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Kinetic Method for Determination of Trace Amounts of Protein Based on its Inhibitive Effect on Potassium Periodate-(Dibromo-*p*-sulfonic acid arsenazo) Indicator Reaction

QING-ZHOU ZHAI*, HUI YU, WEI-HUA HU and PENG-HUI ZHU

Research Center for Nanotechnology, Changchun University of Science and Technology, Changchun 130022, P.R. China

*Corresponding author: Fax: +86 431 85383815; Tel: +86 431 85583118; E-mail: zhaiqingzhou@163.com; zhaiqingzhou@hotmail.com

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A novel kinetic spectrophotometric method for the determination of protein is based on the inhibitive effect of bovine serum albumin (BSA) on the oxidation reaction of dibromo-*p*-sulfonic acid arsenazo (DBS-ASA) by potassium periodate in the medium of $CH_2ClCOOH$-CH_3COONH_4. The maximum absorption peak of BSA-(DBS-ASA)-KIO_4 system is located at 530 nm. The absorbance difference (ΔA) is well linearly related with the concentration of bovine serum albumin over the rage of 2.5-90.0 μg/mL of solution and fitted the equation: ΔA = 0.01091C + 0.0646 (C: μg/mL), with a correlation coefficient γ = 0.9941. The detection limit of the method was 0.48 μg/mL. The method has been successfully used in the determination of the total content of protein in egg white and the relative standard deviation of 15 determinations were less than 5 %. The recovery of standard addition of the method was over the range of 100.1-101.2 %.

Key Words: Bovine serum albumin, Protein, Potassium periodate, Dibromo-*p*-sulfonic acid arsenazo, Inhibitive kinetic spectrophotometry.

INTRODUCTION

Protein is a biological macromolecule that is made up of amino acid as basic unit. Each kind of natural protein has own spacial structure and this kind of spacial structure is usually called the conformation of protein[1]. As a content of protein in body fluid can be used as an important index of the nourishment health of human body and disease diagnosis, thus a quantitative determination of protein has thorough important stations in life science, clinical medicine and chemical investigations[2]. Methods for the determination of protein deal with spectrophotometry, fluorometric method, resonance light scattering photometry, *etc*. Spectrophotometry has the advantages of operation simplicity and low cost of instrumentation, *etc*. and is more suitable to the determination of protein and has higher practical value. The fragrance structure of residue of tyrosine, tryptophan and phenylalanine in protein molecule has absorption action to violet light. Its maximum absorption peak is near 280 nm, which can be used in the quantitative determination of protein content[3]. Dye probe method is namely dye combination method. As the stability of reagent is strong and experimental operation is simple, dye combination method has already become one of the broadest methods for the test of protein. Now it has been applied to the studies of clinical medicine and life sciences[4]. The commonly used dye probes mainly have triphenyl methane type dye, azo type dye, *etc*. Triphenyl methane type dye is an excellent probe reagent of performance for the determination of protein. For example, bromo cresol green, eriochromocyanine R have been reported for the determination of protein[5,6]. The azo type dye that have been reported for the determination of protein have thoron I[7], suffochlorophenol S[8], arsenazo K[9]. Li *et al*.[10], studied the change of spectral performance caused by the interaction of more than ten kinds of chromotropic acid azo dyes and proteins and discussed the effects of experimental conditions, dye structures and their mutual interations. After proteins combined with some dyes with fluorometric characteristic, the changes of fluorometric intensity could be produced and in a definite range the change is proportional to the concentration of protein. Therefore, the determination of protein can be used[2]. The fluorometric dye probes reported have 8-amidoxyl-1-naphthalene monosulfonic acid[11], eosin Y[12], erythrosine B[13], acridine red[14], α, β, γ, δ-tetra(4-carboxyphenyl)porphin[15]. In addition, rare earth complexes such as the complex of terbium can also be used as fluorometric probes to make the quantitative analysis of protein[16]. The commonly used resonance scattering probes organic dyes have:chrome azurol S[17], acid green[18], bromopyogallol red[19], 4-azochromotrope[20], trypan blue[21], quercetin[22] and PSbMo heteropoly blue[23]. Binary complex such as dibromomethyl-arsenazo-Al(III)[24], dibromohydroxyphenyl-fluorone-molybdenum(VI)[25] have been also used as resonance scattering probes. Huang *et al*.[26] method is based on resonance

light scattering enhancement effect of proteins on sodium dodecyl benzene sulfonate and established a method for the determination of proteins. Unfortunatelly, selectivities of these methods were not ideal. Development and establishment of a novel method for the determination of protein still have very important theoretical significance and applied value.

