Effect of Alcohol Addition on Back-Extraction of BSA and Cytochrome c Using AOT Reverse Micellar System

Sung-Sik Lee,* Bong-Kuk Lee, Jin-Sung Choi, and Jong-Pal Lee†

Department of Chemical Engineering, Dong-A University, Pusan 604-714, Korea
†Department of Chemistry, Dong-A University, Pusan 604-714, Korea
Received April 16, 2001

The protein back-extraction processes were discussed from the viewpoint of the micelle-micelle interaction. Bovine serum albumin (BSA) suppressing the cluster formation of reverse micelle (positive value of β_{pc}) has the high back-extracted fraction (E_b), but cytochrome c enhancing the formation of reverse micelle (negative value of β_{pc}) has the low back-extracted fraction, relatively. We have also examined quantitatively the effects of alcohol addition and protein solubilization on the percolation process of reverse micelle. The alcohols suppressing the formation of micellar cluster (high values of β_t), remarkably improved the back-extraction rates of BSA and cytochrome c. The values of β_t, defined by the variation of percolation process, and the back-extraction behavior of proteins have a good linear correlation. These results indicate that the micelle-micelle interaction or micellar clustering plays an important role in the back-extraction process of proteins.

Keywords : Back-extraction, AOT-RVMS, Percolation BSA, Cytochrome c.

Introduction

Reverse micelles are nanometric aggregates formed by surfactants in organic solvents, which have a capability to solubilize a variety of biomolecules such as proteins and enzymes into the water pools surrounded by surfactants. Then, the reverse micelles have been extensively studied for the extraction of these biomolecules.[1-5] Among the variety of publication, anionic surfactants such as sodium bis(2-ethylhexyl) sulfosuccinate (AOT) was mostly employed to form the extractive reverse micellar system (RVMS). In general, using AOT-RVMS for protein extraction, the distribution of proteins between a micellar organic phase and an aqueous phase is largely determined by the conditions of the aqueous bulk phase, namely pH, ionic strength and types of salt. The parameters related to the organic phase also influence the partition of protein, such as the concentration and type of surfactant, presence of cosurfactant, type of solvent and so on.[6] By controlling these parameters, the extracted fraction can be varied *via* variations of protein-micelle electrostatic, hydrophobic and steric interactions. Among these interactions, the electrostatic interaction was considered as the main driving force especially in forward-extraction processes.[7-8] However, there are many problems in back-extraction processes of proteins, such as decreasing back-extracted fractions or activity yields and the rate of back-extraction is much smaller than the rate of forward-extraction.[5,9-10] These problems originate from strong interaction between proteins and micelles.[4,6-7] These interactions between proteins and micelles were influenced by the micelle-micelle interaction.[11-12] Dungan *et al.* explain that in the reverse micellar extraction processes, particularly in the back-extraction process, the micelle-micelle interaction has to be considered one of the important factors.[13] In order to improve the back-extraction process, many studies have been reported using various methods. The strategy of improvement could be considered in two aspects. One is a dealing surfactant-organic phase by concentration and species of surfactant, type of organic solution.[14-15] The other is a dealing stripping aqueous phase by pH, concentration and species of salts or adding various alcohols.[5,9,16-19] More recently, the tendency of research is being concentrated on the affinity-based extraction such as the use of RVMSs added cosurfactant.[20-23] 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. We expect that the alcohol molecule could be a good modifying agent for the reverse micelles, because alcohol molecules have amphiphilic property as a cosurfactant. Hong and Kuboi examined the effect of various alcohols on the RVMS using the percolation process.[24] The percolation threshold can be varied by different additives. It has been also demonstrated that the solubilization of proteins clearly affects the percolation process with an rapid increase in the conductivity at lower or higher water content or temperature, suggesting stronger or weaker attractive interactions between micelles in the presence of proteins.[28-29,31]

