ISSN : 0974 - 7427 Volume 7 Issue 4

BIOCHEMISTRY
An Indian Journal

Regular Paper

BCAIJ, 7(4), 2013 [125-130]

Microdetermination of proteins based on enhancement effect of Rayleigh light scattering of hematoxylin in hot water bath

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ABSTRACT

A highly sensitive Rayleigh light scattering (RLS) method has been developed for the determination of protein concentrations in human serum album (HSA) and bovine serum album (BSA) based on the reactions of HSA and BSA with oxidized hematoxylin. One of the distinguishing features of this method proposed here was heating the mixture of protein and hematoxylin at pH 4.40 in 80 °C water bath for 15 minutes before assay. And another interesting phenomenon observed was that the addition of hematoxylin to the protein solution could prevent protein from deposition in hot water. Under the optimal experimental conditions, the greatly enhanced RLS of dye-protein was linearly. The linear range of the determination is 0-6.2 mg/L for HAS, and the detection limit is 34 μg. The foreign substances, such as urea, amino acids and metal ions, have little effect on the determination.

KEYWORDS

Rayleigh light scattering;
Hot water bath;
Oxidized hematoxylin;
Protein;
Microdetermination.

INTRODUCTION

Elastic light scattering can be divided into two categories[1]: when the scale is much smaller than the incident light wavelength of scattering particles (dimension of the scattering particles smaller than 0.1 λ), it is known as Rayleigh scattering; if the Rayleigh scattering located near the absorption, it is likely to cause scattering intensity increased dramatically, and this phenomenon is known as Rayleigh scattering[2]. Pasternack[3] had put forward a method for the determination of nucleic acid based on the resonance light scattering technique, which use ordinary fluorescent photometer for determination, overcoming traditional light scattering technique sensitivity low, the disadvantages of instruments and experimental conditions, and this method opened up a new field for the research on biological macromolecular as the light scattering probe.

The determination of proteins is a basic requirement in biochemistry, because it is related to nourishment, cellularity, enzyme, hormone, virus, epidemic disease, inheritance, vital genesis, and so on. In recent years, many dyes have been used for protein determination, such as Coomassie Brilliant Blue (CBB), Lowry and Bromcresol Green (BCG) methods. A sensitive method based on enhanced Rayleigh light scattering (RLS)[1-3] has been developed. RLS has been used to measure metal ions[4] and biological molecule[5]. On in-

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vestigating the interaction between hematoxylin (its chemical structure shown in Figure1[6]) and proteins in hot water bath, we surprisingly found that the RLS of dye-protein system was greatly enhanced compared with pure dye. In this paper, detailed study of the appropriate conditions has been conducted and we found that the values of enhancement were linear with the concentrations of proteins.

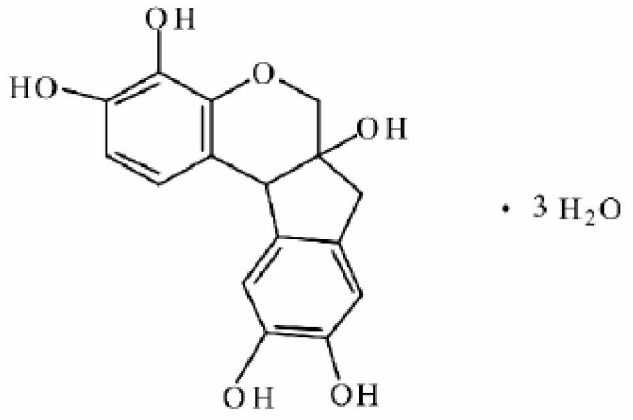

Figure 1

EXPERIMENTAL

Apparatus

The spectrum and the intensity of RLS were obtained with a RF-5301PC fluorescence spectrometer (Shimadzu, Japan) with a 150 W xenon lamp and a 1 cm quartz cell. The absorption spectra in visible light range were recorded with a Shimadzu (Kyoto, Japan) UV-265 Spectrophotometer. The pH measurements were made with a pHS-3C meter (Shanghai, China).

Reagents

Hematoxylin (Hmx) was obtained from Shanghai Chemical Reagent Company (China) and was directly dissolved into water to prepare a solution of 5×10^{-4} mol/L, and then it was heated in 80 °C water bath for one hour. The solution was only used the day it prepared. Tween-20 (Farco, chemical supplies), dodecylbenzene sulfonic acid sodium salt (shanghai chemical reagent purchasing station), and cetyl trimethyl ammonium bromide (CTMAB, Beijing chemical plant) were used. Britton-Robinson buffer (pH=4.40) was composed of 1000mL acid mixture (2.71 mL H_3PO_4, 2.36 mL CH_3COOH, 2.47 g H_3BO_3) and 275 mL NaOH (0.2 mol/L).

