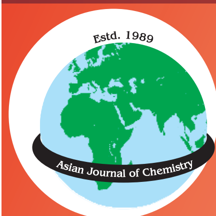

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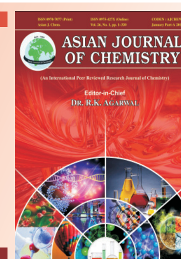

Spectrophotometric Determination of Ascorbic Acid with Hemoglobin as Catalyst

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A novel method was described for the determination of ascorbic acid based on the catalytic effect of hemoglobin for the oxidation of hydrogen peroxide and *o*-phenylenediamine. UV-visible spectrophotometry was used to study the kinetic behaviour of the oxidation reaction. The optimal conditions of the system were found. A linear calibration graph was obtained over the ascorbic acid concentration range of $1.0 \times 10^{-9} – 2 \times 10^{-8}$ mol/L with a correlation coefficient of 0.9996. The results confirmed that the absorbance of the catalytic system can be enhanced obviously in the presence of limited ascorbic acid and the detection method of ascorbic acid with hemoglobin as catalyst is feasible.

Keywords: Catalytic, Spectrophotometry, Hemoglobin, Ascorbic acid, *o*-Phenylenediamine.

INTRODUCTION

Ascorbic acid is an essential micronutrient required for normal metabolic functioning of the body. Many biochemical and clinical studies have indicated that ascorbic acid may be of benefit in chronic diseases such as cardiovascular disease and scurvy [1]. Ascorbic acid is a powerful water-soluble antioxidant and plays a vital role in protecting against oxidative damage [2]. Ascorbic acid also helps maintain capillaries, bones and teeth and acids in the absorption of iron [3]. The determination of ascorbic acid is of great importance for clinical diagnosis and physiological research. Numerous methods have been used to determine ascorbic acid, such as electrochemistry[4], chemiluminescence [5], HPLC [6]. Enzymatic kinetic spectrophotometry was extensively studied owing to its simplicity, rapidity and high selectivity [7].

Horseradish peroxidase (HRP), in which the heme iron acts as the activity center, is the commonly used enzyme in ascorbic acid detection. However, there are shortcomings in horseradish peroxidase. For example, it is expensive, easy to inactivation and has strict experimental conditions[8]. Therefore, the search for a substitute for horseradish peroxidase is greatly significant.

Hemoglobin (Hb) has the similar catalytic characteristics as that of horseradish peroxidase contributes due to they have analogous iron-porphyrin cofactor [9]. Hemoglobin consists of four subunits of polypeptide and each polypeptide chain contains a heme group that serves as the active center[10]. Compared with other replacements for horseradish peroxidase, hemoglobin has inimitable virtues-natural spatial structure, which is essential for the special inclusion behaviour between the enzyme and the substrate [11].

Based on the above, as well as low cost price of hemoglobin, hemoglobin is used as an effective substitute for horseradish peroxidase to detect ascorbic acid by catalyzing the reaction between *o*-phenylenediamine and hydrogen peroxide to form 2,3-diaminophenazine (DAPN[8,11]. Hb-OPDA-H_2O_2 reaction system was established. The enzymatic reaction was studied by measuring the increase in absorbance of 2,3-diaminophenazine at 428 nm.

EXPERIMENTAL

Hemoglobin solution (1.0×10^{-4} mol/L) was prepared by dissolving 0.1612 g of hemoglobin (ASUS Fine Chemicals Co., Ltd. Shanghai, China) in double distilled water and stored below 4 ºC. 0.1 mol/l *o*-phenylenediamine stock solution was prepared by dissolving 1.0814 g *o*-phenylenediamine in a 100 mL flask. Ascorbic acid (1.0×10^{-3} mol/L) was prepared by dissolving 17.85 mg of ascorbic acid (Kaitong Chemical Reagent Company, Tianjin, China) in double distilled water. The working solution was prepared daily by diluting the stock solution to the desired volume. H_2O_2 solution was prepared by diluting 1.1 mL of 30 % H_2O_2 to 1000 mL. Buffer solution was composed of 0.2 mol/L Na_2HPO_4 and 0.1 mol/L citric acid.

