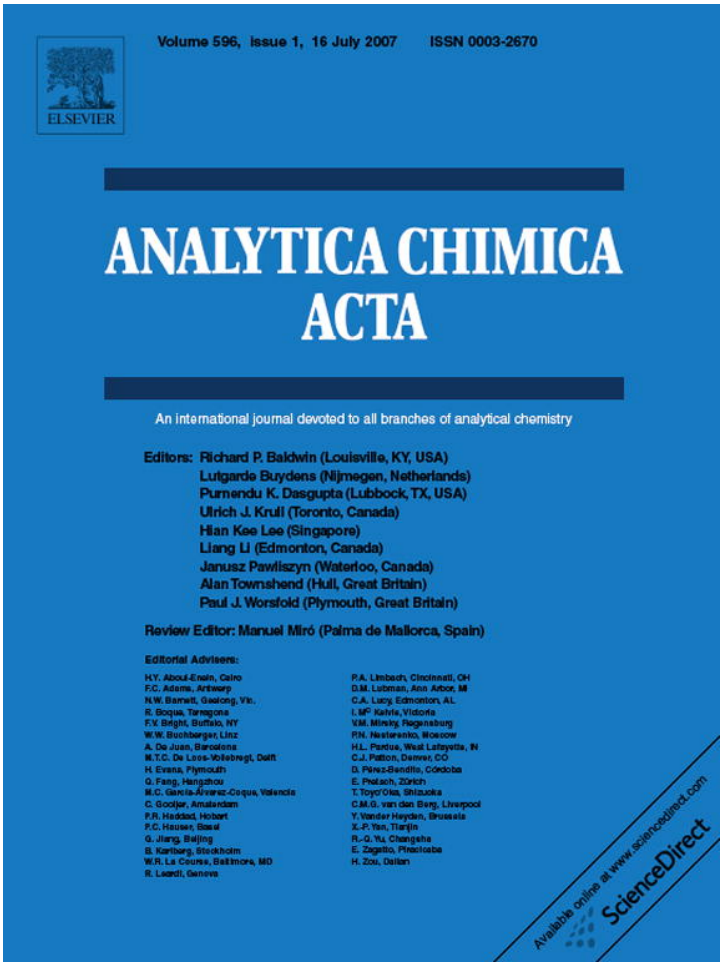

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Interaction of brilliant red X-3B with bovine serum albumin and application to protein assay

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Abstract

The interaction of brilliant red X-3B (BRX) with bovine serum albumin (BSA) in three pH media has been characterized by the spectral correction technique. The binding number maximum of BRX was determined to be 102 at pH 2.03, 82 at pH 3.25 and 38 at pH 4.35 and the binding mechanism was analyzed in detail. The effects of ionic strength from 0 to 1 mol L^{-1} and temperature from 20 to 70 °C on the binding were investigated. The results showed that the interaction of BRX with BSA responded to the Langmuir adsorption isothermal model and the binding constant was determined. From the correlation between the binding number and the number of basic amino acid residues, the ion-pair attraction induced the union of non-covalent bonds including H-bond, van der Waals force and hydrophobic bond and the binding model was illustrated. The binding of BRX to BSA has resulted in change of the BSA conformation confirmed by means of circular dichroism. Using this interaction at pH 2.03, a sensitive method named the absorbance ratio difference spectrometry was established and applied to the protein assay and the limit of detection of protein was only 6 μg L^{-1}. Two samples were determined and the results were in agreement with those obtained by the classical coomassie brilliant blue colorimetry.

Keywords: Brilliant red X-3B; Bovine serum albumin; Non-covalent binding; Conformational change; Molecular spectrometry; Protein assay

1. Introduction

Recently, the small molecule–biomacromolecule interactions are focused increasingly for many biochemists and thousands of new findings are reported every year. Understanding the interaction between organic ligand and biomacromolecule is always helpful for us to recognize the structure, function and activity of macromolecule as well as the toxicity of an organic toxicant, *e.g.* protein–protein [1], protein–DNA [2], protein–glycosaminoglycan [3] and protein–ligand [4] interactions. Though people have clarified the structure, conformation, activity and function of many proteins, the protein interaction with organic substance as well as conformational changes of protein is still in the extensive research so as to be realized the structure-activity relationships and particular biological roles of biomacromolecule. The spatial structure of protein is the representation of the foundation of the biological function. Generally speaking, the structure of crude protein is relatively stable. The structural stability keeps individual function and relative stability of species. Organic substance–protein interactions modulate the structure of protein and thus affect its biological functions. So, structural transformation that occurs in the process of protein folding and functioning is of great significance in biological organisms [5]. In fact, the interaction occurring in protein medium is so complicated. In the formation of complex, small organic compound may insert into the protein inner to regulate their structures and functions [6] through non-covalent union, *e.g.* hydrophobic bond, van der Waals force, dipole effect and hydrogen bond. The non-covalent binding is often weak and non-specific [7]. With the weak binding of the non-covalent interaction, this complex is often affected by the urea, dioxane or electrolyte and high temperature [8].

