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# The Factors Affecting the Backward-Transfer of Bovine Serum Albumin (BSA) from Sodium Bis(2-ethylhexyl) Sulfosuccinate (AOT) Reverse Micellar Solutions

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**Abstract**−The factors affecting the back-extraction efficiency of Bovine Serum Albumin (BSA, 65kDa, pI 4.9) solubilized in an AOT reverse micellar solution, prepared by the injection method, to an excess aqueous phase were investigated. In particular, effects of pH, the type of salt and its concentration in the excess aqueous phase were examined. Furthermore, by comparing CD spectra of the back-extracted BSA with the feed BSA, the structural changes of the protein during the extraction process were determined. The addition of 1 : 1 salt such as KCl or NaCl to the aqueous phase resulted in a 100% recovery of the protein to the aqueous phase at a pH higher than its isoelectric point pI. This high efficiency of the back-extraction might be due to the change in the interactions between the protein and micellar aggregates driven by the added salt. For 1 : 2 salts like $MgCl_2$ or $CaCl_2$, BSA was back extracted with lower than 20% extraction efficiency. Maximum efficiencies were achieved at about pH=7 and pH=8 for monovalent and divalent salts, respectively. From the CD spectra of back-extracted BSA, it was observed that denaturation of BSA was not significant during the extraction process.

Key words: Reverse Micelle, AOT, Injection Method, Back-Extraction, BSA

## INTRODUCTION

Reverse micelles are aggregates of surfactants stabilized in an apolar organic solvent that contain nanometer-scale polar cores of solubilized water. In the reverse micellar system, the polar head groups of surfactants are concentrated in the interior of the aggregates and their hydrophobic moieties extend to the bulk apolar solvents. The ability of these aggregates to solubilize water varies widely with the molecular structure of the surfactants and depends on the organic solvent as well as the ionic strength of the aqueous phase and the concentration of surfactant [Mitchell and Ninham, 1981; Rabie and Vera, 1996; Rabie et al., 1997]. However, as long as water is present, it is localized in the inner core of the micelles forming a so-called "water pool" [Jolivalt et al., 1990]. In recent years, the reverse micellar systems have attracted considerable interest owing to their capability to solubilize, preferably selectively, hydrophilic components such as amino acids, nucleic acids and proteins present in aqueous phase [Goklen and Hatton, 1987; Kadam, 1986; Jolivalt et al., 1990; Dekker et al., 1989]. Because amphiphilic surfactant molecules prevent direct interactions with apolar solvent, biologically active materials encapsulated in the polar interior of the reverse micelles may retain their activity [Goklen and Hatton, 1987; Kadam, 1986; Jolivalt et al., 1990; Dekker et al., 1989; Marcozz et al., 1991; Krei and Hustedt, 1992; Shiomori et al., 1998].

Reverse micelles can be formed in an organic phase by the phase-transfer method or by the injection method [Dekker and Leser, 1994]. In the phase transfer method, a bulk excess aqueous phase is contacted with an organic solvent containing surfactant and cosurfactant. The excess aqueous phase usually contains an electrolyte to prevent the transfer of the surfactant from an organic phase to the aqueous phase. After mixing and settling, the organic phase is in equilibrium with the excess aqueous phase, thus forming a Winsor II system. In the injection method, on the other hand, a small amount of aqueous stock solution of a hydrophilic component is directly added to an organic solvent containing a surfactant and cosurfactant. The mixture is shaken until total solubilization has occurred. The maximum solubilization is determined from the appearance of permanent turbidity. However, relatively large monomeric and oligomeric proteins such as BSA have been found to be rather difficult to be solubilized in the reverse micelles by the phase transfer method [Kadam, 1986; Jolivalt et al., 1990; Dekker and Leser, 1994; Wolbert et al., 1989]. In fact, difficulty of the reverse micellar extraction of BSA may originate from its large molecular size compared with the average size of the reverse micelles at relatively low concentration of the surfactant. In most of the studies, a small amount of BSA with a prescribed condition was directly solubilized into the micelles by the injection method [Shiomori et al., 1995].

