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Extraction of Bovine Serum Albumin Using Reverse Micelles Formed by Hexadecyl Trimethyl Ammonium Chloride

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Abstract The extraction of bovine serum albumin (BSA) has been investigated using reverse micelles of hexadecyl trimethyl ammonium chloride/n-octanol/isooctane. Forward extraction process parameters such as the surfactant concentration, co-solvent concentration, pH, ionic strength, and species of the initial aqueous phase were important factors affecting the extraction performance. These parameters were varied to optimize the extraction efficiency. Under the optimized conditions, forward extraction efficiencies of BSA can reach practically 99.55%. The thermodynamic study revealed that the extraction of BSA is controlled by entropy changes. Maximum back-extraction efficiency of 85.16% can be obtained at low pH values and high salt concentrations. The structures of BSA during reverse micelle extraction did not change by comparing the circular dichroism spectra of BSA back-extracted to the aqueous phase with that of feed BSA.

Keywords BSA · Reverse micelles · Forward extraction · Back extraction · CD spectra

Introduction

Reverse micelles are nanometric aggregates of surfactant molecules in a continuous organic solvent medium. A variety of biologically active materials such as amino acids, proteins, and enzymes can be solubilized into water pools inside reverse micelles [1]. These biologically active materials may retain their activity because surfactant molecules can prevent direct interactions with apolar solvent [2–4]. Extraction of proteins into reverse micelles is mainly influenced by surfactant concentration, solution pH, ionic strength, and phase volume ratio. The mechanism of protein extraction has been expounded by the interaction between reverse micelles and proteins, such as electrostatic, steric, and hydrophobic interactions [5]. It has been reported that the back extraction of protein from reverse micelles to the aqueous solution is relatively slow due to high interfacial resistance in mass transfer [6, 7]. Conditions of high ionic strength and

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pH within a certain range could not be used for the complete recovery of proteins solubilized in the forward extraction. The back-extraction efficiency can be improved by addition of various alcohols like methanol, ethanol, and propanol to the fresh aqueous phase [8].

Bovine serum albumin (BSA; molecular weight (MW)=67 kDa, pI=4.8) has been widely researched as a model protein with high helix content in the study of interactions with surfactants. BSA has been found to be rather difficult to be solubilized in the reverse micelles owing to its large molecular size (3.5–3.6 nm) compared with the average size of reverse micelles [9]. Although, a few reports on reverse micelle extraction of BSA have been reported, extraction efficiency of BSA that has been reported is different. Wolbert et al. reported the unsuccessful extraction of BSA in sodium bis (2-ethyl-hexyl) sulfosuccinate (AOT)/isooctane reverse micellar system owing to the extraction conditions employed that were not sufficient enough to provide the required energy for the extraction of BSA [10]. BSA could be easily extracted by a cetyl trimethyl ammonium bromide (CTAB)/n-hexanol/octane reversed micellar system. Forward and backward transfer can be achieved under certain conditions [11]. It has been reported that BSA is more difficult to extract into AOT reversed micelles than CTAB reversed micelles [12]. Although the size of the Triton X-100 reverse micelle in toluene was large enough to host BSA molecule in the hydraulic core, the overall extraction efficiency was found to be low, which might be due to lack of strong driving force [13]. The affinity extraction of proteins with reverse micellar system composed of cibacron blue (CB)-modified lecithin was studied and concluded that the failure to extract BSA was due to its large molecular weight [14]. The extraction of BSA was greatly enhanced by the addition of alkyl halides to the reverse micellar system which is made of the cationic surfactant CTAB soluble in hexanol [15]. The extraction efficiency of CTAB/CB/hexane reverse micelles can be enhanced by adding alcohol to the aqueous phase during back extraction of BSA [16]. Hong et al. [17] reported that the effect of addition of different alcohols on the extraction of BSA using AOT/isooctane reverse micelles.

In this work, BSA was chosen as a model protein. BSA was found to be effectively extracted into reverse micelle phase which was made of the cationic surfactant hexadecyl trimethyl ammonium chloride (CTAC) soluble in isooctane and n-octanol mixture. The process parameters of forward extraction such as the surfactant concentration, co-solvent concentration, pH, ionic strength, and species of the initial aqueous phase have been optimized. The effects of process parameters on forward extraction of BSA as well as on back extraction and BSA structural changes during the extraction process were examined in detail. The reusability of the reverse micelles solution has been determined. The Gibbs energy of the BSA transfer process at a given temperature has been calculated.

