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Conformational transition and mass transfer in extraction of proteins by AOT–alcohol–isooctane reverse micellar systems

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Abstract

We examined quantitatively the effect of alcohols on protein and reverse micellar structure. We used circular dichroism (CD) to compare the effects of various alcohols on the protein structure, and percolation phenomena to evaluate the effects of various alcohols on reverse micellar structure. Upon the addition of alcohols to the bulk aqueous phase, proteins were denatured significantly, depending on the alcohol species and concentration, suggesting that use of alcohol directly to the stripping solution is not effective in back-extraction processes of proteins. In the present study, a new method, a small amount of alcohol is added to the surfactant–organic solution to improve the back-extraction behaviors of proteins. Practically, in the back-extraction process, the alcohols suppressing the cluster formation of reverse micelles (high value of β_t), remarkably improved the back-extraction behavior of proteins. In addition, the same alcohol molecules showed a positive effect on the rate and fraction of protein back-extraction. From a result of the CD measurement of the back-extracted proteins, it was known that the alcohols added to reverse micellar solution allowed the proteins to back-extract safely without causing structural changes. These results show that the values of β_t, defined by the variation of percolation processes, and the back-extraction behaviors of proteins have a good relationship, suggesting that the back-extraction processes were controlled by the micellar–micellar and protein–micellar interactions.

Keywords: Circular dichroism; Proteins; Alcohol; Isooctane

1. Introduction

Reverse micellar systems (RVMSs) have been widely used for protein extraction systems composed of the bulk aqueous and the micro aqueous phase (i.e., a kind of aqueous two-phase system), which is surrounded by surfactants and dispersed in the organic phase [1–6]. Goklen and Hatton [2] carried out the extraction of amino acid and proteins from bulk aqueous solution to reverse micelles, and demonstrated the effects of operating parameters. 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. Among these interactions, electrostatic interactions were considered as the main driving force especially in forward-extraction processes. Unfortunately, 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 lower than the rate of forward-extraction [8–10].

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These problems originate from the structural change of proteins and micelles due to strong interaction between proteins and micelles. In order to improve the back-extraction process, many studies have been reported using various methods. The strategy of improvement could consider three aspects. One deals with the surfactant–organic phase by concentration and species of surfactant or type of organic solution [11–13], another with the stripping aqueous phase by pH, concentration and species of salts or adding various alcohols [7,8,10,14–16], and the third deals with the whole system by temperature or pressure [9,17,18]. For example, Carlson and Nagarajan [15] showed that the addition of 10–15% isopropanol to the stripping aqueous phase increased the rate of protein release and allowed for nearly complete back-extraction of porcine pepsin and 70% back-extraction of bovine chymosin. Dekker et al. [17] also studied the method using the effect of temperature. They showed that by increasing the temperature of the reverse micellar phase, after it has been saturated with the aqueous phase during the forward-extraction, a separate aqueous phase was formed in which most of enzyme was concentrated. Unfortunately, alcohols or high temperature conditions are also known to have destructive effects on native proteins [19–22]. We expect, however, that the alcohol molecule could be a good modifying agent for the reverse micelles, because our previous paper showed that the reverse micellar properties were influenced sensitively by alcohol molecules [23].

The back-extraction process on RVMSs is a difficult process, but it may be carried out successfully to control the properties or structures of reverse micelles. In our previous papers [13,24], we have shown that the interaction between micelles, reflecting the properties or structures of reverse micelles, can be easily evaluated by a percolation phenomenon of the RVMS. We have also examined the effect of various alcohols on the RVMS using the percolation process in our previous paper [23]. The percolation processes clearly reflect the micellar–micellar interaction and it can be quantified easily by the measurement of the electrical conductivity of the RVMS. Electrical conductivity measurements have been used to assess reverse micellar formation and to probe the structural changes occurring in such systems [13,24–27].

In the present paper, we have examined the effect of various alcohols on the structures of proteins and back-extraction behaviors in terms of their back-extraction rate and back-extracted fraction. We have also studied the percolation processes of RVMSs to evaluate the protein–micellar interactions.

2. Experimental

2.1. Materials

AOT (sodium di[2-ethylhexyl] sulfosuccinate) of 95% purity from Tokyo Kasei (Tokyo, Japan) was used without further purification. 2,2,4-Trimethylpentane (isooctane) was purchased from Ishizu Seiyaku (Osaka, Japan). Bovine serum albumin (BSA, p*I* 4.9), carbon anhydrase from bovine (CAB, p*I* 5.8) and β-lactoglobulin (β-LG, p*I* 5.2) were purchased from Sigma (St. Louis, MO, USA). Lysozyme (LYS, p*I* 11.1) from egg white was purchased from Wako (Osaka, Japan). Abbreviations used for alcohols are summarized in Table 1. MeOH, EtOH, PrOH, BuOH, PenOH, HexOH, OctOH and HFIP

Table 1
The β_t values of various alcohols

Alcohol	Abbreviation used	β_t (±error) (l mol^{-1})
Ethanol	EtOH	−1.00 (±0.03)
1-Propanol	PrOH	−0.50 (±0.03)
1-Butanol	BuOH	−0.23 (±0.02)
1-Pentanol	PenOH	0.44 (±0.01)
1-Hexanol	HexOH	1.16 (±0.02)
1-Octanol	OctOH	2.15 (±0.03)
2,2,2-Trifluoroethanol	TFE	0.36 (±0.03)
1,1,1,3,3,3-Hexafluoro-2-propanol	HFIP	3.57 (±0.22)

were purchased from Wako. TFE was purchased from Aldrich (Milwaukee, WI, USA) at the highest purity available.