Double distilled water was used throughout. All the chemicals used were of analytical-reagent grade.

The UV-visible spectra and relative absorbance were measured with a 721 spectrophotometer (Shanghai Third Instrument Factory, China) with a 1cm quartz cell. All pH values were measured with a PHS-5320 precision pH meter.

Procedure: In a set of 10 mL volumetric tubes containing 0.6 mL of 1.0×10^{-2} mol/L H_2O_2, 0.4mL of 0.1 mol/L *o*-phenylenediamine, 3 mL pH 5 of citric acid-phosphate buffer solution were added orderly and diluted with water to 10 mL. The reaction solution was kept at 25 ºC for 10 min and then 0.2 mL of 1×10^{-4} mol/L hemoglobin and various amount of ascorbic acid were added. The change in absorbance was measured at 428 nm after 5 min and the blank solution is without ascorbic acid.

RESULTS AND DISCUSSION

Effect of pH and volume of buffer solution: Enzymatic activity was greatly influenced by the solution pH. Thus the pH dependence of the system was investigated over the range of 3.0-8.0 using Na_2HPO_4-citric acid buffer. The results are shown in Fig. 1. It can be seen that absorbance increased with increasing pH up to 5.0 and above 5.0 a sharp decrease of absorbance was observed.

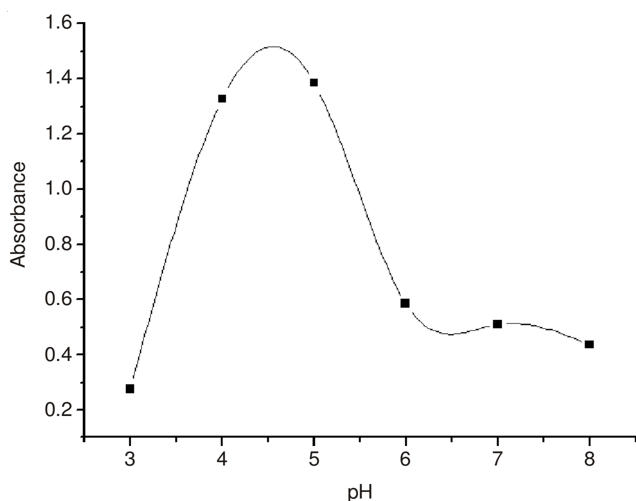

Fig. 1. Effect of pH on the absorbance. Solution: $[H_2O_2] = 5 \times 10^{-4}$ mol/L; $[OPDA] = 5 \times 10^{-3}$ mol/L; $[Hb] = 2 \times 10^{-6}$ mol/L

The effect of the Na_2HPO_4-citric acid buffer concentration was also tested at pH 5. Within a certain range, the absorbance of the blank and sample both increased with increasing buffer capacity, ΔA reached the maximum between 3 and 4 mL, indicating excessive buffer inhibit the catalytic rate of the system (Fig. 2). Thus, 3 mL pH 5.0 Na_2HPO_4-citric acid buffer was chosen in the recommended procedure.

Effect of concentration of *o*-phenylenediamine: The effect of *o*-phenylenediamine concentration on ΔA of the system was investigated and the results were shown in Fig. 3, illustrating that high concentrations of *o*-phenylenediamine are favorable to the reaction. However, excessive large concentration will increase the absorbance of the blank whereas have little effect on the sample, so ΔA will gradually decrease with *o*-phenylenediamine increasing concentration.

Effect of hydrogen peroxide concentration: The dependence of ΔA on hydrogen peroxide concentration was illustrated in Fig. 4. At low concentrations of hydrogen peroxide, ΔA increased linearly and reached a maximum at 6×10^{-4} mol/L and then decreased when the hydrogen peroxide concentration exceeded the level. It can be interpreted as high concentration of hydrogen peroxide will lead to the direct oxidation of ascorbic acid, the activate effect of ascorbic acid on the system disappeared.

