ENZYME CATALYTIC RESONANCE SCATTERING SPECTRAL DETECTION OF TRACE HYDROGEN PEROXIDE USING GUAIACOL AS SUBSTRATE

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ABSTRACT. Hydrogen peroxide oxidized guaiacol to form tetramer particles that exhibited a strong resonance scattering (RS) peak at 530 nm in the presence of horseradish peroxidase (HRP) in citric acid-Na$_2$HPO$_4$ buffer solution of pH 4.4. The RS peak increased when the concentration of hydrogen peroxide increased. The increased RS intensity ($\Delta I_{530}$) was linear to the hydrogen peroxide concentration in the range of 0.55-27.6 µM, with a linear regression equation of $\Delta I_{530} = 17.1C + 1.6$, a relative coefficient of 0.9996 and a detection limit of 0.03 µM H$_2$O$_2$. This proposed method was applied to detect hydrogen peroxide in rain water, with sensitivity, selectivity, rapidity, and recovery of 98.0-104 %.

KEY WORDS: HRP, H$_2$O$_2$, Guaiacol, Resonance scattering spectral method

INTRODUCTION

Hydrogen peroxide is an important strong oxidant, sterilant, and disinfectant, and it is a main composition of acid rain [1]. After hydrogen peroxide disinfecting or acid rain, there are undecomposed hydrogen peroxide that can threaten to people’s health. So, it is essential to determination of trace hydrogen peroxides in environmental, chemical, biological and clinical fields [2]. At present, the main assays for detecting the trace hydrogen peroxide include high performance liquid chromatography [3, 4], chemiluminescence [5, 6], fluorescence [7, 8], atomic absorption spectrometry [9, 10], spectrophotometry [11, 12], flow injection [13, 14], and resonance scattering spectrometry [15, 16] methods. Light scattering phenomenon commonly exists in the interaction between photon and particle. It plays a very important role in the research areas of photonics, colloid chemistry and polymer chemistry. Resonance scattering technique has been established by Pasternack et al. [17] in 1993 with a conventional fluorescence spectrophotometer, and have been utilized to study molecular recognition, assemblies and aggregates with sensitivity. In 1990’s, Liu [18] and Huang [19] expanded the application scope of resonance scattering technique, and used it in inorganic and nucleic acid analysis. Since 1998, our research group discovered the resonance scattering effect of some nanoparticles including metal, nonmetal and semiconductor, and it was combined with associated reaction, catalytic reaction, immune reaction and aptamer reaction to establish the corresponding RS methods [20-23]. However, there is no report about organic particle RS effect and RS study of H$_2$O$_2$-guaiacol-HRP catalytic system. In this article, we study the organic particle system with RS technique, and a new RS method has been set up for the determination of H$_2$O$_2$.

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EXPERIMENTAL

Apparatus and reagents

A Cary Eclipse spectrofluorometer (Varian, USA) and a Zetasizer Nano ZS90 (Malvern, UK) were used.

A 500 µg/mL HRP (biochemical reagent, RZ [\(A_{403\,nm}/A_{275\,nm}\) more than 3, 300 U/mg, Huamei Biological Engineering Co.) was prepared by dissolving 5.0 mg HRP to 10 mL with water, and was stored in 4 °C refrigerator. A \(\text{H}_2\text{O}_2\) solutions (8.27 × 10^{-2} M) were standardized by using KMnO\(_4\) standard solution. The pH 4.4 citric acid (\(\text{C}_6\text{H}_8\text{O}_7\))-\(\text{Na}_2\text{HPO}_4\) buffer solution was prepared by mixing of 11.2 mL 0.1 M citric acid and 8.8 mL 0.1 M \(\text{Na}_2\text{HPO}_4\) solutions. The pH buffer solutions of \(\text{C}_6\text{H}_8\text{O}_7\)-\(\text{C}_6\text{H}_5\text{O}_7\)Na, \(\text{HAc-NaAc}\) and \(\text{HCl-NaAc}\) were prepared by mixing of 0.1 M corresponding both components according to a certain volume ratio. A 2 × 10^{-4} M guaiacol solution was prepared. All the reagents were of analytical grade except explained and all the solutions were prepared with double-distilled water.