2.2. Methods

2.2.1. Circular dichroism measurements

Circular dichroism (CD) measurements were done with a Jasco spectropolarimeter (Tokyo, Japan), Model J-720. Far-UV CD spectra were obtained using a cell with a 1-mm light-path depending on the absorption of the solvent. The temperature was controlled with a water-circulating cell-holder (25°C). The data were expressed as molar residue ellipticity (θ) as described [19]. Typically, 50 μl of protein solution at a concentration of 0.1 mg ml^{-1}, dissolved in deionized water, was mixed with various concentrations of alcohols.

2.2.2. Percolation

The conductivity of RVMSs was measured as a function of water content (ϕ_{aq}) with a conductivity meter CM-40V (TOA Electronics, Tokyo, Japan) and a platinum electrode. The electrode was inserted into a test tube containing the reverse micellar solution and the tube was placed in a thermostated water bath (25±0.1°C). Electrical conductivity measurements were performed with dropwise addition of an aqueous phase to AOT–isooctane or AOT–alcohol–isooctane solution until the percolation phenomenon was observed. The percolation threshold with (ϕ_t) and without (ϕ_p) alcohol was defined as the starting point of the sharp increase in conductivity and used to evaluate the effect of alcohols on micellar–micellar interactions, based on the previous papers [13,24,25]. The values of ϕ_t or ϕ_p were determined by extrapolation for finding an intersecting point between constant line and increasing line of the curve (see Fig. 5a).

2.2.3. Back-extraction of proteins

The proteins were solubilized into AOT–isooctane solution by the injection method following the description in previous papers [13,24]. The buffer solution containing the protein was injected into the AOT–isooctane solution and the mixture was shaken vigorously until a clear solution was obtained. Back-extraction of the protein from the reverse micelles was carried out by contacting the protein containing reverse micellar solution with buffer solution (pH_{aq}) containing 0.1 *M* KCl. Similar experiments were also carried out for AOT–alcohol mixed RVMSs. 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 previous work [13,24]). The proteins concentrations were determined by spectroscopy (UV-1600A, Shimadzu, Tokyo, Japan) at 280 nm. The activity of CAB was determined by measuring the ester hydrolysis rate using *p*-nitrophenyl acetate (*p*-NPA) as the substrate [33].

3. Results and discussion

3.1. Effect of alcohol on protein structure

Fig. 1 shows the alcohol-induced conformational transitions of β-LG, measured by the ellipticity at 222 nm. The native structure of β-LG, a predominantly β-sheet protein, is denatured and transforms to an α-helical denatured state by the addition of alcohols. Fig. 1 denotes that the effectiveness of

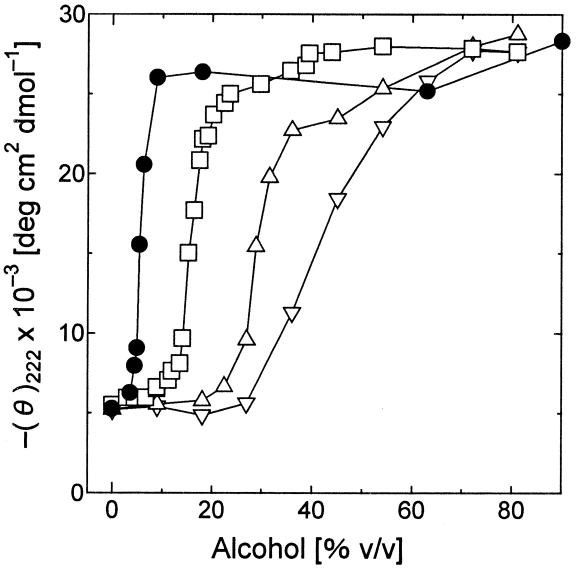

Fig. 1. Alcohol-induced conformational transition of bovine β-LG A measured by the ellipticity at 222 nm. Methanol (▽), ethanol (△), TFE (□) and HFIP (●). The data are taken from Hirota et al. [19] with permission.

alcohols on the protein denaturation varies markedly depending on their species. HFIP is an alcohol with a strong potential of protein denaturing. This result suggests that the use of an alcohol directly in the stripping solution is not good in the back-extraction process of the proteins and it needs an appropriate choice of alcohol molecule. In this study, we used an alcohol addition concentration of 0.01–0.1% (v/v) where the alcohol-induced protein denaturation is negligible. Our other study, which clarified the mechanism of the alcohol effect on the native proteins, suggested that aggregation of alcohol molecules was a critical factor enhancing the protein denaturation [28].

3.2. Effect of alcohol addition on the back-extraction behaviors of proteins

Proteins are usually back-extracted by putting into contact the organic phase loaded with protein with a new aqueous phase at high ionic strength (up to 1 *M* salt) [1,17]. However, the proteins are likely to denature under such a high ionic strength condition. We have examined the back-extraction behaviors of proteins using various alcohols under low ionic strength conditions (0.1 *M* KCl).