Kinetic spectrophotometry is a photometric analytical new method established based on the kinetic characteristic of colour reaction. Due to its high sensitivity and the simplicity of required instrumentation, all the time a rapid developing tendency has been kept and will be lasting. The range of its determination covers inorganic substance, organic substance. Especially, it is gained to the application in analysis of biology and medicine[27]. Kinetic spectrophotometry has a series of advantages of high sensitivity and low detection limit, *etc*. and this has already caused people's concern[28,29]. However, an application to study of the determination of protein is few[30]. In this study, dibromo-*p*-sulfonic acid arsenazo (DBS-ASA) was used as chromogenic agent. In the aryl group of DBS-ASA with -N=N- and many ligands containing N and O, the reagent not only has strong ability of chelation and can chelate with metallic ions to form various water soluble complexes, but also DBS-ASA contains -N=N- groups which themselves can produce colour. On oxidation or reduction, -N=N- group will be broken. This makes the colour of solution become weak or even colourless. This paper reports that in a $CH_2ClCOOH$-NH_4OAc buffer solution at pH 2 protein inhibits a decolouring reaction of DBS-ASA oxidized by KIO_4 and based on this a novel method for the determination of protein is developed. The sensitivity of the method is high, the operation is simple and the analytical cost is low. It has been used in the determination of total protein in egg white with satisfactory results.

EXPERIMENTAL

A 722S spectrophotometer (Shanghai Lingguang Technique Co. Ltd., China) and a HH4-digital thermostat water bath kettle (Jiangsu Jintan Ronghua Apparatus Manufacture Co. Ltd., China) were used for absorbance measurement and temperature control, respectively.

Bovine serum albumin (BSA, biochemical reagent, Beijing Aoboxing Biotechnology Co. Ltd., China), 500 μg/mL aqueous solution: an amount of 0.2500 g of BSA was weighed, placed into a 500-mL calibrated flask and diluted up to the mark with water to get 500 μg/mL BSA working solution, kept in cold storage at 2 ºC. Dibromo-*p*-sulfonic acid arsenazo (DBS-ASA, Shanghai Changke Reagent Research Institute, China) solution (5.0×10^{-4} mol/L): 0.0429 g of DBS-ASA was dissolved in 100 mL of water. KIO_4 solution (1.0×10^{-2} mol/L): 0.2300 g of KIO_4 was dissolved in 100 mL of water. $CH_2ClCOOH$-NH_4OAc buffer solution at pH 2.0:0.4725 g of $CH_2ClCOOH$ and 0.0385 g of NH_4OAc were dissolved in 100 mL of water. Emulsifier OP solution (0.5 %, v/v): 0.5 mL of OP was dissolved in 100 mL water. Absolute ethanol. The reagents were of analytical grade and the water was deionized water.

Recommend procedure: Two 10-mL comparison tubes were taken. To one of them was added 1.0 mL of 500 μg/mL BSA solution (inhibitive system), while to another one no BSA solution was added (non-inhibitive system). In turn, were added 0.50 mL of 0.5 % (v/v) OP solution, 1.2 mL of 5.0×10^{-4} mol/L DBS-ASA solution,1.5 mL of pH 2.0 $CH_2ClCOOH$-NH_4Ac buffer solution, 1.2 mL of absolute ethanol, 1.0 mL of 500 μg/mL BSA solution, 0.50 mL of 1.0×10^{-2} mol/L KIO_4 solution. The mixtures were diluted to the mark with water and mixed well. A water bath of 75 ºC was used to heat for 5 min. The mixtures were rapidly taken out and cooled by running water for 10 min. The abosrbance of non-inhibitive system (A_1) and the absorbance of inhibitive system (A_2) were measured at 600 nm with 1 cm cells using water as reference and $\Delta A = A_2 - A_1$ was calculated.

RESULTS AND DISCUSSION

Absorption spectra: Fig. 1 is the absorption curves of inhibitive system and non-inhibitive system. It can be known from the curve C the absorbance difference ΔA was maximum at 600 nm. At this time the sensitivity of the inhibitive reactive system was the highest. Therefore, this paper selected 600 nm as the measurement wavelength.

