Korean J. Chem. Eng., **22**(4), 611-616 (2005)

Interaction between Reverse Micelles as a Key Factor Governing Back-Extraction of Proteins and Its Control Systems

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(*Received 23 December 2004 • accepted 25 April 2005*)

Abstract−The interfacial transport processes of proteins from a reverse micellar phase to an aqueous phase have been investigated focusing on micellar-micellar interaction. The proteins solubilized into reverse micelles were back-extracted to the aqueous phase depending on the concentration of reverse micelles in organic phase. This fact seems to suggest the importance of micellar-micellar interactions in back-extraction processes. The interactions induced by various alcohol addition and temperature change could be evaluated easily and quantitatively by the percolation phenomena in reverse micellar systems (RVMS). The interactions were influenced considerably by the presence of small amount of alcohol and temperature in the RVMS. The addition of alcohols promotes the back-extraction of proteins depending on their species and concentrations. In particular, the alcohols that suppress the cluster formation of reverse micelles, remarkably improve the back-extraction processes. With a small amount of alcohol (20 mM OctOH), Bovine carbonic anhydrase (CAB) can be back-extracted completely from reverse micelles to aqueous solution at the optimal temperature, in which the so high concentration of salt is not necessary.

Key words: Micellar-micellar Interaction, Back-extraction, Alcohols, Temperature

INTRODUCTION

The reverse micellar systems (RVMS) have a capability to solubilize a variety of biomolecules such as proteins and enzymes into the nanometer-size water pools surrounded with a monolayer of surfactant, which can be utilized for protein extraction systems [Luisi et al., 1988; Hatton 1989; Leser and Luisi 1990; Kuboi et al., 1990]. Reverse micelles are so small that they do not scatter light, so they are optically transparent. Furthermore, they are thermodynamically stable. Sodium di[2-ethylhexyl] sulfosuccinate (AOT) is the surfactant most often used.

Since it was discovered that proteins could be solubilized in reverse micelles, many researches have been studied for the protein extraction using RVMS. For this extraction system to be feasible, several requirements have to be met: the protein of interest has to be active and stable in the system of choice, the system has to be scaled up and the product has to be separated without contamination from the surfactant. Among various factors, pH and ionic strength are dominant factors for the reverse micellar extraction process. By controlling these parameters, the extracted fraction can be varied via variations of protein-micellar electrostatic, hydrophobic and steric interactions. However, since the proteins are subjected to denaturation, the recovered (back-extracted) fraction decreases significantly when the protein-micellar interactions are strong. In order to improve the back-extraction process, many studies have been reported using various methods [Dekker et al., 1990, 1991; Aires-Barros et al., 1991; Carlson and Nagarajan, 1992; Pires et al., 1993; Carneiro-da-Cunha et al., 1994; Nishiki et al., 1993, 1995; Sun et al., 1998]. For example, Carlson and Nagarajan [1992] showed that the addition of 10-15% isopropyl alcohol to the stripping aqueous phase increased the rate of protein release and allowed nearly complete back-extraction of porcine pepsin and 70% back-extraction of bovine chymosin. Carneiro-da-Cunha et al. [1994] also studied the method using the temperature effect. They showed that the recovery of cutinase from the reverse micellar phase (100 mM AOT/iso-octane, W_o=20) into a new aqueous phase increased until the temperature reaches at the maximum of 20 °C. Although the back-extraction process on RVMS is a difficult process, it may be carried out successfully to control the properties or structures of reverse micelles. Dungan et al. [1991] explain that in the reverse micellar extraction processes, particularly in the back-extraction processes, the micellar-micellar interaction has to be considered to be one of the important factors. It is expected that the alcohol molecule could be a good modifying agent to control the micellar-micellar interaction.