Some works [7–10] studied the protein extraction process by reverse micelles in terms of the mass transfer behaviors. They showed that the rate-determining step is the desolubilization at the interface in the back-extraction process, suggesting the diffusional resistances in the reverse micellar phase and the aqueous phase can be neglected. Thus, when we assumed simply that the overall rate constant associated with the back-extraction process is *K*, the equation of back-extraction rate is:

$$\ln\left[\left\{C^0_{org} - (1+m)C_{aq}\right\}/C^0_{org}\right] = (1+m)Kt \qquad (1)$$

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 conditions.

Fig. 2a shows that the time course of the back-extracted fraction of BSA is exponential and reached equilibrium. There is a clear difference depending on the species of alcohol added to the RVMS. For example, in the case of the addition of PrOH, the

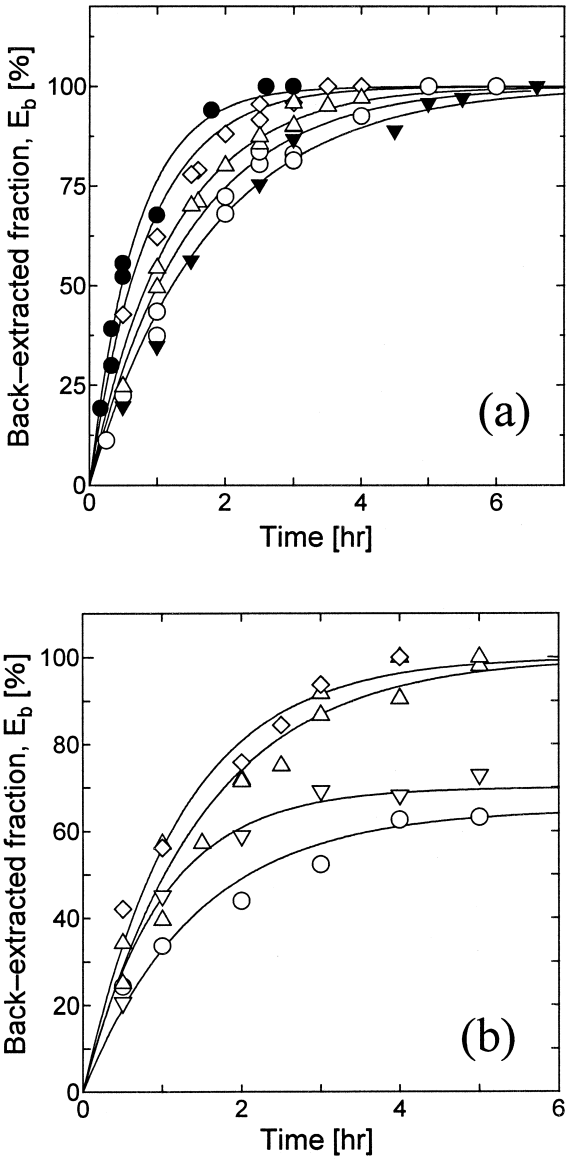

Fig. 2. (a) Time course of the back-extraction fraction of BSA for the AOT (0.2 *M*)–alcohol RVMSs. Alcohols are 0 m*M* (○), PrOH 25 m*M* (▼), HexOH 25 m*M* (△), OctOH 25 m*M* (◇), and HFIP 25 m*M* (●). (b) Time course of the back-extraction fraction of β-LG for the AOT (0.2 *M*)–alcohol RVMSs. Alcohols are 0 m*M* (○), PenOH 20 m*M* (▽), HexOH 20 m*M* (△), and OctOH 20 m*M* (◇). The pH values of pH_{inj} and pH_{aq} are 8.0 and 8.2, respectively and the W_o value is 20. Salt concentration in stripping aqueous solution is 0.1 *M* KCl.

back-extraction rate is reduced slightly. In adding OctOH or HFIP, however, the back-extraction rates are accelerated remarkably with the alcohol concentration. This is an interesting result indicating a possibility that the protein back-extraction process can be regulated by a small amount of alcohol added to the RVMS. It is considered that this variation of the back-extraction behavior can be induced by the alcohol effect on the micellar–micellar interaction and protein–micellar interaction described in the next section. Fig. 2b also shows the plot of the back-extraction fraction of β-LG against time for the various RVMSs and a improvement of an equilibrium back-extraction fraction by alcohols. Therefore, we can clearly conclude that the method of a addition of a small amount of alcohol to the organic solvent, is good to reform the back-extraction behavior of proteins and to control the properties of the micellar membrane.

Fig. 3a shows the effects of alcohol 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 concentrations increase in the back-extracted fraction, BuOH and PrOH do not show such a good effect. Alcohol additions have good effects on the activity yields as well as the back-extracted fractions, indicating a decrease in the resistance of micellar or interface membrane by the alcohol molecules. We have also obtained good rates of back-extraction by using of alcohol in the back-extraction processes of LYS. Fig. 3b shows that LYS cannot be back-extracted entirely under various pH_{inj} conditions at low concentration of salt (KCl, 0.1 *M*) and high concentration of AOT (0.2 *M*). The back-extracted fraction of LYS, however, is increased with addition of alcohols to about 20% of back-extracted fraction.