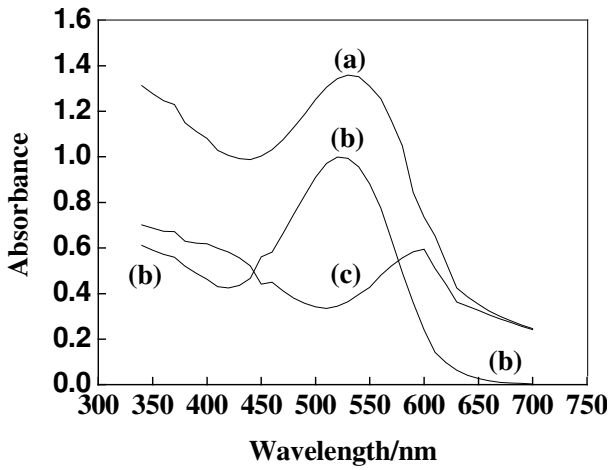

Fig. 1. Absorption spectra: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0×10^{-5} mol/L; [KIO_4] = 5.0×10^{-4} mol/L; [OP] = 0.025 % (v/v); [C_2H_5OH] = 2.1 mol/L; heating temperature T = 75 ºC; heating time t = 5 min

Effect of acidity: According to the procedure different pH buffer solution was added for experiments. By compsrison it is found (Fig. 2) that for the system at pH 2.0 ΔA was the largest. At this time net inhibitive reactive sensitivity was the largest. This paper selected pH 2.0 $CH_2ClCOOH$-NH_4OAc buffer solution system.

Effect of the amount of buffer solution: According to the standard procedure, 0.80, 1.0, 1.2, 1.5, 1.8 and 2.0 mL of pH 2.0 $CH_2ClCOOH$-NH_4OAc buffer solutions were, respectively added to make experiments. By comparison it is found (Fig. 3) that when the volume of $CH_2ClCOOH$-NH_4OAc buffer solution was 1.5 mL, ΔA showed a peak value. At this time net inhibitive reactive sensitivity was the largest. This paper selected the amount of $CH_2ClCOOH$-NH_4OAc buffer solution to be 1.5 mL.

Effect of the amount of OP: According to the standard procedure, 0.20, 0.30, 0.50, 0.80, 1.0, 1.2, 1.5 and 1.8 mL of 0.5 % (v/v) OP solution were, respectively added to make

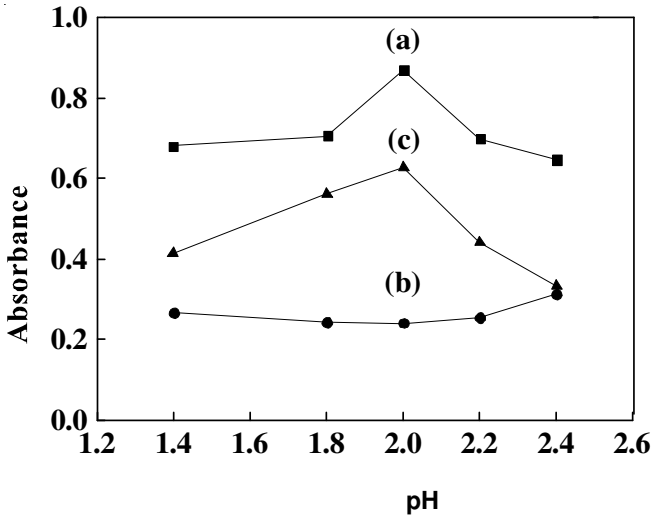

Fig. 2. Effect of acidity: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0 × 10^{-5} mol/L; [KIO_4] = 5.0 × 10^{-4} mol/L; [OP] = 0.025 % (v/v); [C_2H_5OH] = 2.1 mol/L; heating temperature T = 75 ºC; heating time t = 5 min

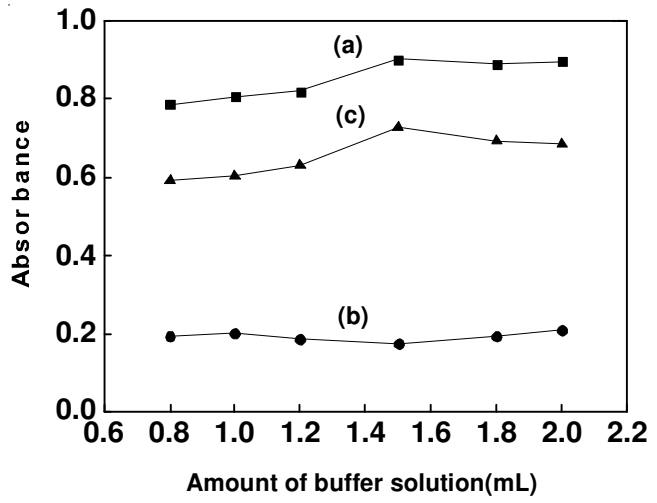

Fig. 3. Effect of the amount of buffer solution: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0 × 10^{-5} mol/L; [KIO_4] = 5.0 × 10^{-4} mol/L; [OP] = 0.025 % (v/v); [C_2H_5OH] = 2.1 mol/L; heating temperature T = 75 ºC; heating time t = 5 min

