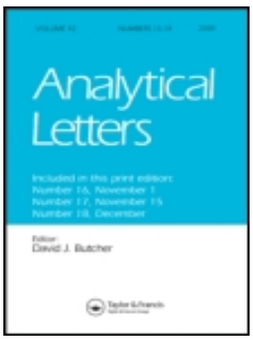

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# Study on Fluorometric Determination of Hydrogen Peroxide Catalyzed by Iron(III)-Tetrasulfonato-Phthalocyanine with Thiamine Hydrochloride as a Substrate

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# STUDY ON FLUOROMETRIC DETERMINATION OF HYDROGEN PEROXIDE CATALYZED BY IRON(III)-TETRASULFONATO-PHTHALOCYANINE WITH THIAMINE HYDROCHLORIDE AS A SUBSTRATE

**Key Words:** Mimetic enzyme, Spectrofluorimetric determination, Hydrogen peroxide, Glucose, Thiamine, Iron-tetrasulfonatophthalocyanine

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## ABSTRACT

Iron(III)-tetrasulfonatophthalocyanine(FeTSPc) has been used as a mimetic enzyme in the determination of hydrogen peroxide with thiamine hydrochloride as a fluorogenic substrate. The determinations were carried out in both acidic and basic environments, with different limits of detection and linear ranges. In

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acidic condition, the linear calibration graph was obtained from $5.0x10^{-8}$ mol/L to $8.0x10^{-6}$ mol/L, with a detection limit of $2.1x10^{-8}$ mol/L $H_2O_2$ when $Na_2HPO_4$-citric buffer solution (pH 2.8) was used as the reaction medium. It was also found that using one of the three polybasic carboxylic acids such as citric acid, tartaric acid and malonic acid as the catalytic reaction medium can lead to particularly sensitive systems, permitting a detection limit as low as $3.5x10^{-9}$ mol/L $H_2O_2$; whereas in basic reaction medium ($Na_2CO_3$-$NaHCO_3$ buffer solution, pH = 10.0), the linear range of the calibration graph was from $5.0x10^{-8}$ mol/L to $2.0x10^{-6}$ mol/L $H_2O_2$ with a detection limit of $1.4x10^{-8}$mol/L. The applicability of the method to the determination of glucose in human serum was demonstrated by investigating the recovery of the known glucose added to human serum.

## INTRODUCTION

Trace determination of hydrogen peroxide is of considerable importance in environmental and clinical chemistry. Up to now, peroxidase-catalyzed reactions between hydrogen peroxide and hydrogen-donating substances have been utilized in biochemical analysis. Enzymatic determinations of $H_2O_2$ have drawn increasing interests due to their unusual sensitivity, selectivity and simplicity. However, natural enzymes have their potential deficiencies of unstability and high cost. Hence, the study of enzyme-mimicking compounds is indispensable for enzyme chemistry. In recent years, application of peroxidase-mimetics in analytical chemistry was often dominated by metal porphyrin compounds in the catalytic oxidation of chromogenic, chemiluminogenic and fluorogenic substrates[1]. Results showed that they were quite efficient compared with natural HRP[2] and might find some application in immunoassays[3,4] and DNA hybridisation assays[5]. Metal phthalocyanine compounds, as the structural analogue to metal porphyrins, have similar catalytic activities and were proposed

as a set of new sensitive catalysts in the reaction between luminol and $H_2O_2$[6]. Metal phthalocyanine compounds are cheap and more stable than HRP. Furthermore, FeTSPc (see Fig.1) contains four available sulfonic groups that may be used in immobilized enzyme reactor or in a flow injection procedure as a label, like HRP. As far as we know, they haven't been used in fluorogenic reactions yet. In our work, thiamine hydrochloride was chosen for FeTSPc as an inexpensive fluorogenic substrate. Reports showed that the oxidations of thiamine to fluorescent thiochrome were often carried out in alkaline media[7,8,9,10]. However, it was found that peroxidase-like FeTSPc could catalyze the oxidation of thiamine to fluorescent thiochrome in both acidic and basic conditions. Thus, trace amounts of hydrogen peroxide and glucose can be determined by using FeTSPc as a catalyst for thiamine oxidation in both acidic and basic conditions. The applicability of the catalytic reaction in acidic condition was adopted to the determination of glucose in human serum. Satisfactory results were obtained.

## EXPERIMENTAL

### Apparatus

The fluorescence spectra and relative fluorescence intensity were measured with a Hitachi 650-10S fluorescence spectrophotometer with a 10 mm silica cell.