Materials and Methods

Instrument and Materials

The following are the materials used in this study: vibrator (made by Yancheng Science Instrument Factory, Jiangsu Province), with a vibration frequency of 275±5 times/min and controlling temperature precision of ±1 K, UV-754 type grating spectrophotometer (Shanghai Third Analysis Instrument Factory), and J-810 circular dichroism (CD) spectrometer (Jasco Corporation, Japan). BSA (MW=67 kDa, pI=4.8), ethanol, and Coomassie Blue G250 were from Sinopharm Chemical Reagent Co., Ltd. CTAC (dye content approximately 98.0%) was obtained from Tianjin Fuchen Factory (China). N-octanol and isooctane were supplied by Beijing Damao Chemical Reagent Company (analytic grade). Other chemicals were analytical grade.

Experimental Procedure

Forward Extraction

The organic phase was prepared by dissolving CTAC in isooctane containing n-octanol as co-solvents. The initial aqueous phase was containing 1.0 mg/mL BSA. Except for temperature control experiments, the experimental temperature was maintained at 298±1 K. Equal volumes (usually 4 mL each) of aqueous and organic solutions were poured gently into a 10-mL tube. The mixtures were shaken mechanically for 5 min (The extraction of BSA has reached the equilibrium after 4 min, and no further transfer of BSA was observed). The mixtures were then centrifuged at 3,200 r/min for 2 min to obtain a clear separation of two phases with no precipitation at the interface. Blank experiments were performed simultaneously with an aqueous phase containing no protein.

Back Extraction

Back extraction was carried out by mixing the organic phase of the forward extraction with an equal volume of fresh aqueous phase in a tube. The organic phase and fresh aqueous phase were shaken mechanically for 60 min. Then, the mixtures were centrifuged at 3,200 r/min for 2 min. The two phases were then collected for further analysis. Blank experiments were performed simultaneously on the upper organic phase obtained in the forward extraction with the aqueous phase containing no protein.

Analytical Methods

BSA concentrations of aqueous phases were determined by the Bradford method [18] using UV-754 type spectrophotometer. CD spectra of the native and the back-extracted BSA were measured with a J-810 CD Spectrometer (Jasco Corporation, Japan). Efficiencies of forward (E_f) and back (E_b) extraction were estimated using the equation given below:

$$\textit{Extraction efficiency } \mathrm{E}_f(\%) = \frac{\textit{BSA concentration in organic phase after forward extraction } (mg/mL)}{\textit{BSA concentration in feed } (mg/mL)} \times 100$$

$$\text{Back extraction efficiency } \mathrm{E_b}(\%) = \frac{\text{BSA concentration in back extracted aqueous phase } (\mathrm{mg/mL})}{\text{BSA concentration in forward extracted organic phase } (\mathrm{mg/mL})} \times 100$$

Results and Discussion

Forward Extraction

Effect of Co-solvent Concentration

N-octanol was selected as a co-solvent owing to its solubility in water being relatively low, and hence the amount lost is reduced for practical application. Effect of co-solvent

concentration on the extraction efficiency was shown in Fig. 1. As the n-octanol concentration was increased above 25% (*v*/*v*), extraction efficiency decreased. This may probably be due to the reduction in electrostatic repulsion between the charged surfactant head groups at higher concentration of n-octanol, which gives rise to weak hydrophobic interaction between the hydrophobic tails of surfactant molecules, leading to a denser packing of surfactant molecules in reverse micelles [19]. The organic phase was cloudy, and a white precipitate was observed when n-octanol concentration is lower than 15% (*v*/*v*). At 25% (*v*/*v*), the extraction efficiency was highest (99.55%), and the rate of phase separation is fast enough. In reversed micellar systems, a co-solvent (usually an alcohol) plays two roles [20]. Firstly, the solubility of the surfactant in the hydrocarbon can be increased. Secondly, the strong repulsive ion–ion interaction between the surfactant head groups may be buffered, thus allowing their close packing in order to form the inner core of a reverse micelle.