Fig. 4 shows the dependency of the back-extraction rate constant, *K*, against the concentrations of various alcohols added to the RVMS. There are two types of alcohols. The first type promotes back-extraction rates with increasing alcohol concentration. These types of alcohols, such as HexOH, OctOH and HFIP, usually have an effect of reducing the interaction between micelles. The second type of alcohols reduces the back-extraction rates slightly with increasing alcohol concentration. They have a tendency of acting the interaction between micelles,

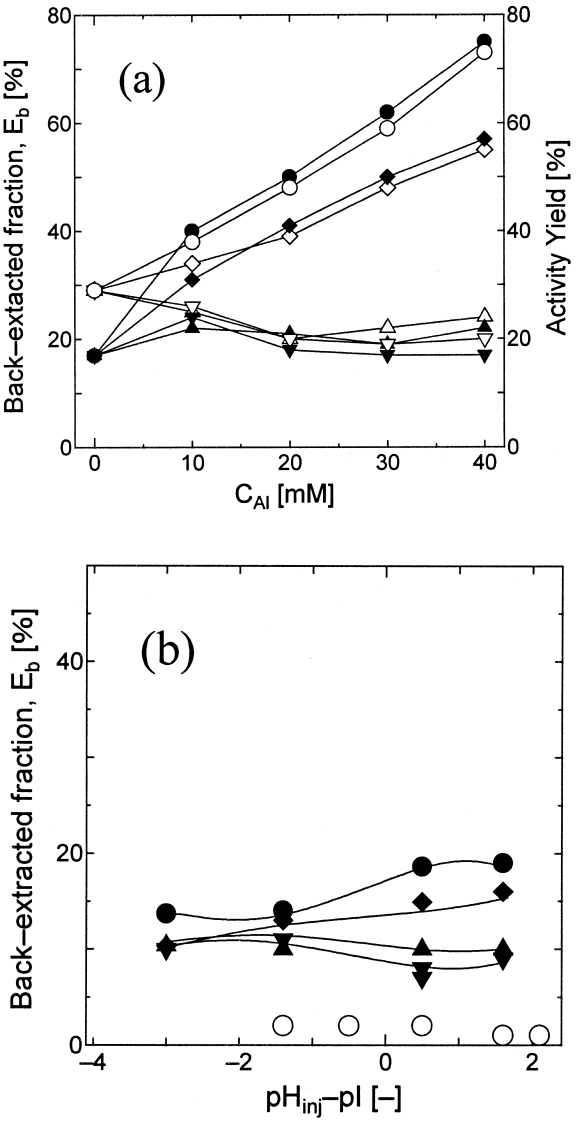

Fig. 3. (a) Effect of alcohol addition on the back-extracted behaviors of CAB for AOT (0.1 *M*) RVMSs. The pH values of pH_{inj} and pH_{aq} were 8.0 and 8.2, respectively and the W_o value was 20. Added alcohols are PrOH (▽, ▼), BuOH (△, ▲), HexOH (◇, ◆), and OctOH (○, ●). The open and closed keys are the back-extraction fraction and activity yield, respectively. (b) Effects of pH_{inj} and alcohol addition on the back-extracted fraction of LYS for AOT (0.2 *M*) RVMSs. Alcohols are 0 m*M* (○), OctOH 10 m*M* (▼), OctOH 30 m*M* (▲), HFIP 10 m*M* (◆), and HFIP 20 m*M* (●). The pH value of pH_{aq} is 11.5 and the W_o value was 20.

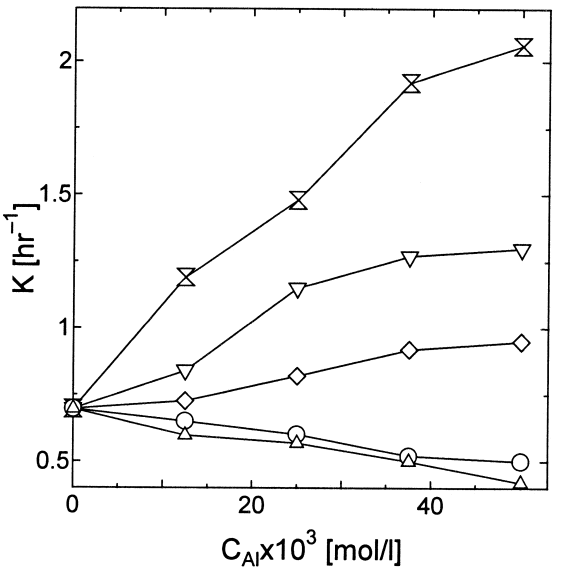

Fig. 4. Dependencies of alcohol concentration on the back-extraction rate constants (K) of BSA. (△) PrOH, (○) BuOH, (◇) HexOH, (▽) OctOH and (⧖) HFIP.

e.g., PrOH and BuOH [23]. At high concentrations of HexOH, OctOH, or HFIP, the formation of reverse micelles are disrupted when the protein solution is injected into the AOT organic solution. Whereas we do not know clearly the reason, the property, that alcohols have usually been used for destruction of the reverse micelles, may be the reason. At low concentrations of the alcohols, however, the reverse micelles were formed safely. In this study, the back-extraction experiments were carried out under the condition of the transparent reverse micellar solution.