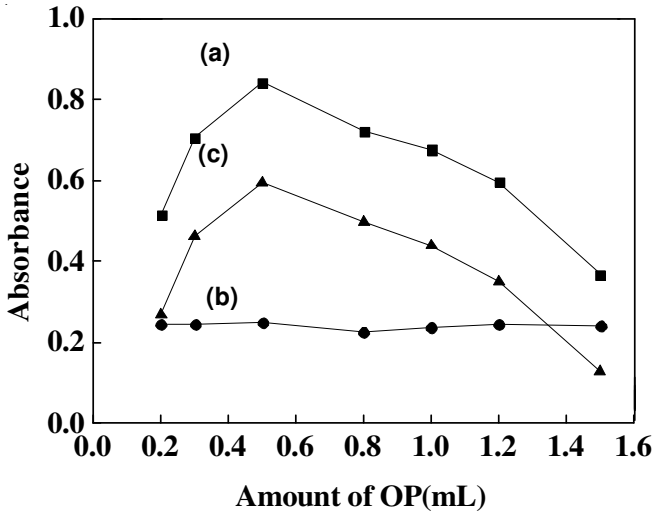

Fig. 4. Effect of the amount of OP: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0 × 10^{-5} mol/L; [KIO_4] = 5.0 × 10^{-4} mol/L; [C_2H_5OH] = 2.1 mol/L; heating temperature T = 75 ºC; heating time t = 5 min

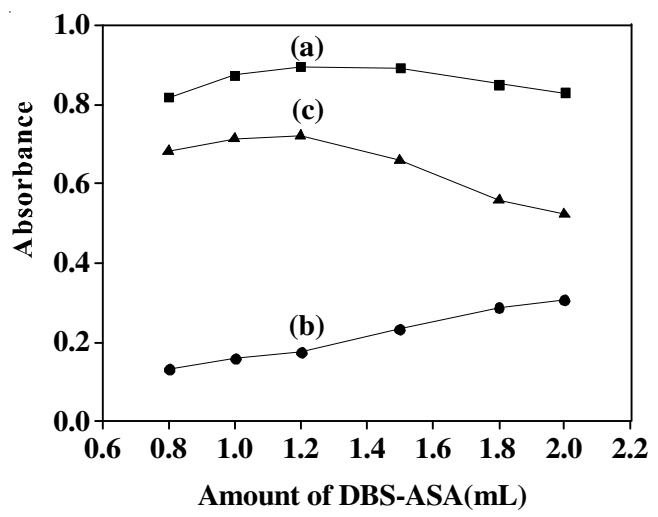

Fig. 5. Effect of the amount of dibromo-*p*-sulfonic acid arsenazo: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 50.0 μg/mL; [KIO_4] = 5.0 × 10^{-4} mol/L; [OP] = 0.025 % (v/v); [C_2H_5OH] = 2.1 mol/L; heating temperature T = 75 ºC; heating time t = 5 min

experiments. By comparison it is found (Fig. 4) that when the addition amount of 0.5 % (v/v) OP solution was 0.5 mL, ΔA was maximum. At this time net inhibitive reactive sensitivity was the highest. This paper selected the amount of 0.5 % (v/v) OP solution to be 0.5 mL.

Effect of the amount of DBS-ASA: According to the standard procedure, 0.80, 1.0, 1.2, 1.5 and 1.8 mL of 5.0 × 10^{-4} mol/L DBS-ASA solution were, respectively added to make experiments (Fig. 5). At 1.2 mL, absorbance difference ΔA was maximum. At this time net inhibitive reactive sensitivity was the largest. This paper selected the amount of 5.0 × 10^{-4} mol/L DBS-ASA solution to be 1.2 mL. At this time in the 10 mL system the concentration of DBS-ASA was 6.0 × 10^{-5} mol/L.

Effect of the amount of ethanol: According to the standard procedure, 0.50, 0.80, 1.0, 1.2, 1.5 and 1.8 mL of ethanol were, respectively added to make experiments (Fig. 6). At 1.2 mL, absorbance difference ΔA was maximum. At this time net inhibitive reactive sensitivity was the largest. In present work, the amount of ethanol to be 1.2 mL. In the 10-mL system the concentration of ethanol was 2.1 mol/L.

Effect of the amount of KIO_4: According to the standard procedure, 0.10, 0.20, 0.50, 0.80 and 1.2 mL of 1.0 × 10^{-2} mol/L KIO_4 solution were, respectively added to make experiments (Fig. 7). At 0.50 mL, ΔA was maximum. At this time net inhibitive reactive sensitivity was the largest. This paper selected the amount of 1.0 × 10^{-2} mol/L KIO_4 solution to be 0.50 mL. At this time, in the 10-mL system the concentration of KIO_4 was 5.0 × 10^{-4} mol/L.