Effect of Surfactant Concentration

The surfactant CTAC concentration in the organic phase was varied in the range of 0.016–0.04 M, while maintaining the aqueous pH and KCl concentration at 11.0 and 0.1 M, respectively (Fig. 2). The increase in surfactant concentration from 0.016 to 0.02 M was found to increase appreciably the extraction efficiency of BSA (from 99.21% to 99.55%). This could be explained based on the fact that an increase in surfactant concentration increased the number of reverse micelles, which in turn enhanced the extraction efficiency of BSA [21]. Beyond surfactant concentration of 0.02 M, the extraction efficiency of BSA was found to decrease. The efficiency dropped to 96.72% at 0.04 M surfactant concentration, which may be due to increased surfactant concentration that causes micellar interactions leading to percolation and interfacial deformation with a change in micellar shape and micellar clustering [22]. The critical micelle concentration (CMC) of CTAC is 0.016 M at 298±1 K. Therefore, the experimental concentration of CTAC should be greater than the CMC.

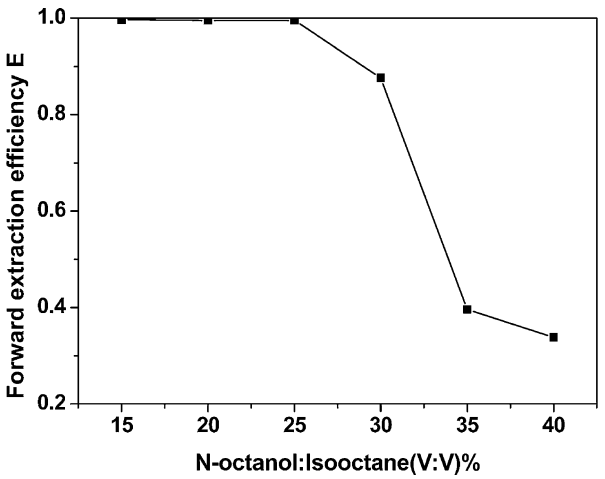

Fig. 1 Effect of co-solvent concentration on extraction efficiency *E* ([CTAC]=0.02 mol/L, [KCl]=0.1 mol/L, pH=11.0, [BSA]=1.0 mg/mL)

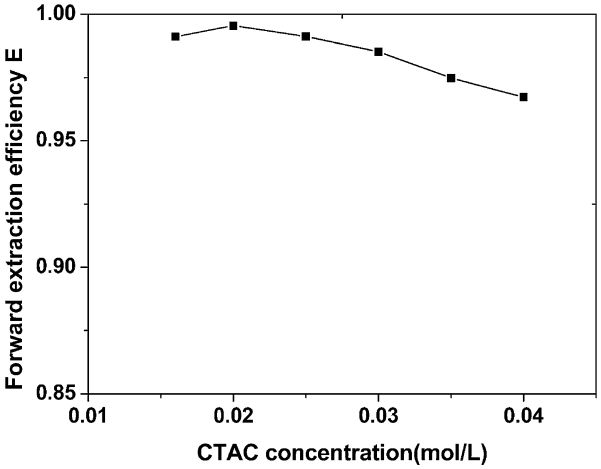

Fig. 2 Effect of surfactant concentration on extraction efficiency *E* (n-octanol/isooctane (*v*/*v*)=25%, [KCl]=0.1 mol/L, pH=11.0, [BSA]=1.0 mg/mL)

Effect of Aqueous Phase pH

Extraction of BSA was carried out at various pH values from 6.0 to 11.5, a salt concentration of 0.1 M (KCl), and a CTAC concentration of 0.02 M (Fig. 3). When the initial aqueous pH is lower than 6.0 or higher than 11.5, white precipitate was observed, and it was difficult to measure BSA concentration due to turbidity in the aqueous phase. When the aqueous phase pH is less than 6.0, the amount of white precipitate decreased with increasing pH. When the aqueous phase pH is more than 11.5, the amount of white precipitate increased with increasing pH. This can be interpreted that electrostatic force between protein molecules and polar heads of surfactant molecules is too strong to form a stable reversed micelle which contains protein. At the pH of initial aqueous phase in the range of 6.0 to 11.0, BSA extraction efficiency increased with an increase in aqueous phase pH, reaching a maximum (99.55%) at 11.0. This result can be interpreted in terms of electrostatic interaction between BSA and the reverse micelles. The cationic surfactant CTAC was used to form the reverse micelles, and the inner surfaces of reverse micelles