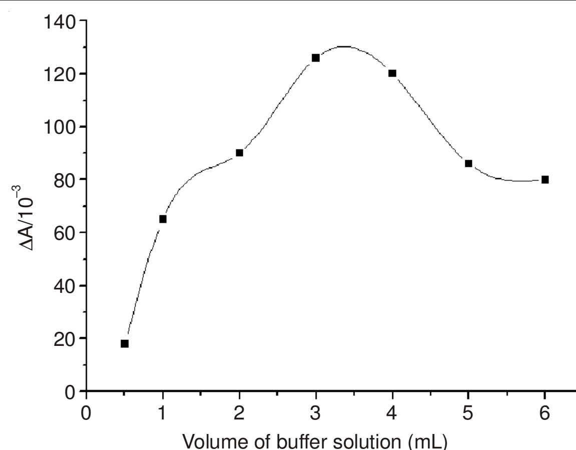

Fig. 2. Effect of the volume of buffer solution. Solution: $[H_2O_2] = 5 \times 10^{-4}$ mol/L; $[OPDA] = 5 \times 10^{-3}$ mol/L; $[Hb] = 2 \times 10^{-6}$ mol/L; $[AA] = 1.0 \times 10^{-8}$ mol/L; $\Delta A = A_{sample} - A_{blank}$

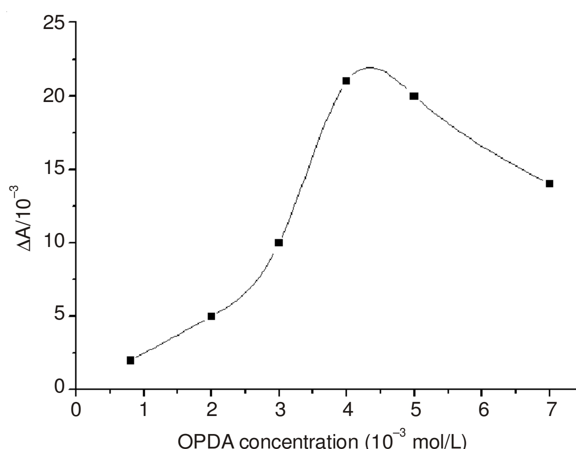

Fig. 3. Effect of *o*-phenylenediamine concentration. Solution: $[H_2O_2] = 5 \times 10^{-4}$ mol/L; $[Hb] = 2 \times 10^{-6}$ mol/L; $[AA] = 1.0 \times 10^{-8}$ mol/L; 3 mL pH 5.0 Na_2HPO_4-citric acid buffer solution; $\Delta A = A_{sample} - A_{blank}$

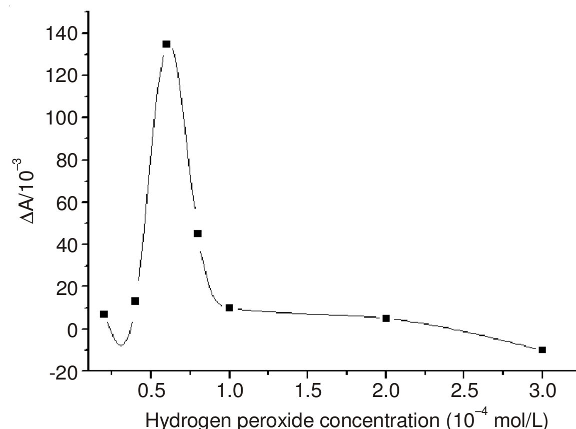

Fig. 4. Effect of hydrogen peroxide concentration. Solution: $[Hb] = 2 \times 10^{-6}$ mol/L; $[AA] = 1.0 \times 10^{-8}$ mol/L; $[OPDA] = 4 \times 10^{-3}$ mol/L; 3 mL pH 5.0 Na_2HPO_4-citric acid buffer solution; $\Delta A = A_{sample} - A_{blank}$

Effect of hemoglobin concentration: The effect of hemoglobin concentration on ΔA of the system was investigated and the results were shown in Fig. 5. It can be seen that ΔA increased with increasing concentrations of hemoglobin. At a high concentration, ΔA increased slowly, moreover the reproducibility of the signal is not ideal that could due to the interference of the blank absorption from the high concentration of hemoglobin[8]. In order to insure the sensitivity as well as the reproducibility, 2×10^{-6} mol/L hemoglobin was selected for the subsequent experiment.