Effect of heating temperature: According to the procedure the water baths of 20, 30, 40, 50, 60, 75, 80, 85, 90 and 100 ºC were, respectively used to heat in experiment using

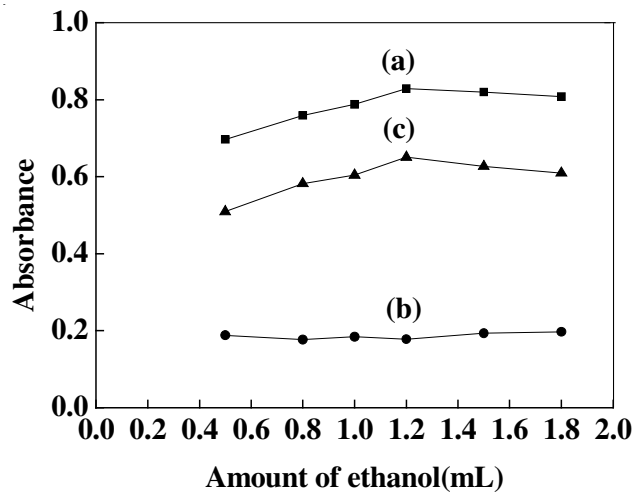

Fig. 6. Effect of the amount of ethanol: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0×10^{-5} mol/L; [KIO_4] = 5.0×10^{-4} mol/L; [OP] = 0.025 % (v/v); heating temperature T = 75 ºC; heating time t = 5 min

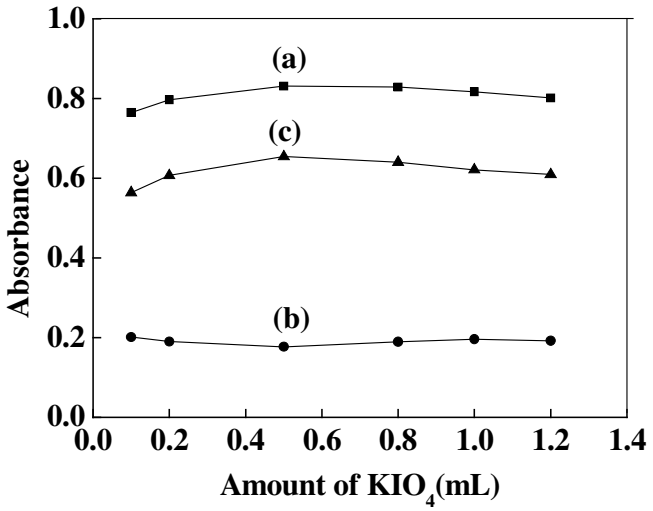

Fig. 7. Effect of the amount of potassium periodate: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0×10^{-5} mol/L; [C_2H_5OH] = 2.1 mol/L; [OP] = 0.025 % (v/v); heating temperature T = 75 ºC; heating time t = 5 min

reagent blanks as references (Fig. 8). ΔA achieved maximum at 75 ºC and at this time the sensitivity of the inhibitive reaction was the highest. The optimum temperature of the water bath is 75 ºC. A regression analysis of the obtained data was made (Fig. 9) to obtain linear regression equation:log (A_2/A_1) = 780.1/T(K) - 2.960 (A_2: absorbance of inhibitive system, A_1: absorbance of non-inhibitive system, T: Kelvin temperature of system), with a correlation coefficient of γ= 0.9947.According to the slope of the equation the activation energy of the inhibitive reaction was obtained to be Ea = 6.49 kJ/mol.

Effect of heating time: According to the procedure heating was made for 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 5.5 and 6 min, respectively and contrast was made with blank reagent (Fig. 10). The results showed that a linear relationship was presented between the range of ΔA, 0.75-5 min and heating time. Its linear regression equation was: ΔA = 0.1106 t + 0.1552 (t: min), with a correlation coefficient of γ = 0.9952. At 5 min,

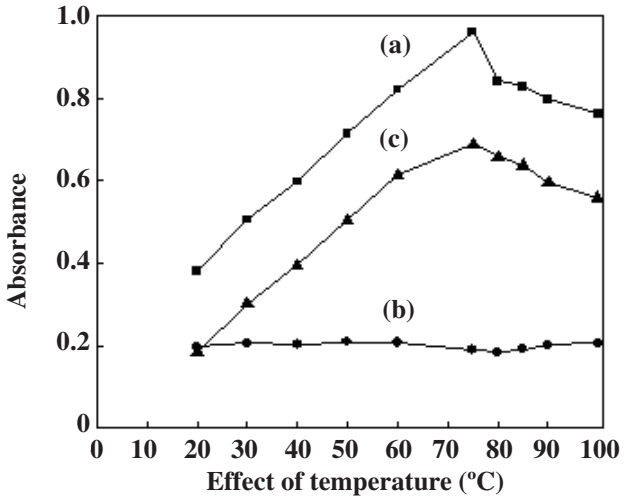

Fig. 8. Effect of temperature: (a) Inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0 × 10-5 mol/L; [KIO_4] = 5.0×10^{-4} mol/L; [C_2H_5OH] = 2.1 mol/L; [OP] = 0.025 % (v/v); heating time t = 5 min

