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Adsorption-desorption of BSA to highly substituted dye-ligand adsorbent: quantitative study of the effect of ionic strength

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Abstract Cibacron Blue 3GA was immobilized on Sepharose CL-6B to obtain a highly substituted dye-ligand adsorbent which dye concentration was 17.4 μmol dye per gram wet gel. This adsorbent had a highly binding capacity for bovine serum albumin (BSA). The effects of ionic strength on the adsorption and desorption of BSA to the adsorbent were studied. Adsorption isotherms were expressed by the Langmuir model. The quantitative relationships between the model parameters and the ionic strength were obtained. The desorptions were performed by adding salt to the BSA solutions in which adsorption equilibria had been reached. Adding salt to the solution resulted in the desorption of the bound protein. It was found that the isotherm obtained from the desorption experiments agreed well to the isotherm obtained from the adsorption experiments at the same ionic strength. The result demonstrated that the adsorption of BSA to the highly substituted adsorbent was reversible.

List of symbols

C	mg cm^{-3}	liquid phase concentration of BSA at equilibrium
C_0	mg cm^{-3}	the concentration of the initial protein solution
C_1	mg cm^{-3}	liquid phase concentration of BSA at first equilibrium
C_2	mg cm^{-3}	liquid phase concentration of BSA after desorption
C_s	mol dm^{-3}	the salt concentration
K_d	mg cm^{-3}	dissociation constant in Langmuir model
q	mg g^{-1}	adsorbed density of BSA at equilibrium
q_1	mg g^{-1}	adsorbed BSA density at first equilibrium
q_2	mg g^{-1}	adsorbed BSA density after desorption
q_m	mg g^{-1}	Langmuir parameter, adsorption capacity for BSA
V_0	cm^{-3}	volume of the initial protein solution
V_1	cm^{-3}	withdrawn volume of protein solution for earlier adsorption determination
V_2	cm^{-3}	added volume of the buffer solution for desorption
W	g	wet weight of the adsorbent

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1 Introduction

Dye-ligand adsorbents for protein purifications have been developed extensively because they offer the chance of simple and rapid isolations of proteins, typically 20 to 80 fold purification with about 80% recovery from a crude extract without prior treatment [1]. There are now examples of at least 30 different dyes for the purification of a great variety of proteins ranging through recombinant polypeptide hormones, blood proteins, and most classes of enzymes [2]. Dyes have often replaced nucleotides as affinity ligand in large-scale purification of biomolecules [3]. There are several examples of large-scale purification of proteins using dye ligand affinity chromatography, such as glycerokinase, ATP: AMP phospho-transferase [4, 5]. More recent alternative exploitations of dye-protein interactions such as affinity cross-flow filtration, affinity precipitation and affinity extraction also play an important role in such isolations [6–9]. Among all of the dyes, the mostly utilized one is a reactive dye, Cibacron Blue F3GA (Fig. 1), which is a mixture of two isomers [3, 10, 11].

There are many factors affecting the interactions between a protein and an immobilized dye, such as immobilized dye density, ionic strength, temperature, pH and buffer composition [1, 8, 9, 12]. It was found that the adsorption capacity of a dye-ligand adsorbent was approximately proportional to the immobilized dye density [8]. Moreover it was observed that more highly substituted adsorbents exhibited higher affinity for proteins [8].

In spite of the extensive studies and applications of dye-ligand adsorbents, adsorption isotherm data remained rare [9]. Although NaCl was generally used as an eluant [13], little work has been published to give a quantitative analysis of the effect of ionic strength on the adsorption isotherm. Boyer et al. studied the effects of pH and ionic strength on the adsorption of lysozyme and BSA to Blue Sepharose [9]. However, the information provided by their report was qualitative. Moreover, because the immobilized dye density of the commercial Blue Sepharose they used was very low, the adsorption capacity was relatively small.

