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TECHNICAL NOTE

Extraction Behavior of Proteins Using AOT Reversed Micellar Systems Formed by Kerosene and Silicone Oil

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ABSTRACT

The formation of AOT/kerosene and AOT/silicone oil reversed micelles was confirmed by measurements of the interfacial tension between two phases and the water content in the reversed micelles. The extraction behavior of lysozyme and cytochrome c was investigated using the formed reversed micellar systems. It was found that the forward extraction fraction of the two proteins increased with an increase in AOT concentration in the organic phase and with a decrease in KCl concentration in the aqueous phase. The fraction of backward extraction of lysozyme increased with an increase in pH value and KCl concentration in the stripping aqueous phase. It was also found that the fraction of backward extraction depended on the pH value of the initial aqueous phase used for the forward extraction. It was suggested that the AOT/kerosene and AOT/silicone oil systems can be used for the large-scale development of the reversed micellar extraction of proteins.

Key Words. AOT; Extraction; Kerosene; Silicone oil; Proteins; Reversed micellar systems

INTRODUCTION

The extraction and purification of proteins using reversed micellar systems have been studied extensively in recent years (2, 4, 6, 10). The reversed

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micelles are spontaneously formed aggregates of amphiphilic molecules in organic solvents. Proteins in aqueous solution can be transferred into the reversed micellar organic phase under appropriate conditions. Several surfactants have been used to form reversed micelles in apolar solvents, most notably AOT (di-2-ethylhexyl sodium sulfosuccinate), CTAB (cetyltrimethylammonium bromide), and TOMAC (trioctylmethylammonium chloride). Organic solvents such as isooctane, octane, *n*-heptane, cyclohexane, and chloroform (3, 10, 11) were employed for the formation of reversed micellar solutions. However, these solvents are either expensive or unsafe for industrial applications.

Safe organic solvents of low cost need to be developed because of the large quantities used during extraction processes. For this study we chose kerosene and silicone oil as the organic solvents. The former is cheap and has been widely used as an extractant both in research work and industry. The silicone oil series has the advantage of physiological safety. Both solvents have a higher flash point than isooctane (12). In order to determine the applicability of the above solvents, we focused on the formation of AOT/kerosene and AOT/silicone oil reversed micelles. We also studied the extraction behavior of lysozyme and cytochrome c by using the two kinds of reversed micellar systems.

MATERIALS AND METHODS

Reagents

The surfactant was di-2-ethylhexyl sodium sulfosuccinate, AOT, a product of Nacalai Tesque Co., Kyoto, Japan. Isooctane and kerosene were purchased from Wako Chemical Co., Tokyo, Japan. Silicone oil [KF96L-1, dynamic viscosity 1 cSt, formula $(CH_3)_3SiO(CH_3)_2SiOSi(CH_3)_3$] was provided by Shin-Etsu Chemical Co., Tokyo. The detailed properties of kerosene and silicone oil used in this study can be found elsewhere (12). Lysozyme (MW = 14,300, pI = 11) and cytochrome c (MW = 12,384, pI = 10.6) were purchased from Sigma Chemical Co., St. Louis, MO, USA. All reagents were used without further purification.

Formation of AOT/Kerosene and AOT/Silicone Oil Reversed Micelles

AOT was dissolved into kerosene or silicone oil with various concentrations from 2 to 200 mM. The organic phase with AOT was mixed with 0.2 M KCl aqueous phase for 20 minutes and then separated by centrifugation. The water content in the organic phase was measured by Karl-Fischer titration. The interfacial tension between the AOT/kerosene or AOT/silicone oil or-

ganic phase and the aqueous phase was determined using the drop-weight method.

Extraction of Proteins

The initial aqueous phase was prepared by dissolving lysozyme or cytochrome c into a 0.2 M KCl aqueous solution. The pH value of the aqueous phase was adjusted by 0.1 M HCl or 0.1 M NaOH. The initial protein concentration was 10 μM for both lysozyme and cytochrome c. The reversed micellar organic phase with various AOT concentrations in kerosene or silicone oil was made in advance.

The forward extraction of proteins was carried out by mixing the two phases for 20 minutes. Then the mixture of the two phases was centrifuged for 15 minutes at 2500 rpm. The backward extraction was performed by mixing the protein-containing organic phase (only for 50 mM [AOT] reversed micellar systems) and the stripping aqueous phase with various KCl concentrations from 0.5 to 2.0 M for 20 minutes. The two phases were centrifuged and separated. The protein concentrations in the aqueous phase and the AOT/silicone oil reversed micellar organic phase were measured directly by spectrophotometry at 280 nm. Direct UV analysis was not possible for protein concentration in the AOT/kerosene organic phase since the solvent is a mixture of various hydrocarbons. We carried out backextraction for the AOT/kerosene samples three times and then determined the protein concentration in the stripping aqueous phase at 280 nm. Experimental data within 10% error in the mass balance were used for analysis.