In the present work, we undertook in an attempt to clarify the general principle involved in the protein–organic substance

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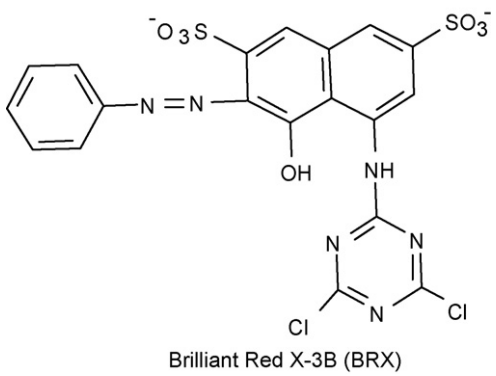

Fig. 1. Chemical structure of BRX.

interaction, characterization of the complex and effect of organic substance on protein conformation. Bovine serum albumin (BSA) obtained easily with well-known properties is often used as a model protein [9]. In an acidic medium, the basic amino acid residues (AARs) of protein are often protonated to form the poly-cationic amino groups. Brilliant red X-3B (BRX) as a kind of azo dye is commonly used in textiles and its chemical structure is given in Fig. 1. Absorption of BRX through the skin is a possible exposure route. We investigated the non-covalent interaction of BRX with BSA by means of molecular spectrometry. The BRX binding product with BSA was characterized by the combination of the spectral correction technique and the Langmuir isothermal adsorption. The binding number is corresponded to the number of basic AARs. A new model is advanced for the ion-pair electrostatic attraction inducement to promise the protein–organic substance binding by the co-action of the non-covalent poly-bonds, *i.e.* the ion-pair electrostatic interaction pulls BRX molecule to insert near the basic AARs and then the other non-covalent bindings form a union to bind BRX between BRX and AARs. By means of circular dichroism (CD) spectrometry [10], the secondary structure of BSA was measured in the presence of BRX and the increase of α-helix transferred from β-sheet was analyzed. Although the target acceptor of BRX may not be BSA, the aim of the research was to study non-covalent bridging of BRX with protein so as to provide a theoretical basis and methodology for estimation of target toxicity of an organic toxicant. In addition, though BRX is only a kind of industrial dyestuffs but not a normal chromogenic agent, it was found to be very sensitive to bind proteins. As a result, the determination of trace amounts of protein was advanced using the interaction of BRX with protein.

2. Experimental

2.1. Apparatus and instruments

The absorption spectra of BRX and its BSA solutions were recorded with a Model Lambda-25 spectrometer (Perkin-Elmer, USA) equipped with a thermostatic cell holder attachment to link with a Model TS-030 water-circulated thermostatic oven (Yiheng Sci. Technol. Shanghai, China). The spectrometer was computer controlled using UV WinLab software (Version 2.85.04). The pH of solutions was measured with a Model pHS-25 acidity meter (Shanghai Precise Sci. Instrum., China). The temperature of solutions was warmed with a Model HHS-11-2 thermostat water bath (Shanghai Precise Sci. Instrum., China). A Model BCD-196 refrigerator freezer (Meiling Production, Hefei, China) was used to store the protein solutions. A Model J-715 Circular Dichroism Spectropolarimeter (JASCO, Japan) was used to measure the conformation of proteins. The spectropolarimeter was computer controlled by J-715 Control Drover software (Version 1.00, JASCO). A Model JY92-II Ultrasonic Cell Crusher (Xinzhi Instrum., Ningbo, China) was used to break down the bacterial cell to prepare a protein sample. A Centrifuge (Shanghai Sci. Instrum., China) was used in the separation of protein sample.

2.2. Reagents and solutions

Bovine serum albumin (BSA) was purchased from the BioDev Biological Gene Technol. of Beijing. 0.20 g of BSA was dissolved in 100 mL of deionized water and then diluted to 1000 mL. The protein concentrations were determined by the UV method [11]. The solution must be stored in a refrigerator freezer at less than 4 °C.