### Reagents

All the chemicals used were of analytical grade commercially available and all aqueous solutions were prepared in twice de-ionized water.

Hydrogen peroxide solution for analysis was freshly prepared by appropriate dilution of a stock solution which was standardized with potassium permanganate.

FeTSPc was synthesized and purified according to[11]. The stock solution was made to be $1.0 \times 10^{-3}$ mol/L, it was stable in room temperature for at least six months

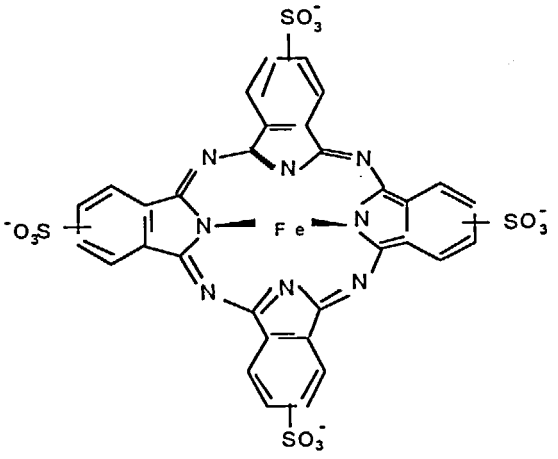


Fig.1 The molecular structure of FeTSPc

Thiamine hydrochloride was obtained from Shanghai Chemical Reagent Co. The stock solution was prepared at a 0.1mol/L concentration with water. The solution was stable for weeks when kept refrigerated at 0-4°C, working solutions of lower concentrations were prepared daily by diluting with water.

$Na_2HPO_4$-citric buffer and $Na_2CO_3$-$NaHCO_3$ buffer were used for pH 2.2 to 5.4 and pH 9.5 to 10.8, respectively.

Citric acid, tartaric acid and malonic acid solutions were all made to be 0.1mol/ L with water.

Glucose stock solution (0.1mol/L) was prepared by dissolving glucose in water. The solution was further diluted as required.

Human serum samples from healthy donors were obtained from the hospital of Xiamen University. Deproteinization of the samples was essential because the interference of protein macromolecules resulted in serious interference.

**Procedure for the determination of $H_2O_2$ in acidic medium**

To a set of 10-mL standard tubes containing 1.0mL of pH 2.8 $Na_2HPO_4$-citric buffer (or 1.0 mL 0.1mol/L of one of the three acids, citric, tartaric and malonic,

when the reaction was carried out in polybasic carboxylic acid), various volumes of $H_2O_2$ working solution, 1.0mL of 0.1 mol/L thiamine hydrochloride and 1.0 mL of $1.0x10^{-4}$ mol/L FeTSPc were added consecutively. The mixtures were diluted to the mark with water and kept from light at room temperature. The intensity of the emitted fluorescence ($\lambda$ex=395nm, $\lambda$em=455nm) was measured 10 minutes after mixing the solution.

**Procedure for the determination of $H_2O_2$ in basic buffer system**

In a set of 10-mL standard tubes, 0.50 mL of $1.0x10^{-4}$ mol/L FeTSPc, 0.20 mL of 0.01 mol/L thiamine hydrochloride, different amounts of $H_2O_2$ standard solution and 1.0 mL $Na_2CO_3$-$NaHCO_3$ buffer were added accurately and diluted to the mark. The light-protected mixtures were allowed to stand at room temperature for 5 minutes. Then the fluorescence was measured at the excitation wavelength of 370nm and emission wavelength of 440nm.

**Procedure for glucose determination and sample analysis in acidic buffer**

The catalytic reaction can easily be coupled to the determination of biochemical substances that produce $H_2O_2$ in the oxidation reaction catalyzed by oxidase. Glucose in human serum, for instance, can be determined in that way. Take appropriate amounts of glucose standard solution into a 10-mL volumetric tube, add 0.50 mL of 1U/mL of glucose oxidase solution and 1.0 mL of 0.2mol/L phosphate buffer solution (pH 6.0). Keep the mixture at room temperature for 5 minutes and cool down immediately to stop the oxidation reaction. Then, $Na_2HPO_4$-citric buffer, thiamine and FeTSPc solutions were added sequentially, and the rest of the procedure was the same as the above mentioned.

As to sample determination, the serum was pretreated with 0.6 mol/L trichloroacetic acid to eliminate the interference of protein macromolecules by means of protein precipitation.