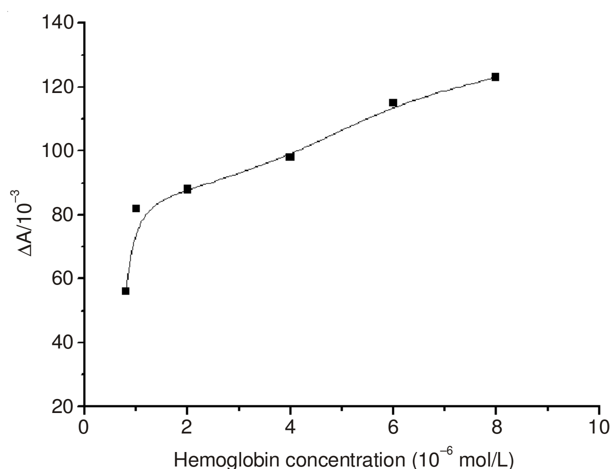

Fig. 5. Effect of concentration of hemoglobin. Solution: $[H_2O_2] = 6 \times 10^{-4}$ mol/L; $[AA] = 1.0 \times 10^{-8}$ mol/L; $[OPDA] = 4 \times 10^{-3}$ mol/L; 3 mL pH 5.0 Na_2HPO_4-citric acid buffer solution; $\Delta A = A_{sample} - A_{blank}$

Effect of order of reagent addition: The effect of different order of reagent addition on absorbance was indicated in Table-1 It is clearly seen by No. 4 reagent addition the absorbance of the system reached maximum in that such a sequence resulted in the least oxidation of ascorbic acid by H_2O_2 and the most cooperative effect of ascorbic acid on the reaction[12].

TABLE-1
EFFECT OF REAGENTS ADDITION ON THE REACTION

No.	System	Absorbance
1	H_2O_2 + AA + OPDA + buffer solution + Hb	0.188
2	H_2O_2 + OPDA + AA + buffer solution + Hb	0.157
3	H_2O_2 + OPDA + buffer solution + AA + Hb	0.180
4	H_2O_2 + OPDA + buffer solution (5 min later) + Hb + AA	0.240
5	H_2O_2 + OPDA + buffer solution (5 min later) + Hb	0.133
6	H_2O_2 + AA + buffer solution + Hb (5 min later) + OPDA	0.100
7	H_2O_2 + OPDA + buffer solution + Hb	0.055
8	OPDA + buffer solution + AA + Hb	0.028
9	H_2O_2 + buffer solution + AA + Hb	0.030
10	H_2O_2 + OPDA+ buffer solution + AA	0.024

AA = Ascorbic acid; OPDA = *o*-Phenylenediamine; Hb = Hemoglobin

Comparing with Nos. 4 and 5, the result demonstrated ascorbic acid has significant activation on the reaction. In addition, if ascorbic acid had reacted with H_2O_2 for 5 min then *o*-phenylenediamine was added, as No. 6 reagent addition, the absorbance of the system was lower perhaps owing to the oxidation of ascorbic acid by H_2O_2.

Calibration curve: According to the optimized experimental conditions, ΔA is proportional to the ascorbic acid concentration in the range of 1.0×10^{-9}-2×10^{-8} mol/L by the regression equation: $\Delta A = 0.112C$ (10^{-8} mol/L)-0.0015 and the correlation coefcient was $R^2 = 0.9992$.

Conclusion

It was found that hemoglobin can catalyze the redox reaction for hydrogen peroxide and *o*-phenylenediamine. Different concentration of ascorbic acid lead to opposite effect on the system. High concentrations of ascorbic acid restrain the reaction, whereas low concentrations of ascorbic acid activate the catalytic system. Depend on this principle, the results investigated that the appropriate use of the UV-visible spectroscopy allows determining trace amount of ascorbic acid utilizing the unique catalytic activity of natural enzyme hemoglobin. To ensure satisfactory results, some conditions such as pH and volume of buffer solution, concentration of the reagents, the orders of reagent addition were optimized. In summary, a simple, rapid and specific method for the determination of ascorbic acid was proposed and it can be used for real samples.

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