The percolation processes clearly reflect the micelle-micelle interaction and it can be quantified easily by the measurement of electrical conductivity of the RVMS as a function of water content or temperature. Electrical conductivity measurements have been used to assess a reverse micelle formation and to probe the structural changes occurring in such systems.[25-27] The interaction between the micelle membrane and protein in reverse micelles has been also studied by several authors using percolation processes of RVMS. It has demonstrated that the solubilization of proteins clearly

*To whom correspondence should be addressed. e-mail: sslee@mail.donga.ac.kr

affects the percolation process with rapid increase of the conductivity at lower or higher water content or temperature. This indicates the stronger or weaker attractive interactions between micelles in the presence of proteins.[12,28,29]

In the present paper, the behaviors of protein back-extraction and their percolation phenomena have been studied to focus on the formation of micellar cluster *via* protein-micelle interactions by the solubilization of proteins. We have also investigated the relationship between the behaviors of protein back-extraction and their percolation phenomena by the addition of a small amount of alcohol.

Experimental Section

Materials. AOT (sodium di[2-ethylhexyl] sulfosuccinate) was purchased from Tokyo Kasei Co. and used without further purification. Bovine serum albumin (BSA, pI = 4.9) and cytochrome c (pI = 10.1) were purchased from Sigma Chemical Company. The buffer solutions employed were acetic acid/acetate (pH 4-6), tris/HCl (pH 7-8) and glycine/NaOH (pH 10-11.7) and the concentration of each buffer salt was 10 mM. Propanol (PrOH), Butanol (BuOH), Pentanol (PenOH), Hexanol (HexOH) and Octanol (OctOH) were purchased from Wako Pure Chemical.

Percolation processes. The conductivity of RVMS was measured as a function of water content with conductivity meter CM-40V from TOA Electronics Ltd 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 solution containing proteins to the 200 mM AOT/iso-octane until the percolation phenomenon was appeared. The percolation threshold with and without proteins, defined as the starting point of the sharp increase of conductivity is abbreviated as $\phi_{p,p}$ or ϕ_p according to the previous paper.[11] The percolation threshold with and without alcohols and protein defined as the starting point of the sharp increase in conductivity is abbreviated as $\phi_{t,p}$ or ϕ_t, respectively. The percolation thresholds were determined by the extrapolation method which consist of finding an intersecting point between the constant line and the increasing line and the decreasing line of the curve as shown in Figure 1.

Back-extraction of proteins. The proteins were solubilized into 100 mM AOT/iso-octane solution by the injection method. The buffer solution of protein was injected into AOT/iso-octane solution, and shaken vigorously until a clear solution was obtained. The value of W_o (=[H_2O]/[AOT]) of the reverse micellar solution was kept at 20 in all the back-extraction experiments. Back-extraction of the protein from the reverse micelles was carried out by contacting the reverse micellar solution containing proteins with new buffer solution containing 0.1 M KCl. Similar experiments were also carried out for AOT-alcohol mixed RVMS. The pH value in the feed solution injected into reverse micelles, pH_{inj}, was varied. The protein concentration was determined by spectroscopy (UV-1600A, Shimadzu) at 280 nm.

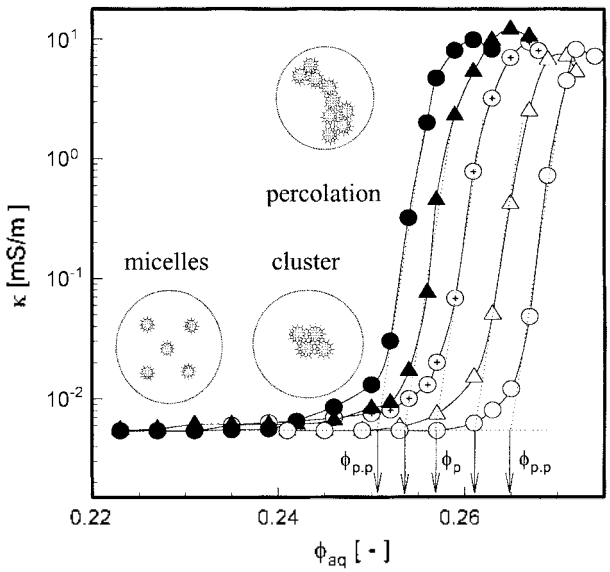

Figure 1. Effect of cytochrome c (open key) and BSA (closed key) solubilization on the percolation process of AOT-RVMS (0.2 M). Protein concentrations are 0 mg/mL (⊕), 3 mg/mL (▲, △) and 5 mg/mL (● , ○), respectively at pH 8.