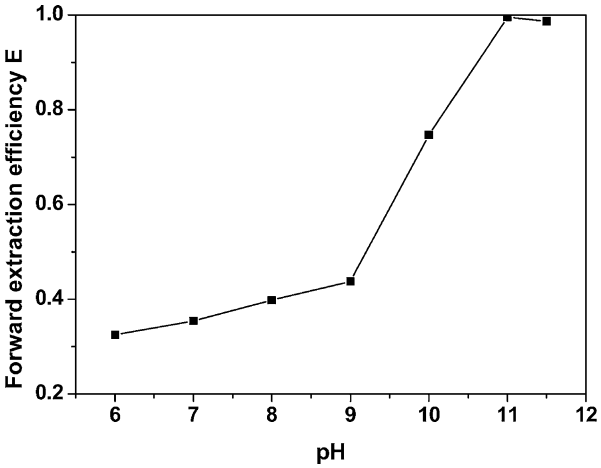

Fig. 3 Effect of aqueous phase pH on efficiency *E* ([CTAC]= 0.02 mol/L, n-octanol/isooctane (*v*/*v*)=25%, [KCl]=0.1 mol/L, [BSA]=1.0 mg/mL)

were positively charged. When the initial aqueous pH is higher than the isoelectric point of the BSA that will be negatively charged, the transferring protein into reverse micelles will be observed [23]. The more the number of protein negative charge carried by the greater initial aqueous pH, the stronger the electrostatic attraction between the inner surface of reverse micelle and BSA.

Effect of Ionic Strength and Species

The effect of ionic strength (0.025–1.0 M KCl) on the degree of extraction was shown in Fig. 4. The surfactant CTAC concentration and initial aqueous pH were maintained at 0.02 M and 11.0, respectively. When the salt concentration is less than 0.025 M, the phenomenon of two-phase precipitate appears, and the two phases cannot be separated. The ionic strength may be too weak to form stable reverse micelles which contain proteins. At salt concentration in the range of 0.025 to 0.15 M, the extraction efficiency was almost constant with increasing salt concentration, all around 99%. The BSA extraction efficiency decreased with salt concentration increasing from 0.15 M to 1.0 M. This phenomenon can be explained by two reasons. On one hand, the variation in extraction efficiency with salt concentration indicated the existence of electrostatic attraction between the BSA and surfactant molecules. The role of the shielding which affects the electrostatic attraction between BSA and surfactant molecules is enhanced with ionic strength increase. On the other hand, the expulsion of the BSA from the core due to reduction in reverse micelle sizes with increased salt concentration (squeezing out effect) and reduction in Debye length [24] might have resulted in the lower extraction efficiency at higher salt concentrations.

Figure 4 shows the effect of ionic species on extraction of BSA. Changing cation species (K^+ Na^+) had no significant effect on the extraction efficiency. The effect of anion species on the extraction efficiency of BSA is more significant than that of cation species. The existence of F^-, Cl^-, and Br^- ions in the system can affect the extraction of BSA in two ways: one is to decrease solubility of BSA in aqueous phase due to salting out effect, and another is the screen effect due to their electrostatic attraction with CTAC as mentioned before [25]. At low concentration, the salting out effect dominates, the partition effect

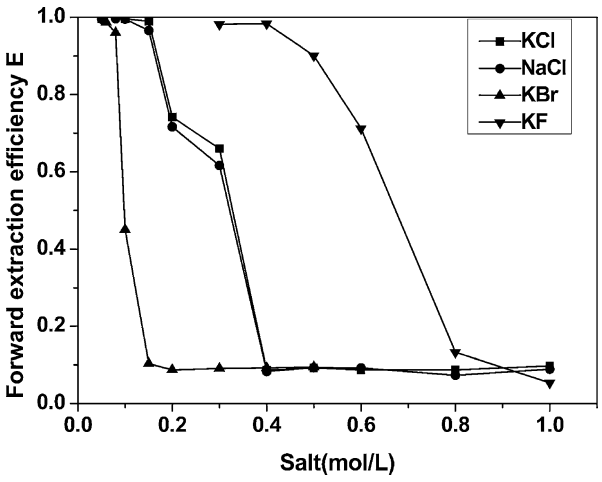

Fig. 4 Effect of ionic strength and species on extraction efficiency *E* ([CTAC]=0.02 mol/L, n-octanol/isooctane (*v*/*v*)=25%, pH=11.0, [BSA]=1.0 mg/mL)

increases with an increase of anion concentration. At higher anion concentration, the screen effect dominates, the partition effect decreases with a further increase of anion concentration. The smaller ions with larger charges have better salting out effect, and the bigger ions with larger charges have stronger screen effect. In the same ionic strength, the influence of anion species on the extraction efficiency follows Hofmeister series of anions: $F^- < Cl^- < Br^-$. The BSA extraction is relatively sensitive to the anion species, and the order of extraction easiness is KBr < KCl < KF. Anion species have also an impact on the separation characteristics of the two phases. Experiment observed that the phase separation is not easy when the aqueous phase is solution of KF.