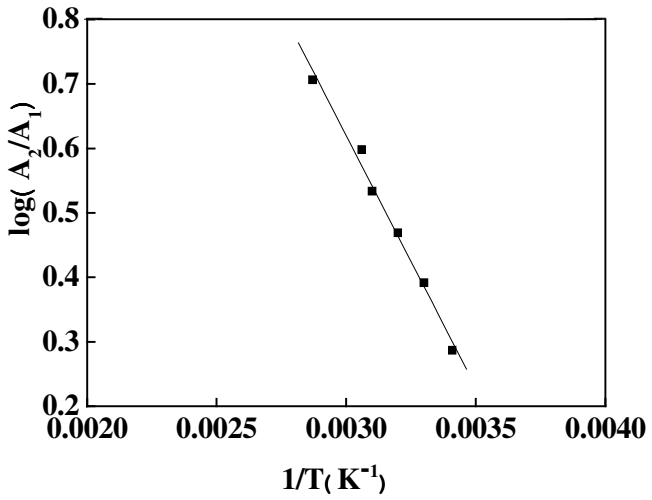

Fig. 9. Effect of temperature: [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0×10^{-5} mol/L; [KIO_4] = 5.0×10^{-4} mol/L; [C_2H_5OH] = 2.1 mol/L; [OP] = 0.025 % (v/v); heating time t = 5 min

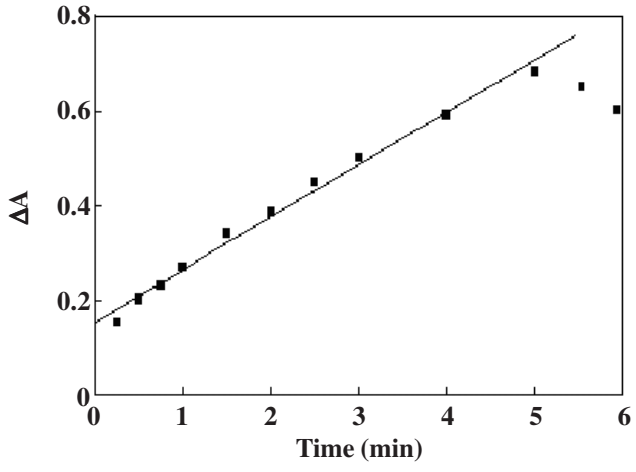

Fig. 10. Effect of heating time: (a) inhibitive reaction; (b) non-inhibitive reaction; (c) net inhibitive reaction; [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0×10^{-5} mol/L; [KIO_4] = 5.0×10^{-4} mol/L; [C_2H_5OH] = 2.1 mol/L; [OP] = 0.025 % (v/v); heating temperature T = 75 ºC

ΔA was maximum. The net inhibitive reactive sensitivity was the highest at this time. The optimum time was 5 min. Plotting of log (A_2/A_1) against t was made (Fig. 11) and its linear regression equation obtained was log (A_2/A_1) = 0.0765 t (min) + 0.2450 (A_2 is the absorbance of inhibitive system, A_1 is the absorbance of non-inhibitive system, t was heating time) with a correlation coefficient of γ = 0.9941. The rate constant of reaction was $K = 1.86 \times 10^{-3}$ s^{-1}. The half-period was $t_{1/2}$ = 1.64 min.

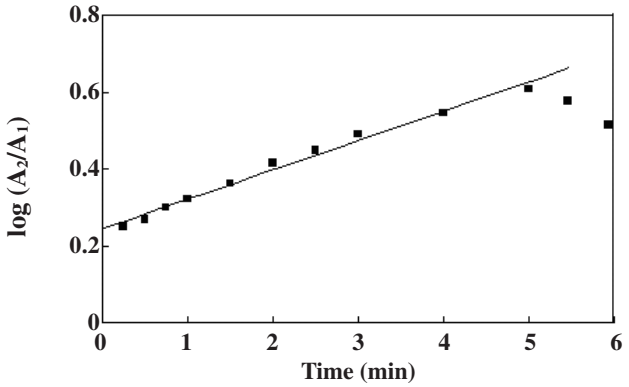

Fig. 11. Effect of heating time: [BSA] = 50.0 μg/mL; [DBS-ASA] = 6.0×10^{-5} mol/L; [KIO_4] = 5.0×10^{-4} mol/L; [C_2H_5OH] = 2.1 mol/L; [OP] = 0.025 % (v/v); heating temperature T = 75 ºC

Stability of system: Under the optimum experimental conditions, for the determination of 50.0 μg/mL BSA the results showed that with 3 h, ΔA was stable and its variation was within ± 5 %.