The effectiveness of reverse micellar extraction processes will depend on the ease with which the protein can be stripped (back-extracted) from the loaded organic phase into an aqueous phase, and on the degree to which biological function of the recovered product is retained. Back-extraction includes mixing of the laden organic phase with an excess aqueous phase where the protein is recovered after settling. In general, solubilization and thus the back-extraction of proteins is mainly governed by steric, electrostatic, and hydrophobic interactions between proteins and micelles, and the dominant factors for the reverse micellar extraction process include pH, ionic strength and type of ions present in the system, type of surfactant and organic solvent used, and physicochemical properties of the proteins such as isoelectric point (pI), hydrophobicity, size, charge density, and charge distribution [Goklen and Hatton, 1987; Kadam, 1986; Dekker and Leser, 1994]. The optimal conditions of back extraction can be conjectured from conditions under which

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the solubilization (forward transfer) is minimal. Alternatively, addition of polar solvents such as alcohols or acetates [Marcozz et al., 1991; Hilhorst et al., 1995; Hong et al., 2000] and an insoluble material such as silica or molecular sieve [Leser et al., 1993; Gupta et al., 1994] or formation of gas hydrate [Nagahama et al., 1996] could lead to an enhanced recovery with significant destruction of the protein activity.

Surfactants are molecules that consist of a polar head group, which can be anionic, cationic, zwitterionic, or nonionic, and apolar moiety, which may contain more than one hydrocarbon chains. Among all surfactants developed for reverse micellar extraction of biomolecules, sodium bis(2-ethylhexyl) sulfosuccinate (AOT), an anionic surfactant, has received the greatest attention because of its ability to form microemulsions containing large amount of water without adding any cosurfactant over a wide range of surfactant and water concentrations [Jolivalt et al., 1990; De and Maitra, 1995].

The purpose of this work is to examine the back-extraction efficiency of BSA from the laden organic phase by varying the pH, salt type and its concentration added to the excess aqueous phase. In this effort, BSA was solubilized into AOT reverse micelles dispersed in isooctane by the injection method. Back-extraction was followed by contacting an excess aqueous phase with the organic phase in which BSA has been incorporated. Further, structural change of the recovered BSA was determined by the circular dichroism (CD) method.

## EXPERIMENTAL

BSA (Mw=65 kDa, pI=4.9, Type-7906), AOT from Sigma Chemical Co. and isooctane (>99%) from YAKURI Co. were used without further purification. NaCl and KCl were purchased from Sigma Chemical Co. and Junsei Co., respectively. $MgCl_2$ and $CaCl_2$ of guaranteed reagent grade from YAKURI Co were used as received. Alcohols such as methanol, ethanol, and propanol were purchased from Sigma Chemical Co. Acetic acid-sodium acetate (pH 4-6) and Tris-HCl (pH 7-8) buffers were used for the pH adjustment of the aqueous phase. The concentration of these buffers was 10 mM. The water used for preparation of brine protein solution was doubly distilled and deionized.

The protein solution (0.24 mL) dissolving 4 mg/mL of BSA was injected into 3.76 mL of the organic phase comprised of AOT and isooctane. The initial AOT concentration of the organic phase was 200 mM and pH of the feed solution, denoted by $[pH]_{inj}$, was held at 5.0. The initial water content of the protein-incorporated organic phase $W_o$, which is usually represented by ratio of water concentration to that of AOT, was estimated as 16.7. The solution thus prepared was shaken vigorously until completely clarified. No salts were added in the BSA stock solution to prevent reduction of the value of $W_o$.

By contacting 4 mL of the laden organic solution with 4 mL of brine buffer solution, the protein was stripped from the reverse micellar phase to the excess aqueous phase of $[pH]_{aq}$. The solution was centrifuged at 360 rpm for 30 min and subsequently left to stand for 2 days at 20 °C in a temperature-controlled bath. The protein concentration of the organic and the aqueous phases were determined by spectrophotometry at 280 nm using Spectronics 21 (Bausch & Lamb). Water content of the organic phase at equilibrium was determined by using a Karl-Fisher titrator (Model 150 Denver Instrument, U.S.A.). Since the BSA concentrations of both phases were directly determined, any precipitation or aggregation of the protein in the interface, which has been often observed in the case of relatively massive proteins, can be easily detected.