Results and Discussion

Effect of protein solubilization on the percolation processes of AOT-RVMS. The percolation phenomenon of reverse micelles is changed by the solubilization of various materials (*i.e.* enzyme, protein and polymer). The percolation processes are effective for the evaluation of the micelle-micelle interactions.[11-12,27] A sharp increase in electrical conductivity caused by the percolation process demonstrates well the existence of the strong micellar-micellar interaction inducing a micellar clustering. It is generally accepted that percolation in AOT-RVMS with a spherical droplet structure is a result of reverse micellar droplet clustering.[27] The variation of the electrical conductivity of the AOT reverse micellar solution is plotted in Figure 1 against the volume fraction of water in the organic phase, ϕ_{aq}. In the case of BSA solubilized into the RVMS, the electrical percolation threshold ($\phi_{p,p}$) is increased with solubilizing BSA into the reverse micelles. This result indicates an increase in the attractive interaction between micelles as BSA solubilized into the RVMS. In contrast, for the RVMS solubilized cytochrome c, the percolation threshold ($\phi_{p,p}$) was decreased in the lower value of ϕ_{aq} than that of the protein-free system. These results suggest that the micelle-micelle interactions are notably influenced by the protein species and concentration solubilized into the reverse micelles. Cytochrome c in reverse micelles has been studied by several authors.[30-31] There are indications that cytochrome c interacts with the AOT surfactant layer. Therefore, the protein-micelle (electrostatic attractive) interactions seem to decrease the stability of RVMS by decreasing electrostatic repulsive interaction between micelles. The formation of micellar clusters shows a larger hydrophobic attraction than an electrostatic repulsive force between the micelles.

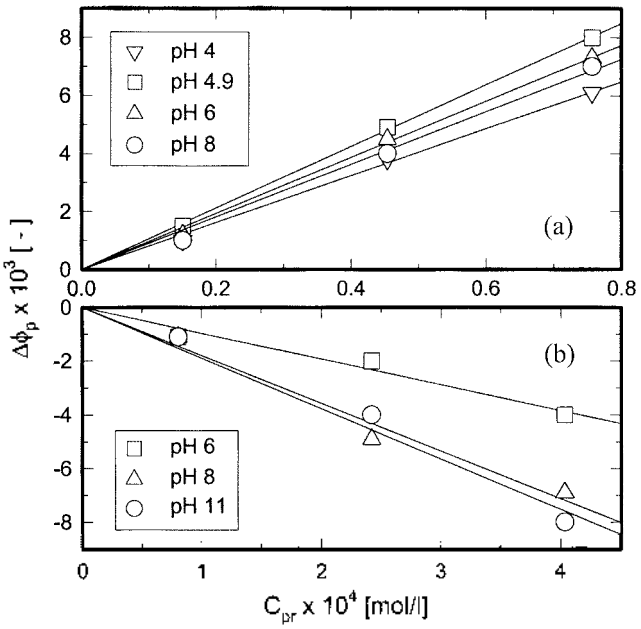

Figure 2. Effect of protein concentrations on the percolation threshold: (a) BSA, (b) cytochrome c.