Procedure

A 0.2 mL pH 4.4 \(\text{C}_6\text{H}_8\text{O}_7\)-\(\text{NaH}_2\text{PO}_4\) buffer solution, a 0.1 mL 2 × 10^{-4} M guaiacol solution, a certain amount of \(\text{H}_2\text{O}_2\), 100 µL 5 µg/mL HRP solution were pipetted into a 5 mL marked tube, mixed well and diluted to 1.5 mL with water. Then it was put into water-bath at 37 °C for 30 min. The RS spectrum was scanned synchronously (\(\lambda_{em} - \lambda_{ex} = 0\,nm\)) on fluorescence spectrophotometer with the excited and emission slit width of 5.0 nm, and the PMT voltage of 400 V. The RS intensity at 530 nm (\(I_{530\,nm}\)) and a reagent blank \(I_0\) was recorded. The value of \(\Delta I_{530\,nm} = I_{530\,nm} - I_0\) was calculated.

RESULTS AND DISCUSSION

Principle

In pH 4.4 \(\text{C}_6\text{H}_8\text{O}_7\)-\(\text{NaH}_2\text{PO}_4\) buffer solution, HRP catalyzed \(\text{H}_2\text{O}_2\) to form •OH. Then it oxidize guaiacol to generate tetramer complex [24]. This tetramer complex molecules can aggregate into particles with an average size of 463 nm tested by Zetasizer Nano ZS90 (Figure 1). The particle exhibited a RS peak at 530 nm, and the RS value was linear to the concentration of \(\text{H}_2\text{O}_2\). On this basis, a new RS method for the quantification of \(\text{H}_2\text{O}_2\) was established. The principal reactions were following:

1. \(\text{H}_2\text{O}_2\) catalyzed by HRP → •OH

2. •OH + 4 \(\text{H}_2\text{CO}\) → \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\)

3. \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) → \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\) \(\text{H}_2\text{CO}\)
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**Figure 1.** Laser scattering image of \( \text{C}_6\text{H}_8\text{O}_7\)-Na\( _2 \)HPO\( _4 \)-guaiacol-H\( _2 \)O\( _2 \)-HRP system.

**RS spectra**

The scattering signal of the \( \text{C}_6\text{H}_8\text{O}_7\)-Na\( _2 \)HPO\( _4 \), guaiacol, HRP systems is very weak. When a certain \( \text{H}_2\text{O}_2 \) was added into the system of \( \text{C}_6\text{H}_8\text{O}_7\)-Na\( _2 \)HPO\( _4 \)-guaiacol-HRP, the catalytic oxidation reaction took place rapidly, and formed tetramer complex particle in dark brown color. There is a resonance scattering peak at 530 nm (Figure 2). The color becomes deeper and the RS value increased at 530 nm when \( \text{H}_2\text{O}_2 \) concentration increased. So, 530 nm was selected for use in this assay.

**Figure 2.** Resonance scattering spectra. a: pH 4.4 Na\( _2 \)HPO\( _4 \)-\( \text{C}_6\text{H}_8\text{O}_7 \)-1.33 \( \times 10^{-5} \) M C\( _7\text{H}_8\text{O}_2 \)-0.33 \( \mu \)g/mL HRP; b: a - 1.1 \( \times 10^{-6} \) M \( \text{H}_2\text{O}_2 \); c: a - 5.51 \( \times 10^{-6} \) M \( \text{H}_2\text{O}_2 \); d: a - 11.03 \( \times 10^{-6} \) M \( \text{H}_2\text{O}_2 \); e: a - 27.56 \( \times 10^{-6} \) M \( \text{H}_2\text{O}_2 \).