Bovine serum albumin (BSA, Beijing Aoboxing Biology Technology Company, the purity grade inferior 99.9%) and egg albumin (Alb) were directly dissolved in water to prepare stock solutions of 620 μg/mL, and stored at 0-4 °C. Working solutions were freshly prepared by appropriate dilution with water before use. The precise concentrations were determined spectrophotometrically at 280 nm with the $\varepsilon^{1\%}$ (the absorbency of 1% m/v solution with a 1 cm cell, $cm^{-1}mL^{-1}g$) values for BSA: 6.6[7,8], HSA: 5.3[7], Alb: 7.5[8]. The concentrations of the stock solutions were that BSA: 61 mg/L, HSA: 58 mg/L, Alb: 52 mg/L.

All other chemicals were analytical-reagent grade or the best grade commercially available. Human serum samples were obtained from Hebei workers' Medical College (Baoding, China). The serum samples were diluted 100,000-fold with water to prepare stock standard solutions, which were stored at 0-4 °C. All stock solutions of proteins and chemicals were prepared in doubly de-ionized water.

Standard procedure

A certain volume of proteins standard solutions, oxidized hematoxylin, 2.0 mL buffer and 0.3mL dodecylbenzene sulfonic acid sodium salt solution were added to a 10 mL volumetric flask, diluted with water, and then put it into 80 °C water bath for 15 minutes. The RLS spectra were obtained with the excitation and emission monochromators of the spectrofluorimeter scanned synchronously (0.0 nm interval between excitation and emission wavelength) with the wavelength range 200-700 nm. All measurements were obtained against a blank treated in the same way without proteins. Based on these spectra, the intensity of RLS was measured with excitation and emission wavelengths at 510.0 nm.

RESULTS AND DISCUSSION

Reaction and spectral characteristics

The absorption spectra of hematoxylin (Figure 2) have been studied. There were two peaks in ultraviolet light range, while a peak appeared at 443.0 nm after hematoxylin solution was heated and the two peaks in ultraviolet light range became higher. When

protein was added in hematoxylin solution, the absorption at 443.0 nm became lower. It indicated that BSA was bounded to hematoxylin, but the change was too slight to determine. Hematoxylin is an unsteady dye. It can be oxidized by hydrogen peroxide in weakly acidic medium or by trace ruthenium (III)[9]. Water bath can also promote its oxidation, so 80 °C water bath was chosen. The oxidized hematoxylin spectrum changed slightly when BSA was added; this change was more distinct when the system was heated in 80°C water bath for 15 minutes. It can be seen from figure 3, which the scattering intensity of oxidized hematoxylin was greatly enhanced when BSA was added. It indicated that there was interaction between oxidized hematoxylin and proteins after the mix solution was heated. According to the comparison of absorption and RLS spectra of the heated or unheated mixed solution system, hot water bath has a great influence on the RLS spectrum of the system.

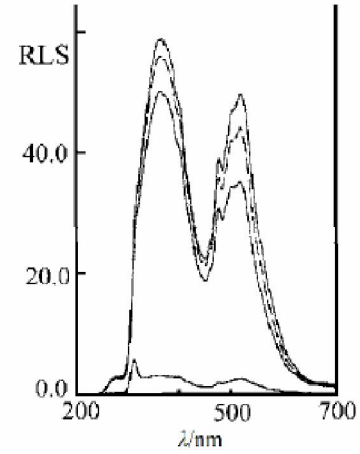

Figure 2

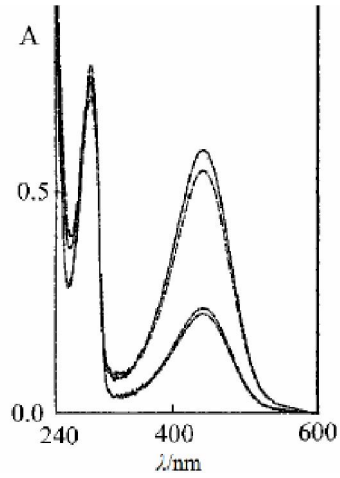

Figure 3

Temperature and time of the reaction

According to the experiment results, we found that the rate of the reaction between oxidized hematoxylin and proteins was very slow and hot water bath can accelerate it. As is known to all that the changes of the temperature can affect the structure of the protein, but the effect may be reversible when the pH of the solution was below 6.0. Some experiments were made in different temperatures (50, 60, 70, 80, 90 °C) with different reaction time including 3, 5, 10, 15, 20, 30, 50 minutes, but no obvious rule was obtained. When hematoxylin was added into protein, protein flocculation and precipitation can be prevented efficiently in high temperature. According to the experiment using oxidized hematoxylin to determine the concentration of Fe[10], we chose 80 °C water bath for 15 minutes in the whole experiment.