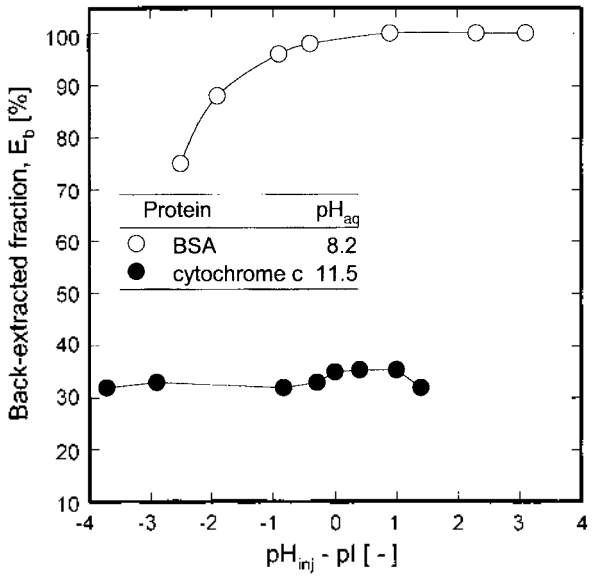

Figure 3. Effect of pHinj on the back-extracted fractions of cytochrome c (open key) and BSA (closed key). [AOT] = 0.1 M, [KCl] = 0.1 M.

The difference, $\Delta\phi_p(=\phi_{p,p}-\phi_p)$, reflects the effect of the protein concentration on the percolation process. Here, $\phi_{p,p}$ and ϕ_p are the values of the percolation threshold with and without protein, respectively. The relationship between the relative percolation thresholds and solubilized protein concentrations is examined. $\Delta\phi_p$ is plotted against the concentration of BSA and cytochrome c solubilized into the RVMS under different pH conditions in Figure 2. There is a linear correlation between $\Delta\phi_p$ and the concentration of protein (C_{pr}). The slope, β_{pr}, is the measure of the effect of protein solubilization on the micellar clustering occurred by protein-micelle interactions. The values of β_{pr} are affected by pH of the solution. That is by some electrostatic interactions between the micelles and proteins. A positive value of β_{pr} means the stabilization of RVMS with the solubilization of protein, suppressing the micellar clustering. On the other hand, a negative β_{pr} means the destabilization of RVMS with protein solubilization, promoting the micellar clustering.

Back-extraction of proteins solubilized into reverse micelles. After the protein was solubilized at a particular pH_{inj}, back-extraction was carried out under the same condition of pH_{aq}. The results for BSA and cytochrome c with various pH_{inj} values are shown in Figure 3. The fraction of the proteins back-extracted to the aqueous phase, E_b, is plotted against the pH deviation from pI (isoelectric point) of each proteins. For example, BSA was easily back-extracted into the new aqueous phase at the pH range above the pI, but the back-extracted fraction of BSA was decreased at the pH range below the pI. On the other hand, cytochrome c was comparatively difficult to back-extract. These results can be explained by the electrostatic interaction between protein and the anionic surfactant, AOT. In general, a net charge of proteins become a positive at the pH range below the pI, and it is known that cytochrome c have a localized positively charged site as a membrane protein. Thus, the decrease of the back-extracted fraction (E_b) of BSA at the pH range below the pI and the low back-extracted fraction (E_b) of cytochrome c in the contrast with BSA can explain the result of the strong electrostatic interaction between the protein and anionic surfactant, AOT. It is very interesting result to compare with the back-extraction process and the percolation process. BSA with the positive values of β_{pr} has the relatively high back-extracted fraction (E_b). On the contrary, cytochrome c with the negative values of β_{pr} has the low E_b. It was found that a strong interaction between the solubilized protein and micelles induces the micelle-micelle interaction or micellar cluster formation, resulting in the decrease of back-extracted fractions. Therefore, the control of micelle-micelle interaction may become a very important factor for the success of the back-extraction processes of proteins.