**Effect of pH buffer solution**

The enzyme active part commonly contains acidic group and basic group, the catalytic reaction is very sensitive to the pH value, and its fitting pH range was narrow. As the pH value was changed, these groups were in different dissociation state, and the activity for HRP changed.
Thus, HRP has its optimal pH. The influence of four pH buffer solutions including Na₂HPO₄-C₆H₈O₇, C₆H₈O₇-C₆H₅O₇Na₃, HAc-NaAc, and HCl-NaAc on the $\Delta I_{530 \text{ nm}}$ was tested. Figure 3 showed that the sensitivity decreased in order of Na₂HPO₄-C₆H₈O₇, C₆H₈O₇-C₆H₅O₇Na₃, HAc-NaAc, and HCl-NaAc. The Na₂HPO₄-C₆H₈O₇ buffer solution is most sensitive, and was chosen for use. The $\Delta I_{530 \text{ nm}}$ reached maximum when the pH value was 4.4 that is its optimal pH. Thus, a pH 4.4 Na₂HPO₄-C₆H₈O₇ buffer solution was chosen for use. The effect of buffer solution volume was suggested. When the volume of buffer solution was 0.2 mL, the $\Delta I_{530 \text{ nm}}$ was maximal. Thus, a 0.2 mL pH 4.4 Na₂HPO₄-C₆H₈O₇ buffer solution was chosen.

![Figure 3. Effect of pH. a. Na₂HPO₄-C₆H₈O₇-1.33 × 10⁻⁵ M guaiacol-0.33 μg/mL HRP-5.51 × 10⁻⁵ M H₂O₂; b. C₆H₈O₇-C₆H₅O₇Na₃-1.33 × 10⁻⁵ M guaiacol-0.33 μg/mL HRP-5.51 × 10⁻⁵ M H₂O₂; c. HAc-NaAc-1.33 × 10⁻⁵ M guaiacol-0.33 μg/mL HRP - 5.51 × 10⁻⁵ M H₂O₂; d. HCl-NaAc-C₆H₈O₇-1.33 × 10⁻⁵ M guaiacol-0.33 μg/mL HRP-5.51 × 10⁻⁵ M H₂O₂.](image)

### Effect of guaiacol

With the concentration of guaiacol increasing, the $\Delta I_{530 \text{ nm}}$ value increased at first and then decreased slowly. The $\Delta I_{530 \text{ nm}}$ value was maximum when guaiacol was 1.33 × 10⁻⁵ M. Thus, a 1.33 × 10⁻⁵ M guaiacol was chosen for use.

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Figure 4. Effect of guaiacol concentration. pH 4.4 Na$_2$HPO$_4$$-$$C_6$H$_8$O$_7$$-0.33$ µg/mL HRP $-5.51 \times 10^{-5}$ M H$_2$O$_2$.

Effect of HRP

The $\Delta$I$_{530}$ nm value of the system increased with HRP concentration increasing in the range of 0.05-0.3 µg/mL, and then decreased as in Figure 5. The $\Delta$I$_{530}$ nm reaches the maximum when the concentration of HRP was 0.33 µg/mL. Thus, 0.33 µg/mL HRP was chosen for use.

Figure 5. Effect of HRP concentration. pH 4.4 Na$_2$HPO$_4$$-$$C_6$H$_8$O$_7$$-1.33 \times 10^{-5}$ M guaiacol $-5.51 \times 10^{-5}$ M H$_2$O$_2$.
Effect of catalytic reaction time and temperature

In general, enzyme reaction has an optimal temperature, because high temperature resulted to enzyme denaturalization, and the velocity increased with the temperature. The effect of temperature on the HRP catalytic reaction is shown in Figure 6. The $\Delta I_{530\text{ nm}}$ value reached maximum between 30-50 °C. A 37 °C was chosen. Results showed that the $\Delta I_{530\text{ nm}}$ value reached maximum when reaction time was 30 min and was stable for 50 min. Thus, 30 min was selected for use.