For the *n*-alcohols, Fig. 4 also shows that the back-extraction rates increased with increasing the number of alkyl chain per alcohol molecule added to RVMSs of the same alcohol concentration. Even though HFIP has a short alkyl chain, it shows a more remarkable effectiveness on the back-extraction rate compared with other alcohols at the same concentration. HFIP has a high β_t value which means decreasing the micellar–micellar interaction in the RVMS [23]. The β_t values of various alcohols are summarized in Table 1. These results explain that the back-extraction rate is more accelerated or reduced by the addition of alcohols, suggesting that the back-extraction rate could be controlled by using alcohol molecules. The back-extraction rates are considered to be governed by the resistance at the interface more than by the diffusional resistance in the reverse micellar phase and the aqueous phase [8–10]. From the above results, therefore, the alcohol molecules added to RVMSs play a role in decreasing the interfacial resistance because the back-extraction rate for the system with alcohol addition can also be explained by Eq. (1).

3.3. Effect of protein solubilization on the percolation processes of RVMSs with added various alcohols

The variation of percolation threshold by solubilizing various proteins into reverse micelles, is clearly reflecting the protein–micellar interactions [24]. In this study, we focused on the concentration of BSA solubilized in reverse micelles kept at $5\cdot10^{-5}$ *M*, under the same conditions of back-extraction. The effect of protein solubilization on the percolation processes of RVMSs added to HexOH is shown in Fig. 5a. The protein solubilization shifted the percolation thresholds (ϕ_p^p) to a higher value of ϕ_{aq} than for the protein-free systems. The interaction between the micellar membrane and protein in reverse micelles has been studied by several authors using percolation processes of RVMSs [29–31]. We have evaluated quantitatively the protein–micellar interaction from the variation of the percolation processes and the protein concentration solubilized the reverse micelles [24]. In order to discuss the effect of protein solubilization, the relationships between the relative percolation thresholds and the alcohol concentration on the RVMSs with added various alcohols were also investigated.

The difference, $\Delta\phi_t$ ($=\phi_t-\phi_p$), shows the effect of the alcohol concentration on the percolation process. Here, ϕ_t and ϕ_p are the values of the percolation threshold with and without alcohol, respectively. The relationship between the relative percolation thresholds and added alcohol concentrations is also examined. $\Delta\phi_t$ is plotted against the alcohol concentration added in the reverse micellar solution and denoted as an open key in Fig. 5b. There is a linear correlation between $\Delta\phi_t$ and the concentration of each alcohol. The slope, β_t, is a measure of the effect of alcohol addition on the

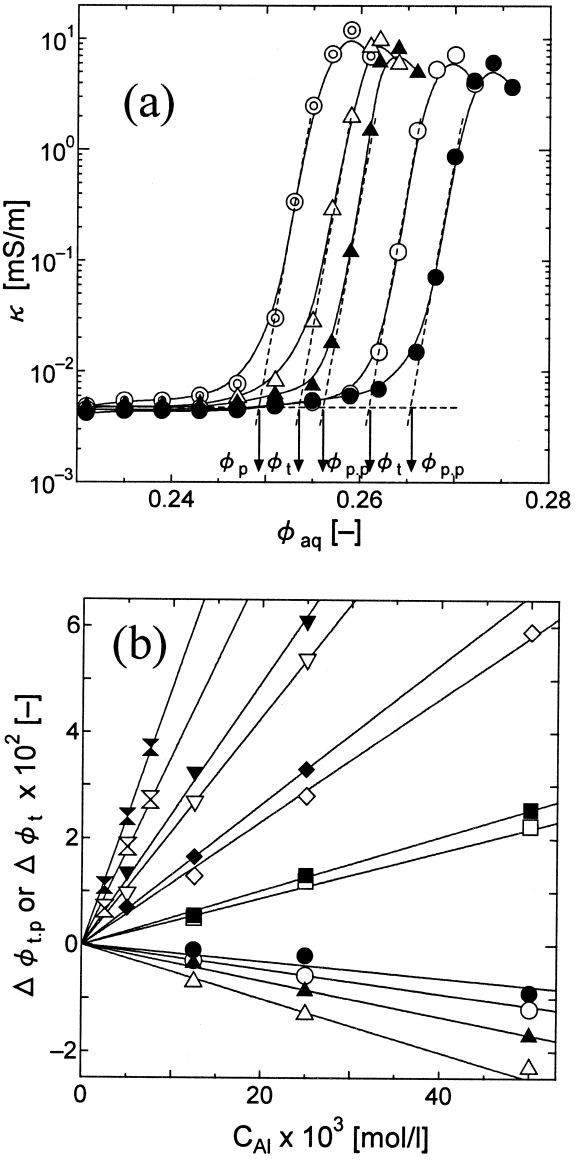

Fig. 5. (a) Effect of BSA solubilization on the percolation process of AOT (0.2 *M*) and AOT–HexOH RVMSs. Alcohol concentrations are 0 m*M* (◎), 5 m*M* (△, ▲) and 12.5 m*M* (○, ●). Without protein (◎, △, ○), with protein (▲, ●). Protein concentration is $5\cdot10^{-5}$ *M*. (b) Dependencies of BSA solubilization and alcohol concentration on the percolation threshold in AOT and AOT–alcohol mixed RVMSs. Open and closed keys are without and with protein, respectively. PrOH (△), BuOH (○), PenOH (□), HexOH (◇), OctOH (▽), and HFIP (⧖).

micellar–micellar interaction. The value of β_t denotes the stability of the RVMS or ability of water solubility with the addition of alcohols. A positive value of β_t means the stabilization of the RVMS or the decrease of micellar–micellar interactions with the addition of alcohol to the RVMS. On the contrary, a negative β_t means the destabilization of the RVMS or the increase of micellar–micellar interactions with alcohol addition to the RVMS. These results are identical to those of type A and B polymers solubilized into reverse micelles considered in previous papers [13,24]. These results show that the values of β_t are markedly changed by the alcohol species. In our previous study [23], we have examined the effect of alcohols on the suppression of the micellar–micellar interaction using a percolation process of RVMS and found that variation in these effects can be explained by the additive contribution of each constituent group of the alcohol; the hydrocarbon (CH) group and any halogen substituents make a suppressive contribution and the hydroxyl (OH) group contributes expeditiously to the effect.

