

Inhibitive Kinetic Spectrophotometric Determination of Traces of Protein Using *m*-Acetylchlorophosphonazo as an Indicator

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A novel kinetic spectrophotometric method for the determination of protein is developed based on the inhibitory effect of bovine serum albumin (BSA) on the oxidation reaction of *m*-acetylchlorophosphonazo (CPAmA) by potassium periodate. In the medium of 6×10^{-3} mol L⁻¹ sulfuric acid and at maximum absorption peak of 550 nm, the absorbance difference (ΔA) is linearly related with the concentration of bovine serum albumin over the range of 0.20-6.0 $\mu\text{g/mL}$ of solution and fitted the equation: $\Delta A = 0.1105C$ ($C: \mu\text{g mL}^{-1}$) + 0.0115, with a regression coefficient of 0.9936. The detection limit of the method was 0.028 $\mu\text{g mL}^{-1}$. The method was used to determine protein in white egg. The relative standard deviation was 3.13 % for 13 replicate determinations. The recovery of the standard addition was 103.7 %. The operation of the method is simple, fast and of high sensitivity and the linear range is wide, so it is suitable for the determination of protein.

Key Words: Inhibitive kinetic spectrophotometry, Bovine serum albumin, *m*-Acetylchlorophosphonazo, Potassium periodate, White egg.

INTRODUCTION

Protein, serving as a physical bearer of genetic information in the organism, is one of the main content of biological chemistry. A Dutch chemist Mulder first proposed the term "protein", whose original intention was "ranked No. 1", recognized as the most important substance in human body and without it there is no life¹. Protein is made from different combinations of 20 sorts of amino acids. Humans have as many as 100,000 different protein molecules, of which small protein molecules consist of hundreds of amino acids and large protein molecules consist of thousands of amino acids. Protein is a main composition in constructing and recovering body tissue, in the formation of enzymes, hormones and antibodies and is needed for regulating fluid balance, transporting materials, maintaining normal function of nervous system and providing energy, *etc.* The methods for the determination of protein² deal with spectrophotometry³, fluorescence spectrometry⁴, resonance light scattering technique^{5,6}, *etc.* The kinetic spectrophotometric method is widely used

in trace-elemental analysis for its advantages such as high sensitivity and simple equipment. *m*-Acetylchlorophosphonazo (CPAmA)⁷ has been used for the determination of iron by kinetic spectrophotometric method. In this research a new method is proposed for the determination of protein based on the inhibitory effect of bovine serum albumin on the oxidation reaction of CPAmA by KIO₄ in the medium of 6×10^{-3} mol L⁻¹ sulfuric acid. This method has been successfully applied to the analysis of protein in hen white egg.

In the aryl derivative of CPAmA, there are -N=N- and ligands containing N and O (Fig. 1). The reagent has the strong ability of chelation and can chelate with metallic ion to form various aqueous complexes. The colour of solution become weak even colourless when -N=N- group is oxidized or reduced. In terms of the oxidation reaction of CPAmA by KIO₄ (Fig. 2), the side chain amino groups of bovine serum albumin are protonated under the acidic conditions and they associate with CPAmA through electrostatic attraction. Thus, the interaction of amino groups of proteins and -N=N- of CPAmA make CPAmA enter the structure of protein (Fig. 3), which gives protection to chromophore -N=N- and inhibits its decolorization process (Fig. 4).