BRX was provided by Shanghai Dyestuff Chemicals Factory. After purified by recrystallization, 0.500 mmol L^{-1} BRX was prepared in de-ionized water. It was used to react with BSA.

A series of Britton-Robinson (B-R) buffer solutions, pH 2.03, 2.21, 2.78, 3.25, 3.88, 4.35, 5.04 and 5.68, were prepared to adjust the acidity of solution in order to find a proper and highly sensitive complexation between BRX and BSA. The electrolyte solution, 5.0 mol L^{-1} $NaNO_3$, was prepared to adjust ionic strength of the complexation solution in order to investigate the effect of electrolyte on the binding. The masking reagent, 0.1 mol L^{-1} EDTA, was prepared and added into a sample to mask metals, *e.g.* Zn(II), Fe(II, III) and Ca(II) possibly existing in sample from interference of detection of proteins.

2.3. Measurements

2.3.1. Photometric characterization of interaction of BRX with BSA

All studies were carried out in a 10.0 mL calibrated flask. Into the flask, a known volume of the BSA solution, 1.0 mL of B-R buffer solution (pH 2.03, 3.25, and 4.35) and a known volume of the BRX solution were added. The solution was diluted to 10.0 mL with deionized water and mixed well. After reacting for 5 min, the absorbance ($A^0_{\lambda 2}$ and $A^0_{\lambda 1}$) of the reagent blank and $A_{\lambda 2}$ and $A_{\lambda 1}$ were measured at 539 nm (λ_2) and 571 nm (λ_1) against water. Thus, the parameters A_c, η and γ of each solution above were calculated.

2.3.2. CD measurement of protein solution

1 mL of B-R buffer solution (pH 2.03, 3.25, and 4.35) and 0.40 mg of BSA were added into five flasks. Then, 0.0, 0.40, 0.60, 0.80 and 1.00 mL of 0.500 mmol L^{-1} BRX were added into these flasks, respectively. The solutions were diluted to 5.00 mL with deionized water. Each was injected into a 0.1-cm

light path cell and the mean residue ellipticity (MRE) of BSA was measured between 200 and 250 nm on the spectropolarimeter. From the MRE curves, the secondary structure factors of BSA, *e.g.* α-helix, β-sheet, β-turn and the random coil forms in all the solutions were worked out by meaning of the Secondary Structure Estimation-Standard Analysis Measurement software (715/#B014460524, JASCO).

2.3.3. Protein assay

A known volume of a sample, 50 μL of 0.1 mol L^{-1} EDTA, 1 mL of pH 2.03 B-R buffer solution and 0.20 mL of 0.500 mmol L^{-1} BRX were added in a 10 mL flask. The solution was diluted to 10.0 mL with deionized water. After 5 min, the absorbances ($A_{571\,nm}$ and $A_{539\,nm}$) were measured at 571 and 539 nm against water. With the same method, a reagent blank without any protein was prepared and the absorbances ($A^0_{571\,nm}$ and $A^0_{539\,nm}$) were measured. The difference (ΔA_r) of absorbance ratio of the BRX–BSA solution was calculated by the relation:

$$\Delta A_r = \frac{A_{571nm}}{A_{539nm}} - \frac{A^0_{571nm}}{A^0_{539nm}} \quad (1)$$

ΔA_r is linear with the concentration (C_{M0}) of protein as follows:

$$\Delta A_r = pC_{M0} + q \quad (2)$$

where both p and q are constants which were obtained by linearly regressing plots ΔA_r versus C_{M0}. p is the sensitivity factor and the inverse ratio of L concentration C_{L0}. The less L is added, the higher the sensitivity is obtained. However, too low L will cause an obvious error in measurement because of noise of instrument background. This method is named as the absorbance ratio difference (ARD) method [12] and it may increase the sensitivity as over 10 times as ordinary spectrophotometry.

3. Results and discussion

3.1. pH dependence and spectral analysis

The BSA–BRX complex solutions were measured in various pH mediums and their absorption spectra are shown in Fig. 2. The interval between the positive peak and the negative valley increases with increase of the acidity of solution. It is well known that the isoelectric point of BSA is at pH 4.60. At pH less than 4.60, the protonation of AARs of BSA occurs so it is favorable for the binding of BRX with the peptide chains by ion-pair attraction. This has been confirmed early [13]. From curves in Fig. 2, pH 2.03 brings the most sensitive complexation. From curve 1, two wavelengths 539 and 571 nm were used in the following work.

