Determination of Bovine Serum Albumin by Its Enhancement Effect of Nile Blue Fluorescence

Sang Hak Lee,* Jung Kee Suh,† and Ming Li

Department of Chemistry, Kyungpook National University, Daegu 702-701, Korea
†Korea Research Institute of Standards and Science, P.O. Box 102, Daejon 305-600, Korea
Received August 5, 2002

A novel fluorimetric method has been developed for the determination of microgram quantities of bovine serum albumin (BSA) based on its enhancement effect of Nile Blue fluorescence at 670 nm, caused by binding of Nile Blue to BSA to produce a stable water soluble complex. The binding constant of micromole Nile Blue-BSA complex was estimated by Scatchard plot method. Under the optimal conditions, the increased fluorescence intensity was linearly related to BSA concentration in the range of 0.5-12.0 μg/mL. The detection limit was 0.2 μg/mL, and the relative standard deviation of six replicate measurements was 1.4% for 10.0 μg/mL BSA. There was little interference from amino acids, sugars and most of metal ions.

Key Words : Bovine serum albumin, Nile Blue, Fluorimetry

Introduction

The determination of protein is very important in clinical diagnostics, pathology, immunology and biochemistry because it is often used as a reference for the measurements of other components in biological system. A variety of methodologies to determine proteins have been used, such as Biuret method, Lowry method,[1] Bradford method[2-4] and Bromocresol Green method.[5] However, these methods are limited by sensitivity, dynamic range, detection limit or sample treatment. The Lowry method has low sensitivity, poor selectivity and a complicated measurement procedure. The Bradford method has disadvantages such as poor linearity between the absorbance of the dye (Coomassie Brilliant Blue G-250)-protein complex and the concentration of protein and the inconvenience in operation. The Bromocresol Green method is liable to disturbance by turbidity and is not sensitive enough for trace analysis. These limitations are now partially overcome by some new methods, such as mass detection method, Rayleigh light scattering method,[6-8] electrochemical method,[9-13] chemiluminescence method.[14-16] Electrochemical methods have only seldom been used in the protein analysis because of a strong protein adsorption at electrode surfaces, even with rather dilute solution. LC-ESI-MS[17] and LC-MALDI-MS[18-21] also have been used for the determination of proteins to obtain low detection limit but these instruments are usually highly expensive. Moreover, these methods are still not free from various types of inherent interferences. Rayleigh light scattering methods are unsuitable for the protein determination in an unknown sample because the method encounters interference by many chemicals.

Since organic dyes can serve as effective probes of the structures and functions of biological macromolecules, interest has been raised in the study of the interaction of dyes with proteins or nucleic acids in recent years. Various new dye-binding methods have been put forward for protein analysis by chemiluminescence method, spectrophotometric and fluorimetric methods. Some dyes such as Magdala Red[22] and Rose Bengal[23] have been used to develop the protein quantification.

In this paper, we used Nile Blue as a ligand which is a cationic dye with planar and rigid structure. Nile Blue emits at 670 nm when it is excited at 635 nm. We also used Triton X-100 to further increase the fluorescence intensity of Nile Blue-BSA complex. This phenomenon can be exploited for development of a new method for determination of BSA. The optimum analytical conditions for pH, concentration of Nile Blue and concentration of surfactant to determine BSA were investigated. The binding constant of Nile Blue-BSA complex was estimated. Interferences from selective amino acids, sugars and some metal ions were also studied.

Experimental Section

Reagents. Bovine serum albumin (fraction V) was purchased from Sigma (St. Louis, MO, USA). The stock solution of 100.0 μg/mL bovine serum albumin in redistilled deionized water was prepared and stored in refrigerator. Nile Blue and Triton X-100 was obtained from Aldrich Co. (Milwaukee, WI, USA). The stock solution of 5.0×10^{-4} M Nile Blue was prepared by dissolving an appropriate amount of reagent in 5% ethanol solution. These stock solutions were kept in amber-colored bottles in the dark. The stock solution of 1.0×10^{-2} % Triton X-100 was prepared by dissolving an appropriate amount of reagent in deionized water. All the other chemicals were of analytical reagent grade and were used as received. Deionized water was obtained by means of a Millipore (Bedford, MA, USA) Milli-Q water system.