Hong et al. [1997, 1999] have shown that the interaction between micelles, reflecting the properties or structures of reverse micelles, could be easily evaluated by a percolation phenomenon of RVMS. They have also examined the effect of various alcohols on the RVMS using the percolation process. The percolation processes clearly reflect the micellar-micellar interaction and they can be quantified easily by the measurement of the electrical conductivity of the RVMS. Electrical conductivity measurements have been used to assess a reverse micellar formation and to probe the structural changes occurring in such systems [Jada et al., 1989, 1990; Lang et al., 1991; Alexandridis et al., 1995; Kuboi et al., 1996; Hong et al., 1997]. The interaction between the micellar membrane and protein in reverse micelles have been also studied by several authors using percolation processes of RVMS [Huruguen et al., 1991; Holovko and Badiadi 1993; Larsson and Pileni 1993; Cassin et al., 1994].

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Recently, we proposed that the values of β, defined by the variation of percolation processes, and the back-extraction behavior of proteins have a good relationship, suggesting that the micellar-micellar interaction may be one of the important factors in the protein back-extraction processes [Hong et al., 2000].

In this study, to further understand the relationship between the micellar-micellar interaction and the back-extraction behavior, we studied the micellar-micellar interaction in RVMS by using the percolation processes as key factor governing the protein back-extraction processes and investigated its control systems by using various alcohol molecules or temperature conditions.

MATERIALS AND METHODS

1. Materials

AOT(sodium di[2-ethylhexyl] sulfosuccinate) of purity 95% was purchased from Tokyo Kasei Co. and used without further purification. Bovine carbonic anhydrase (CAB, MW=30 kDa, pI=5.9) was purchased from Sigma. Lysozyme (MW=14.4 kDa, pI 11.1) from egg white was purchased from Wako Pure Chemical (Osaka, Japan). Propanol (PrOH), Butanol (BuOH), Pentanol (PenOH), Hexanol (HexOH) and Octanol (OctOH) were purchased from Wako Pure Chemical.

2. Methods

2-1. Percolation Processes

The conductivity of RVMS was measured as a function of water content (ϕ_{aq}) with a TOA Electronics Ltd. Conductivity meter CM-40V and a platinum electrode. The electrode was inserted into the test tube containing the reverse micellar solution and the tube was placed in a thermostated water bath. Electrical conductivity measurements were performed with dropwise addition of an aqueous phase to 200 mM AOT/isooctane or AOT-alcohol/isooctane solution until the percolation phenomenon was observed. We have also examined the effect of temperature on the percolation processes.

2-2. Back-extraction of Proteins

The proteins were solubilized into AOT/isooctane solution by the injection method following the description in the previous paper [Kuboi et al., 1996; Hong et al., 1997]. The buffer solution containing the protein (100 μM) was injected into AOT/isooctane solution (100 mM AOT/isooctane, W_o=20) and the mixture as shaken vigorously until a clear solution was obtained. Back-extraction of the protein from the reverse micelles were carried out by contacting the protein containing reverse micellar solution with buffer solution (pH_{aq}) containing 0.1 M KCl for 4 hours reached an equilibrium state [Hong et al., 1999]. Similar experiments were also carried out for AOT-alcohol mixed RVMS. The protein back-extraction behavior depends on the pH value in the feed solution injected into reverse micelles, pH_{inj} (the optimal pH_{inj} was used in this research based on the previous work [Kuboi et al., 1996; Hong et al., 1999]. The protein concentrations were determined by spectroscopy (UV-1600A, Shimadzu) at 280 nm. The activity of CAB was determined by measuring the ester hydrolysis rate using *p*-NPA as the substrate [Kuboi et al., 1993]. CAB-containing sample (50 μL) was added to 1 mL of 50 mM Tris-HCl buffer solution (pH 7.5) containing 5 mM EDTA and 1 mM *p*-NPA. After mixing, the increase in absorbance at 348 nm, which reflects the formation of *p*-nitrophenol, was monitored continuously with a UV spectrophotometer at room temperature. The initial rate of hydrolysis (measured for the first 25 s after a 5 s lag time) was taken as a measure for the activity of CAB. As a measure of lysozyme activity, the rate of enzymatic lysis of M. lysodeikticus cells (0.15 mg/ml), which were suspended in 50 mM phosphate buffer (pH 6.2), was taken [Jolles, 1962]. Lysozyme-containing sample (20 μL) was added to the cell suspension (980 μL), and the decrease in the absorbance at 450 nm was continuously monitored spectrophotomerically at room temperature for 60 s after 10 s of lag time. Enzymatic activities were determined from the slope of the straight line obtained by plotting absorbance *vs* time. The activity yield is defined as the activity of back-extracted solution relative to that of the control solution containing native protein.