Effect of Extraction Temperature

The temperature is an important physical parameter involved in the reverse micelle extraction. The extraction efficiency during forward extraction (from 59.02% to 94.17%) was found to increase with an increase in temperature from 288.15 to 313.15 K. From the viewpoint of thermodynamics, extraction of a protein can be regarded as a transfer process of the protein from the aqueous phase to the organic phase. The Gibbs energy of such a transfer process at a given temperature can be calculated from the partition data by:

$$\Delta G^0{}_T = -RT \ln K \tag{1}$$

where K is partition coefficients of the BSA between two phases, T is the temperature (K), and R is the gas constant. The enthalpic change ($\Delta H^0{}_T$) and entropy change ($\Delta S^0{}_T$) can be calculated from the slope and intercept of the linear Eq. (2) [26]. The linear relationship between ln K and $1/T$ was shown in Fig. 5.

$$\ln K = \frac{-\Delta H_T^0}{RT} + \frac{\Delta S_T^0}{R} \tag{2}$$

$\Delta G^0{}_T$, $\Delta H^0{}_T$, and $\Delta S^0{}_T$ values were included in Table 1. It can be seen that values of $\Delta G^0{}_T$ are negative whereas those of $\Delta H^0{}_T$ and $\Delta S^0{}_T$ are positive, and $T\Delta S^0{}_T$ is always

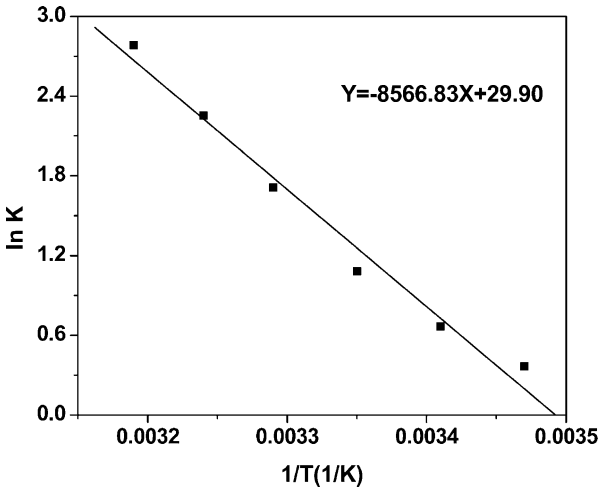

Fig. 5 Effect of extraction temperature on extraction efficiency E ([CTAC]=0.02 mol/L, n-octanol/isooctane (v/v)=25%, [KCl]=0.1 mol/L, pH=10.0, [BSA]=1.0 mg/mL)

Table 1 The transfer thermodynamic properties for BSA from the aqueous phase to organic phase

T (K)	K	$\Delta G^0{}_T$ (kJ mol^{-1})	$T\Delta S^0{}_T$ (kJ mol^{-1})	$\Delta H^0{}_T$ (kJ mol^{-1})
288.15	1.44	−0.87	7.16	
293.15	1.95	−1.63	7.29	
298.15	2.95	−2.68	7.41	7.12
303.15	5.55	−4.32	7.54	
308.15	9.52	−5.77	7.66	
313.15	16.15	−7.24	7.78	

[CTAC]=0.02 mol/L, n-octanol/isooctane (*v*/*v*)=25%, [KCl]=0.1 mol/L, pH=10.0, [BSA]=1.0 mg/mL

greater than $\Delta H^0{}_T$ in value. This indicates that extraction of BSA is controlled by entropy changes, which is the characteristic of hydrophobic interactions [27].