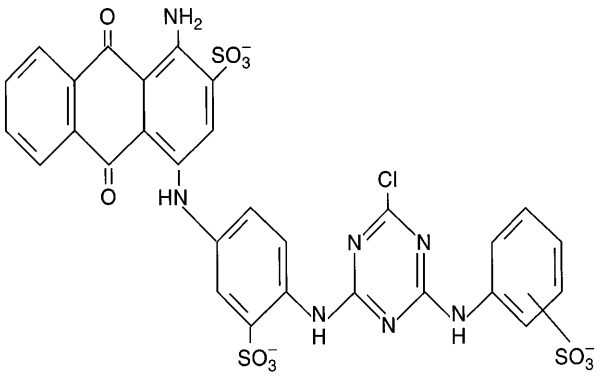

Fig. 1. Molecular structure of Cibacron Blue F3GA

Thus, in this work we immobilized Cibacron Blue 3GA (ortho isomer of Cibacron Blue F3GA) on Sepharose CL-6B to obtain a highly substituted adsorbent. Sepharose was employed because the open structure of the agrose made it bind protein more efficiently [14, 15]. BSA was chosen as a model protein. Both adsorption and desorption isotherms were studied and compared. The effect of ionic strength on the adsorption isotherm was quantitatively determined.

2
Materials and methods

2.1
Materials

Albumin bovine (BSA, Fraction V, minimum 98%), Cibacron Blue 3GA (65%) were purchased from Sigma Chemical Company. Sepharose CL-6B was obtained from Pharmacia Biotech. All other reagents were of analytical grade.

Tris-HCl buffer solutions (10 mmol dm^{-3}) were prepared by adjusting a Tris solution to pH 7.5 by addition of HCl (1.0 mol dm^{-3}) at 25 °C. The ionic strength of the buffer was adjusted by adding sodium chloride.

2.2
Cibacron blue immobilization

Cibacron Blue 3GA was immobilized on Sepharose CL-6B by the method of Atkinson et al. [16]. Typically, seventy-five milliliters of settled Sepharose was washed extensively with deionized water to remove ethanol that had been added to prohibit microbial contamination. Four grams of the blue dye and 12 g NaCl were added to the Sepharose, and water was introduced to give a 180-cm^3 slurry. The Suspension was shaken for 30 min to allow the complete diffusion of the dye into the particles. Coupling of the dye was carried out by adding 20 cm^3 of 1.0 mol dm^{-3} NaOH. The reaction was carried out for 20 h at 45 °C in a shaking incubator. The blue Sepharose was washed on a funnel under suction with a series of solvents as described by Atkinson et al. [16]. The initially introduced amount of the dye was varied to obtain various levels of the dye substitution [8].

2.3
Determination of immobilized dye density

The density of the dye immobilized on the Sepharose was determined spectrophotometrically as follows: In a 50-cm^3 flask were added nearly 0.2 g blue Sepharose (wet weight) and 15 cm^3 HCl (1.0 mol dm^{-3}). The suspension was heated to 80 °C to solutilize the Sepharose. After neutralizing the solution by NaOH (1.0 mol dm^{-3}) titration, 20 cm^3 phosphate buffer (0.2 mol dm^{-3}, pH 7.5) was added. Then deionized water was introduced to give a 100-cm^3 solution. After gently shaking the solution several times, its absorbance at 620 nm was determined.

2.4
Adsorption isotherm studies

The amount of the Cibacron Blue Sepharose was measured in terms of wet weight. The blue Sepharose previously equilibrated in the Tris-HCl buffer with a predetermined ionic strength was added to a 10-cm^3 centrifuge tube. The suspension was centrifuged at 30 000 m s^{-2} for 5 min. After removing the supernatant, approximately 0.3 g of the highly packed wet adsorbent was withdrawn from the tube and added into a 50-cm^3 flask that had been weighted previously. The weight of the flask containing the adsorbent was measured again to determine the precise wet weight of the adsorbent. Then 20 cm^3 of buffered BSA solution with known concentration was introduced to the flask. The suspension was allowed to equilibrate for 20 h at 25 °C and 100 rpm on a shaking incubator. After settling the suspension, 5 cm^3 of the supernatant was withdrawn from the flask to determine BSA concentration by ultraviolet absorbance at 280 nm. The adsorbed BSA density was calculated according to the following equation:

$$q_1 = \frac{V_0 \cdot (C_0 - C_1)}{W} \quad . \tag{1}$$

2.5
Desorption isotherm studies

After 5 cm^3 of the supernatant was withdrawn from the flask where adsorption equilibrium had been reached, 5 cm^3 of the Tris-HCl buffer with a predetermined ionic strength was added to the flask to make the ionic strength of the solution increase 0.5 mol dm^{-3}. The suspension was allowed to equilibrate for another 20 h at the same conditions as in the adsorption stated above. Thereafter, free protein concentration was measured as described above. The adsorbed BSA density was calculated as follows:

$$q_2 = q_1 - \frac{(V_0 - V_1 + V_2) \cdot C_2 - (V_0 - V_1) \cdot C_1}{W} \quad . \tag{2}$$

3
Results and discussion

3.1
Cibacron blue sepharose CL-6B

The typical ligand density of Cibacron Blue on the prepared Cibacron Blue Sepharose CL-6B was determined to be 17.4 μmol g^{-1}. This value was much higher than that of commercial Blue Sepharose provided by Pharamacia Bitech (Blue Sepharose CL-6B, code No. 17-083001, approximately 2 μmol cm^{-3} swollen gel, Manufacturer's specification). In comparison, Blue Sepharoses containing different levels of dye substitution were prepared. It is

Table 1. Adsorption capacities of Blue Sepharoses prepared in this work and that of commercial one[a]

	This work				Commercial one
Blue dye density (μmol g^{-1})	2.04	5.72	12.7	17.4	2.0
Adsorption capacity (mg g^{-1})	11.8	22.2	37.9	59.0	5

[a] The adsorption capacity of the Blue Sepharose prepared in this work was determined by the Langmuir isotherm (Eq. (3)) using the experimental data. The adsorption capacity of the commercial Blue Sepharose was that of the Manufacturer's specification

interesting to compare the adsorption capacity of these adsorbents with the commercial one. The results are presented in Table 1. As expected, high levels of protein adsorption were observed in the highly substituted Blue Sepharose. This agreed to that reported by Boyer et al. [8]. The adsorption capacity of the Blue Sepharose with the immobilized dye density of 17.4 μmol g^{-1} prepared in this work was 59 mg g^{-1}. However, the adsorption capacity of the commercial Blue Sepharose was only 5 mg cm^{-3} swollen gel (Manufacturer's specification). Since the density of the swollen gel was close to unity, the volume of 1 g wet gel should be approximately equal to that of 1 cm^3 swollen gel. This means that only less than 10% of the Blue Sepharose prepared in this work was needed to adsorb the same amount of BSA as the commercial adsorbent was.

3.2 Adsorption isotherm

The isotherms of BSA adsorption to the highly substituted Blue Sepharose (17.4 μmol g^{-1}) at different ionic strengths are shown in Fig. 2. All isotherms were measured in Tris-HCl buffers (0.01 mol dm^{-3}, pH 7.5) at 25 °C. A low buffer concentration was employed to adsorb more protein [1]. A mediocre temperature was chosen to obtain a relatively high adsorption capacity of the adsorbent and to avoid

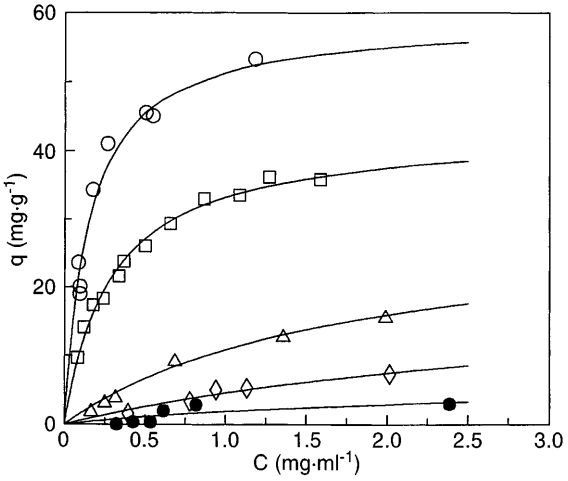

Fig. 2. Adsorption isotherms of BSA to the highly substituted Cibacron Blue Sepharose CL-6B (dye density = 17.4 μmol g^{-1}) in Tris-HCl buffer solutions (0.01 M, pH 7.5) with different salt concentrations. Salt concentrations (M): 0.05 (○), 0.25 (□), 0.5 (△), 1.0 (◇) and 1.5 (●)