RESULTS AND DISCUSSION

1. Effect of Micellar-Micellar Interaction on Back-Extraction Behavior

The reverse micellar size is dependent on the parameter W_o(=[H_2O]/[surfactant]), and if W_o is known, the average size and concentration of micelles can be obtained [Yamada et al., 1994]. It has also been found that the average size of micelles and the micellar size distribution are not affected by the solubilization of 50 μM protein [Kuboi et al., 1990b]. The value of W_o can be modulated conveniently by using the injection method. Fig. 1 shows the back-extraction behavior of CAB and lysozyme as a function of the total micellar concentration [$C_{m,tot}$, Yamada et al., 1994] at the same micellar size and ratio of empty micelles and micelles filled with proteins. Here, we have assumed that only single protein can be solubilized into a micellar water pool. For both CAB and lysozyme, the sharp decreases in back-extraction were observed with increasing micellar concentration, in particular, at 0.012 M of micellar concentration ([AOT]=

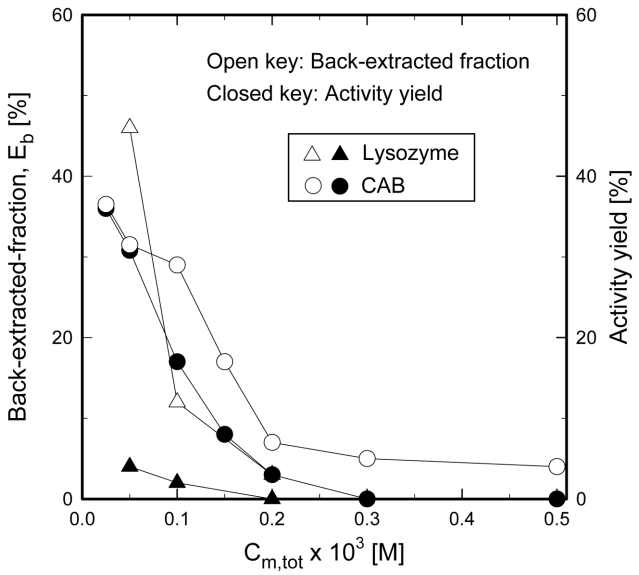

Fig. 1. Effect of micellar concentration on the back-extraction behavior of proteins. For CAB, the pH values of pH_{inj} and pH_{aq} are 8.0 and 8.2, respectively, and for lysozyme, 11.5 and 11.5, respectively. Salt concentration in stripping aqueous solution is KCl 0.1M.

0.2 M). Furthermore, the activity yields of proteins are decreased with the increase of $C_{m,tot}$. This is an interesting result, suggesting the importance of micellar-micellar interaction on the back-extraction processes. It will be discussed later in detail.