Back Extraction

Effect of Fresh Aqueous Phase Salt Concentration on Back Extraction

The effect of salt (KCl) concentration on the back-extraction efficiency of BSA was presented in Fig. 6. In the present case, the extraction efficiency of BSA was found to increase with an increase in salt concentration up to 0.5 M because an increase in salt concentration reduces the repulsive interactions between the surfactant head groups and decreases the size of the micelles, which help in the recovery of BSA during back extraction. When salt concentrations were greater than 0.5 M the back-extraction efficiency decreased. BSA with hydrophobic surface patches exhibit similar behavior and may be retained in the reverse micelle phase even at unfavorable conditions of higher ionic strength [28].

Effect of Fresh Aqueous Phase pH on Back Extraction

The protein extracted into the reverse micelles can be recovered by the backward transfer through the result of electrostatic repulsion by changing the solution pH [29]. Effect of

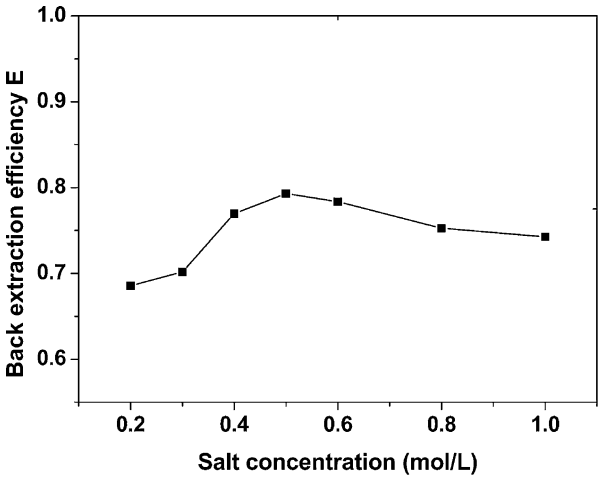

Fig. 6 Effect of fresh aqueous phase salt concentration on back-extraction efficiency E ([CTAC]= 0.02 mol/L, n-octanol/isooctane (*v*/*v*)=25%, ethanol concentration (*v*/*v*)=15%, pH=6.0)

aqueous phase pH used for the backward extraction of the BSA was shown in Fig. 7. In the present study, the aqueous phase pH was varied from 4.0 to 8.0. For pH<pI, back-extraction efficiency was almost constant between pH 4.0 and 5.0, and the extraction efficiency of BSA (85.16%) was found to be highest at pH 5.0. However, at pH>pI, the electrostatic attraction between BSA and CTAB would increase with increasing pH, which will decrease the BSA transfer back into the fresh aqueous phase. The back-extraction efficiency dropped to 70.34% at pH 8.0.

The CD Spectra of BSA Back-Extracted

The back extraction of protein in reversed micellar systems is more difficult to accomplish. This process was reported to be slower than the forward extraction. For backward extraction of BSA, adding ethanol to the fresh aqueous phase can improve back-extraction efficiency. This may be due to the interaction between surfactant molecule and protein that is weakened by ethanol, which helps in the release of BSA from reverse micelles. The experiments revealed that ethanol concentration 15% (*v*/*v*) in the aqueous phase is appropriate. Under the optimized backward conditions, the back-extraction efficiency of BSA (85.16%) was found to be highest using a fresh aqueous phase (KCl 0.5 M, aqueous pH 5.0, and ethanol/fresh aqueous phase 15%)/the organic phase volume ratio of 1.

Successful reverse micelle extraction aims at obtaining high extraction (forward and back) efficiency without affecting the structure/activity of the biomolecule. In order to determine the structural changes in BSA during the extraction process, the CD spectra of BSA back-extracted to the aqueous phase from the organic phase were measured. The CD spectrum in the far-UV region, which reflects the secondary structure of a protein [17], was compared with that of feed BSA (Fig. 8). As can be seen from the spectrum, the ellipticity of the back-extracted BSA was almost similar to that of the feed BSA over the observed range. This indicates the maintenance of the structural integrity of BSA during reverse micelle extraction.