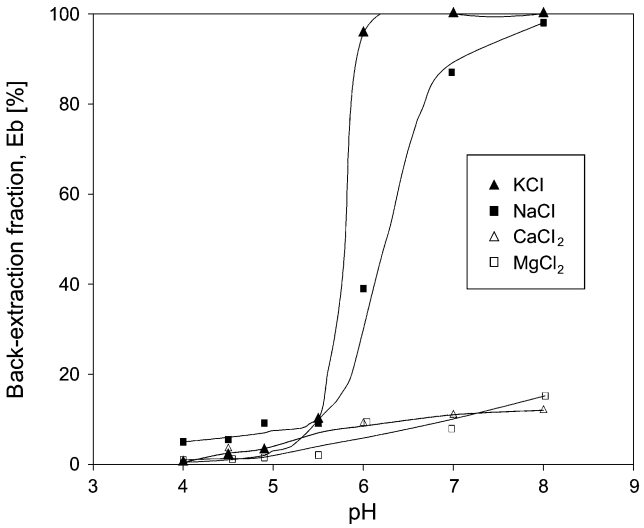


**Fig. 1. Effects of pH on back-extraction of BSA; $[pH]_{inj}$=5.0, [salt]= 0.1 M.**

## RESULTS AND DISCUSSION

Fig. 1 presents the effects of the initial pH of the aqueous phase $[pH]_{aq}$ on the back-extraction efficiency of BSA into the aqueous phase. The fractions of BSA stripped from the organic phase and precipitated in the interface are all based on the amount of the protein injected into the organic phase. For all cases, the initial salt concentration of the aqueous phase was set at 0.1 M. For 1 : 1 salts such as NaCl and KCl, the back-extraction efficiency showed a drastic increase at $[pH]_{inj}=[pH]_{aq}$; most of the proteins incorporated in the reverse micelles were stripped out when $[pH]_{aq}$ exceeded the pI of

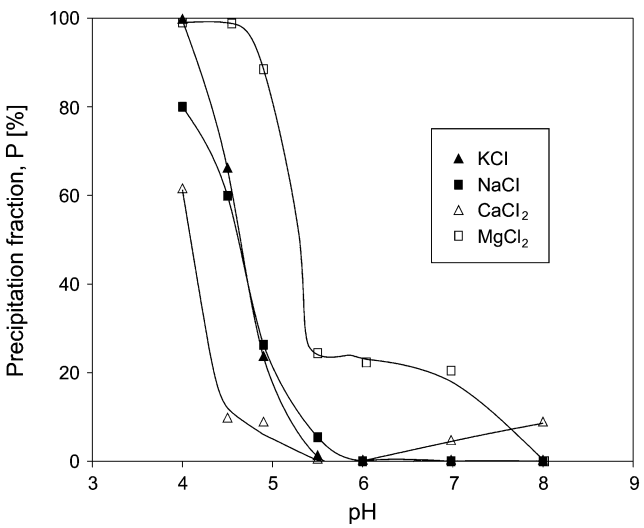


**Fig. 2. Effects of pH on precipitation of BSA; $[pH]_{inj}$=5.0, [salt]= 0.1 M.**

BSA. This result is consistent with the literature despite different experimental conditions [Shiomori et al., 1995; Hong et al., 1997]. In the case of 1 : 2 salts, no significant improvement of the efficiency was observed at higher $[pH]_{aq}$ than the pI. The amount of precipitate of the protein can be estimated from the measured concentrations of the protein of the conjugate phases. As shown in Fig. 2, a steep decrease in the amount of precipitate appeared around $[pH]_{inj}=[pH]_{aq}$ for all salts tested. Compared to the case of the 1 : 1 salts, a considerable amount of precipitate remained far above the pI in case of $MgCl_2$. With increasing $[pH]_{aq}$ beyond the pI, precipitation of the protein was resumed for $CaCl_2$. This result can be interpreted in terms of electrostatic interactions: repulsive interactions between the protein and the surfactant head groups induced stripping. However, divalent cations such as $Ca^{2+}$ and $Mg^{2+}$ may bridge the negatively charged head group of surfactant and the protein and the proteins can be retained in the water-pool beyond the pI.