The difference, $\Delta\phi_{t.p}$ $(=\phi_p^p-\phi_p)$, also shows the effect of the alcohol and protein on the percolation process. Here, ϕ_p^p and ϕ_p are the values of the percolation threshold with and without alcohol and protein, respectively. Fig. 5b shows the plot, for the representative alcohols, of the $\Delta\phi_{t.p}$ against the alcohol concentration, C_{Al}, added to the reverse micellar solution (denoted as a closed key). There also is a linear correlation between $\Delta\phi_{t.p}$ and C_{Al}. Though the slopes, $\beta_{t.p}$, defined the effect of solubilizing protein, are larger than the values of β_t, they show a same tendency of variation of back-extraction rate on each alcohol concentration. This increasing slope denotes the effect of the solubilizing protein on the percolation processes or the protein–micellar interactions. In the case of PrOH or BuOH, even though the proteins are solubilized into the reverse micelles, the slopes still show a negative tendency. It is 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 [23,24,29,32]. Therefore, this result shows that the protein–micellar interactions are influenced notably by the alcohol added RVMS as a co-surfactant. As

for the alcohol molecule is a good agent for modifying the micro-membrane of reverse micelles and controlling the protein–micellar interaction.

3.4. Relationship between back-extraction and percolation behaviors

We have examined the relationship between the protein back-extraction and the percolation behaviors. The rate constant of back-extraction (K) is directly plotted against the variation of percolation processes (β_t) at the same alcohol concentration in Fig. 6. It shows that the increasing β_t value promotes the back-extraction rate constant, K. In other words, decreasing of the micellar–micellar interaction accelerates the proteins back-extraction rate, explaining the role of alcohol in the back-extraction process of protein in RVMSs. We are considering that because the alcohol molecules added to surfactant organic solvent may act as a co-surfactant when the reverse micelle is formed, the micellar property is changed by addition of alcohols, affecting the micellar–micellar and protein–micellar interactions. According to Fig. 6, BSA and β-LG back-extraction rates are estimated easily using the following equation:

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

where, A and B are the proportionality coefficients. This equation is very simple but it is important to see that Eq. (2) can explain the effect of various alcohols on the back-extraction processes of protein and to evaluate the effect of other alcohols.

The back-extracted fractions of CAB and LYS have been also compared with the percolation processes, β_t, in Fig. 7. There are good linear correlations between β_t values and the back-extracted fraction of proteins at the same alcohol concentration. While both CAB and LYS back-extracted fractions increased with increasing value of ($\beta_t C_{Al}$), they show different slopes. In a previous work, we proposed that there are two types for proteins such as type A and B by the relation between the percolation processes and the back-extraction behavior of proteins [24]. Type A proteins, such as CAB, β-LG and BSA, suppress the percolation processes or micellar clustering at the pH both above and below the pI and for which back-extracted fraction are comparatively

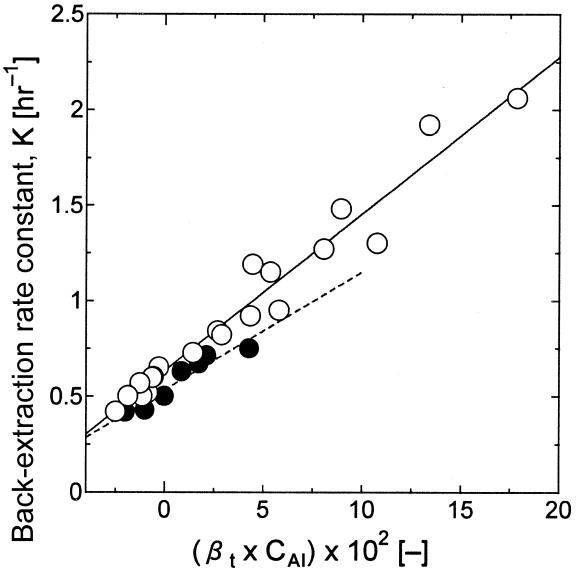

Fig. 6. Correlation between the percolation processes (β_t) and the back-extraction rate constants (K) of BSA (○) and β-LG (●). The back-extraction rate constants of β-LG were calculated from initial change of back-extracted fractions.