Effect of solution acidity

Figure 4 shows that the values of pH significantly affected the RLS intensity of blank oxidized hematoxylin and Hmx-BSA. At the range of 1.81-3.12, the RLS intensity was very high and changed slightly. While in 3.12-4.46, the RLS intensity decreased quickly with the values of pH increasing. The experiment results indicated that the peak of Hmx-BSA complex at 510 nm will move to 470 nm at low values of pH. When the system was heated, some proteins would be metamorphosed and the RLS intensity became very large, the RLS spectrum of Hmx-BSA complex at low values of pH was the same as the spectrum of blank proteins. At pH 4.40 the peak was sharp and far from the peak of degenerative proteins. In order to avoid the interference of degenerative proteins, pH 4.40 was chosen even if some sensitivity would be lost.

Effect of oxidized hematoxylin concentration

The RLS intensities of solutions containing various concentrations of oxidized hematoxylin and BSA were measured (Figure 5). We found that the concentration of oxidized hematoxylin affected the RLS sensitivity. When the concentration was below 1.5×10^{-4} mol/L, the RLS intensity was greatly high. So 1.5×10^{-4} mol/L hematoxylin was selected.

Effect of surfactants

Surfactants affected the intensity of RLS seriously.

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When the concentration of CTMAB is below 0.03%, it can reduce the ability of hematoxylin bounding to proteins. As CTMAB bearing negative charge neutralized the positive charge of the proteins, these resulted in a decrease of scattering intensity. But the influence of Tween-20 was slightly in hematoxylin-proteins system. When dodecylbenzene sulfonic acid sodium salt was added into the system (Figure 6), the RLS intensity of system was affected especially in the concentration ranging from 0.2×10^{-3} g/L to 0.4×10^{-3} g/L. So 0.3×10^{-3} g/L for dodecylbenzene sulfonic acid sodium salt was selected in the following experiments.

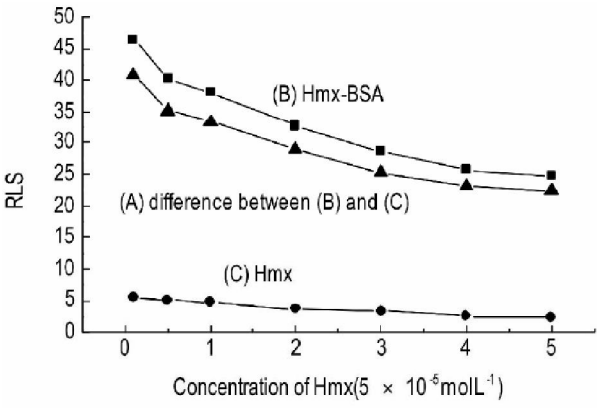

Figure 5

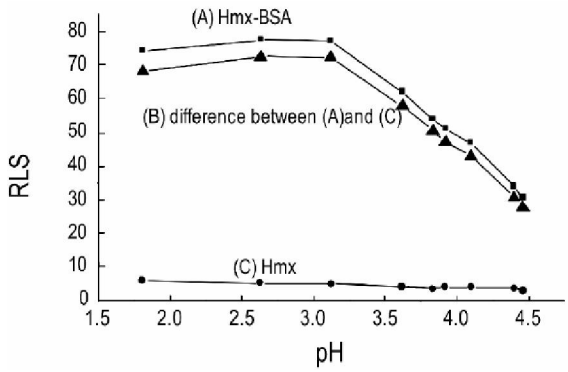

Figure 4

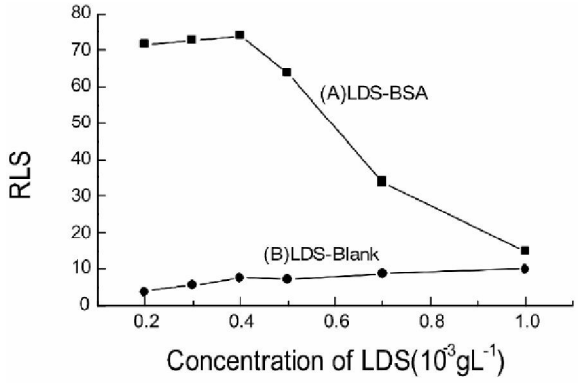

Figure 6

Effect of interfering substances on RLS

The interference of various metal ions has been studied in detail and the statistical evaluation of the experimental results was reported. Some of the metal ions, such as Al^{3+} [11], Ni^{2+} [12], Pb^{2+} [13], Ru^{3+} [9] can react with hematoxylin, but the concentrations of these ions were too low to interfere the determination. As is shown in TABLE 1, a few of basic amino acids (arginine, lysine, histidine, etc.) interfered in this assay. However, it can be ignored after the addition of the buffer. So the special prepara-