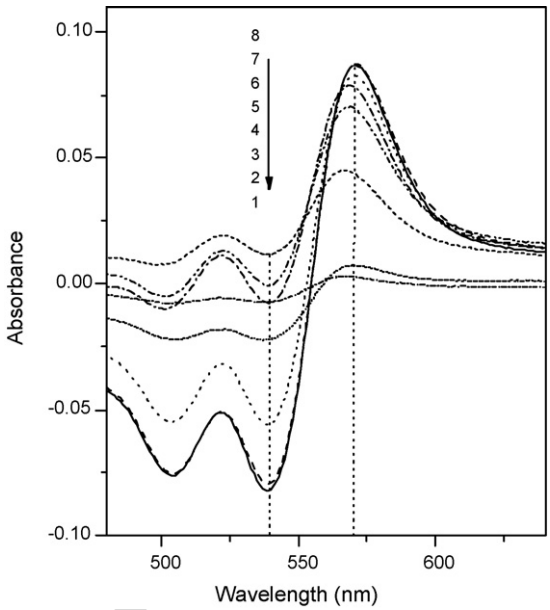

Fig. 2. Absorption spectra of the BSA–BRX solutions containing 0.050 mmol L^{-1} BRX and 12.0 mg L^{-1} BSA at pH (from 1 to 8) 2.03, 2.21, 2.78, 3.25, 3.88, 4.35, 5.04 and 5.68. All of them were measured against the reagent blank.

3.2. Determination of the BRX–BSA interaction

The spectral correction technique [14] is a very useful approach to characterize the interaction of BRX with BSA. The interaction of BSA (M) with BRX (L) is merged as follows:

	***n* BRX**	**+**	**BSA**	⇌	**BSA(BRX)$_n$**
Initiation	C_{L0} ($A^0\lambda 2$)		C_{M0}		0
Equilibrium	$C_L = C_{L0} - nC_{M0}$ ($A\lambda 1$ and $A\lambda 2$)		C_M - - ➤ 0		C_{M0} (A_c at $\lambda 2$)

The effective fraction (η) of BRX binding in protein and its binding ratio (γ) to protein are calculated by the relations:

$$\eta = \frac{A_c - A_{\lambda 2}}{A^0_{\lambda 2}} + 1 \quad (3)$$

and

$$\gamma = \eta \times \frac{C_{L0}}{C_{M0}} \quad (4)$$

where

$$A_c = \frac{A_{\lambda 2} - \beta A_{\lambda 1}}{1 - \alpha\beta} \quad (5)$$

where C_{M0} and C_{L0} are the initial concentrations of BSA and BRX. C_{M0} and C_{L0} are the equilibrium concentrations of BSA and BRX. n is the binding number of BRX and A_c the real absorbances of only the BSA–BRX complex at wavelength λ_2, which cannot be measured directly. $A_{\lambda 2}$ and $A_{\lambda 1}$ are the absorbances of the BSA–BRX solution, respectively, measured at λ_2 and λ_1 against water. Both α and β are the correction constants.

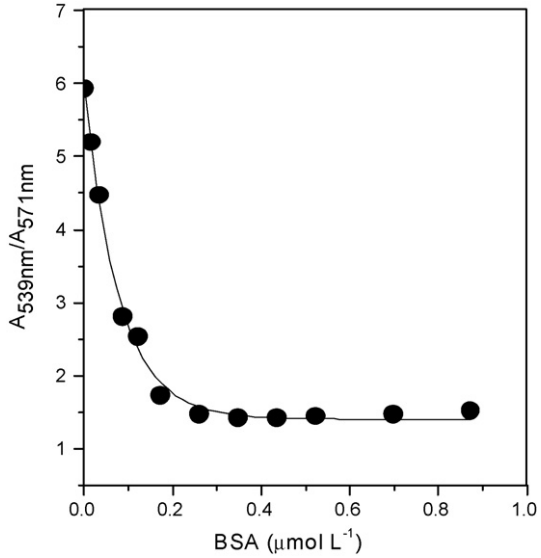

Fig. 3. Variation of the absorbance ratio ($A_{539\,nm}/A_{571\,nm}$) of the BSA–BRX solution at pH 2.03. The solutions contained 0.025 mmol L^{-1} BRX, with the increasing concentration of BSA, the absorbance ratio decreased and at last it reached equilibrium and remained unchanged. Then all the BRX bound to protein and there were no free BRX in the solvent.