Instrumentation. All fluorescence measurements were made with a Spex (Edison, NJ, USA) Model FL111 spectrofluorimeter. A 450 W xenon lamp and a Hamamatsu Model

*To whom correspondence should be addressed: Phone: +82-53-950-5338; Fax: +82-53-950-6330; e-mail: shlee@knu.ac.kr

R928 photomultiplier tube were used. The voltage used for the photomultiplier tube was 900 V. The acquisition mode used was scan mode. The integration time and slit width was 1 s and 1 mm, respectively. A Mettler MA235 pH/Ion analyzer was used for pH measurements.

Measurement procedure. To a 10 mL volumetric flask, 2.0 mL phosphate buffer solution of pH 6.8, 1.0 mL of 3.3×10^{-5} M Nile Blue solution, 1.0 mL of 1.5×10^{-3} % Triton X-100 solution, 1 mL of 0.1 M NaCl solution and an appropriate amount of BSA working solution were added in turn. NaCl solution was used to adjust the ionic strength of the aqueous solution. The mixture was diluted to 10 mL with water and mixed throughly. The final solution was allowed for 2 min to incubate the solution. The fluorescence intensities of the sample and the blank (prepared in a similar manner without protein) were measured at 670 nm at 20 °C. The excitation wavelength used was 635 nm.

Results and Discussion

Spectral characteristics. The emission spectra of a 3.3×10^{-5} M Nile Blue solution at pH 6.8 containing 1.5×10^{-3} % Triton X-100 and different amounts of BSA are shown in Figure 1. The excitation wavelength was chosen since the fluorescence excitation spectrum showed its maximum at 635 nm. The emission spectra of the Nile Blue-BSA-Triton X-100 system give a broad band with its maximum at 670 nm. As shown in Figure 1, the emission wavelength did not appreciably changed with the addition of BSA in the concentration range of 0-12.0 μg/mL. However, the emission intensity was found to increase with the addition of BSA. In micro-environments, complexed BSA hindered the dye's rotational relaxation processes, and increase quantum yield of the dye.[23]

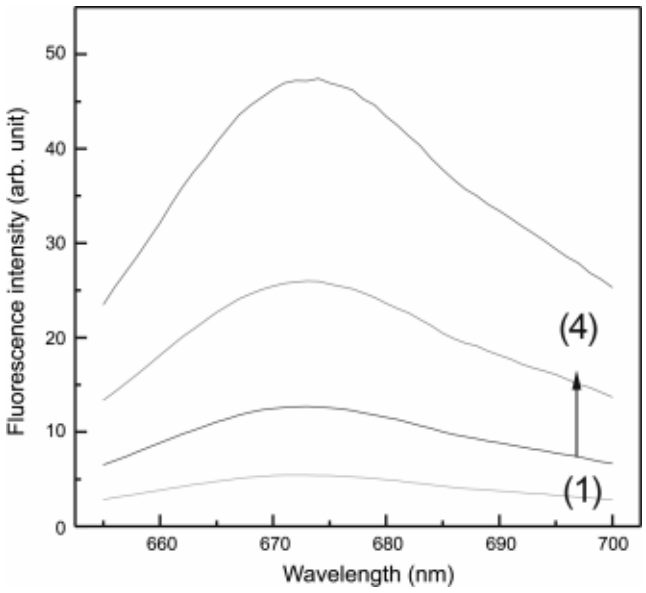

Figure 1. Emission spectra of the Nile Blue-BSA-Triton X-100 system: λ_{ex} = 635 nm; [Nile Blue], 3.3×10^{-5} M; [Triton X-100], 1.5×10^{-3} %; pH 6.8. [BSA]: (1) 1.0; (2) 2.0; (3)5.0; (4) 10.0 μg/mL.

Signal stability. The emission intensity of Nile Blue-BSA-Triton X-100 system was stable within 2 min at room temperature after mixing all the solutions, and kept constant for at least 20 min.