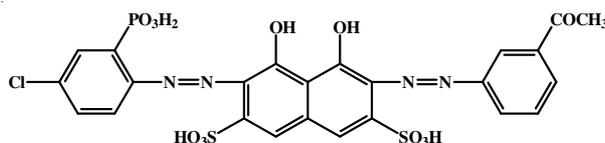


Fig. 1. Structure of CPAmA

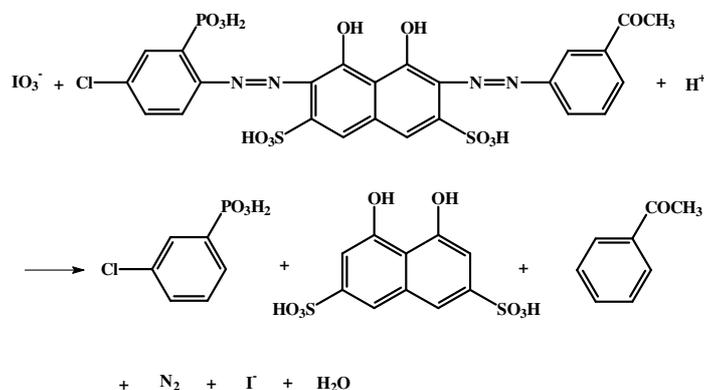


Fig. 2. Oxidation reaction

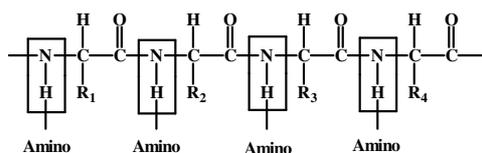


Fig. 3. First structure of protein

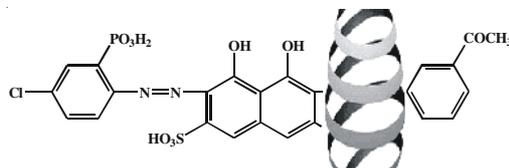


Fig. 4. Inhibitory reaction

EXPERIMENTAL

A 722S spectrophotometer (Shanghai Lingguang Technique Co., Ltd., China) and a HH-2 thermostat water bath kettle (Jiangsu Jintan Ronghua Apparatus Manufacture Co., Ltd., China) were used for absorbance measurement and temperature control, respectively.

0.2500 g of Bovine serum albumin (BSA) was dissolved in 250 mL of water to get 1 mg mL^{-1} BSA stock solution. The required concentration working solution ($20, 10 \text{ } \mu\text{g mL}^{-1}$) was obtained by diluting the above stock solution and kept in cold storage at $2 \text{ } ^\circ\text{C}$. *m*-Acetylchlorophosphonazo (CPAmA) solution was prepared by dissolving 0.0858 g of CPAmA in 250 mL of water to get $5 \times 10^{-4} \text{ mol L}^{-1}$ CPAmA solution. $1 \times 10^{-2} \text{ mol L}^{-1}$ KIO_4 and 0.2 mol L^{-1} sulfuric acid solutions were used. The water was deionized water and the reagents used were of analytical grade.

Procedure: Two 10 mL calibrated flasks were taken. A suitable amount of BSA solution (inhibitory reaction, for optimization conditional experiment using $30 \text{ } \mu\text{g}$) was added into the one, while the other was not added (non-inhibitory reaction). 0.30 mL of H_2SO_4 solution, 2.2 mL of CPAmA solution and 1 mL of KIO_4 solution were subsequently placed into two 10 mL calibrated flasks, respectively, which were diluted up to the mark with water. The mixed solutions were shaken well, heated at $100 \text{ } ^\circ\text{C}$ for 10 min, then removed from the boiling water, cooled down by running water for 4 min. The absorbance values of both non-inhibitory reaction (A_0) and inhibitory reaction (A) were measured at 550 nm in 1 cm cells against water with the spectrophotometer and $\Delta A = (A - A_0)$ was calculated.

RESULTS AND DISCUSSION

Absorption spectra: Fig. 5 shows the absorption spectra of the solutions measured under the optimum experimental conditions. The maximum absorption wavelengths of the inhibitory reaction solution and the non-inhibitory reaction solution are 530 nm, but there is maximum absorbance difference at 550 nm. Thus 550 nm was selected as the measurement wavelength.

Optimization of reaction conditions

Effect of the amount of sulfuric acid: The experimental results (Fig. 6) of acidity effect showed that with the increase in amount of the H_2SO_4 solution in the range of 0.10-0.30 mL, the value of ΔA gradually increased and reached a maximum and the sensitivity of the reaction was the highest at 0.30 mL. When the

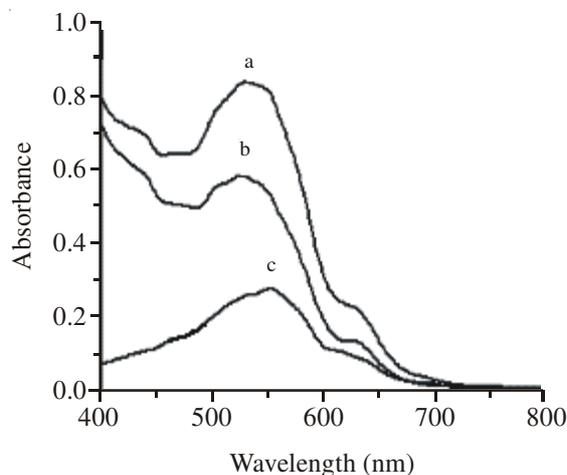


Fig. 5. **Absorption spectra:** (a) CPAmA + KIO₄ + BSA (against water) - inhibitory reaction A; (b) CPAmA + KIO₄ (against water) - non-inhibitory reaction A₀; (c) net inhibitory reaction ΔA; [BSA] = 3 μg mL⁻¹; [CPAmA] = 1.1 × 10⁻⁴ mol L⁻¹; [KIO₄] = 1 × 10⁻³ mol L⁻¹; [H₂SO₄] = 6 × 10⁻³ mol L⁻¹; reaction temperature T = 100 °C; heating time t = 10 min

