent, thus has been successfully used to determine trace biological substances in serum using appropriate enzyme combinations to produce H_2O_2 through enzymatic oxidation. Matsubara and Takamura (1980)

used the Ti-PAR reagent in combination with glucose oxidase, uricase and cholesterol oxidase for the determination of glucose, urate and cholesterol, respectively, in human serum. Similarly, Matsubara *et al.* (1983) used a combination of acyl-CoA synthase and acyl-CoA oxidase to assay for free fatty acid in serum.

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