## RESULTS AND DISCUSSION

### Reaction of thiamine with $H_2O_2$ catalyzed by FeTSPc

The oxidation of thiamine to fluorescent thiochrome(TC) was always accompanied by the simultaneous formation of non-fluorescent thiamine disulfide(TDS), a condensation product of two thiamine molecules. The ratio of TC to TDS is affected by the pH, solvent and oxidizing agent. It was said that $H_2O_2$ was one of the oxidizing agents that favoured the product of non-fluorescenct TDS, but the oxidation of thiamine by $H_2O_2$ under the catalysis of HRP at pH 8.5 led to a greater than 95% yield of TC, which has a strong fluorescence at 440nm with the excitation at 375nm[12]. Similarly, FeTSPc as a mimetic enzyme to HRP can also catalyze the oxidation of thiamine by $H_2O_2$ to TC in basic medium ($\lambda ex$=370nm,$\lambda em$=440nm) with a high yield.

Reports showed that the reactions of thiamine in basic solution had been studied in detail. The possible pathways and intermediates which had previously been identified to be important above pH 11 in aqueous solutions were listed in Fig. 2[8].

In acidic media containing FeTSPc and $H_2O_2$, the oxidation product of thiamine had a strong fluorescence at 455nm with the excitation wavelength of 395nm. It was suggested that thiamine was firstly oxidized to fluorescent product oxodihydro-thiochrome, an intermediate with a tricyclisemi-uinone form, which was then oxidized to TC by $H_2O_2$ in acidic conditions[13]. The fluorescent spectra of the final product red-shifted compared to those in basic medium. This might be due to the protonization of sulful atom in TC .

### Optimization of experimental parameters

The effects of pH, reaction time and temperature, order of mixing reagents as well as the optimum amounts of reactants were investigated in both acidic and basic conditions. The conditions were optimized by changing each parameter in turn while keeping the others unchanged.

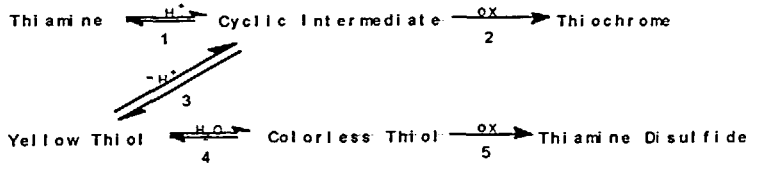


Fig. 2 Pathways for the oxidation of thiamine in basic solution

Effect of pH and buffer

The effect of pH and various buffers on the reaction system were studied. In acidic condition, three kinds of buffer systems, that is glycine - HCl, o-phthalic acid - HCl and $Na_2HPO_4$ - citric buffers, were tested from pH 2.2 to 3.6. Results showed that their background fluorescences were almost the same and the fluorescence intensities of products all reached their maximum around pH 3. As shown in Fig. 3(A), among the three buffers $Na_2HPO_4$-citric buffer solution can render the maximum fluorescence of the product at pH 2.8. Interestingly, we found that using certain polybasic carboxylic acids such as citric acid, tartaric acid or malonic acid as the reaction medium instead of the buffer solutions above-mentioned could lead to slightly lower background and remarkably higher final fluorescence, and the enhancement of fluorescence reached its maximum when 1.0 mL of 0.1mol/L of the acids was used (See Fig. 3), this might implied that the presence of polybasic carboxylic acids might favour the catalytic reaction to some extent.

When the catalytic reaction was performed in basic condition, the net fluorescence increased with increasing pH and reached its maximum in the pH range of 10.0 - 10.8. Results showed that the $Na_2CO_3$-$NaHCO_3$ buffer solution (pH 10.0) was the optimum reaction medium compared with the other two basic buffers, glycine - NaOH and $NaB_2O_4$ - NaOH.

Reaction time and temperature

In acidic media, the final fluorescence was maximal and constant after reacting for 10 minutes, while in basic cases, the final fluorescence reached its plateau in