TABLE 1 : Effect of interfering substances on RLS

Interference	Concentration /μg·mL^{-1}	Change in RLS /%	Interference	Concentration /μg·mL^{-1}	Changes in RLS /%
L-His	40	-7.83	L-Leu	40	-0.37
L-Ile	40	-2.25	L-Arg	40	-7.9
L-Asp	40	-0.23	L-Lys	20	-5.2
L-Gln	40	4.4	L-α-Ala	40	-0.51
L-Val	40	-6.3	Glucose	100	-1.2
L-Thr	40	-3.2	Urea	800	-4.42
L-Pro	20	-2.5	HCO^-_3, Na^+	10	-3.6
L-Tyr	40	-3.0	Zn^{2+}, SO_4^{2-}	10	4.2
L-Met	40	0	SO_4^{2-}, Na^+	10	2.2
L-Trp	40	-14.5	K^+, Cl^-	100	-0.26
L-Gly	40	-5.52	Na^+, Cl^-	10	4.8
L-Ser	40	-2.38	Ca^{2+}, Cl^-	10	6.27
L-Asn	40	-4.2	$NH_4Fe(SO_4)_2$ •$12H_2O$	0.2	0.4
L-Phe	40	-4.0			

tion was not performed before sample determination. The concentration of the interference we investigated was greatly larger than that in blood of human being, which had diluted 100,000 fold (TABLE 2).

TABLE 2 : Concentration of electrolyte in blood[14]

		In blood / mmol·L^{-1}	Diluted 100,000 fold /μg·mL^{-1}
Positive ions	Na^+	145	3.3×10^{-2}
	K^+	4.5	1.8×10^{-3}
	Mg^{2+}	0.8	1.9×10^{-4}
	Ca^{2+}	2.5	1.0×10^{-3}
Negative ions	Cl^-	103	4.6×10^{-4}
	HCO_3^-	27	1.6×10^{-2}
	HPO_4^{2-}	1	9.6×10^{-4}
	SO_4^{2-}	0.5	4.8×10^{-4}

Calibration graphs and sensitivity

The results for the determination of the proteins (BSA, HSA, Egg albumin) are shown in TABLE 3. A satisfactory linear relationship between changes of RLS and proteins concentrations with a high regression coefficient and a wide linear range was obtained.

TABLE 3 Standard regression equations of various proteins

Protein	Standard Regression equation	Linear range /mg·L	Regression coefficient	Determination Limits /μg·L
BSA	I=11797C(g/L)－4.766	0~15.5	0.9993	30.7
HSA	I=10650C(g/L)－1.677	0~6.2	0.9967	34
Alb	I=2121C(g/L)－0.176	0~15.5	0.9975	171

Application to sample analysis

By using the present method and choosing HSA as the standard, proteins in human serum were measured. Compared with the data from Hebei workers' Medical College (Baoding, China), the results are satisfactory (TABLE 4).

TABLE 4 : Determination results for human serum samples

Sample No.	Albumin		
	C^a/g·L	C^b/g·L	RSD/%
1	72.4	68.9	5.62
2	65.6	68.2	8.69
3	72.1	70.2	7.78
4	62.1	67.7	6.26
5	74.3	68.5	3.53

[a] Data obtained from the Hospital of Hebei Zhigong Medical College; [b] This work

CONCLUSIONS

In this paper, a highly sensitive Rayleigh light scattering (RLS) method has been developed for the determination of protein concentrations in human serum album (HSA) and bovine serum album (BSA) based on the reactions of HSA and BSA with oxidized hematoxylin. One of the distinguishing features of this method proposed was heating the mixture of protein and hematoxylin at pH 4.40 in 80 °C water bath for 15 minutes before assay. Without this procedure the interaction between protein and protein was so weak that it was difficult to observe their change in the Rayleigh light scattering spectra. And another interesting phenomenon observed was that the addition of hematoxylin to the protein solution can prevent protein from deposition in hot water. The greatly enhanced RLS of hematoxylin after addition of protein was linearly dependent on the protein concentration in the range of 0-6.2 mg/L for HSA. The foreign substances, such as urea, amino acids and metal ions, have little effect on the determination of proteins RLS. This method has been applied to the determination of proteins in human serum, and the results were extremely close to those obtained by Biuret method.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of National Science Foundation of China (Grant no. 20675024) and Hebei Provincial Key Basic Research Program (Grant no. 10967126D).

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