Effect of protein solubilization on the percolation processes of the AOT-alcohol mixed RVMSs. We have examined the effect of protein solubilization on the percolation processes of RVMS added PenOH as shown in Figure 4. The addition of PenOH shifted the percolation threshold (ϕ_t) to a higher value of ϕ_{aq} than that of protein-free system. Hong and Kuboi observed that the alcohol with long alkyl chains than PenOH suppressed the micellar clustering more than PenOH, and alcohol with shorter alkyl chains than BuOH enhanced the micellar clustering better than BuOH.[24] These results are similar to the our study, suggest that the micelle-micelle interactions are notably influenced by the alcohol species and concentrations added into an organic solution. It is expected that the alcohol molecule would be a good modifying agent for the micellar membrane. For the protein solubilizing into RVMS added PenOH, the electrical percolation threshold ($\phi_{t,p}$) is increased with solubilizing BSA into the PenOH added RVMS. This result indicates an increase in the attractive interaction between micelles as

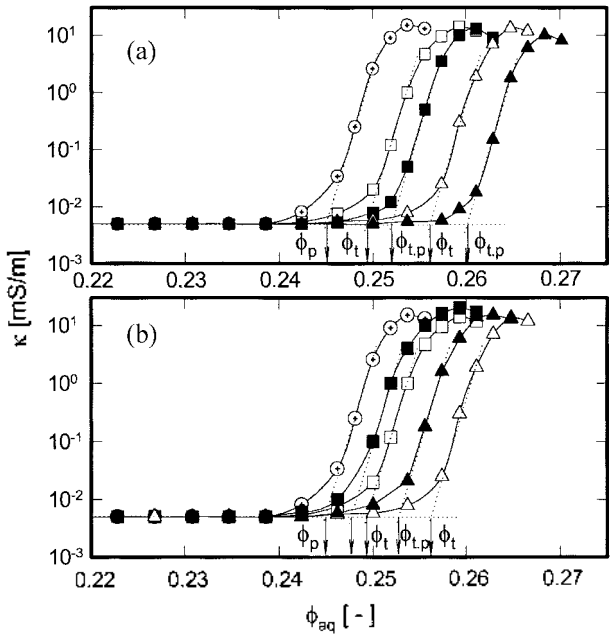

Figure 4. Effect of BSA (a) and cytochrome c (b) solubilization on the percolation process of AOT (0.2 M) and AOT/PenOH-RVMS. Alcohol concentrations are 0 mM (⊕), 5 mM (△ , ▲) and 12.5 mM (○ , ●). Open and closed key are without and with protein, respectively. Protein concentration is 5×10^{-5} M.

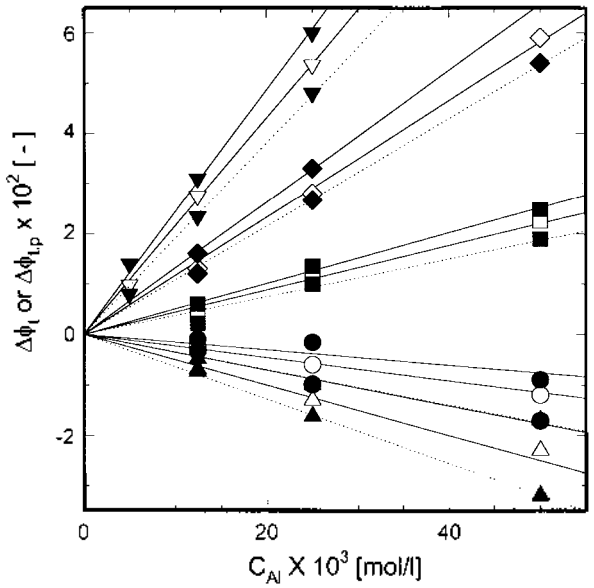

Figure 5. Dependencies of BSA (–closed key–) and cytochrome c (···closed key···) solubilization and alcohol concentration on the percolation threshold in AOT and AOT/Alcohol mixed RVMSs. Open and closed key are without and with protein, respectively. PrOH (△), BuOH (○), PenOH (□), HexOH (◇) and OctOH (▽).

BSA solubilized into the RVMS including PenOH. On the other hand, for the cytochrome c solubilized to the RVMS with penOH, the percolation threshold (ϕ_t) has been decreased to the lower value of ϕ_{aq} than for the PenOH added protein-free system. In order to discuss the effect of protein solubilization, the relationship between the relative percolation threshold and the alcohol concentration on the RVMSs including various alcohols was investigated.