RESULTS AND DISCUSSION

Formation of the Reversed Micelles

Figure 1 shows the dependence of interfacial tension σ between the organic phase and the 0.2 M KCl aqueous phase on the AOT concentration. The AOT/isooctane experimental data are also presented for comparison. It was found that the values of σ decrease with an increase in [AOT] for all three systems. The interfacial tensions of the systems without AOT were much larger than those of the systems with [AOT] above their critical micelle concentration (cmc). However, from the cmc point toward higher values of [AOT], the interfacial tensions barely decreased. This implies that reversed micelles of AOT were formed in kerosene and silicone oil.

Figure 2 shows the dependence of the water content W_0 (defined as the molar-water-to-surfactant ratio, $W_0 = [H_2O]/[AOT]$) in the reversed micellar systems on the AOT concentration with different KCl concentrations. It was found that the value of W_0 increases slightly with an increase in [AOT].

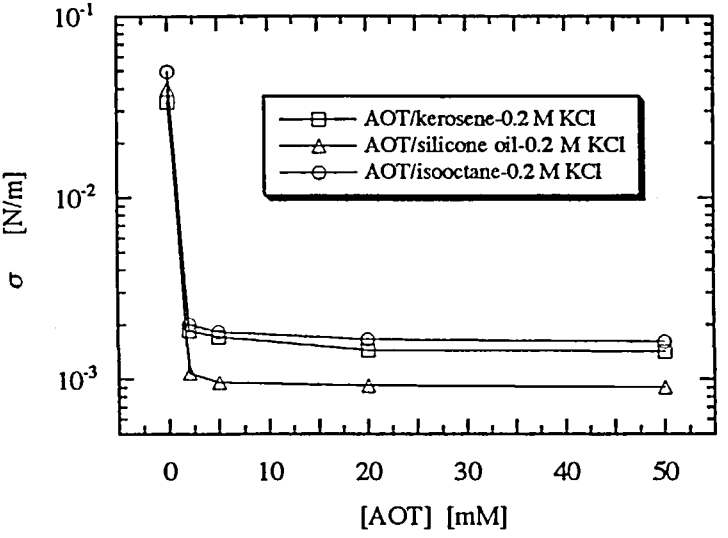

FIG. 1 Dependence of the interfacial tensions on AOT concentration.

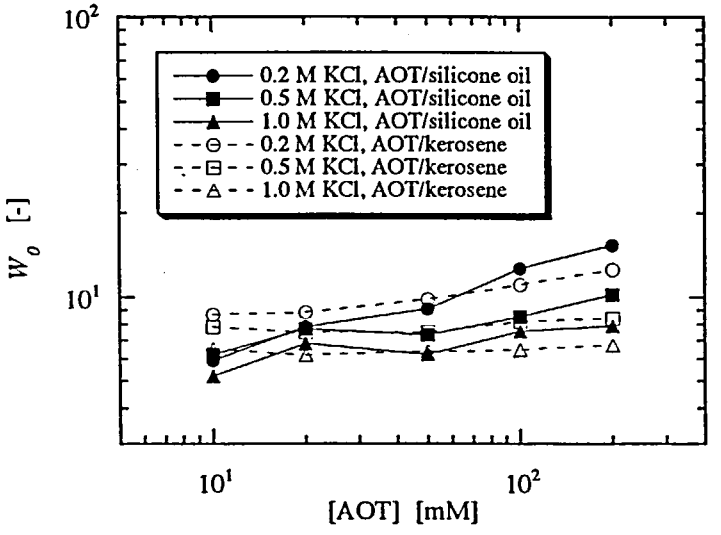

FIG. 2 Dependence of water content in reversed micelles on AOT concentration.

Compared to the AOT/kerosene reversed micellar system, W_0 in the AOT/silicone oil system increases a little faster. On the other hand, W_0 decreases with an increase in KCl concentration of the aqueous phase. As the salt concentration increases, the effects of the electrostatic shield and hydrophobicity increase. Then the repulsion between the head groups of the surfactant molecules decreases. Thus, the reversed micelles were dehydrated and became small. The values of W_0 of the two systems were in the same range compared to the AOT/isooctane reversed micelles (9). It should be noted that a white precipitate was formed in the organic phase near the interface after it was mixed with the aqueous phase when [AOT] was higher than 100 mM for both solvents. The precipitate might be caused by AOT aggrègation at the interface (1). The W_0 data at high [AOT] in Fig. 2 was obtained from samples when the precipitate disappeared in the organic phase after the samples were allowed to stand for about a day.