2. How to Control the Micellar-Micellar Interaction

The percolation processes are effective for the evaluation of the micellar-micellar interactions [Alexandridis et al., 1995; Kuboi et al., 1996; Hong et al., 1997]. A sharp increase in electrical conductivity induced by the percolation process demonstrates well the existence of the strong micellar-micellar interaction inducing a micellar clustering [Alexandridis et al., 1995]. Figs. 2a and 2b show the effects of alcohol addition and temperature on the percolation processes of RVMS. The electrical percolation threshold decreases with the addition of BuOH into the RVMS in Fig. 2a. This result indicates an increase in the attractive interaction between micelles when BuOH is added into the RVMS. However, the addition of PenOH shows a decreasing effect on the attractive interaction between micelles. These results suggest that the micellar-micellar interactions are notably influenced by the alcohol species and concentration. Alcohols with longer alkyl chains than PenOH suppressed the micellar clustering better than PenOH, and alcohols with shorter alkyl chains than BuOH enhanced the micellar clustering better than BuOH [Hong and Kuboi, 1999]. Hong and Kuboi [1999] also examined the effect of the various alcohols such as diols or halogenols, indicating that the alcohol effects can be explained by the additive contribution of each group where hydrocarbon and halogen groups contribute positively and OH groups contribute negatively.

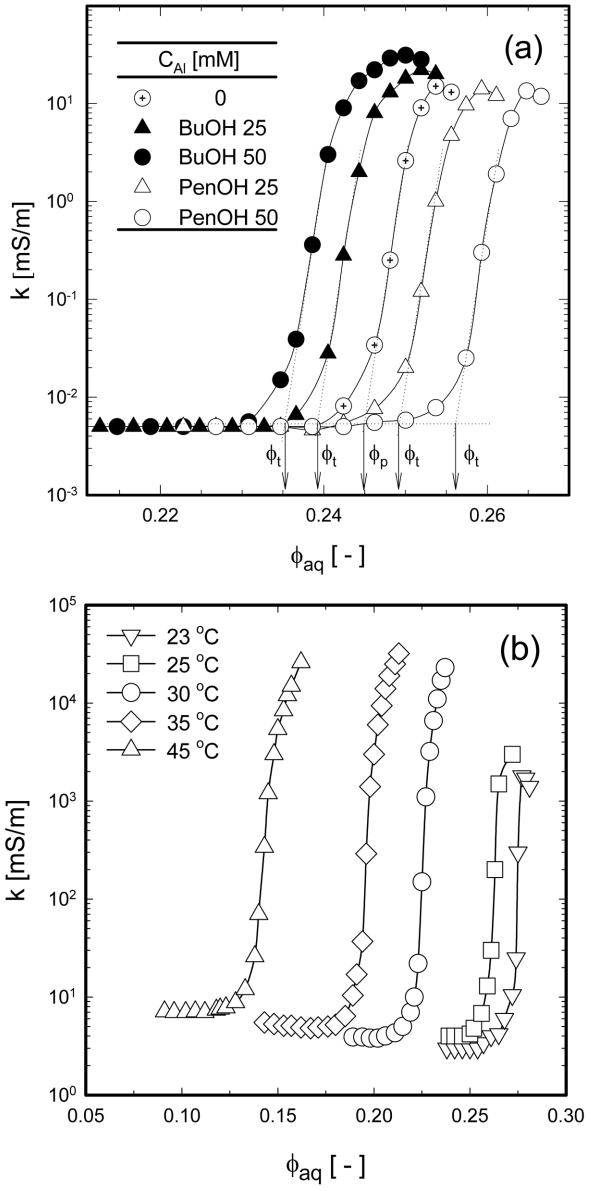

Fig. 2. Effects of (a) alcohol addition and (b) temperature on the micellar-micellar interactions. The percolation experiment of alcohol addition is carried out at 25 °C. The values of temperature in panel b are 45 °C (▽), 35 °C (◇) , 30 °C (□), 25 °C (○) , 23 °C (△).

The effect of temperature on the percolation and the interactions between the micelles is shown in Fig. 2b. The micellar-micellar interactions are strongly temperature dependent. An increase in the temperature increases the probability of AOT ionization, the range of ion diffusion and the frequency of AOT RVMS collision caused by the hydrophobic interaction between the micelles [Alexandridis et al., 1995]. Upon decreasing temperature, although the micellar clustering is suppressed, the maximum electronic conductivity decreases when the percolation phenomenon is caused. The reason is not known clearly, but it is considered probably as one reason that a fluctuation of micellar membrane decreases in low temperature. Therefore, the temperature modulation as well as the alcohol addition may be good methods to control the micellar-micellar interactions.