The difference, $\Delta\phi_t$ ($=\phi_t-\phi_p$), reflects the effect of the alcohol concentration on the percolation processes. Here, ϕ_t and ϕ_p are the values of the percolation threshold with and without alcohol, respectively. The difference, $\Delta\phi_{t,p}$ ($=\phi_{t,p}-\phi_p$), shows the effect of the alcohol and protein on the percolation processes. Here, $\phi_{t,p}$ and ϕ_p are the values of the percolation threshold with and without alcohol and protein, respectively. Figure 5 shows the plot of the $\Delta\phi_t$ and $\Delta\phi_{t,p}$ for the representative alcohols versus the alcohol concentration, C_{Al}, added in the reverse micellar solution (closed key) and without protein systems (open key). There is linear correlation between $\Delta\phi_t$ and the concentration of each alcohol as well as $\Delta\phi_{t,p}$ and the concentration of each alcohol. The value of β_t means the stability of RVMS or ability of water solubility with the addition of alcohol. A positive β_t means the stabilization of RVMS or the decrease of micelle-micelle interaction by the addition of alcohols to RVMS. On the contrary, the negative β_t means the destabilization of RVMS or the increase of micelle-micelle interaction with the addition of alcohols to RVMS. The slop, $\beta_{t,p}$, defines the effect of solubilized protein. In the case of BSA, the slopes of $\beta_{t,p}$ are larger than the values of β_t. This result reflects that the solubilization of BSA into the RVMS containing alcohols decrease in the protein-micelle interaction by inducing the formation of micellar clustering. On the other hand, the $\beta_{t,p}$ of cytochrome c are smaller than the values of β_t. This result indicates that the solubilization of cytochrome c can not be decreased by the protein-micelle interaction by inducing the formation of micellar cluster in the alcohol added RVMS. It has been also known that the solubilization of proteins favors the percolation process with an increase in the conductivity at lower or higher water content of percolation threshold, suggesting stronger or weaker interactions between micelles and proteins.[11,28-29]

Effect of alcohol addition on the back-extraction behaviors of proteins. Several papers have been presented on the interfacial transport processes of proteins between a bulk aqueous and a reverse micellar phase.[5,9,15,19] They showed that the rate-determining step is the desolubilization at the interface in the back-extraction process. In our previous paper, therefore, we assumed simply that the overall rate constant associated with the back-extraction process is K, the equation of back-rate is:

$$\ln[\{C^{\circ}_{org} - (1+m)C_{aq}\}/C^{\circ}_{org}] = (1+m)Kt \qquad (1)$$

where, m is a partitioning equilibrium constant ($=C^{*}_{aq}/C^{*}_{org}$). The variation of K values allows one to easily understand the back-extraction behavior depending on the various condition. Figure 6 shows the effect of alcohol addition on the time course of the back-extracted fraction of BSA and cytochrome c. In the case of the addition of PrOH, the back-extraction rate is reduced than that of free alcohol system. However, in the addition of HexOH and OctOH, the back-extraction rates are accelerated. There is a clear difference depending on the species of alcohols added to RVMS. The