Extraction Behavior

The effect of AOT concentration on the forward extraction fraction E of proteins at different aqueous phase salinity is shown in Fig. 3. The pH value

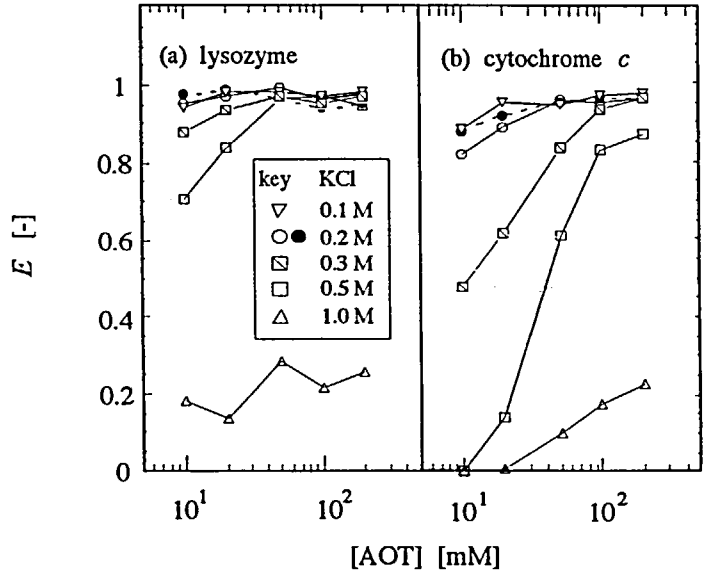

FIG. 3 Effect of AOT concentration on the fraction of forward extraction of proteins. Aqueous phase pH 8.5. Open keys for AOT/kerosene; solid keys for AOT/silicone oil system.

of the aqueous phase was kept at 8.5. Since this value is lower than the pI, the two proteins had net positive charges and could be extracted to the anionic AOT reversed micellar systems. The open keys represent the data of AOT/kerosene and the solid keys are those of the AOT/silicone oil reversed micellar system. It was found that the extraction fractions of the two proteins were high at a low KCl concentration. When the KCl concentration increased, the reversed micellar systems of high AOT concentration were required to extract cytochrome c. For lysozyme, the value of E was around 0.2 when the KCl concentration was 1.0 M. The decrease of E can be attributed to the effects of the electrostatic shield and hydrophobicity induced by the increase in salt concentration (4). It was also noticed that the effects of KCl concentration on the extraction fractions of lysozyme and cytochrome c are in different manners, which is similar to the results obtained by Göklen and Hatton in AOT/isooctane reversed micellar system (5). It is considered that the difference is probably due to the different charge distribution on the surfaces of the two proteins and the difference in their relative hydrophobicities (5). The CD spectra of lysozyme (8) and cytochrome c (6) also reflected their different extraction behavior in AOT/isooctane reversed micellar systems.

We have also studied the effect of pH in the initial aqueous phase on E for lysozyme with the 50 mM AOT reversed micellar systems. It was found that the value of E remained high ($E > 0.95$) within a wide pH range from 4 to 11 for the AOT/kerosene system. However, for the AOT/silicone oil system, the precipitate formed at the interface after extraction when the pH value was lower than 6. One might think that the cmc data of AOT in kerosene and in silicone oil should have an effect on the formed precipitate which is attributed to the aggregate of AOT (1). Unfortunately, we could not obtain these data exactly from our experiments. Also, it might be due to the relative high hydrophobicity of the silicone oil solvent. The interfacial tension between the aqueous phase and the AOT/silicone oil phase is much smaller than that between the aqueous phase and AOT/kerosene, as shown in Fig. 1.

The dependence of the fraction of backward extraction of lysozyme on KCl concentration of the stripping aqueous phase was studied at a pH value of about 12. It was found that complete backward extraction was achieved at 2.0 M KCl rather than at 1.0 M KCl as for the AOT/isooctane reversed micellar system (6). Therefore, the 2.0 M KCl stripping aqueous phase was used during the backward extraction of proteins. The value of [AOT] was kept at 50 mM during the backward extraction experiments.

Figure 4(a) illustrates the effect of the initial pH of the aqueous phase on E_b. The data in Fig. 4(a) was obtained by following these steps.

1. The lysozyme aqueous phase of 0.2 M KCl with various pH values was mixed with 50 mM AOT/kerosene and AOT/silicone oil phases.