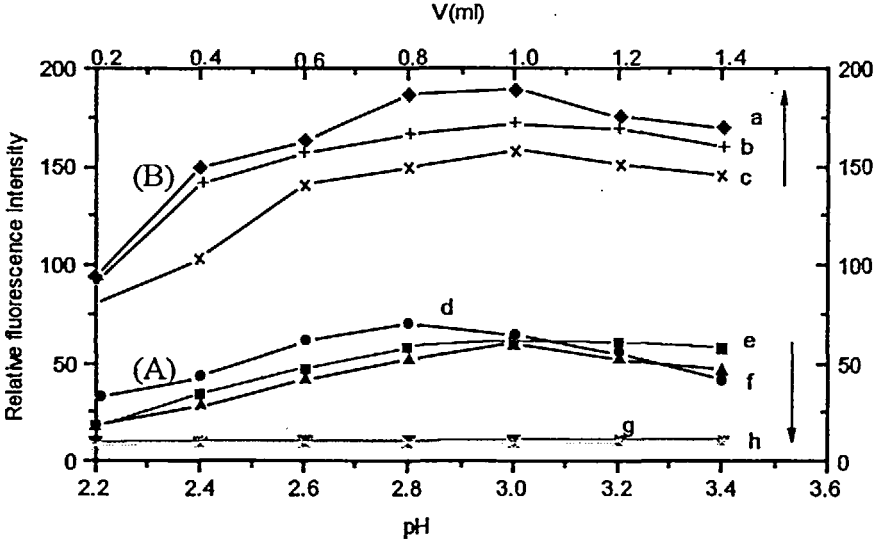


Fig.3 Effect of pH and buffer on the fluorescence intensity (A) and the dependence of fluorescence intensity on the amount of polybasic carboxylic acid(B) in the reaction of 0.01mol/L thiamine with $1.0\times10^{-6}$mol/L $H_2O_2$ in the presence of $1.0\times10^{-5}$ mol/L FeTSPc. a: in 0.1 mol/L citric acid, b: in 0.1mol/L tartaric acid, c: in 0.1 mo/L malonic acid, d: in $Na_2HPO_4$-citric buffer, e: in glycine -HCl buffer, f: in o-phthalic acid buffer, g: blank fluorescence in buffers, h: blank fluorescence in polybasic carboxylic acids.

the reaction time range of 4-8 min. So the equilibrium method was chosen to measure the fluorescence of product in both acidic and basic cases.

The catalytic reaction was also temperature-dependent. The net fluorescence intensity was maximal and constant in the range of 15-35°C. Therefore, room temperature was recommended for reaction and measurement in acidic and basic cases.

Order of adding reagents

The order of adding reagents in the thiamine system was previously reported to be a result of the reaction mechanism proposed[8]. We found that the FeTSPc –

thiamine - $H_2O_2$ system showed a similar behavior. The adding sequence of reaction medium, $H_2O_2$, thiamine and FeTSPc was critical in both acidic and basic conditions. In acidic condition, the optimal sequence of adding reagents was medium, $H_2O_2$, thiamine and FeTSPc. On the contrary, the adding sequence of FeTSPc, thiamine, $H_2O_2$ and medium was proved to be suitable in basic condition.

The optimum amounts of catalyst and substrate

In acidic media, the maximum fluorescence was reached when the final concentrations of FeTSPc and thiamine were over the range of $7.5x10^{-6}$ - $1.2x10^{-5}$ mol/L and 0.008 - 0.015 mol/L, respectively. Therefore, 1.0ml $1.0x10^{-4}$ mol/L FeTSPc and 1.0ml 0.1 mol/L thiamine were chosen.

In basic medium, the results indicated that the fluorescence was maximal and constant in the final concentration ranges of $3.0x10^{-6}$ - $7.0x10^{-6}$ mol/L for FeTSPc and $1.0x10^{-4}$ - $3.0x10^{-4}$ mol/L for thiamine. Therefore, 0.50ml of $1.0x10^{-4}$ mol/L FeTSPc and 0.20 ml of 0.01 mol/L thiamine were recommended.

It can be seen that the optimum amount of thiamine in acidic conditions was much larger than that in basic medium. This confirms the assumption that TC was practically the only product of thiamine oxidation at high initial concentration of thiamine in acidic medium[13]. So a high initial concentration of thiamine is required for the formation of TC in thiamine oxidation under acidic conditions.

**Features of the analytical method**

The calibration graphs for the determination of $H_2O_2$ obtained under different reaction media were listed in Table 1, where the detection limits of $H_2O_2$ ($3\sigma/k$) were calculated from the standard deviation of the blank and the slope of the calibration graph. It is shown in Table 1 that the graphs obtained under acidic conditions usually have a wider linear range and lower detection limit than those