The aggregation of organic molecules in BSA often obeys the Langmuir isothermal adsorption equation [15]:

$$\frac{1}{\gamma} = \frac{1}{N} + \frac{1}{KNC_L} \quad (6)$$

where

$$C_L = (1-\eta)C_{L0} \quad (7)$$

where K is the stability constant of complex and N is the maximal binding number of L. By regressing Plots γ^{-1} versus C_L^{-1}, both K and N are calculated. So interestingly, the Scatchard equation [16] is similar to Eq. (6). Possibly, the Langmuir adsorption isotherm should provide the Scatchard model with a theoretical basis.

From the absorbance ratio of the BSA–BRX solutions measured at 571 and 539 nm in Fig. 3, it decreases with the increase of BSA and then approaches to almost constant when BSA is over 0.3 μmol L^{-1}. This means that more and more BRX molecules will bind in BSA with the increase of BSA up to no excess and free BRX, *i.e.* the color substance in such solutions is only the BRX–BSA product. Thus, the constant minimum at 1.41 should be α of the BSA–BRX complex. Besides, from the inflexion point of curve, the gross binding number maximum of BRX in BSA was predicted at approximately 110 by the molar ratio method [17]. Its precise values will be determined below.

3.3. Effect of electrolyte and temperature

The electrolyte, $NaNO_3$, was added into the BSA–BRX solution in order to investigate the effect of electrolyte on the non-covalent interaction. The results are given in Fig. 4. With increase of $NaNO_3$, γ of BRX always decreases at various pH media. γ in over 0.2 mol L^{-1} $NaNO_3$ approaches only half of that

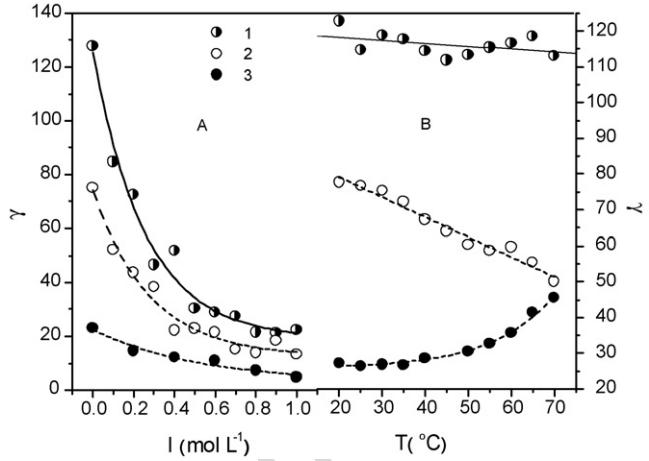

Fig. 4. Effects of electrolyte (A) and temperature (B) on γ of the solutions containing 0.025 mmol L^{-1} BRX and 12.0 mg L^{-1} BSA at (1) pH 2.03, (2) pH 3.25 and (3) 4.35.

in the absence of $NaNO_3$. When ionic strength of the solution increases, the self-aggregation of the lipophilic groups occurs so the steric effect of BSA inner becomes serious. It is unfavorable for insertion of BRX into BSA.

A high temperature may have two opposite effects on the non-covalent interaction. On one side, BSA would expand with rise of the temperature. Such an expansion could weaken the three-dimensional steric effect of BSA so as to be favorable for insertion of organic molecules. On the other side, expansion of the peptide chain could result in a farer distance between the adjacent peptide chains. Thus, the effective binding sites of BRX would reduce to cause the easier desorption of BRX from the peptide chain. Such two opposite actions decided the final effect of temperature on the non-covalent interaction. From curve 1 in Fig. 4B, γ has only a little change. It demonstrates that such two effects are almost equivalent at pH 2.03. From curve 2, γ decreases obviously with increase of temperature. It indicates that the second effect is primary at pH 3.25. However, from curve 3, γ increases with increase of temperature at pH 4.35. Because as the pH approaches to the isoelectric point of BSA, only a little fraction of basic AARs binds with BRX at room temperature. The other basic AARS could bind with acidic AARs by internal non-covalent bonds. With increase of temperature, the original internal bindings were destroyed and the basic AARs were released. Thus, BRX may insert into BSA to bind with the released basic AARs.