By varying the salt concentration, while keeping $[pH]_{aq}$ constant, back-extraction was performed and the results are shown in Figs. 3 and 4. As shown in Fig. 3, the protein, which was incorporated in the organic phase, was completely recovered when the salt concentration was larger than 0.1 M for 1 : 1 salts. However, the back-extraction efficiency remained very low with addition of 1 : 2 salts beyond 1.0 M. Except for $MgCl_2$, however, the amount of the precipitate in the interface was not observed over the tested range. When the amount of added $MgCl_2$ was less than 0.1 M, the protein precipitation was considerable. The higher the salt concentration (or ionic strength), the more pronounced the shielding effects. This effect leads to reduction of the micellar size and thus induction of steric exclusion effects. However, since divalent cations can play a role of "bridging agents" between negatively charged groups, the steric effects can be diminished to a considerable extent.

Fig. 5 presents improvement of the back-extraction efficiency with addition of various alcohols like methanol, ethanol, and propanol to the aqueous phase. In fact, cosurfactants may participate in forming reverse micelles and reduce the repulsive interactions between the negatively charged head groups. Furthermore, cosur-

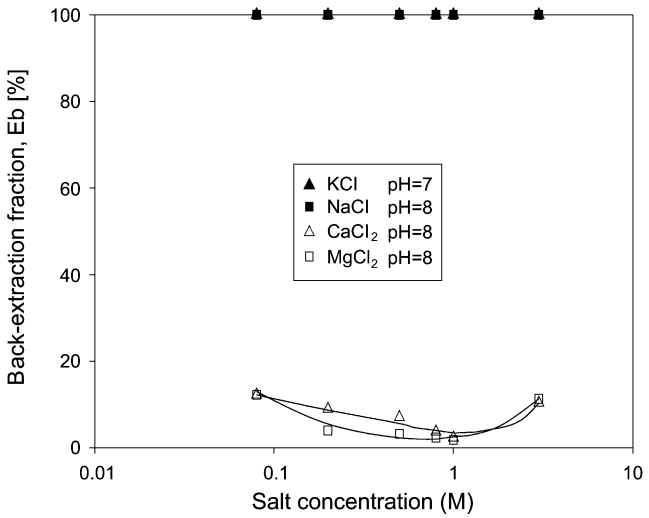


**Fig. 3. Effects of salt concentration on back-extration of BSA; $[pH]_{inj}$=5.0.**

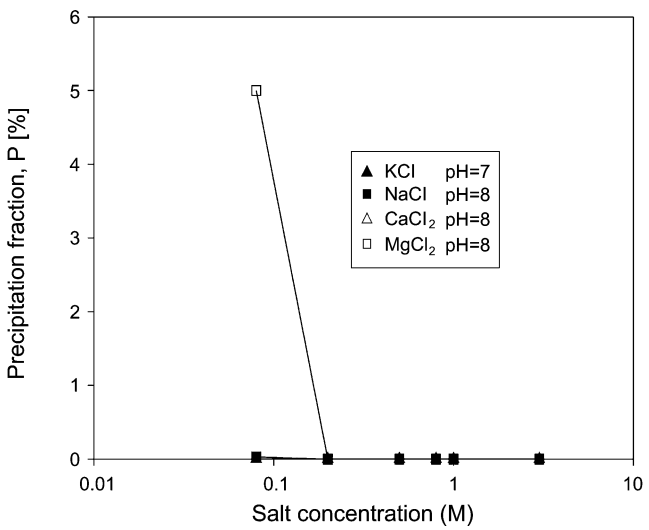


**Fig. 4. Effects of salt concentration on precipitation of BSA; $[pH]_{inj}$=5.0.**

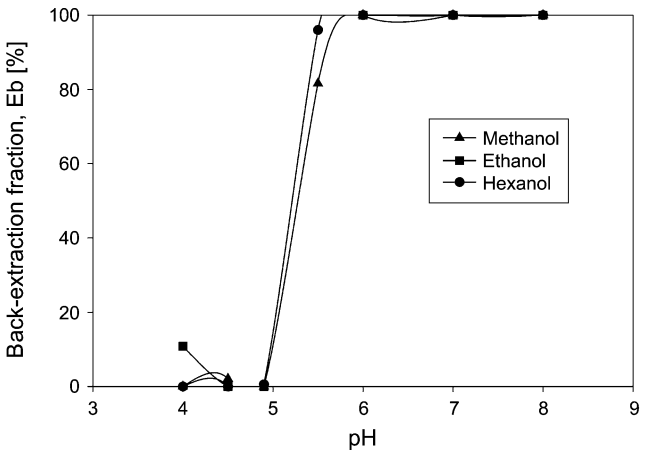


**Fig. 5. Effects of alcohol on extraction of BSA; $[KCl]_{aq}$=0.1 M, [Alcohol]=4 vol%.**

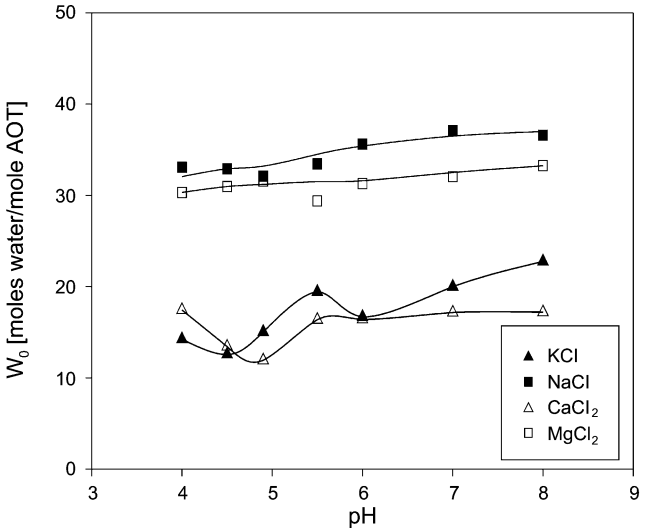


**Fig. 6. Effects of pH on water content of the organic phase; $[pH]_{inj}$= 5.0, [salt]=0.1 M.**

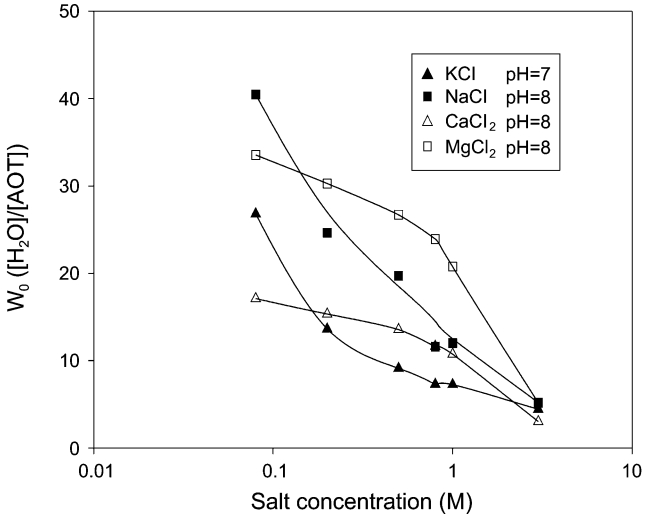


**Fig. 7. Effects of salt concentration on water content of the organic phase; $[pH]_{inj}$=5.0.**

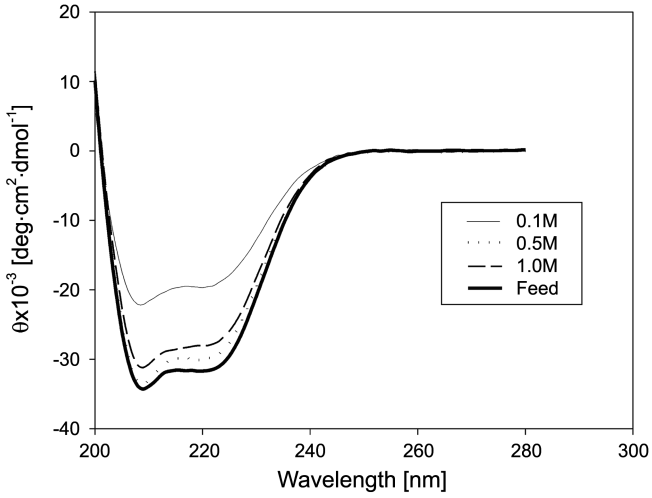


**Fig. 8. Comparison of CD spectra of feed BSA ($[pH]_{inj}$=5.0]) with those extracted from organic phase at various salt concentration of aqueous phase ($[pH]_{aq}$=8.0, NaCl). $\theta$ represents the mean residue ellipticity.**