Effectiveness of Reverse Micelle Solution after Cycling Use

An important factor in using reversed micellar systems for protein separation is the reusability of the reverse micelle phase. In these experiments, the BSA solution was firstly

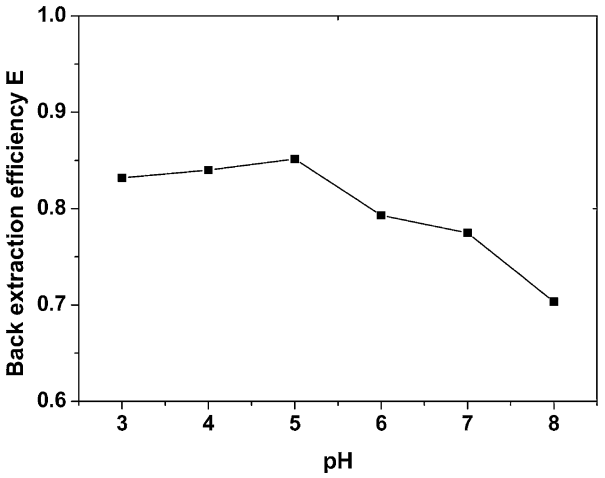

Fig. 7 Effect of fresh aqueous phase pH on back-extraction efficiency *E* ([CTAC]=0.02 mol/L, n-octanol/isooctane (*v*/*v*)=25%, ethanol concentration (*v*/*v*)=15%, [KCl]=0.5 mol/L)

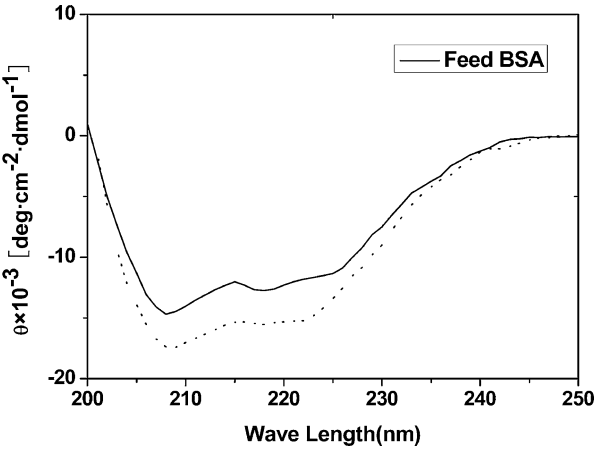

Fig. 8 Comparison of CD spectra of feed BSA with the BSA back-extracted from organic phase.

contacted with reverse micelle solution. After reaching forward extraction equilibrium, the two phases were separated. The BSA content of each phase was determined, and forward extraction efficiency could be calculated. The protein-laden reverse micelle solution was then contacted with an aqueous stripping solution. After reaching back-extraction equilibrium, the back-extraction efficiency could be available. Thirdly, following phase separation, the used reverse micelle solution was reused to treat another fresh BSA aqueous solution. In this way, the reverse micelle solution was repeatedly used, and the results are shown in Table 2. It can be seen that the extraction efficiency slightly decreased after two cyclic operations of forward and back extraction. This may be due to the surfactant losses during extraction process.

Conclusions

In conclusions, effects of different parameters on the forward- and back-extraction efficiency have been researched. Extraction efficiency of BSA was found to depend upon the reverse micelle system selected as well as the processing conditions adopted for forward and back extractions. Effects of surfactant concentration, pH, ionic strength, and species of the aqueous phase on forward extraction were obvious. Under the right conditions, forward extraction equilibrium can be achieved very quickly, and extraction efficiency (99.55%) was maximum using pH 11.0 containing 0.10 M KCl in aqueous phase and 0.02 M CTAB in organic phase consisting of 25% (*v*/*v*) n-octanol/isooctane. The experimental study

Table 2 Effectiveness of reverse micelle solution after cycling use

Effectiveness	Number of cycle	
	1	2
Forward extraction *E* (%)	99.55	98.53
Back extraction *E* (%)	85.16	78.94

Forward extraction: [CTAC]=0.02 mol/L, n-octanol/isooctane (*v*/*v*)=25%, [KCl]=0.1 mol/L, pH=11.0; back extraction: ethanol/fresh aqueous phase (*v*/*v*)=15%, [KCl]=0.5 mol/L, pH=5.0

revealed that the major driving force in the BSA extraction was electrostatic interaction. Although the hydrophobic interactions were also important for extraction of BSA, higher back-extraction efficiency (85.16%) was obtained using a fresh aqueous phase (KCl: 0.5 M, aqueous pH 5.0, and ethanol/fresh aqueous phase 15%)/the organic phase volume ratio of 1. We will use different methods to improve the BSA back-extraction efficiency and rate of backward extraction in the future. The CD spectra analysis of BSA indicates the maintenance of the structural integrity of BSA during reverse micelle extraction. Extraction of BSA using the reverse micelles has an excellent extractability, and the reverse micelle solution is reusable.

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