3. Effect of Alcohol Addition on the Back-Extraction Processes of Proteins

Proteins are usually back-extracted by contacting the organic phase loaded with protein and a new aqueous phase at high ionic strength, i.e., the strength of up to 1 M salt solution [Hatton 1989; Dekker et

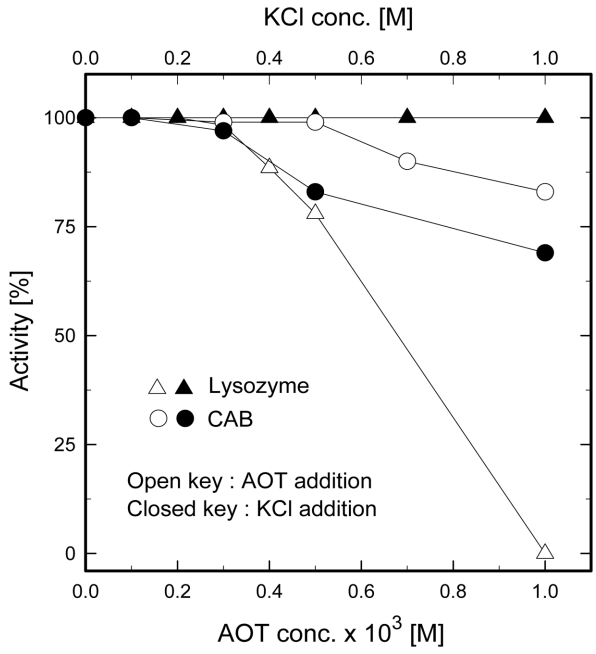

Fig. 3. Effects of AOT and KCl concentrations added into buffer solution on the denaturation of proteins.

al., 1991]. However, the proteins are likely to denature in such a high ionic strength condition. We have examined the effects of salt and surfactant addition on the denaturations of CAB and lysozyme. Fig. 3 shows the plot, for the KCl or AOT, of the activity of proteins related to that of the control solution against the salt and surfactant concentration added in the buffer solution. The activities shown in Fig. 3 were obtained after 4 hours since the preparation of protein solution. In the case of lysozyme, while the activity decreases sharply with an increase of AOT concentration as shown marked denaturation at 1 mM AOT, the denaturation of lysozyme by KCl is not shown in a concentration less than 1 M. Lysozyme is a hydrophobic protein, leading easily to the hydrophobic interaction with the hydrophobic site of surfactant. On the other hand, CAB is denatured by both AOT and KCl in high concentrations of them. The denaturation of proteins by AOT or KCl in low concentration is not shown, suggesting that the low concentration of KCl or AOT is favorable to the back-extraction processes of proteins.

We have examined the back-extraction of CAB using various alcohols at low ionic strength conditions ([KCl]=0.1-0.5 M). Fig. 4 shows the effects of alcohols on the back-extracted fractions and activity yields of CAB. The back-extracted fraction varies with the addition of a small amount of alcohol. While HexOH and OctOH concentration increases the back-extracted fraction, BuOH and PrOH do not show such a good effect. This result is interesting, suggesting that the back-extraction behavior is improved by a small amount of alcohol that controls the micellar property such as micellar-micellar interactions. Addition of alcohols has a good effect on the activity yields as well as on the back-extracted fractions, indicating a decrease in the resistance of micellar or interface membrane by the alcohol molecules. At high concentrations of HexOH or OctOH, the formation of reverse micelle is disrupted when the protein solution is injected to AOT organic solution. Whereas, we do not know