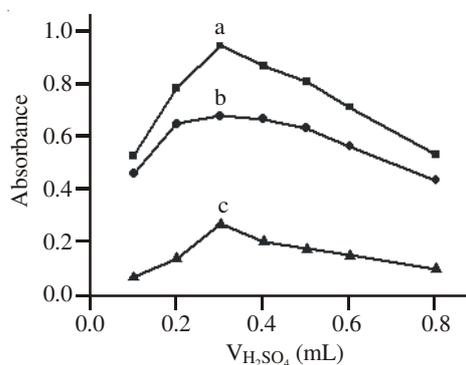


Fig. 6. **Effect of acidity:** (a) CPAmA + KIO₄ + BSA (against water) - inhibitory reaction A; (b) CPAmA + KIO₄ (against water) - non-inhibitory reaction A₀; (c) net inhibitory reaction ΔA; [BSA] = 3 μg mL⁻¹; [CPAmA] = 1.1 × 10⁻⁴ mol L⁻¹; [KIO₄] = 1 × 10⁻³ mol L⁻¹; reaction temperature T = 100 °C; heating time t = 10 min; λ = 550 nm

amount of H₂SO₄ solution was more than 0.30 mL, ΔA began to decrease, thus 0.30 mL of 0.2 mol L⁻¹ H₂SO₄ solution was selected. The concentration of the H₂SO₄ solution was 6 × 10⁻³ mol L⁻¹ in the reactive system at this time.

Effect of the amount of CPAmA: The experimental results (Fig. 7) of CPAmA effect showed that with the increase in amount of CPAmA solution over the range of 0-2.2 mL, the value of ΔA increased and reached a maximum at 2.2 mL. When the amount of CPAmA solution was more than 2.2 mL, ΔA began to decrease, thus 2.2 mL of 5 × 10⁻⁴ mol L⁻¹ CPAmA solution was selected.

Effect of the amount of KIO_4 : The effect of the KIO_3 showed (Fig. 8) that with the increase in amount of KIO_4 solution over the range of 0.20-1.0 mL, the value of ΔA increased and reached a maximum at 1 mL. When the amount of KIO_4 solution was more than 1 mL, ΔA began to decrease. Thus, 1 mL of 0.010 mol L^{-1} KIO_4 solution was selected.

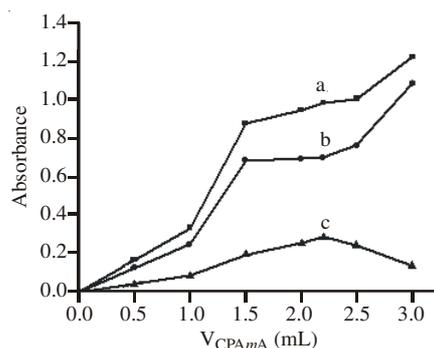


Fig. 7. **Effect of amount of CPAmA:** (a) CPAmA + KIO_4 + BSA (against water) - inhibitory reaction A; (b) CPAmA + KIO_4 (against water) - non-inhibitory reaction A_0 ; (c) net inhibitory reaction ΔA ; $[BSA] = 3 \mu\text{g mL}^{-1}$; $[H_2SO_4] = 6 \times 10^{-3} \text{ mol L}^{-1}$; $[KIO_4] = 1 \times 10^{-3} \text{ mol L}^{-1}$; reaction temperature $T = 100 \text{ }^\circ\text{C}$; heating time $t = 10 \text{ min}$; $\lambda = 550 \text{ nm}$

