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Affinity-Based Reversed Micellar Bovine Serum Albumin (BSA) Extraction with Unbound Reactive Dye

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ABSTRACT

A quick and simple method has been introduced for affinity extraction with an unbound reactive dye in a reversed micellar system. Bovine serum albumin (BSA) and Cibacron Blue 3GA (CB) were chosen, respectively, as a model protein and an unbound ligand. CB in the aqueous phase was directly transferred to the reversed micelles due to electrostatic interaction between anionic CB and cationic cetyltrimethylammonium bromide. The BSA transfer to the reversed micelles increases significantly by affinity interaction with the addition of a small amount of CB to the aqueous phase. For solutions with pH close to pI, the selectivity of proteins can be expected in the presence of affinity CB as very little BSA is extracted into the reverse micelles without the addition of CB. Most of the BSA (~82%) extracted into the reversed micelles can be backextracted into the stripping aqueous phase by increasing of *n*-hexanol concentration in the organic phase while no CB is transferred to the aqueous solution at the same time.

Key Words. Affinity extraction; Reversed micelles; Cibacron Blue 3GA; Bovine serum albumin

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INTRODUCTION

Affinity-based separations have exquisite selectivity and have been used to purify proteins. The dominating affinity purification technique applied is connected with chromatography. While affinity chromatography has been carried out on a large scale, for the most part such systems operate discontinuously and the economy of scaleup has not often been realized (1). The affinity-based reversed micellar extraction and separation (ARMES) has advantages over other affinity separation techniques due to the inherent simplicity and scalability of liquid–liquid extractions. Moreover, ARMES affords selectivity in both the affinity interaction step and the extraction step (2).

In recent years, several research groups have investigated the separation of proteins with reversed micellar systems with the addition of bioaffinity ligands (2–10). Significant improvement on process selectivity can be achieved in most of these studies. Anionic surfactants such as AOT have often been performed with alkyl glucoside as the ligand in affinity reversed micellar systems. One of the most popular ligands for use in other affinity techniques such as chromatography and aqueous two-phase extraction has been reactive dyes, especially Cibacron Blue F3G-A (CB). It seems that the reactive dyes have been paid little attention for separation with reversed micellar systems (11). Sun et al. used immobilized CB to extract low molecular weight proteins such as lysozyme with the result of a significant increase of the solubilization of lysozyme and selectivity. The reversed micellar system has been shown to give low protein extraction capacity and no solubilization of high molecular weight proteins such as BSA (bovine serum albumin) (11).

In this work, BSA was chosen as a model protein. The CB in the aqueous phase was directly introduced to the reversed micelles formed with a cationic surfactant, cetyltrimethylammonium bromide (CTAB), due to electrostatic interaction between anionic CB dye and cationic surfactant. Various parameters, such as concentrations of CB, BSA, and hexanol, as well as the ionic strength of the aqueous phase on the forward and backward transfer of BSA were studied.

MATERIALS AND METHODS

Bovine serum albumin (67 kDa, pI4.7) was purchased from Beijing Hongxing Huaxue Factory, China. Cibacron Blue 3GA (dye content approximately 55%) was obtained from Sigma. Beijing Chemical Reagents Company supplied cetyltrimethylammonium bromide (CTAB), *n*-hexanol, and *n*-hexane (analytical grade). Other chemicals were all commercially available reagents of analytical grade.

CTAB can be dissolved in apolar solvents to form reversed micelles with the addition of a cosolvent (12, 13). *n*-Hexanol was chosen as the cosolvent for