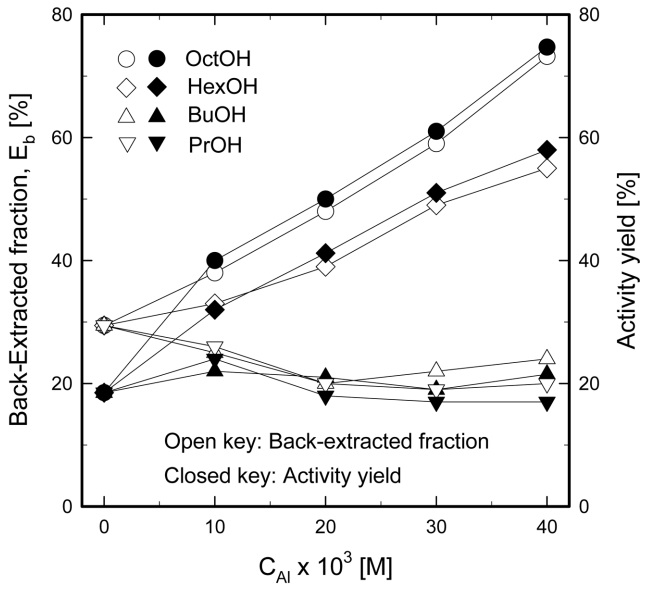

Fig. 4. Effect of alcohols addition on the back-extracted behavior of CAB for 0.1 M AOT/isooctane and 0.1 M KCl. Added alcohols are PrOH (▽, ▼), BuOH (△, ▲), HexOH (◇, ◆), and OctOH (○, ●). The open and closed keys are the back-extracted fraction and activity yield, respectively.

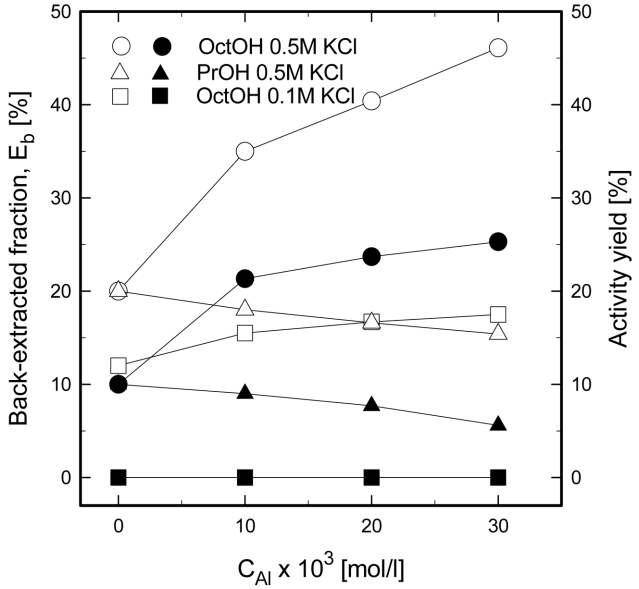

Fig. 5. Effect of alcohol addition on the back-extracted behavior of lysozyme for 0.1 M AOT/isooctane and 0.5 M KCl. Added alcohols are PrOH (▽, ▼), and OctOH (○, ●). The open and closed keys are the back-extracted fraction and activity yield, respectively. The keys (□, ■) indicate the addition of OctOH in the condition of 0.1 M KCl.

clearly the reason, the property, that alcohols have been used for the destruction of the reverse micelles in usual, may be the reason. At low concentration of the alcohols, however, the reverse micelles were formed safely. In this study, the back-extraction experiments were carried out in transparent reverse micellar solution.

As shown in Fig. 5, we have also obtained improved back-extractions by using alcohol in lysozyme back-extraction processes. Upon addition of OctOH, while the back-extracted fraction of lysozyme is increased slightly in low concentration of KCl (0.1 M), a sharply increasing effect in 0.5 M KCl concentration is observed. In the case of HexOH addition, the back-extracted fraction was increased slightly, as found for CAB (data not shown). On the other hand, the increase of PrOH concentration decreases the back-extracted fraction of lysozyme. These findings indicate that using special alcohols can improve the back-extraction processes probably due to the effect of alcohol on the micellar-micellar interaction.