**Table 1 Analytical characteristics of FeTSPc-thiamine system in different reaction media**

| Media | Linear range. (mol/L) | Detection limit(mol/L) | Correlation coefficient |
|---|---|---|---|
| $Na_2CO_3$-$NaHCO_3$ (pH 10.0) | $5.0x10^{-8}$-$2.0x10^{-6}$ | $1.4x10^{-8}$ | 0.996 |
| Citric-$Na_2HPO_4$ (pH 2.8) | $5.0x10^{-8}$-$8.0x10^{-6}$ | $2.1x10^{-8}$ | 0.999 |
| 0.1 mol/L | $1.0x10^{-6}$-$3.0x10^{-6}$ | | 0.999 |
| citric acid | $1.0x10^{-8}$-$1.0x10^{-6}$ | $3.5x10^{-9}$ | 0.994 |
| 0.1mol/L | $1.0x10^{-7}$-$3.0x10^{-6}$ | | 0.999 |
| tartaric acid | $1.0x10^{-8}$-$1.0x10^{-7}$ | $8.1x10^{-9}$ | 0.995 |
| 0.1mol/L | $2.0x10^{-7}$-$4.0x10^{-6}$ | | 0.999 |
| malonic acid | $1.0x10^{-8}$-$2.0x10^{-7}$ | $4.4x10^{-9}$ | 0.996 |

under basic conditions. The analytical characteristics for the determination of $H_2O_2$ in other sensitive catalytic systems were summarized in Table 2. Compared to those systems shown in Table 2, it can be seen that the FeTSPc-thiamine system shows the remarkable merits of having both advantages of wide linear range and low detection limit at the same time.

## Interference

Interferences from some foreign ions were studied in $Na_2HPO_4$-citric buffer system. An ion was considered not to interfere if the relative error it caused was less than 5%. Among the common ions only $Mn^{2+}$ and $Cu^{2+}$ caused serious interference. Bilirubin, a natural substance in serum, was also found to interfere with the determination. The results are shown in Table 3.

## Application

Since the determination of $H_2O_2$ in citric-$Na_2HPO_4$ buffer solution had a wider linear range and a suitable detection limit, it was applied to the determination of glucose in human serum. The inorganic ions in dilute human serum did not

**Table 2 Analytical characteristics for the determination of $H_2O_2$ with other catalysts and/or substrates**

| Catalyst | Substrate | Linear Range ($10^{-7}$ mol/L) | LOD ($10^{-7}$ mol/L) | Reference |
|---|---|---|---|---|
| HRP | Thiamine | 5.0-1000 | 1.0 | 12 |
| Hemin | Thiamine | 0.0-50 | 0.3 | 14 |
| HRP | p-HPPA | 0.04-0.8 | 0.021 | 15 |
| Hemin | p-PPA | 0.08-0.8<br>1.0-10 | 0.046 | 15 |
| Mn-TAPP | L-tyrosine | 0.0-0.32 | 0.035 | 16 |
| Mn-TPPS4 | HVA | 0.0-25 | 0.85 | 17 |
| Hematin | p-cresol | 0.25-1000 | 0.05 | 2 |

**Table 3 Tolerance of foreign substances in the determination of $5.0x10^{-7}$ mol/L $H_2O_2$**

| Substances added | Tolerance (molar ratios) |
|---|---|
| $Na^+$,$K^+$,$Cl^-$ | 10000 |
| $Al^{3+}$,$Ni^{2+}$,$Ca^{2+}$,$Mg^{2+}$,$Co^{2+}$ | 500 |
| $Pb^{2+}$ | 200 |
| $Fe^{3+}$,$Cd^{2+}$ | 100 |
| $Zn^{2+}$ | 50 |
| $Ag^+$ | 20 |
| $Mn^{2+}$,$Cu^{2+}$ | 0.5 |
| Bilirubin | 0.5 |

interfere with the determination. In addition, bilirubin in serum did not cause interference either because the glucose content far exceeded that of bilirubin in human serum. The results are summarized in Table 4. The possibility of using this method for the analysis of real samples was tested by determining the recovery of known amounts of glucose added to the sample. The precisions

**Table 4 Determination of glucose in human serum**

| Sample No. | *Content of Glucose (μg/mL) | Glucose added (μg) | Glucose** found (μg) | RSD(%) Within-day | Recovery (%) |
|---|---|---|---|---|---|
| 1 | 0.104 | 5.00 | 5.76 | 4.4 | 94.4 |
| 2 | 0.102 | 5.00 | 6.20 | 3.4 | 99.0 |

* The content of glucose in the original serum following 5000 times dilution.

** The mean of determinations (n=3)

(relative standard deviation) of the method for the sample analysis were all below 4.5% for within-day (n=7) as shown in Table 4.

## CONCLUSION

The proposed method can be used as a sensitive and inexpensive method for the determination of $H_2O_2$, or indirect determinations of glucose and other substances, in either acidic or basic condition.

## ACKNOWLEDGMENT

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