CTAB, and all reversed micellar solutions contained 50 mM CTAB and 15% (v/v) *n*-hexanol in *n*-hexane. The aqueous phase was 10 mM citric acid–sodium citrate buffer stocks. The ionic strength of the aqueous phase was adjusted to the desired value by the addition of KBr or KCl. Protein solutions were prepared by dissolving BSA in buffer stocks. The concentration of BSA in the initial aqueous phase was 1.0 mg/mL before forward extraction. CB (typical 0.947 mmol/l) with a molar ratio of CB to CTAB (50 mM) of 0.0189 was dissolved in buffer solution before the forward extraction. The phase transfer experiments were carried out in tightly stoppered 50 mL glass flasks. During the forward extraction, equal volumes (usually 3 mL) of reversed micellar solution and aqueous phase were mixed in a flask and agitated horizontally in a water bath at 30.0 ± 0.5°C with rate of 2.91 s^{-1} for 10 minutes. The mixtures were then centrifuged at 50 s^{-1} (∼6000*g*) for 5 minutes to reach a clear separation of the two phases; no precipitation appeared at the interface. The lower aqueous phase was separated and analyzed. Blank experiments were performed simultaneously with the aqueous phase containing no protein. The unbound CB in the initial aqueous phase will transfer into the organic phase in all experiments studied on forward extraction. CB could not be detected in the aqueous phase after forward extraction, which indicated that the amount of CB remaining in the aqueous phase can be considered to be very small (as low as 3.8 nmol CB in the aqueous phase can be detected by visible light spectroscopy). BSA concentration in the aqueous phase was determined by UV spectroscopy and in the reversed micellar phase by mass balance because the UV absorbance could not be measured with the organic phase due to its extremely high contents of CB. To assure the correct estimation of BSA in the reversed micelles, the mass balance of BSA was made after forward and backward extraction with the two examples shown in Table 1. The error in BSA estimation in the reversed micellar phase is usually lower than 3.0% by mass balance.

TABLE 1
Mass Balance of BSA after Forward and Backward Extraction

BSA in initial aqueous phase (mg)	3.0	3.0
BSA in aqueous phase after forward extraction (mg)	0.0[a]	2.2626[b]
BSA in reversed micellar phase by mass balance (mg)	3.0	0.7374
BSA in aqueous phase after backward extraction (mg)[c]	2.9541	0.7182
Error, %	−1.53	−2.60

[a] Forward extraction conditions: pH 7.70, CB and KCl concentrations were 0.71 and 75 mmol/L, respectively.

[b] Forward extraction conditions: pH 4.50, CB and KCl concentrations were 0.95 and 66.7 mmol/L, respectively.

[c] Backward extraction conditions: pH4.50, KBr and isopropanol concentrations were 1.5 mol/L and 17.5% (v/v), respectively.

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During the backward extraction, the reversed micellar phase loaded with protein from the forward extraction was mixed with an equal volume of fresh stripping solution at 30.0 ± 0.5°C with an rate of agitation of 3.75 s^{-1} for 60 minutes. To study the influence of operating conditions on backward transfer, KBr for ionic strength adjustment and the effect of an increase of hexanol concentration in the organic phase were tested. Blank experiments were performed simultaneously on the upper organic phase obtained in the forward extraction with the aqueous phase containing no protein. The mixture was then centrifuged at 50 s^{-1} (~6000*g*) for 5 minutes and the protein concentration in the stripping solution was determined.

The concentration of unbound CB in the aqueous phase was measured by visible light spectroscopy at 605 nm on a model 751G UV/Vis spectrophotometer (190–800 nm, Shangfen Analytical Instrument Factory of China). BSA concentrations in the aqueous solution were determined by measuring the absorbance at 278 nm, or by the Bradford method with the calibration curves prepared (14).

RESULTS AND DISCUSSION

Effect of CB Concentration on BSA Solubilization

CB has a strong binding affinity to BSA whose dissociation constant was reported as 8.5×10^{-6} mol/L (15). Figure 1 shows the BSA solubilization in the reversed micellar solution as a function of initial aqueous CB concentra-