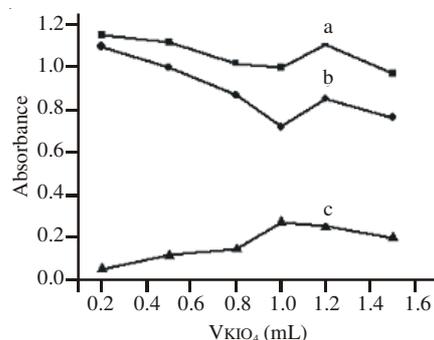


Fig. 8. **Effect of amount of KIO_4 :** (a) CPAmA + KIO_4 + BSA (against water) - inhibitory reaction A; (b) CPAmA + KIO_4 (against water) - non-inhibitory reaction A_0 ; (c) net inhibitory reaction ΔA ; $[BSA] = 3 \mu\text{g mL}^{-1}$; $[H_2SO_4] = 6 \times 10^{-3} \text{ mol L}^{-1}$; $[CPAmA] = 1.1 \times 10^{-4} \text{ mol L}^{-1}$; reaction temperature $T = 100 \text{ }^\circ\text{C}$; heating time $t = 10 \text{ min}$; $\lambda = 550 \text{ nm}$

Effect of the adding order of the reagents: The different adding order of the reagents had no effect on experimental results. In this paper, the adding order was $BSA + H_2SO_4 + CPAmA + KIO_4$.

Stability of system: For the determination of $3 \mu\text{g mL}^{-1}$ BSA the change of ΔA was less than 5 % within 3.5 h and the system remain stable.

Effect of heating temperature: The results (Fig. 9) showed that ΔA increased gradually with temperature and got a maximum and its highest sensitivity reached

at 100 °C. Therefore, 100 °C was selected as optimum experimental temperature. The data measured over the range 65-95 °C was processed by regression and the linear regression equation obtained was as follows: $\log(A_0/A) = -1301.288/T(K) + 4.9541$, $\gamma = 0.9912$. The apparent activation energy calculated by the slope of the equation was $E_a = 24.90 \text{ kJ mol}^{-1}$.

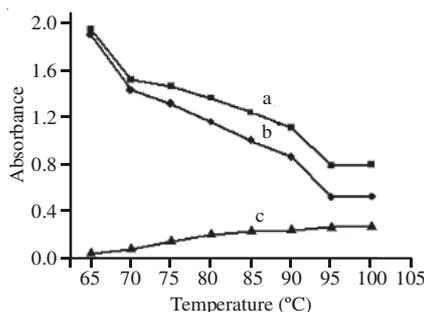


Fig. 9. **Effect of heating temperature:** (a) CPAmA + KIO₄ + BSA (against water)-inhibitory reaction A; (b) CPAmA + KIO₄ (against water) - non-inhibitory reaction A₀; (c) net inhibitory reaction ΔA; [BSA] = 3 μg mL⁻¹; [H₂SO₄] = 6 × 10⁻³ mol L⁻¹; [CPAmA] = 1.1 × 10⁻⁴ mol L⁻¹; [KIO₄] = 1 × 10⁻³ mol L⁻¹; heating time t = 10 min; λ = 550 nm

Effect of heating time: The results (Fig. 10) showed that ΔA and t show a good linear relationship over the range of 4-10 min. The ΔA obtained reached a maximum value at 10 min and then began to decrease gradually. Thus, 10 min was selected as optimum time in the experiment. The linear regression equation obtained was as follows: $\log(A/A_0) = 0.0114t(\text{min}) - 0.0196$, $\gamma = 0.9977$. The reactive rate constant calculated was $k = 5.163 \times 10^{-4} (\text{s}^{-1})$ and the half-life period was $t_{1/2} = 27.76 \text{ min}$.

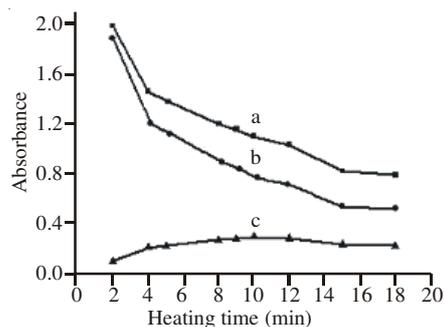


Fig. 10. **Effect of heating time:** (a) CPAmA + KIO₄ + BSA (against water) - inhibitory reaction A; (b) CPAmA + KIO₄ (against water) - non-inhibitory reaction A₀; (c) net inhibitory reaction ΔA; [BSA] = 3 μg mL⁻¹; [H₂SO₄] = 6 × 10⁻³ mol L⁻¹; [CPAmA] = 1.1 × 10⁻⁴ mol L⁻¹; [KIO₄] = 1 × 10⁻³ mol L⁻¹; heating time T = 100 °C; λ = 550 nm