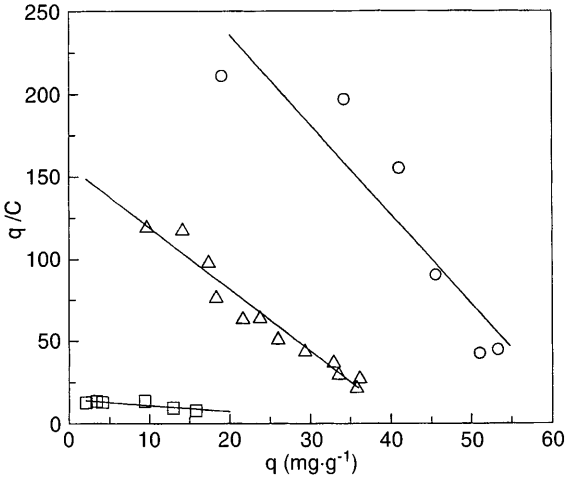

Fig. 3. Scatchard analysis of BSA adsorption to the highly substituted Cibacron Blue Sepharose CL-6B (dye density = 17.4 μmol·g^{-1}) at lower ionic strengths. Salt concentrations (M): 0.05 (○), 0.25 (△) and 0.5 (□)

protein denaturation. Each set of the equilibrium data was fitted to the Langmuir model by nonlinear Simplex method:

$$q = \frac{q_m \cdot C}{K_d + C} \quad . \tag{3}$$

In general, the model fitted the data well except the equilibrium data deviated slightly from the model at higher ionic strength. Scatchard analysis of the data is shown in Figs. 3 and 4. As shown in Fig. 3, three straight lines were obtained at lower ionic strength. The linearity of the plots indicated the presence of a homogeneous binding affinity and an equilibrium of the Langmuir type [17]. The result did not agree to that reported by Boyer et al. [9], in which the commercial Blue Sepharose with a dye density of 2 μmol ml^{-1} was used. This might be attributed to the fact that the two kinds of Blue Sepharoses had different surface structures due to the different dye densities.

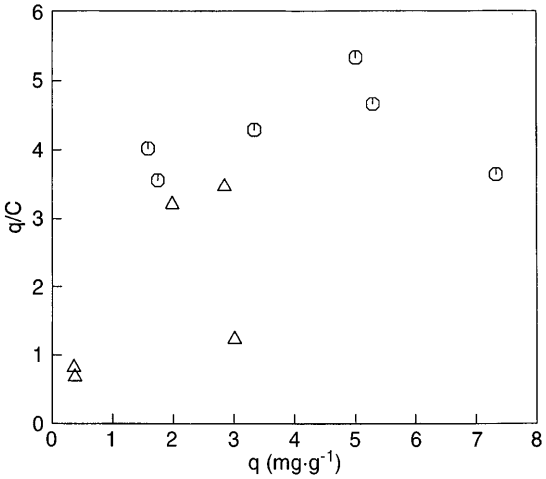

Fig. 4. Scatchard analysis of BSA adsorption to the highly substituted Cibacron Blue Sepharose CL-6B (dye density = 17.4 μmol g^{-1}) at higher ionic strengths. Salt concentrations (M): 1.0 (○) and 1.5 (△)

However, the plots were nonlinear in Fig. 4, where the ionic strengths were higher than those in Fig. 3. This indicated that the adsorption at higher ionic strength did not follow the Langmuir model and the binding involved heterogeneous interactions. Because higher ionic strength would promote the adsorption of the immobilized dye to the Sepharose surface [18–20], it is considered that surface of the Sepharose become heterogeneous at higher ionic strength. In spite of this, the Langmuir model was used in this study because it adequately described the adsorption isotherms as indicated in Fig. 2.