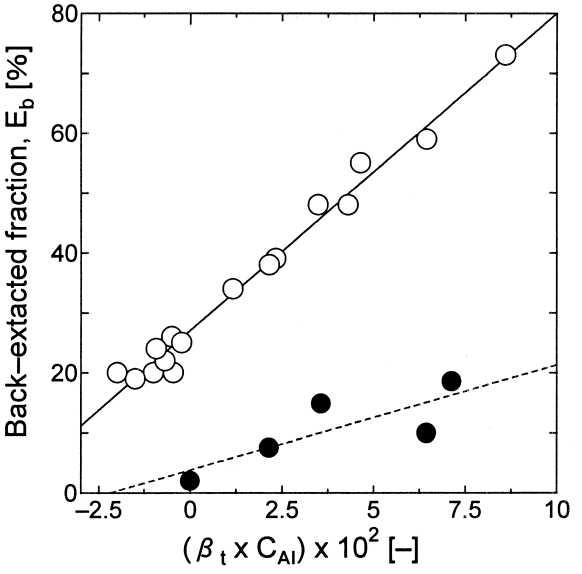

Fig. 7. Correlation between the percolation processes (β_t) and the back-extracted fraction of CAB (○) and LYS (●). The data for LYS indicate the data at pH_{inj} 11.5 in Fig. 3b.

higher. Type B proteins such as LYS, however, in which the back-extraction process is relatively difficult, promote the percolation processes. The difference of slopes in Fig. 7 has to be considered to be due to these reasons. For the type B proteins, further study on the protein–micellar interaction inducing conformation change in reverse micelles should be made in the future.

3.5. Secondary structure of BSA back-extracted from various RVMSs

We assayed the secondary structure of BSA recovered by back-extraction. The far-UV CD spectra of BSA back-extracted from various RVMSs and native BSA were examined in Fig. 8. Because the CD spectrum in the far-UV region reflects the secondary structure of a protein, and the secondary structure of the stripped BSA was only slightly changed after the back-extraction, it does not reflect the effect of alcohol addition to the RVMS. This result suggests that the alcohols added to RVMSs may improve the back-extraction behaviors of proteins, but not influence the protein structure in this range of concentration. However, at high concentrations of alcohols, it should be important to the protein structure as Fig. 1 shows. Therefore, addition of a small amount of alcohol to the organic solvent as a co-surfactant, is a good method to improve the back-extraction behavior of proteins.

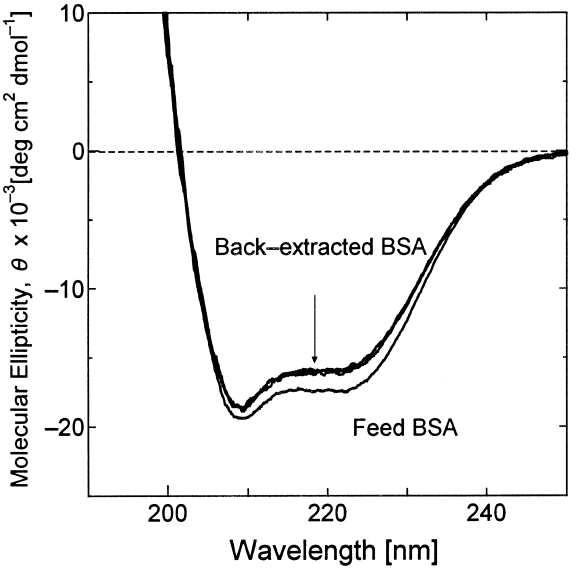

Fig. 8. Circular dichroism spectra of back-extracted BSA from AOT RVMSs and AOT RVMSs with added PrOH or OctOH or HFIP. Added alcohol concentration is 25 m*M*.

4. Conclusions

We examined quantitatively the effect of alcohols on protein and reverse micellar structure. We used CD to compare the effects of various alcohols on the protein structure, and percolation phenomena to evaluate the effects of protein solubilization on AOT–alcohol reverse micellar structure. The native structure of β-LG is denatured and transforms to an α-helical denatured state by the addition of alcohols, suggesting that the use of an alcohol directly in the protein solution is not effective in the back-extraction process of the proteins. An interesting result about the effect of various alcohols on the back-extraction behaviors 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 behaviors of proteins depending on the concentration and species of alcohol. Therefore, this method is effective to control the properties of the micellar interface as well as to improve the back-extraction processes of proteins. The percolation processes with alcohols could be utilized to evaluate and understand the micellar–micellar interactions (β_t), showing a good relationship with the back-extraction behaviors of various proteins.

5. Abbreviations

AOT	Sodium di[2-ethylhexyl] sulfosuccinate, anionic surfactant
BSA	Bovine serum albumin
CAB	Carbonic anhydrase from bovine erythrocytes
CD	Circular dichroism
HFIP	1,1,1,3,3,3-Hexafluoro-2-propanol
β-LG	β-Lactoglobulin
LYS	Lysozyme
p-NPA	*p*-Nitrophenyl acetate