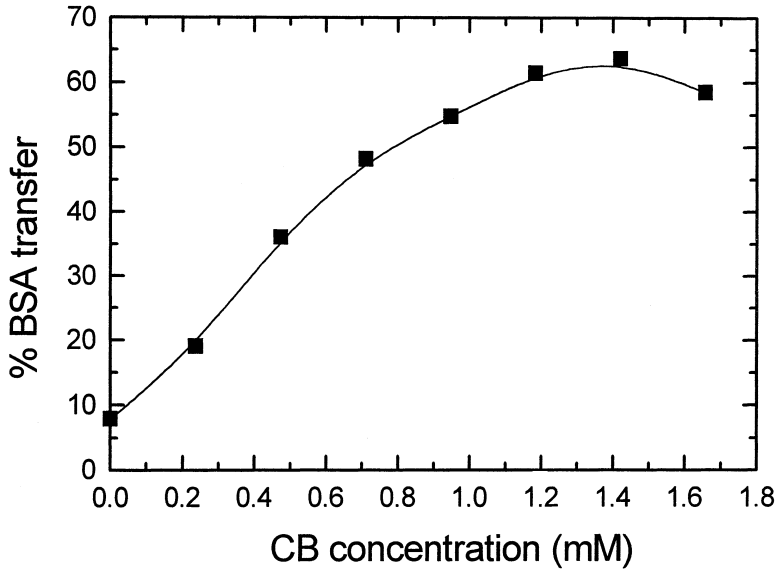

FIG. 1 Effect of CB concentration on BSA transfer. Forward extraction conditions: pH 4.83; KBr and BSA concentrations were 45 mM and 1.0 mg/mL, respectively; CTAB (50mM)/hexanol (15% v/v)/hexane.

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tion where the forward transfer is defined as the percentage of BSA transferred to the micellar phase based on the total BSA initially present in the aqueous phase. At pH 4.83 (pH near pI), little transfer of BSA (7.88%) without CB addition was observed due to weak electrostatic attraction between BSA and CTAB. As expected, the transfer of BSA increases with an increase of CB concentration, which indicates the affinity effect of CB. The enhancement of BSA transfer with CB addition indicated that the affinity interaction provided the main driving force for BSA transfer. However, the BSA transfer is not very high, which might be a limitation of the reversed micellar system and is worthy of further study. At the maximum of BSA transfer (~63%), which is much higher than that obtained (7.88%) without the addition of CB, the molar ratio of CB to BSA is 95.3. Excess CB is necessary due to the relatively weak affinity of CB to BSA (the dissociation constant is 8.5×10^{-6} mol/L) as well as the strong tendency of CB for its own transfer into the reversed micellar phase, resulting in a lowering of the concentration of CB available to bind to BSA in the aqueous phase.

Effect of BSA Concentration on BSA Solubilization

Figure 2 shows the solubilization of BSA in reversed micellar solution as a function of initial aqueous BSA concentration. For cases with and without the

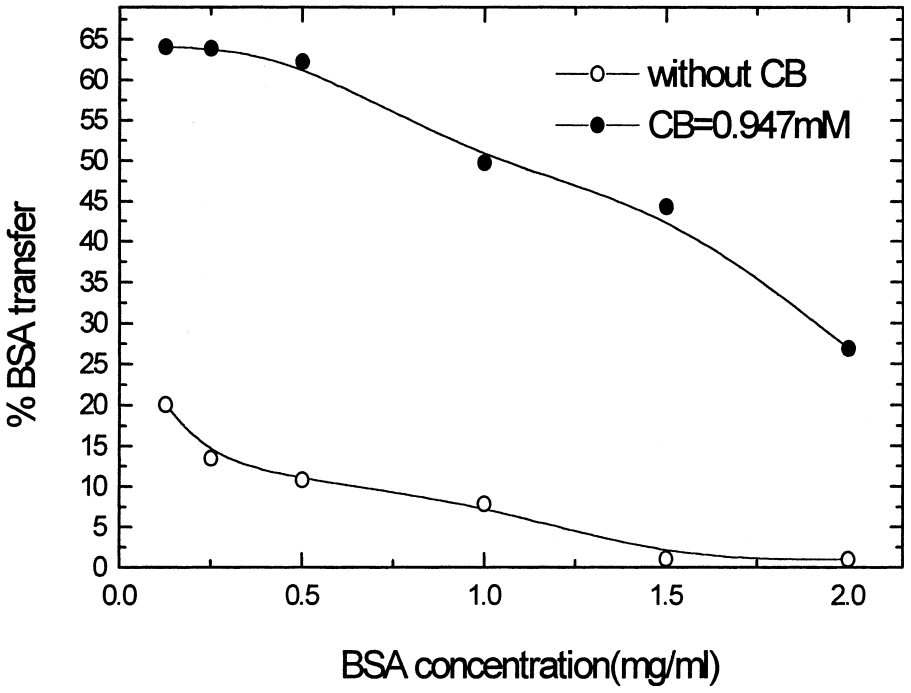

FIG. 2 Effect of BSA concentration on BSA transfer. Other forward extraction conditions are the same as indicated for Fig. 1.