3.4. Photometric characterization of complexation

Fig. 5A shows the variation of γ of BRX in BSA solutions. From curves, γ always increases with increase of BRX less than 25 μmol L^{-1}. More and more BRX are bound on BSA. γ approaches the maximal constants at about 105 at pH 2.05, 82 at pH 3.25 and 38 at pH 4.35 when BRX is more than 25 μmol L^{-1}. It indicates that the bindings of BRX in BSA reach the saturated status. In spite of increase of BRX, the fraction of excess BRX will go greater and greater and γ will not increase yet. C_L of each solution is calculated from Eq. (7) and all plots γ^{-1} versus C_L^{-1}

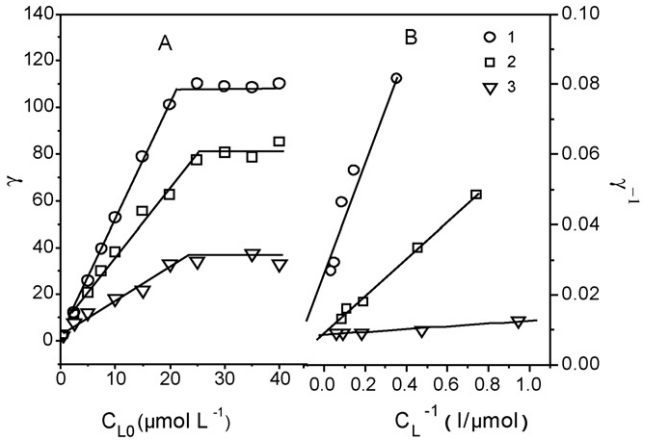

Fig. 5. (A) Variation of γ of the solutions containing BRX and 12.0 mg L^{-1} BSA at pH 2.03 (1), pH 3.25 (2) and pH 4.35 (3). (B) Plots γ^{-1} vs. C_L^{-1} of the above solutions.

of the BRX–BSA solutions are shown in Fig. 5B. All of them have very good linearity relationships. Thus, the aggregations of BRX on BSA obeyed the Langmuir adsorption isotherm. N of BRX and K of the BRX–BSA complex were calculated from the slopes and intercepts of lines in Fig. 5B and are given in Table 1. N is always close to N' in various pH media. Moreover, N is just equal to N_{AARs} at pH 2.03. It demonstrates that all 102 of basic AARs of BSA were protonized at pH 2.03 and each binds with BRX until the saturated status. Therefore, the ion-pair electrostatic attraction plays an allocation role for BRX to close the basic AARs. Then it is said that the electrostatic attraction induces the co-action of non-covalent bonds to result in BRX binding with BSA. From curves 2 and 3, N is less than the number of basic AARs. It indicates that a fraction of basic AARs charged positively and bound with BRX. From Table 1, K is maximal at pH 2.03. It indicates that the peptide chain at pH 2.03 has the most binding sites. Moreover, BRX has the potential binding points including 1 of –N=N– group, 1 of –OH groups and 2 of $–SO_3^-$ groups to form hydrogen bonds with the peptide chain and naphthyl and phenyl groups to form hydrophobic bonds, *e.g.* π–π interaction with non-polar AARs. The sketch for BRX binding in the peptide chain is shown in Fig. 6. The co-action of non-covalent bonds resulted in a firm binding of BRX.

3.5. Change of protein conformation

The specific conformation of a protein with special function results from the covalent and non-covalent interactions among

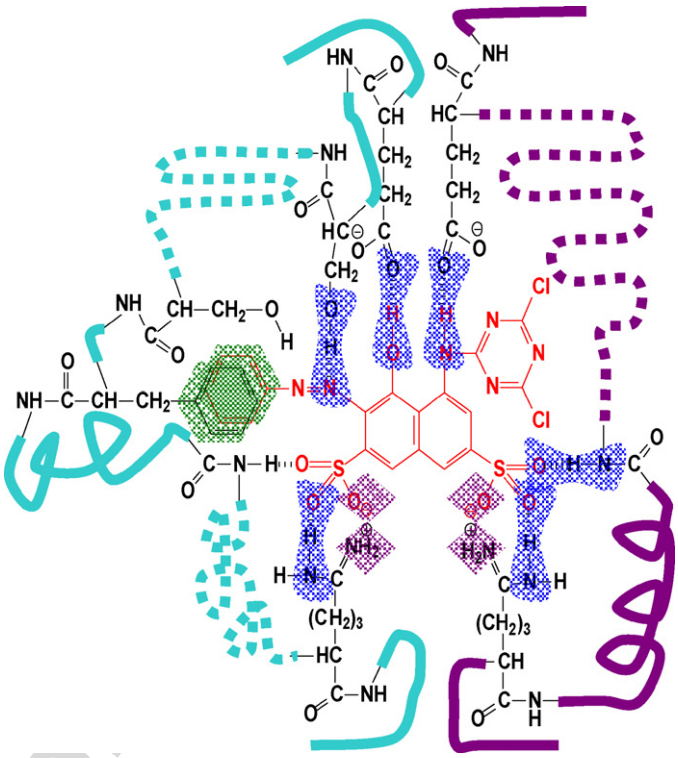

Fig. 6. Cartoon illustrating the binding of BRX with amino acid residues through co-action of non-covalent poly-bonds.