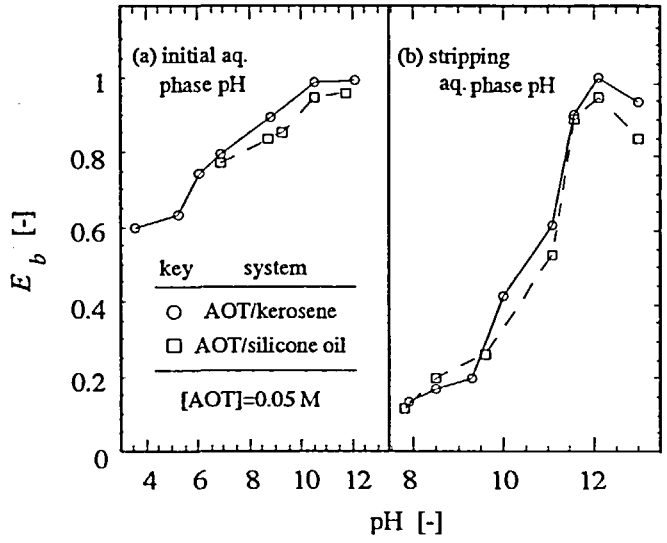

FIG. 4 Effects of the initial aqueous phase pH (a) and the stripping aqueous phase pH (b) on the fraction of backward extraction of lysozyme, (a) Initial aqueous phase: 0.2 M KCl; stripping aqueous phase: 2.0 M KCl, pH 12.1. (b) Initial aqueous phase: 0.2 M KCl, pH 10.5; stripping aqueous phase: 2.0 M KCl.

2. The reversed micellar phase containing the solubilized lysozyme was separated. (The extraction fraction of lysozyme after the forward extraction was kept over 95% within a wide pH range from 4 to 11 for the AOT/kerosene system and from 6 to 11 for the AOT/silicone oil system. No precipitate was formed in the AOT/silicone oil system above pH 6. On the other hand, at ca. pH 12, only ca. 35% lysozyme was extracted into both AOT/kerosene and AOT/silicone oil reversed micellar systems.)
3. The protein-containing reversed micellar phase was contacted with the 2.0 M KCl stripping aqueous phase at pH 11.5.

The value of E_b increased with the increase in the pH value of the initial aqueous phase. It was considered that the pH in the water pools of the reversed micelles after extraction depended on the initial aqueous phase pH. The higher the pH value of the initial aqueous phase, the higher the pH value in the water pools. It was indicated that lysozyme transferred easily from the water pools to the stripping aqueous phase when the pH difference between the extraction and stripping aqueous phases was small. It should be noted that at a pH value higher than the pI of lysozyme, the fraction of forward extraction was low,

as mentioned above, but the reversed micellar phase after extraction was well stripped as shown in Fig. 4(a).

On the other hand, Fig. 4(b) shows the dependence of E_b on the pH value of the stripping aqueous phase. Here, the lysozyme aqueous phase of 0.2 M KCl (pH 10.5) was used to produce the protein-containing reversed micellar organic phase at 50 mM AOT concentration. It was found that the value of E_b increased with an increase in pH of the stripping aqueous phase. At ca. pH 12, E_b reached its maximum value.

The relative specific activity (8) after backward extraction of the extracted lysozyme was measured using the method described by Imoto and Yagishita (7). It was found that the AOT/kerosene system maintained a relative specific activity of 83.5% of the native lysozyme, while the AOT/silicone oil system kept ca. 90.5% activity after backward extraction.

From the results demonstrated above and after consideration of the relative cheapness and safety of the two organic solvents, it is suggested that AOT/kerosene and AOT/silicone oil reversed micellar systems can be used for large-scale protein extraction.

CONCLUSION

The formation of AOT/kerosene and AOT/silicone oil reversed micelles was confirmed by measurements of the interfacial tension between two phases and the water content in reversed micelles.

It was found that the forward extraction fraction of lysozyme and cytochrome c increased with an increase in AOT concentration in the organic phase and with a decrease in KCl concentration in the aqueous phase. The fraction of backward extraction of lysozyme increased with an increase in pH value and KCl concentration in the stripping aqueous phase. It was also found that the fraction of backward extraction depended on the pH value of the initial aqueous phase used for forward extraction.

As kerosene and silicone oil are relatively cheap and/or safe, and have shown satisfactory reversed micellar properties and protein extraction ability using AOT as a surfactant, it is suggested that the AOT/kerosene and AOT/silicone oil systems can be used for the large-scale development of reversed micellar extraction of proteins.

NOMENCLATURE

[AOT]	AOT concentration in the organic phase (mM)
E	fraction of forward extraction of proteins based on the initial aqueous phase concentration (—)

E_b fraction of backward extraction of proteins based on the reversed micellar phase concentration after the forward extraction (—)
$[H_2O]$ water concentration in the organic phase (mM)
W_0 water content, $[H_2O]/[AOT]$ (—)

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