RVMS	Reverse micellar system
TFE	2,2,2-Trifluoroethanol

6. Nomenclature

[AOT]	Concentration of AOT (mol l^{-1})
C_{pr}	Concentration of protein (mol l^{-1})
C_{Al}	Concentration of alcohol (mol l^{-1})
C^0_{org}	Protein concentration in organic phase at time 0 (mol l^{-1})
C_{aq}	Protein concentration in aqueous phase at time t (mol l^{-1})
C^*_{org}	Protein concentration in organic phase at equilibrium (mol l^{-1})
C^*_{aq}	Protein concentration in aqueous phase at equilibrium (mol l^{-1})
E_b	Fraction of back-extraction = $100[\text{Protein}]_{aq}/[\text{Protein}]_{org}$ (%)
t	Time (h)
K	Overall rate constant of back-extraction (h^{-1})
m	Partitioning equilibrium constant ($=C^*_{aq}/C^*_{org}$) (–)
pH_{inj}	pH of the protein solution injected into reverse micelles (–)
pH_{aq}	pH of aqueous phase used for back-extraction (–)
pI	Isoelectric point of protein (–)
W_o	Water content, molar ratio of H_2O to AOT = $[H_2O]/[AOT]$ (–)
β_t	Stability parameter of RVMS with alcohol (l mol^{-1})
$\beta_{t.p}$	Stability parameter of RVMS with alcohol and protein (l mol^{-1})
κ	Electrical conductivity (mS m^{-1})
ϕ_p	Percolation threshold without protein and alcohol (–)
$\phi_{p.p}$	Percolation threshold with protein and alcohol (–)
ϕ_t	Percolation threshold with alcohol (–)
$\Delta\phi_{t.p}$	$\phi_{p.p} - \phi_p$ (–)
$\Delta\phi_t$	$\phi_t - \phi_p$ (–)

Superscripts

0	Initial
*	Equilibrium

Subscripts

inj	Injected solution
aq	Aqueous solution used for back-extraction
org	Organic solution
pr	Protein
Al	alcohol

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References

[1] T.A. Hatton, in: J.F. Scamehorn, J.H. Harwell (Eds.), Surfactant-Based Separation Processes, Marcel Dekker, New York, 1989, pp. 55–90.
[2] K. Goklen, T.A. Hatton, Sep. Sci. Technol. 22 (1987) 831.
[3] M.E. Leser, P.L. Luisi, Chimia 44 (1990) 270.
[4] R. Kuboi, K. Hashimoto, I. Komasawa, Kagaku Kogaku Ronbunshu 16 (1990) 335.
[5] R. Kuboi, Y. Mori, I. Komasawa, Kagaku Kogaku Ronbunshu 16 (1990) 763.
[6] R. Kuboi, Y. Mori, I. Komasawa, Kagaku Kogaku Ronbunshu 16 (1990) 1060.
[7] M. Dekker, K.V. Riet, B.H. Bijsterbosch, R.B.G. Wolbert, R. Hilhorst, Chem. Eng. Sci. 45 (1990) 2949.
[8] T. Nishiki, A. Sato, T. Kataoka, Solv. Ext. Process Industries 2 (1993) 840.
[9] T. Nishiki, A. Muto, T. Kataoka, Kagaku Kogaku Ronbunshu 21 (1995) 916.
[10] T. Nishiki, A. Muto, T. Kataoka, D. Kato, Chem. Eng. J. 59 (1995) 297.
[11] Y. Yamada, R. Kuboi, I. Komasawa, Solv. Extr. Res. Dev. Jpn. 1 (1994) 167.
[12] Y. Yamada, R. Kuboi, I. Komasawa, Biotechnol. Prog. 11 (1995) 682.
[13] D.-P. Hong, R. Kuboi, I. Komasawa, Korean J. Chem. Eng. 14 (1997) 334.
[14] M.R. Aires-Barros, J.M.S. Cabral, Biotechnol. Bioeng. 38 (1991) 1302.
[15] A. Carlson, R. Nagarajan, Biotechnol. Prog. 8 (1992) 85.
[16] M.J. Pires, J.M.S. Cabral, Biotechnol. Prog. 9 (1993) 647.
[17] M. Dekker, K.V. Riet, J.J. Van Der Pol, Chem. Eng. J. 46 (1991) B69.
[18] M.J. Pires, J.M.S. Cabral, J. Chem. Tech. Biotechnol. 61 (1994) 219.
[19] N. Hirota, K. Mizuno, Y. Goto, Protein Sci. 6 (1997) 416.

[20] N. Hirota, K. Mizuno, Y. Goto, J. Mol. Biol. 275 (1998) 365.
[21] R. Kuboi, K. Yamahara, H. Ota, J. Chem. Eng. Jpn. 30 (1997) 1119.
[22] K. Yamahara, H. Ota, R. Kuboi, J. Chem. Eng. Jpn. 31 (1998) 795.
[23] D.-P. Hong, R. Kuboi, Biochem. Eng. J. 4 (1999) 23.
[24] R. Kuboi, D.-P. Hong, I. Komasawa, K. Shiomori, Y. Kawano, S.-S. Lee, Solv. Extr. Res. Dev. Jpn. 3 (1996) 223.
[25] A. Jada, J. Lang, R. Zana, J. Phys. Chem. 93 (1989) 10.
[26] A. Jada, J. Lang, R. Zana, J. Phys. Chem. 94 (1990) 387.
[27] P. Alexandridis, J.F. Holzwarth, T.A. Hatton, J. Phys. Chem. 99 (1995) 8222.
[28] D.-P. Hong, M. Hoshino, R. Kuboi, Y. Goto, J. Am. Chem. Soc. 121 (1999) 8427.
[29] J.P. Huruguen, M. Authier, J.L. Greffe, M.P. Pileni, Langmuir 7 (1991) 243.
[30] K.M. Larsson, M.P. Pileni, Eur. Biophys. J. 21 (1993) 409.
[31] G. Cassin, S. Illy, M.P. Pileni, Chem. Phys. Lett. 221 (1994) 205.
[32] M. Holovko, J.P. Badiadi, Chem. Phys. Lett. 204 (1993) 511.
[33] R. Kuboi, K. Yano, H. Tanaka, I. Komasawa, J. Chem. Eng. Jpn. 26 (1993) 286.