AARs. When an organic compound such as pollutant, drug or toxicant is added in protein, the internal non-covalent interaction would be destroyed to cause change of the original conformation. Especially, a strong binding ligand could result in the irrecoverable transition of the conformation and loss of the original function. The CD spectrometry is often used to evaluate the secondary conformation of a protein such as β-sheet, α-helix and β-turn forms of a protein. The secondary structure factors of BSA in presence of BRX are determined and shown in Table 2. The β-sheet of BSA always decreases with the increasing pH from 2.03 to 4.35 in absence of BRX and α-helix increases. The increase of solution acidity has caused the protonization of AARs and destroyed the original internal hydrogen bonds. The β-sheet fraction of BSA decreases rapidly with increasing BRX from 0 to 0.10 mmol L^{-1} at pH 2.03. On the contrary, the α-helix increases. The same phenomena were found at pH 3.25 and 4.35. Thus, the addition of BRX has transferred the β-sheet of BSA into helix form. The possible reason is that BRX with two negatively charged groups binds with two diagonal side-groups

Table 1
Determination of the characteristic factors of the BSA–BRX complex

M	Length/N_{AARs} [a]	pH	N' [b]	N [c]	K, $\times 10^5$	Complex formed
BSA	607/102	2.03	102	110	21.5	BSA·BRX_{102}
		3.25	82	90	1.62	BSA·BRX_{82}
		4.35	38	40	1.50	BSA·BRX_{38}

[a] Number of AARs and number of basic AARs.
[b] From Fig. 5A.
[c] From Fig. 5B.

Table 2
Effect of BRX on the conformational factors of BSA in various pH media

pH	Factor	Fraction (%)				
		BRX (mmol L^{-1})				
		0.00	0.04	0.06	0.08	0.10
2.03	α-Helix	16.4	20.2	27.3	31.5	38.1
	β-Sheet	25.2	22.0	18.3	13.9	7.7
	β-Turn	20.4	21.4	21.1	22.2	21.7
	Random	37.9	36.5	33.3	32.4	32.5
3.25	α-Helix	28.1	31.3	30.8	47.3	81.7
	β-Sheet	19.7	17.4	15.9	0.0	0.0
	β-Turn	20.2	20.2	21.5	22.1	0.0
	Random	32.0	31.0	31.8	30.7	18.3
4.35	α-Helix	30.9	32.3	41.6	40.9	78.4
	β-Sheet	15.3	15.2	0.0	0.0	0.0
	β-Turn	21.2	21.3	24.6	25.1	0.0
	Random	32.6	31.4	33.8	34.0	21.6

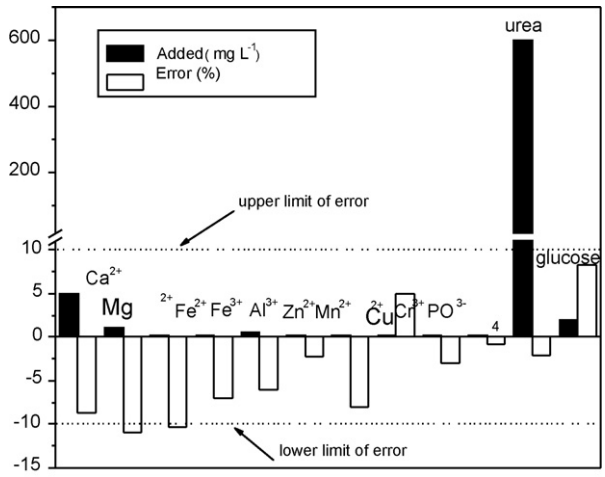

Fig. 7. Effect of some foreign substances on the determination of 2.0 mg L^{-1} BSA at pH 2.03 with the existence of 5.0 μmol L^{-1} BRX.

of basic AARs on a β-sheet. The electrostatic attraction causes such two side-groups of β-sheet to rotate around BRX molecule. Thus, the hydrogen bonds of two peptide chains forming the β-sheet were destroyed and a helix conformation was formed. Without doubt, basic AARs in random form could bind with BRX, too. In a solution containing a high BRX concentration, *e.g.* 0.100 mmol L^{-1} as shown in Table 2, all of β-turn and β-sheet forms at pH 3.25 and 4.35 were changed into the helix form. The interval between the random and helix sectors became short. BRX might bridge one basic AAR in a random coil sector with the other one in the helix sector. Thus, a random form sector would climb along a helix sector. As a possible result, some random coils were transferred into the helix form.