3.3
Quantitative expressions of the Langmuir model parameters

The adsorption capacity and the dissociation constant at different ionic strengths were estimated using the experimental data shown in Fig. 2. The dependency of the adsorption capacity on ionic strength is shown in Fig. 5. In order to describe the relationship between the adsorption capacity and the ionic strength, an exponential equation was employed. Simplex fitting gives:

$$q_m = 59.5 \exp\ (-1.1 \cdot C_s)\ . \qquad (4)$$

Fig. 6. shows the effect of ionic strength on the dissociation constant. The dissociation constant increased with increasing the ionic strength. The relationship can be described as follows:

$$K_d = 2.2\ (0.27 + C_s)^2\ . \qquad (5)$$

It has been assumed that a protein has arginine or lysine residues could interact with the sulfonate group of the dye [21]. Scopes suggested that histidine residues played an important role in the dye-protein electrostatic interaction [1] and BSA has a relatively high level of histidine residues [9]. From the results shown in Figs. 5 and 6, it was also likely that BSA was bound to the Blue Sepharose by electrostatic interaction because the adsorption became weak with the increase of the ionic strength. However, the isoelectric point of BSA is 4.9, lower than the buffer pH. BSA should present a net negative charge in the buffer. Although arginine or lysine residues might carry local positive charge for their high isoelectric point (10.76 and 9.74, respectively), it was difficult for the protein presenting a negative charge to be bound by the sulfonate groups of the dye. Boyer et al. supposed that there were biospecific interactions between BSA surface and the dye [9]. But it was known that the biospecific interaction was mostly involved in the binding of blue dyes to enzymes such as dehydrogenases at the dinucleotide binding sites [11, 22, 23]. BSA has no such binding sites.

Therefore, it was most likely that the ionic strength affected protein adsorption isotherm principally by affecting the interaction between the immobilized dye and the gel matrices. Increasing the ionic strength could promote the adsorption of the dye molecules to the matrix surface by hydrophobic interaction [18–20]. Moreover, the hydrophobic interaction between the immobilized dye molecules themselves would also become strong, because it has been observed that the salt addition to a dye solution caused the stacking of the free dye molecules [21]. Thus, the density of the immobilized dye accessible to protein would decrease as the ionic strength

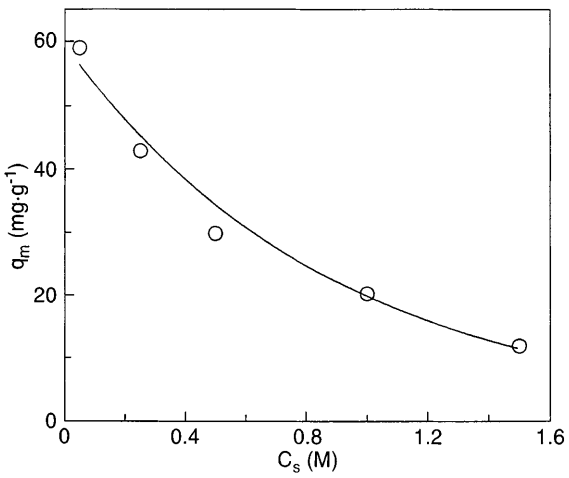

Fig. 5. Effect of salt concentration on the adsorption capacity in the Langmuir equation

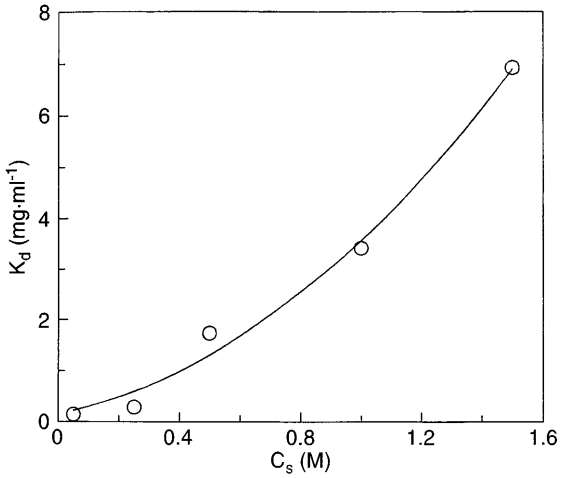

Fig. 6. Effect of salt concentration on the dissociation constant in the Langmuir equation