Effect of co-exisitng ions: The effect of co-existing substances on the determination of 400.0 μg BSA in 10 mL system was studied under the optimum conditions. A relative error of ± 5 % was controlled and the allowable amounts of coexisting substances (multiple in mass, m/m) are as follows: glycine (5); Li^+, malic acid (2.5); ascorbic acid (2); Zn^{2+}, glucose (0.5); Ti^{4+}, Fe^{2+}, Mo^{6+} (0.1); Al^{3+}, Co^{2+}, W^{6+}, Br^-, PO_4^{3-}, citric acid (0.05); urea (0.02); Ce^{4+}, Th^{4+}, Si^{4+}, Eu^{3+}, La^{3+}, Sr^{2+}, Ni^{2+}, VO_3^-, S^{2-}, EDTA, acetic acid (0.01); Fe^{3+}, Cr^{3+}, Ga^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, I^- (0.005); Cu^{2+} (0.002); BO_3^-, Cr^{6+}, Hg^{2+}, Pb^{2+}, Ba^{2+}, oxalic acid (0.001); MnO_4^- (0.0005); Bi^{3+} (0.0001).

Working curve: Under the optimum conditions the experiments of linear range were made. A definite amount of protein standard solution was, respectively added and blank reagent was used for contrast. The results showed (Fig. 12) that under the optimum conditions in a 10-mL solution the mass of BSA over the range of 25-900 μg (2.5-90.0 μg/mL) with ΔA presented a linear relationship and its linear regression equation was: ΔA = 0.01091C + 0.0646 (C: μg/mL), with a correlation coefficient of γ = 0.9941. Thirteen parallel determinations of 50.0 μg/mL BSA were made and a relative standard deviation (RSD) = 0.713 %. Eleven determinations of a blank reagent were made and the standard deviation obtained was 0.176 %. The detection limit of the method 3S/K was 0.48 μg/mL (S is the standard deviation of deteminations of 11 blank reagent, K is the slope of working curve).

Reaction mechanism: In the aryl group of DBS-ASA with -N=N- and many ligands containing N and O, the reagent can chelate metallic ions to form various water soluble complexes,

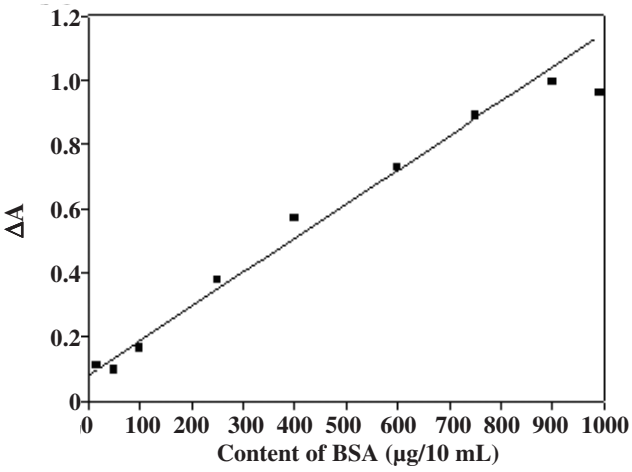

Fig. 12. Calibration curve: [DBS-ASA] = 6.0×10^{-5} mol/L; [KIO_4] = 5.0×10^{-4} mol/L; [C_2H_5OH] = 2.1 mol/L; [OP] = 0.025 % (v/v); heating temperature T = 75 ºC; heating time t = 5 min

but also DBS-ASA contains -N=N- groups which themselves can produce colour. On oxidation or reduction -N=N- group will be broken. This makes the colour of solution become weak or even colourless. Under acidic conditions, the amido group of the side chain in BSA is protonated and for the protonated protein with positive charge an association reaction with DBS-ASA occurred due to electrostatic attraction. As the amido group was uniformly distributed in protein, DBS-ASA interacted with amido group into the protein structure, which protected the chromophore -N=N- and inhibited the process of fading. The first structure of protein and the structure of DBS-ASA are given in Figs. 13 and 14, respectively. The oxidation reaction is given in Fig. 15. The inhibitive reaction is shown in Fig. 16.