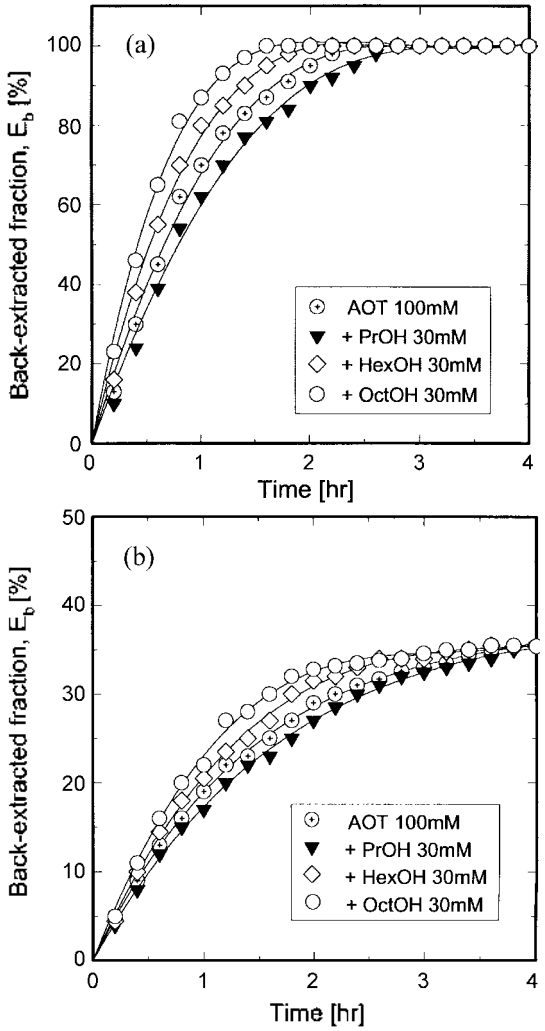

Figure 6. Time course of the back-extracted fraction of BSA (a) and cytochrome c (b) for the AOT (0.1 M)-alcohol RVMSs.

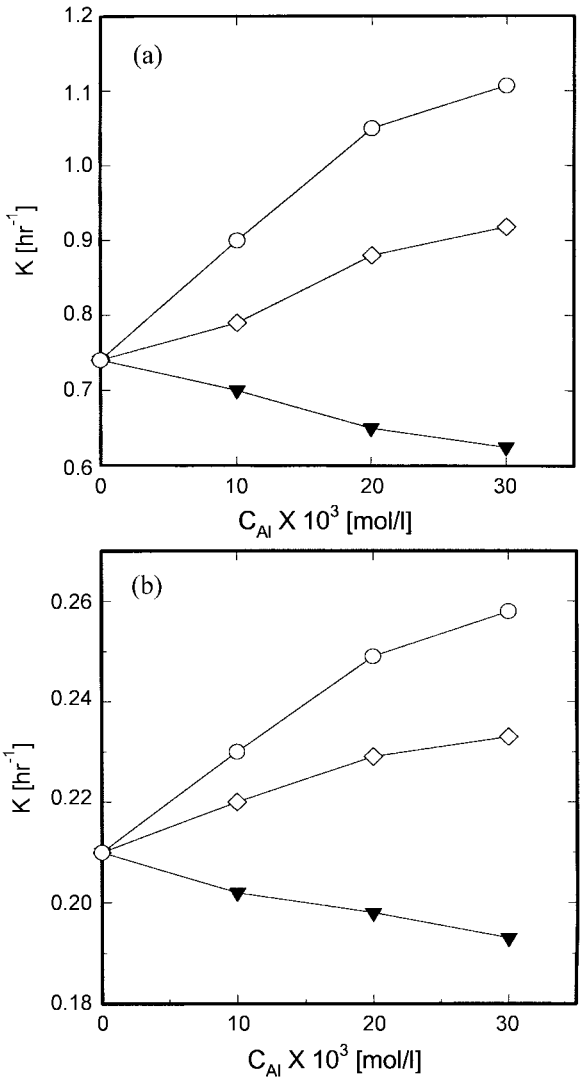

Figure 7. Dependencies of alcohol concentration on the back-extraction rate constants (K) of BSA (a) and cytochrome c (b). PrOH (▼), HexOH (◇) and OctOH (○).

back-extraction rates are increased with increasing of the number of alkyl chain per alcohol molecule added to RVMS in same alcohol concentration. This is an interesting result indicating the possibility that the protein back-extraction process can be controlled by a small amount of alcohol addition to RVMS. It is considered that these variations of the back-extraction behaviors can be induced by the alcohol effect on the micelle-micelle interaction and protein-micelle interaction. It can be clearly concluded that the method of a little alcohol addition to organic solvent, is good to reform the back-extraction behavior of proteins and to control the properties of micellar membrane.