Effect of pH. The effect of pH in the range 2.0-10.0 on the emission intensity for the Nile Blue-BSA-Triton X-100 system is shown in Figure 2. The pH was adjusted by using 50 mM CH_3COOH-0.1 M NaOH, 50 mM NaH_2PO_4-0.1 M NaOH and 50 mM Na_2HPO_4-0.1 M NaOH buffer solutions for 2.0-5.5, 5.5-8.0 and 8.0-10.0, respectively. The concentrations used are 3.3×10^{-5} M, 1.5×10^{-3} % and 10.0 μg/mL for Nile Blue, Triton-X-100 and BSA, respectively. The emission intensity slowly increased with pH up to about 6.8 and then abruptly decreased. Therefore, pH 6.80 was selected for the subsequent studies. Figure 1 and 2 indicate that there are interactions between Nile Blue and BSA. The mechanism of this reaction should be different from the case when metal ions and organic ligands where coordination bonds are formed. In the view of the molecular structure and existing state of Nile Blue and BSA in solution, the interactions between Nile Blue and BSA could be only explained in terms of intermolecular binding force. The change in solution pH may lead to the distribution change of monocationic, neutral or even dicationic molecules. Under the experimental conditions, Nile Blue might be in neutral form, and could only bind to protein by nonelectrostatic forces including van der Waals attraction, hydrogen bonding and hydrophobic forces, since Nile Blue contains planar polycyclic aromatic rings. The planar rings of the dyes can interact with a hydrophobic area of negatively charged BSA (p*I* = 4.7-4.9). Therefore, many dye molecules can position at the specific binding sites of a protein molecule. The emission intensity decreased abruptly about pH 7-8.5, because of both of dye and protein are negatively charged at pH above 7.0, and could not interact with each other effectively.

Effect of Nile Blue concentration. The effect of Nile Blue concentration in the presence of 1.5×10^{-3} % Triton-X-100 and 10.0 μg/mL BSA on the emission intensity was

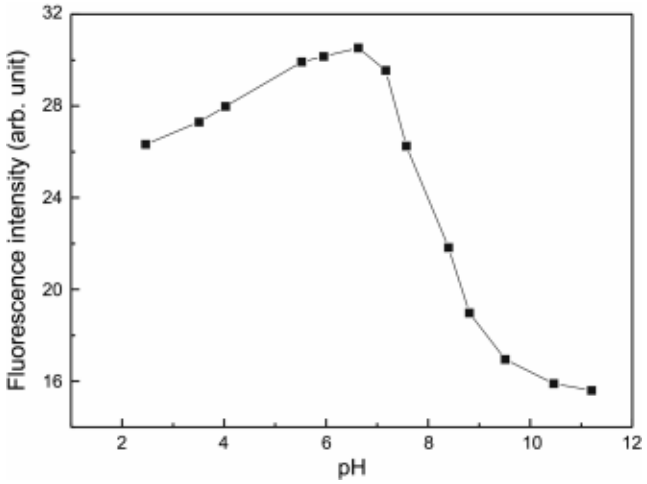

Figure 2. Effect of pH on emission intensity of the Nile Blue-BSA-Triton X-100 system:. [Nile Blue], 3.3×10^{-5} M; [Triton X-100], 1.5×10^{-3} %; [BSA], 10.0 μg/mL.

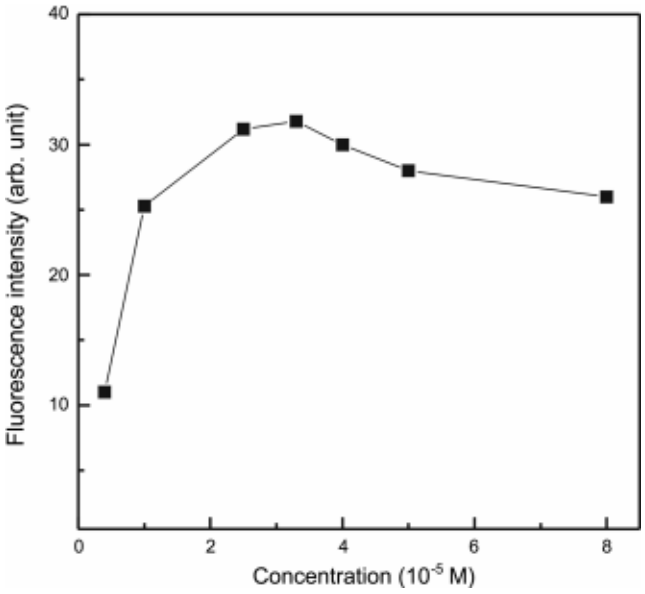

Figure 3. Effect of Nile Blue concentration on emission intensity of the Nile Blue-BSA-Triton X-100 system: [Triton X-100], 2.0×10^{-3} %; [BSA], 10.0 μg/mL; pH 6.8.

investigated. The concentration range of Nile Blue used was 5.0×10^{-7}-8.0×10^{-4} mol/L. The result is shown in Figure 3. The emission intensity abruptly increased with an increase in Nile Blue concentration and the maximum was reached when the concentration of Nile Blue was 3.3×10^{-5} mol/L. Further increase in Nile Blue concentration above this concentration did not appreciably change the emission intensity possibly due to the occurrence of dye aggregation. Ionic dye aggregation may increase with an increase of dye concentration or ionic strength. It was found that the reaction is not affected significantly by ionic strength at the pH 6.8.