![Figure 6. Effect of temperature. pH 4.4 Na$_2$HPO$_4$-C$_6$H$_8$O$_7$-1.33 \times 10^{-5}$ M guaiacol-0.33 µg/mL HRP-5.51 \times 10^{-5}$ M H$_2$O$_2$.](image)

Influence of foreign substances

According to the procedure, influences of some foreign substances including common metal cation and anion on the detection of 5.33 µM H$_2$O$_2$ were tested, with a relative error of ± 5 %. The results showed the tolerance limit as the following, 14 times of Mg$^{2+}$, 36 times of Ni$^{2+}$, NO$_2^-$ may be coming from NO gas, and Fe$^{3+}$ may be coming from dust, 60 times of K$^+$, Ca$^{2+}$ may be coming from dust, NO$_3^-$, CO$_3^{2-}$ may be coming from CO$_2$ and dust, SiO$_3^{2-}$ may be coming from dust, SO$_4^{2-}$ may be coming from dust and SO$_2$ gas, and C$_2$O$_4^{2-}$, 138 times of Li$^+$, 1200 times of F, 3900 times of NH$_4^+$ may be coming from NH$_3$ gas do not interfere with the determination. The proposed RS assay had a good selectivity.

Calibration curve

According to the procedure, the calibration curve between H$_2$O$_2$ and $\Delta I_{530\text{ nm}}$ was drawn, the linear range was 0.55-27.6 µM, with a regression equation of $\Delta I_{530\text{ nm}} = 17.1C + 1.6$, a correlation coefficient of 0.9996, and a detection limit (DL) of 0.03 µM. The DL can be calculated according to the equation DL = 3S/K, where 3 is the factor at the 95% confidence level, S the standard deviation of the blank measurements ($n = 10$), and K is the slope of the calibration curve.

Sample determination

The autumnal rain water sample 1, 2 and 3 were collected in the place of urban (Sanlidian of Guilin city), rural (Caoping village of Guilin city) and factory (Commodity Chemical Factory of Guilin city), respectively, and filtered before analysis. A 100 µL of the sample was measured.
according the procedure. At the same time, the samples were also analyzed with the Fe(II)-ortho-phenylene diamine catalytic spectrophotometric method [11]. The results of both the methods are listed in Table 1. The linear regression analysis revealed a correlation coefficient, slope, and intercept of 0.9997, 0.9759, and 0.0568 µM, respectively. This showed that the RS results were in good agreement with that of the spectrophotometric method, and both methods have good correlation. A 1.30 µM, 0.250 µM and 5.00 µM H$_2$O$_2$ were added in the sample 1, 2 and 3, respectively. Then it was analyzed according to the procedure. The recoveries were in the range of 98.0-104 %. The results indicated that this enzyme catalytic RS assay is accurate and reliable. From Table 1, we can see that the H$_2$O$_2$ content of this assay is lowest in rural rain water sample owing to the least environmental pollution, the H$_2$O$_2$ content is highest in the place of factory that one reason may be serious pollution, and the content of urban sample locates the both samples. Table 1 also showed that this assay results were agreement with that of the reference results.

### Table 1. Analytical results.

| Sample  | Results of this assay ($\mu$M, n = 5) | Added ($\mu$M) | Found ($\mu$M) | Recovery (%) | Reference results [11] ($\mu$M, n = 5) |
|---------|-------------------------------------|---------------|---------------|--------------|----------------------------------------|
| Sample 1| 1.336±0.069                         | 1.30          | 1.35          | 104          | 1.280±0.071                            |
| Sample 2| 0.273±0.012                         | 0.250         | 0.256         | 102          | 0.270±0.011                            |
| Sample 3| 5.006±0.144                         | 5.00          | 4.95          | 98.0         | 5.084±0.125                            |

### CONCLUSIONS

Based on the catalytic effect of horseradish peroxidase on the particle reaction between guaiacol and H$_2$O$_2$, and the RS effect of the organic particles, a new, sensitive, selective and rapid enzyme catalytic RS assay was proposed for the determination of 0.55-27.6 µM H$_2$O$_2$.

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