Linear range and detection limit: The experimental results (Fig. 11) showed that under the optimum experimental conditions ΔA and BSA showed a good linear

relationship over the range of 0.20-6.0 $\mu\text{g mL}^{-1}$ and the linear regression equation was $\Delta A = 0.1105C$ ($C: \mu\text{g mL}^{-1}$) + 0.0115 with a regression coefficient $\gamma = 0.9939$. 3 $\mu\text{g mL}^{-1}$ of BSA was determined 13 times and the relative standard deviation determined was 3.44 %. The reagent blank was determined 11 times and the detection limit determined was 0.028 $\mu\text{g mL}^{-1}$ by 3S/K method (S is the standard deviation of the reagent blank for 11 times determination, K is the slope of the working curve).

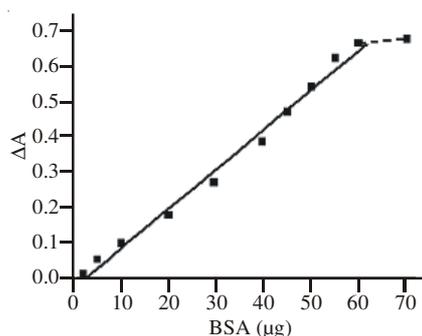


Fig. 11. **Calibration curve:** $[\text{H}_2\text{SO}_4] = 6 \times 10^{-3} \text{ mol L}^{-1}$; $[\text{CPAmA}] = 1.1 \times 10^{-4} \text{ mol L}^{-1}$; $[\text{KIO}_4] = 1 \times 10^{-3} \text{ mol L}^{-1}$; reaction temperature $T = 100 \text{ }^\circ\text{C}$; heating time $t = 10 \text{ min}$; $\lambda = 550 \text{ nm}$

Selectivity of the method: The co-existing ions did not interfere with the determination of 3 $\mu\text{g mL}^{-1}$ BSA and the tolerance limits (weight ratio) of the ions tested (causing $\leq 5 \%$ relative error) were summarized as follows: F^- (40); Cd^{2+} (15); Cr^{3+} (10); Li^+ , PO_4^{3-} , malate (6); Cl^- (5); Si^{4+} , Zn^{2+} , lysine (2); Ni^{2+} , glycine (1.5); Sr^{2+} , Mg^{2+} (1); citric acid, glucose, ascorbic acid, alanine, urea (0.6); Al^{3+} , acetic acid, tartaric acid (0.5); $\text{C}_2\text{O}_4^{2-}$ (0.4); Ca^{2+} , Mn^{2+} , Cu^{2+} (0.3); leucine, S^{2-} (0.2); Ti^{4+} , Co^{2+} (0.1); VO_3^- (0.08); W^{6+} , Zr^{4+} , Fe^{3+} , Bi^{3+} , La^{3+} , Eu^{3+} , Ba^{2+} , I^- (0.05); Mo^{6+} , $\text{S}_2\text{O}_7^{2-}$ (0.03); Ce^{4+} , Pb^{2+} (0.02), Cr^{6+} , Th^{4+} , B^{3+} , Fe^{2+} , MnO_4^- (0.01); Hg^{2+} (0.005).

Analysis of sample: The fresh white egg was stirred, from which 0.50 mL was taken and placed into a 50 mL calibrated flask, diluted up to the mark with water. Then 1 mL of the diluted white egg solution was placed into a 50 mL calibrated flask, diluted up to the mark with water as a sample solution. 0.50 mL of this sample solution was used for the determination of protein according to the experimental procedure. Meanwhile, the standard addition recovery experiments were made. The results of 13 times determination were 545.5, 561.0, 528.5, 545.5, 523.0, 516.0, 539.0, 517.0, 525.5, 547.5, 547.5, 501.5 and 525.5 mg mL^{-1} and the relative standard deviation was 3.13 %. The recovery of standard addition of the method was 96 % with satisfactory analytical results.

Conclusion

The optimum experimental conditions and the properties of reactive system as well as the dynamics parameters of the inhibitory effect of BSA on the oxidation

reaction of CPAmA by KIO_4 were studied. BSA can protect -N=N- of CPAmA from being oxidized by KIO_4 . The linear range for the determination of BSA is 0.20-6.0 $\mu\text{g mL}^{-1}$ at 550 nm and the detection limit determined is 0.028 $\mu\text{g mL}^{-1}$. This method has been successfully applied to the analysis of protein in the hen white egg.

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