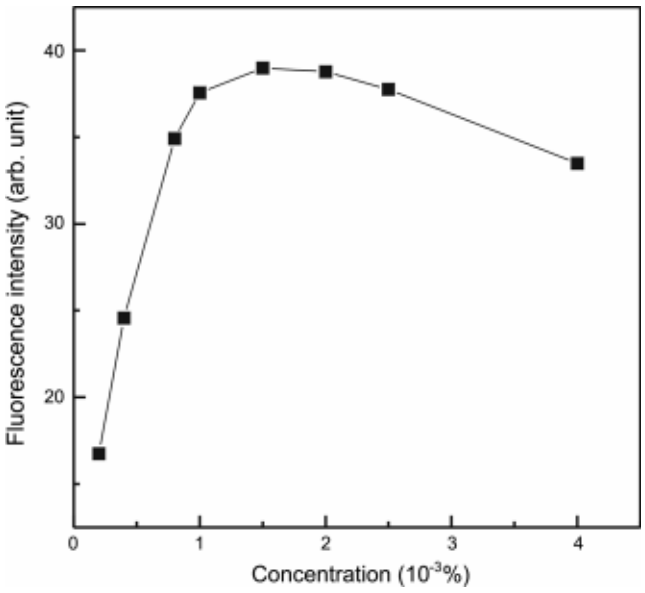

Figure 4. Effect of Triton X-100 concentration on emission intensity of the BSA-Nile Blue system: [Nile Blue], 3.3×10^{-5} M; [BSA], 10.0 μg/mL; pH 6.8.

Therefore, the concentration of Nile Blue was kept at 3.3×10^{-5} mol/L for the subsequent studies.

Effect of Triton X-100 concentration. Figure 4 shows the effect of Triton X-100 concentration in the 3.3×10^{-5} mol/L Nile Blue solution containing 10.0 μg/mL BSA at 20 ^{o}C on the emission intensity. The emission intensity rapidly increased up to about 1.2×10^{-3} % Triton X-100 concentration and then slightly decreased afterwards. The structure of micelle and there effects on certain chemical reactions have been very extensively studied recently. In micelle solution, the quantum yield of the dye molecule increases due to prevention of radiationless deactivation to its ground state. The selected concentration of Triton X-100 (CMC, 1.0×10^{-3} %) was 1.5×10^{-3} % for the optimum surfactant concentration.

Calculation of binding constant. In order to understand the binding properties of the Nile Blue-BSA complex, the modified Scatchard equation[24] was used (Eq. 1).

$$F/[\mathrm{NB}]_f = (F_\infty - F)/K_d \quad (1)$$

where K_d is the binding constant, $[NB]_f$ is the free Nile Blue concentration. F is the measured fluorescence intensity and F_∞ is the fluorescence intensity in the presence of infinite Nile Blue concentration. When BSA concentration was low enough, the $[NB]_f$ was approximately equal to the total Nile Blue concentration. Figure 5 shows the Scatchard plot, which gives a straight line indicating one binding site. The binding constant estimated from the plot was 3.8×10^4 M^{-1}.

Calibration curve for the determination of BSA. Figure 6 shows the calibration curve to determine BSA. The experimental conditions used were 3.3×10^{-5} M, 1.5×10^{-4} % and 6.8 for the concentration of Nile Blue, the concentration of Triton X-100 and pH, respectively. The linear range and the detection limit were 0.5-12.0 μg/mL and 0.2 μg/mL (S/N = 3), respectively. The correlation coefficient for this plot was 0.9992, and the relative standard deviation of six