3.6. Application to protein assay

3.6.1. Calibration graphs and LOD

Four standard series of BSA were prepared according to the measurement method. The absorbances of each solution were measured and ΔA_r was calculated from Eq. (1). Their regression equations according to Eq. (2) are given in Table 3. The LOD of BSA, defined as the blank values plus three times the standard deviation of 10 replicated blanks, was also calculated and is given in Table 3. The less BRX was added and the higher the sensitivity was obtained. However, too low BRX could cause an obvious error in measurement because of noise of instrument background. The LODs of series 3 and 4 are the lowest but the linearity of series 4 is the best. Thus, 10.0 μmol L^{-1} BRX was added to determine the total concentration of proteins in a sample such as food, body liquid, blood, and cell liquid. The corresponding LOD of protein is 6 μg L^{-1}.

3.6.2. Effect of foreign substances

The normal masking reagent, EDTA, was used to mask most of metals possibly existing in samples. The results shown in Fig. 7 indicate that none of the following species affected the direct determination of 2.0 mg L^{-1} BSA (error less than 10%) with BRX: 5.0 mg L^{-1} Ca^{2+}, 1 mg L^{-1} Mg^{2+}, 0.5 mg L^{-1} Al^{3+}, 0.2 mg L^{-1} Zn^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Mn^{2+}, PO_4^{3-} and Cl^-, 2 mg L^{-1} glucose and 600 mg L^{-1} urea. Therefore, the recommended method was selective and fit for quantitative analysis of water-soluble proteins.

3.6.3. Analysis of samples

In the present work, one sample was from the cytosol of *Escherichia coli* (*E. coli*), which was prepared by cytolysis and separation of the cell residue. The supernatant was analyzed and the water-soluble proteins existed in *E.coli* cytosol were determined. The other sample was from a kind of commercial milk. 1.00 mL of milk was diluted in 1000 mL of deionized water. Their determinations are given in Table 4. The analytical results are in agreement with those determined by the colorimetry with coomassie brilliant blue (CBB) [18]. As a result, the proposed method is accurate and credible for practical analysis.

Table 3
The linear regression equations of BSA between 0 and 4.5 mg L^{-1} with BRX and its limit of detection (LOD)

BRX (μmol L^{-1})	Regression equation	R^a	σ^b	LOD^c (μg L^{-1})
1.5	$\Delta A_r = 18.0C_{M0} + 0.0422$	0.9861	0.0532	9
3.5	$\Delta A_r = 11.1C_{M0} + 0.0059$	0.9954	0.0307	8
5.0	$\Delta A_r = 7.07C_{M0} + 0.0292$	0.9904	0.0124	5
10.0	$\Delta A_r = 5.41C_{M0} - 0.0005$	0.9999	0.0100	6

[a] Linear correlation coefficient.
[b] Standard deviation of 10 replicated reagent blanks.
[c] LOD = 3σ/p.

Table 4
Determination of proteins in samples with BRX as reactant at pH 2.03 in the presence of EDTA

Sample	Protein added[a] (mg L^{-1})	Protein found[b] (mg L^{-1})	Recovery (%)	CBB method[c] (mg L^{-1})
Milk	0	2.2 ± 0.2	–	2.1
	2.4	4.6 ± 0.4	100	–
E. coli	0	1.4 ± 0.3	–	1.6
	2.4	3.9 ± 0.1	104	–

[a] 0.5 mL of a sample was added for complexation.
[b] Average of five replicated determinations.
[c] Average of five replicated determinations.

4. Conclusions

Recently, many azo compounds have been used in protein assay by photometric and fluorometric methods [19,20]. However, the binding mechanism was not often emphasized and discussed in detail. From investigation of the BRX–BSA interaction, the non-covalent interaction has supported the Langmuir isothermal aggregation of BRX on BSA. Moreover, the binding number of BRX responds to the number of basic AARs. As a result, ion pair attraction induced the binding of BRX in BSA. The stability of the complex depends upon the number of active connection sites. Though the crystal structure of a number of proteins was understood and the possible binding region was identified [21], we could not distinguish all of the connection forms and accurately determine the non-covalent bond energy. The combination of the Langmuir isothermal adsorption and the spectral correction technique will provide a useful experimental strategy for the interaction of organic ligand with protein. For the quantitative detection of protein, the ARD method is more sensitive for color change complexation than traditional spectrophotometry.

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