factants increase solubility of surfactants in an organic continuum. Due to these effects, reduction of reverse micellar size results in the steric exclusion effect. Unfortunately, any significant difference of the effects of the hydrophobic tail length was not observed in Fig. 5.

In the injection method, there is no excess aqueous phase in equilibrium with the organic phase containing the reverse micelles. However, water content of the organic phase may be altered on contacting with an excess aqueous phase because the capacity for water uptake is generally a strong function of the identity of the surfactant counterions. In Figs. 6 and 7, resulting water content of the organic phase after the backward extraction, represented by $W_o$=[$H_2O$]/[AOT], was plotted against $[pH]_{aq}$ and salt concentration, respectively. Compared with the initial water content of the protein-containing organic phase of 16.7, addition of NaCl or $MgCl_2$ of 0.1 M doubled the water content. For KCl and $CaCl_2$, in contrast, almost a half of water that was initially entrapped in reverse micelles was squeezed out. However, the water content of the organic phase in equilibrium with the excess aqueous phase remained nearly constant over a wide range of $[pH]_{aq}$. On the other hand, increase in salt concentration led to reduction of water content independently of the type of salt as shown in Fig. 7. The results presented in Figs. 6 and 7 may be explained in terms of the solubilization mechanism for larger molecules.

Because surfactant molecules protect the solubilized protein from direct contact with apolar solvents in reverse micellar extraction, biological activity of the protein can be retained. In this work, structural change of BSA during the extraction process was determined by comparing CD spectra of the feed and back-extracted proteins. In Fig. 8, CD spectra of BSA extracted from the laden organic phase were compared with that of feed BSA for various NaCl concentrations. $[pH]_{inj}$ and $[pH]_{aq}$ were 5 and 8, respectively. As shown in the figure, the extracted BSA showed spectra similar to that of feed BSA. Thus, it could be concluded that helicity of BSA was largely retained when NaCl concentration was higher than 0.5 M. Though not presented in this paper, similar effects were shown for other salts.

## CONCLUSION

In this work, effects of pH and salt concentration of the aqueous phase on the back-extraction efficiency were examined. The protein stock solution was solubilized into the AOT reverse micelles dispersed in isooctane by using the injection method. Contacting the laden reverse micellar organic solution with an excess aqueous solution, the solubilized protein in the organic solution was stripped into the aqueous phase. For various salt species including NaCl, KCl, $CaCl_2$, and $MgCl_2$ and their concentrations, back-extraction efficiency was observed.

For 1 : 1 salts such as NaCl and KCl, almost complete back-extraction was achieved when $[pH]_{aq}$ exceeded the pI of BSA while variation of $[pH]_{aq}$ resulted in a slight increase of the efficiency. Below the pI, extensive precipitation of the protein in the interface occurred. As $[pH]_{aq}$ approached the pI, the precipitation decreased very sharply and complete dissolution was attained beyond the pI except for the case of $MgCl_2$. For 1 : 1 salts, the solubilized protein was completely recovered into the aqueous phase when more than 0.1 M of salt was added. However, addition of 1 : 2 salts did not improve the back-extraction efficiency.

Alcohols including methanol, ethanol, and propanol played a significant role in the stripping process. Upon addition of alcohols to the aqueous phase, the solubilized protein in the organic phase was completely back-extracted when $[pH]_{aq}$ was higher than the pI of BSA. Water content of the organic phase was increased by addition of KCl and $MgCl_2$, but decreased for NaCl and $CaCl_2$. The altered water contents remained nearly constant over a wide range of pH. The higher the salt concentration, however, the lower the water content for all salts.

Comparing the CD spectra of the extracted BSA, it was concluded that the secondary structure of the protein remained unaltered and BSA was not denatured to a significant extent during the extraction process.

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