In the previous work, we proposed that there are two types of proteins, such as type A and B, by the relation between the percolation processes and the back-extraction behavior of proteins [Kuboi et al., 1996]. Type A proteins, such as CAB, suppress the percolation processes or micellar clustering at the pH both above and below pI and for which back-extracted fractions are comparatively higher. Type B proteins such as lysozyme, however, in which the back-extraction is relatively difficult, promote the percolation processes. For the difference of the alcohol effect on the protein back-extraction processes, these reasons have to be considered. For the type B proteins, the protein-micellar interaction inducing conformation change in reverse micelles should be studied in the future.

4. Effect of Temperature on the Back-Extraction of Proteins

Changes in temperature have drastic effects on the physicochemical properties of the reverse micellar systems as shown in the per-

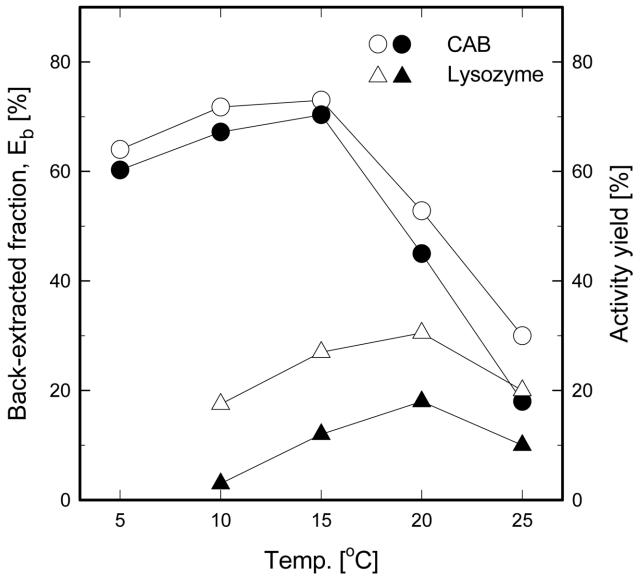

Fig. 6. Effect of temperature on the back-extraction of CAB (○, ●) and lysozyme (△, ▲). The open and closed keys are the back-extracted fraction and activity yield, respectively.

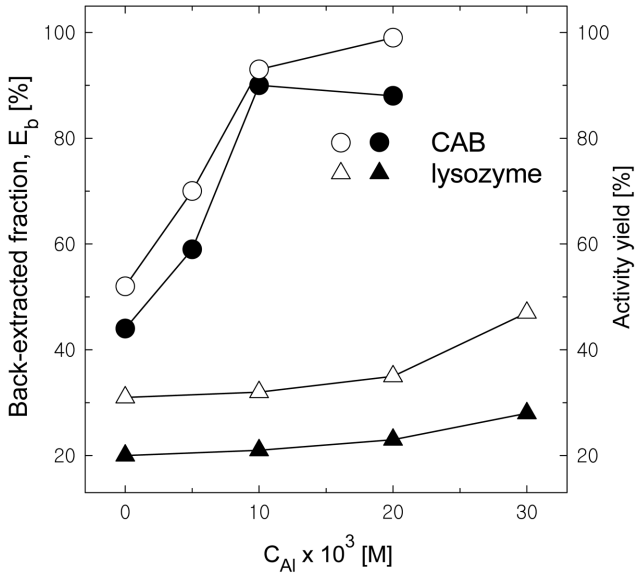

Fig. 7. Effect of OctOH addition on the back-extraction behavior of CAB (○, ●) and lysozyme (△, ▲) at 20 °C. The open and closed keys are the back-extracted fraction and activity yield, respectively.