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addition of CB, percent BSA transfer increases with decreasing BSA concentration. The curve of %BSA transfer to reverse micellar solution with the presence of CB decreases much faster than the curve shown for the BSA transfer without addition of CB.

Backward Extraction

The backward extraction of protein in reversed micellar systems is more difficult to accomplish. This process was reported to be three orders of magnitude slower than the forward extraction (16, 17). In a recent report the expelling of proteins from the reversed micelles was obtained by adding the series of 1-alkanols, e.g., 1-butanol or 1-decanol (in the absence of aqueous stripping solution), into the reverse micellar phase (18). The backward extraction of protein in affinity reversed micellar systems to an aqueous solution has not been fully studied (4, 11). To obtain a high BSA recovery in backward extraction, regulation of the concentration of hexanol in the reversed micellar system could lessen the affinity interaction between protein and BSA.

The effect of hexanol concentration in the organic phase on BSA backward transfer was shown in Fig. 3. A low transfer of BSA (16.5%) into the fresh aqueous phase in backward extraction was observed when the total hexanol concentration in the organic phase was 15% (v/v). The BSA transfer increases with an increasing concentration of total hexanol in the organic phase. No further increase of BSA transfer was observed when the total hexanol concentra-

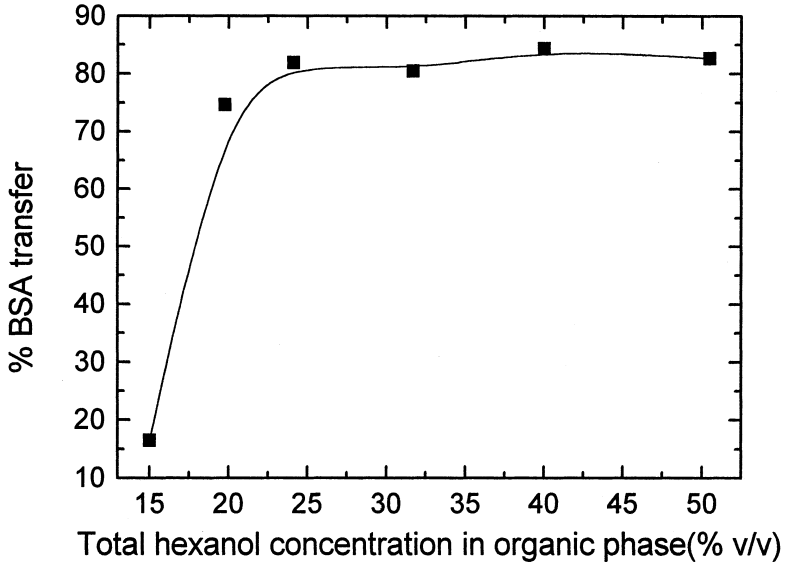

FIG. 3 Effect of hexanol concentration in the organic phase on BSA backward extraction. Forward extraction: CB concentration was 0.947 mM, other conditions were the same as given for Fig. 1. Backward extraction conditions: pH 4.82, KBr concentration was 1.5 M.