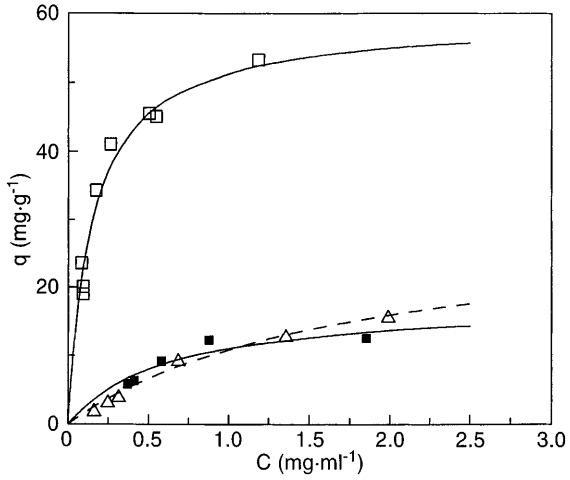

Fig. 7. Comparison of adsorption (△) and desorption (■) isotherms at the same salt concentration (0.5 M NaCl). Batch desorptions were conducted from the equilibrium state at $C_s = 0.5$ M (□) by adding concentrated NaCl solution

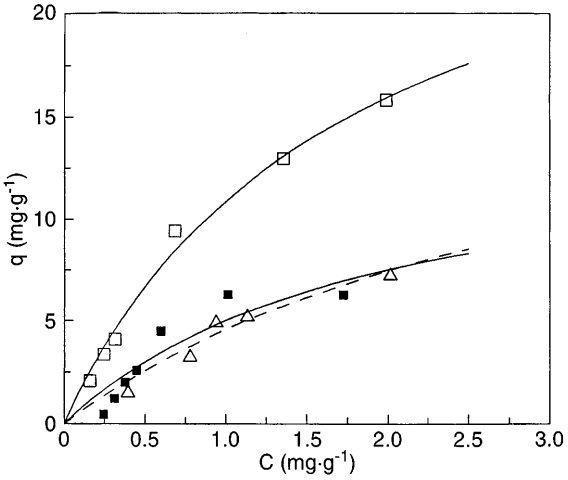

Fig. 8. Comparison of adsorption (△) and desorption (■) isotherms at the same salt concentration (1.0 M NaCl). Batch desorptions were conducted from the equilibrium state at $C_s = 0.5$ M (□) by adding concentrated NaCl solution

increased, and the binding of the protein to immobilized dye became difficult. This resulted in the decrease of the adsorption capacity and the increase of the dissociation constant.

3.4 Desorption

Figs. 7 and 8 show two typical desorption results. In Fig. 7, the upper curve represents the adsorption isotherm at an initial ionic strength of 0.05 mol dm^{-3}, while the lower solid curve represents the desorption isotherm at a final ionic strength of 0.5 mol dm^{-3}. In comparison, the adsorption isotherm at the ionic strength of 0.5 mol dm^{-3} is also illustrated (dashed curve). It is observed that most bound protein was desorbed when the ionic strength was raised from 0.05 to 0.5. Comparison of the adsorption and desorption isotherms at the same ionic strength indicated that the two isotherms fitted well. Similar results were obtained when the ionic strength was raised from 0.5 to 1.0 as shown in Fig. 8.

The results demonstrated that BSA adsorption to the highly substituted Blue Sepharose was completely reversible. Adsorbed BSA could be eluted by increasing ionic strength. So a highly substituted Blue Sepharose could be employed for BSA purification. However, for other proteins there may exist elution difficulty caused by high substitution [4]. In this case, an adsorbent with an intermediate dye density should be employed.

4 Conclusions

It is encouraging that the highly substituted dye-ligand adsorbent had a high adsorption capacity. The identical isotherm obtained by adsorption and desorption experiments at the same ionic strength showed that the adsorption was completely reversible. Hence a highly substituted dye-ligand adsorbent could be employed to adsorb more protein without worrying the difficulty in an elution stage. Quantitative relationships between the Langmuir model parameters and the ionic strength were obtained. The relationships might be useful to the design and analysis of dye-affinity adsorption processes.

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