Figure 7 shows the dependency of the back-extraction rate, K, against the concentrations of various alcohols added to RVMS. There are two types for alcohols. One is the promoting of the back-extraction rates with increasing alcohol concentration. These type's alcohols usually have an effect of reducing the interaction between micelles such as HexOH and OctOH. The other type is the reducing the back-extraction rates slightly with increasing alcohol concentration. They have a tendency of acting the interaction between micelles such as PrOH and BuOH in the percolation process.

The back-extraction rates are considered to be governed by the resistance at the interface more than the diffusional resistance in the reverse micellar phase and the aqueous phase.[9,19]

Relationship between back-extracted rates and percolation behaviors of proteins. The relationship between the protein back-extraction behaviors and the percolation phenomena has been examined. The rate constant of back-extraction (K) is directly plotted against the variation of percolation processes (β_t) at the each same alcohol concentration in Figure 8. It shows that the increasing β_t value promotes the back-extraction rate constant, K. In other word, the decreasing of the micelle-micelle interaction accelerates the proteins back-extraction rate, explaining the role of alcohol to the back-extraction process of protein in RVMS. It is considered that the alcohol molecules added to surfactant

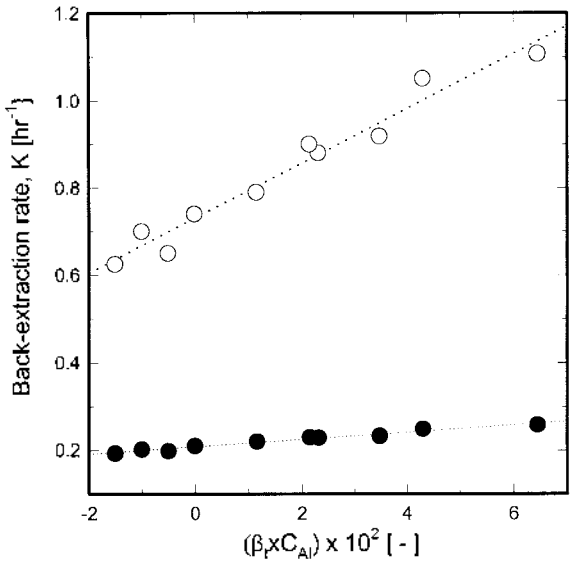

Figure 8. Correlation between the percolation processes (β_t) and the back-extraction rate constants of BSA (○) and cytochrome c (●).

organic solvent may be played as a cosurfactant when the reverse micelle is formed. because the micellar property is changed by addition of alcohols affecting the micelle-micelle and protein-micelle interactions. According to Figure 8, BSA and cytochrome c back-extraction rates are estimated easily as following equation[23]:

$$K = A(\beta_{t,p}C_{Al}) + C = B(\beta_t C_{Al}) + C \quad (2)$$

where. A and B are the proportionality coefficients. This equation is very simple but it is important to evaluate the effect of various alcohols on the back-extraction processes.

Conclusions

The interfacial transport processes of proteins from a reverse micellar phase to an aqueous phase have been investigated focusing on micelle-micelle interaction. BSA suppressing the cluster formation of reverse micelles (positive value of β_{pr}) has the high back-extracted fraction (E_b). but cytochrome c enhancing the cluster formation of reverse micelles (negative value of β_{pr}) has the low back-extracted fraction, relatively. The percolation processes reflecting clearly the micelle-micelle interaction. have been influenced by a small amount of alcohol and the protein solubilization, suggesting to be controlled the interactions. The alcohols suppressing the formation of micellar cluster (high values of β_t), remarkably improved the back-extraction rates of BSA and cytochrome c. The values of β_t, defined by the variation of percolation processes. and the back-extraction behaviors of proteins have a good linear correlation. The micelle-micelle interaction or micellar clustering plays an important role in the back-extraction processes of proteins.

Acknowledgment. This paper was supported by the Dong-A University Research Fund. in 2000.

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