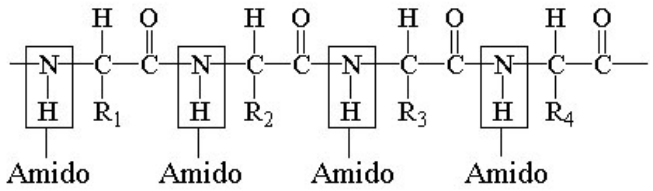

Fig. 13. First structure of protein

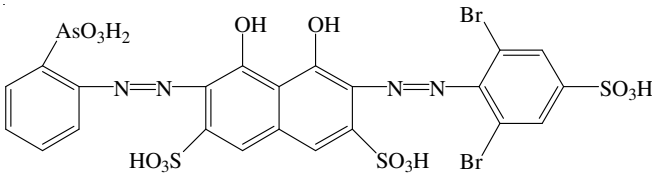

Fig. 14. Structure of dibromo-*p*-sulfonic acid arsenazo

Analysis of sample: 1 mL of egg white was taken, in a 100-mL calibrated flask and diluted to the mark with water. 25 mL of the solution was taken and diluted to 100 mL to obtain the solution that would be determined. 2 mL of the solution was taken and determined according to the standard procedure. The results of determination of total proteins in the samples are seen in Table-1.

Conclusion

The protein inhibitive decolouring reaction of dibromo-*p*-sulfonic acid arsenazo oxidized by KIO_4 and its optimum

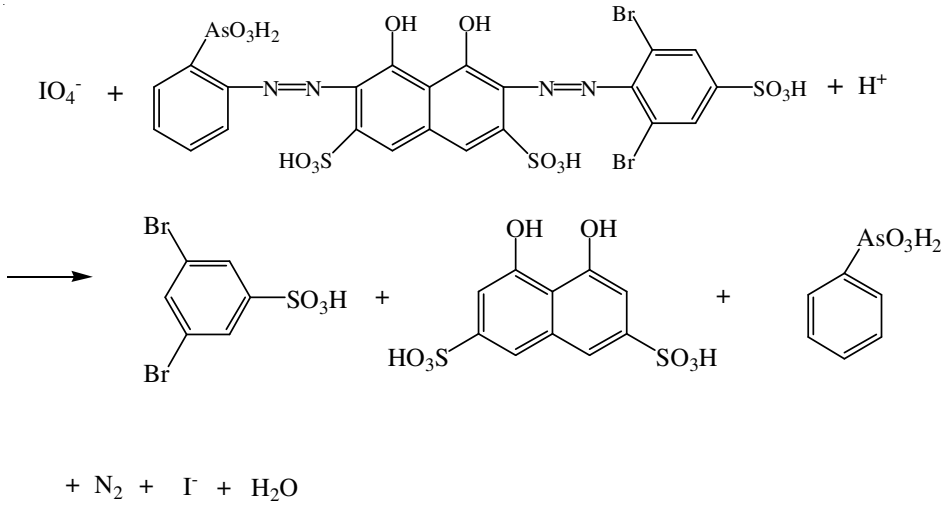

Fig. 15. Oxidation reaction

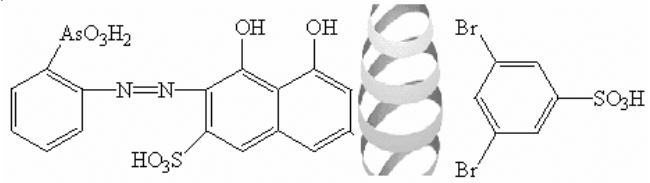

Fig. 16. Inhibitive reaction

experimental conditions were studied in this paper. Protein can protect the -N=N- of DBS-ASA from KIO_4 oxidation. In the $CH_2ClCOOH$-NH_4OAc buffer medium,the maximum absorption peak of BSA-(DBS-ASA)-KIO_4 system is located at 530 nm. The good linear relationship was showed between the range of 2.5-90 μg/mL for protein and the absorbance difference and its linear regression equation of the method was: $\Delta A = 0.01091C + 0.0646$ (C: μg/mL), with a correlation coefficient of $\gamma = 0.9941$. The protein contents in egg whites were successfully determined by the method.

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TABLE-1
ANALYTICAL RESULTS OF SAMPLES

Sample	Found (mg/mL)		Average (mg/mL)	RSD (%)	Added (μg/mL)	Recovered (μg/mL)	Recovery (%)	Contrast method (mg/mL)[30]
1	31.62	31.25	31.44	2.167	25.00	25.31	101.2	31.44
	31.80	30.34						
	32.17	30.89						
	30.52	29.60						
	33.09	28.50						
	32.35	31.07						
	32.17	31.25						
	30.89	–						
2	28.14	28.69	28.32	2.653	25.00	25.03	100.1	28.31
	28.32	29.42						
	30.70	26.12						
	27.77	29.60						
	28.50	27.40						
	28.32	27.59						
	27.77	28.32						
	28.87	–						

*Contrast method was *p*-acetylchlorophosphonazo indicator kinetic spectrophotometry.

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