colation processes of AOT-RVMS (Fig. 2b). The feasibility of temperature modulation for the back-extraction of proteins was verified experimentally. Fig. 6 shows the effect of temperature on the back-extraction behavior for CAB and lysozyme. Over 70% of CAB could be back-extracted at 15 °C. While at a lower temperature than 15 °C, the back-extracted fraction is decreased again. Carneiro-da-Cunha et al. [1994] have shown a similar result where the back-extraction of cutinase increased with the temperature (From 4 to 40 °C) reaching a maximum of 20 °C. They discussed the result in light of a hydrophobic interaction between the protein and the surfactant. They explained that at low temperature (4 and 10 °C), cutinase solubility in aqueous phase decreased, which leads to 40-50% protein precipitation at the interface, and at high temperature (30 and 40 °C), the strong hydrophobic interaction between protein and surfactant denatures the protein leading to a lower protein back-extraction. In this work, however, the protein precipitation at the interface was not confirmed except at 5 °C. The interface was contaminated with small amount of precipitation considered protein aggregation at 5 °C. The denaturation of protein by the hydrophobic interaction at the temperature condition of 20 °C or 25 °C was not expected. Thus, although the protein-micellar hydrophobic interaction is a fundamental factor on temperature effects, we propose the micellar-micellar interactions are markedly influenced by temperature.

5. Improvement of Back-Extraction by Alcohol Molecules and Temperature

We have also examined the effect of alcohol addition on the back-extraction of proteins in the optimal temperature. The back-extraction behavior of CAB and lysozyme was plotted against OctOH concentration added to RVMS at 20 °C in Fig. 7. The addition of 20 mM-OctOH to RVMS allowed nearly complete back-extraction of CAB and 50% back-extraction of lysozyme at OctOH 30 mM. At lower temperature in which the back-extracted faction was decreased as shown in Fig. 6, the effect of OctOH addition was not observed. Although the micellar-micellar interaction is decreased at the low temperature, the molecular interaction between the alcohols and surfactants is also decreased at low temperatures. CAB and lysozyme activity yields of 90 and 30%, respectively, were also obtained for the back-extraction process. This result is good evidence, clearly reflecting the improvement of back-extraction by controlling the micellar-micellar interaction at least for CAB. It is very difficult to back-extract lysozome at low ionic strength because it interacts with the surfactant or micelle strongly, i.e., protein-micellar hydrophobic interaction. Shiomori et al. [1999] showed the complete back-extraction of lysozyme under low ionic strength condition (NaCl 0.1 M) by using long chain alkyl amines, controlling the formation of reverse micelles that is thought to be the controlling of the micellar-micellar interaction. Therefore, the protein-micellar interactions are very important factors in the reverse micellar extraction systems, but, the micellar-micellar interaction has to be considered as an important factor, particularly, in back-extraction processes. From this result, we propose that the phenomenon of reverse micellar cluster formation near the interface may be one of the most important factors for the back-extraction process. If the micelles form micellar clusters as they approach and accumulate on the interface with the bulk aqueous phase, the mass transfer resistance through the interface may increase and cause the difficulty of back-extraction processes.

CONCLUSIONS

The protein back-extraction processes were discussed from the viewpoint of the micellar-micellar interactions. We have also examined quantitatively the effects of alcohol molecules and temperature on the micellar-micellar interactions in RVMS by using percolation processes. The percolation processes reflecting clearly the micellar-micellar interaction, have been influenced by a small amount of alcohol or temperature condition of RVMS, suggesting that the in-

teraction could be controlled by them. The interesting result about the effect of various alcohols on the back-extraction behavior of proteins from a reverse micellar phase to an aqueous phase has been obtained. A small amount of alcohol added to an organic solution improves the back-extraction behavior of proteins depending on the concentration and species of alcohols. A slight falling of temperature from room temperature is effective in the back-extraction of proteins, which the micellar-micellar interaction is suppressed. Therefore, these methods are effective to control the properties of micellar interface as well as to improve the back-extraction processes of proteins at low ionic strength. Our results indicated clearly that the micellar-micellar interaction or micellar clustering plays an important role in the back-extraction processes of proteins.

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