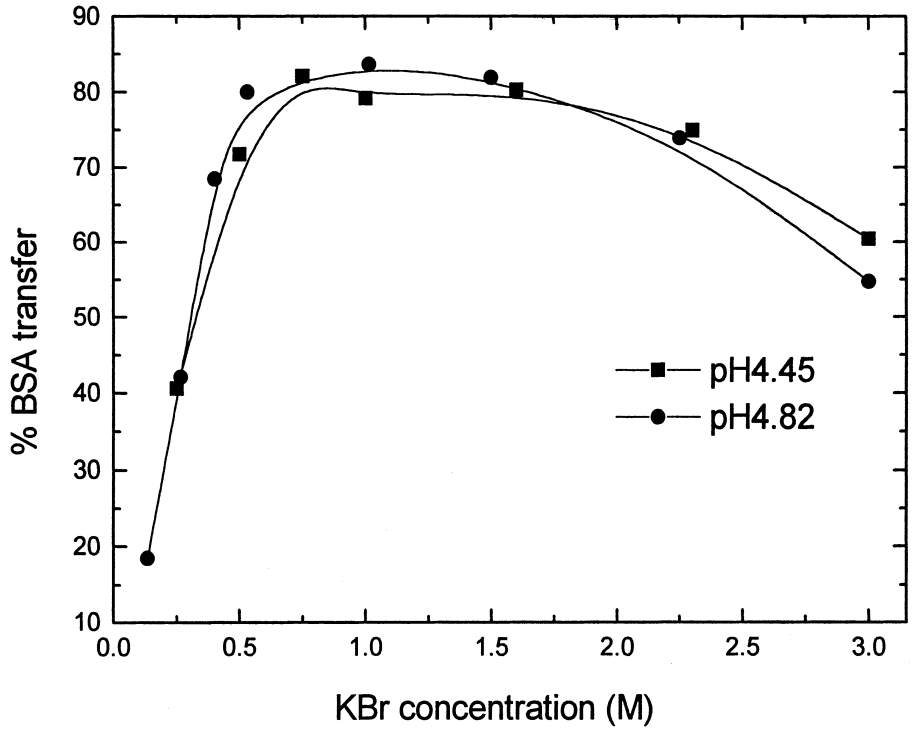

FIG. 4 Effect of salt concentration on BSA backward transfer under different pH conditions. Forward extraction conditions were the same as given for Fig. 3. Backward extraction: Total hexanol concentration in organic phase was 24.1% (v/v).

tion in the organic phase was above 24.1% (v/v). The maximum transfer of BSA in backward extraction is ~81%, which means that it is a quick and simple method for the recovery of BSA by adjusting the hexanol concentration in the reversed micellar system. No CB is observed in the aqueous phase after backward extraction, which indicates that CB remains in the organic phase.

The salt concentration in the aqueous phase can influence the efficiency of protein transfer through a size exclusion effect (19). Without the addition of CB, the addition of KBr into the aqueous phase gives a higher BSA transfer compared to the addition of KCl (13). The effect of KBr concentration on BSA transfer in backward extraction is shown in Fig. 4. The curves given indicate a maximum of BSA transfer (~82%) with about 1.0 M KBr. There is not much difference between pH 4.45 and pH 4.82 (pI 4.7 for BSA) for BSA backward transfer.

BSA should be dissociated from CB in the reversed micelles in the backward extraction. Both high KBr (1.0 M) and hexanol (24.1 %v/v) concentrations are necessary to obtain high BSA transfer in backward extraction (Figs. 3 and 4). A high salt concentration could lessen the affinity interaction between BSA and CB. An increase of hexanol concentration might be favorable

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for BSA transfer into the stripping aqueous solution since a high hexanol concentration is not favorable for BSA transfer in forward extraction (13).

An important factor in using the affinity reversed micellar systems for protein separation is the reusability of the reversed micellar phase. In this work, no other cosolvent was introduced during backward extraction. The reversed micellar phase can be reused following a cycle of forward and backward extractions with the necessary adjustment of hexanol concentration in the organic phase.

CONCLUSIONS

Reversed micelles containing no affinity ligand solubilize little BSA due to weak electrostatic interaction with pH near pI. The addition of a small amount of unbound CB to the aqueous phase will be transferred to the reversed micelles by electrostatic interaction between anionic CB and cationic CTAB.

The enhancement of BSA transfer with the addition of CB indicates a stronger interaction between the extracted BSA and the reversed micellar phase. For pH near pI, the selectivity can be expected in the presence of CB because affinity interaction was the main driving force for BSA transfer. For backward extraction of BSA from the reversed micellar phase with a stripping aqueous solution, the increase of hexanol concentration in the organic phase can increase the amount BSA stripped back to the aqueous phase with CB remaining in the organic phase.

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