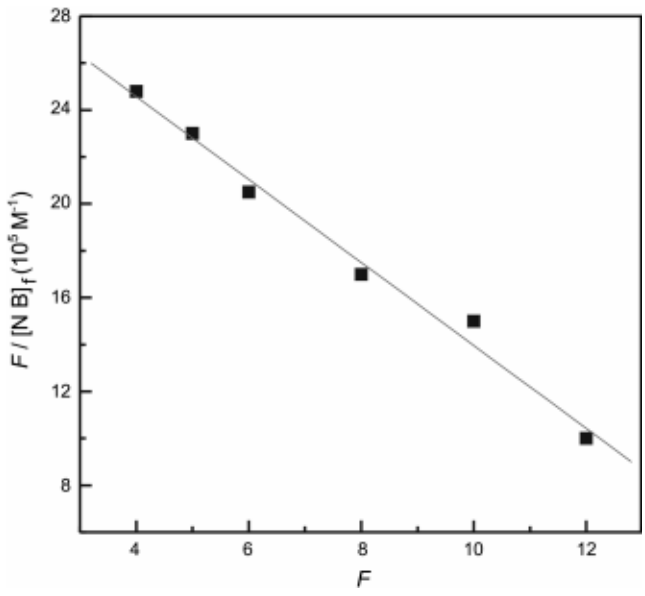

Figure 5. Scatchard plot at pH 6.8.

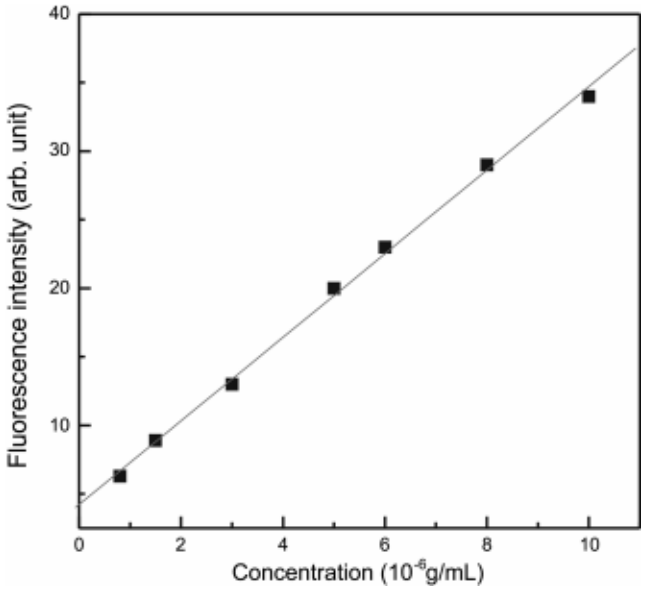

Figure 6. Calibration curve for BSA standard solution: [Nile Blue], 3.3×10^{-5} M; [Triton X-100], 1.5×10^{-3} %; pH 6.8.

replicate measurements was 1.4% for 10.0 μg/mL BSA.

Interfering substances. Both Lowry method and BCA methods are suffered interferences from Cu^{2+} and cysteine, tyrosine, and tryptophan residues. In order to test interferences from foreign substances, emission intensity of a standard solution containing 10.0 μg/mL BSA, 3.3×10^{-5} M Nile Blue and 2.0×10^{-3} % Triton X-100, and interfering species were measured. The results are listed in Table 1

Table 1. Effect of foreign substances on determination of 8.0 μg/mL BSA

Substance	BSA found. (μg/mL)
none	8.0
1 mM Mg^{2+}	7.8
1 mM Ca^{2+}	8.3
1 mM Co^{2+}	8.2
1 mM Mn^{2+}	8.2
1 mM Cr^{2+}	8.1
1 mM Cd^{2+}	8.0
1 mM Cu^{2+}	7.8
1 mM Hg^{2+}	8.1
1 mM Zn^{2+}	7.7
3000 μg/mL L-Cys	7.8
3000 μg/mL L-Val	8.1
3000 μg/mL L-Thr	8.4
3000 μg/mL L-Glu	8.0
3000 μg/mL L-Lys	8.3
3000 μg/mL L-Try	8.1
3000 μg/mL L-Phen	7.6
3000 μg/mL glucose	7.7
1 mM ascorbic acid	8.1
1 mM urea	7.8

(n = 3, RSD = 2.8%). Provided that an error less than 5% is considered acceptable, it shows that little interference from amino acid, sugars and few substances with this assay upon the listed concentration range.

Conclusions

In this work, we demonstrated how fluorescence intensity of Nile Blue could be used for the determination of bovine serum albumin (BSA) in aqueous solution. The fluorescence intensity was found to increase with the addition of BSA. Moreover, the intensity further increased with the addition of Triton X-100. The enhancement of fluorescence intensity was explained in terms of the binding of Nile Blue to BSA. The binding constant of micromole Nile Blue-BSA complex was estimated by Scatchard plot method. Under the optimal conditions, the fluorescence intensity was linearly related to BSA concentration in the range of 0.5-12.0 μg/mL and the detection limit was 0.2 μg/mL. The present method exhibited little interferences from amino acids, sugars and most of metal ions.

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