A REVERSED MICELLAR SYSTEM USING MIXED SURFACTANTS OF SODIUM BIS (2-ETHYLHEXYL) SULFOSUCCINATE AND DI(2-ETHYLHEXYL) PHOSPHORIC ACID FOR EXTRACTION OF PROTEINS

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Protein extraction, including solubilization of water, in a reversed micellar solution has been studied using mixed surfactants of sodium bis(2-ethylhexyl) sulfosuccinate (AOT) and di(2-ethylhexyl) phosphoric acid (DEHPA) in isooctane. It appears that the mixed micelles could be formed from AOT and charged species of DEHPA molecules in the organic phase, while uncharged DEHPA does not take part in forming the micelles. The solubilization of water into the mixed micelles depends on pH and salt concentration in the aqueous solution as well as composition and concentration of surfactants. Hemoglobin having relatively large molecular weight could be successfully extracted by the mixed micelles. Furthermore, it was found that the addition of *n*-hexanol in the micellar solution is useful for stripping of proteins from the micelles into dilute alkaline solution.

Introduction

Reversed micellar solutions have been studied extensively with a view to utilization as extraction media for proteins and enzymes^{4,8,12}. Sodium bis(2-ethylhexyl) sulfosuccinate (AOT)/hydrocarbon is most commonly used as a micellar system for protein separation. AOT is a surfactant forming stable reverse micelles in apolar solvent and the properties of its aggregate have been examined in detail. However, the reverse micelle has a major drawback, namely it is difficult to extract proteins having molecular weights larger than about 30,000. Hence, in practical situations, it is crucial to develop reversed micellar systems for the extraction of proteins having larger molecular weights.

Some studies have been published on the improvement of solubilization of proteins in the reversed micellar phase. Goto *et al.*⁵) developed a new reverse micellar system using dioleoyl phosphoric acid (DOLPA) which could extract large proteins such as hemoglobin (molecular weight = 65,000). Ayala *et al.*²) used polyoxyethylene sorbitan trioleate (Tween 85) of nonionic surfactant, and showed that since the interaction between surfactant and protein is so weak, the protein keeps its native activity. Most recently, the application of a synergistic effect using a secondary surfactant for protein extraction has been reported. Kuboi *et al.*¹¹) used the mixed surfactants of AOT and taurodeoxycholic acid to extract lipase, and found

distinct advantages over the AOT single micelles in terms of activity yield and selectivity. Goto *et al.*⁶) have also reported that the extraction of α -chymotrypsin by the mixed micelles of AOT and DOLPA was enhanced up to 90%, though the extractions for the single micellar systems were 50 and 70%, respectively.

In the present study, a mixed surfactant solution of AOT and di(2-ethylhexyl) phosphoric acid(DEHPA), which are both commercial reagents, in isooctane was used as a novel reversed micellar system for protein extraction. Characteristics of water solubilization and protein extraction in the mixed micelles have been examined for comparatively large proteins. Furthermore, the enhancement of the protein recovery from the micelles was studied as an effect of the addition of alcohols in the organic phase.

1. Experimental

Sodium bis(2-ethylhexyl) sulfosuccinate(AOT), obtained from Nacalai Tesque Inc., was used without further purification. Di(2-ethylhexyl) phosphoric acid (DEHPA), a commercial reagent (DP-8R) supplied from Daihachi Chemical Ind. Co.(Japan), was purified by washing alternately with diluted hydrochloric acid and distilled water three times. An organic solution was prepared by dissolving AOT and DEHPA as the surfactants in GR grade 2,2,4-trimethylpentane(isooctane), selecting an appropriate surfactant composition as defined by

$$X_s = [DEHPA] / C_s \quad (1)$$

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**Extraction ratio by a spent micellar solution was defined as the ratio of amount of protein extracted into the spent organic phase to that of the aqueous feed.

Fig. 1. Effect of surfactant composition on water content of AOT-DEHPA mixed reversed micellar solution. $C_s = 0.05\text{M}$, $[\text{KCl}] = 0.2\text{M}$; pH = 4-4.5 (○), 8-8.5 (△) —: calc.line

where C_s is the total concentration of the surfactant, $[\text{AOT}] + [\text{DEHPA}]$.

An aqueous solution with or without protein was adjusted to the desired pH and ionic strength by adding Kolthoff buffer solution (ionic strength: 0.05) and KCl, respectively. Proteins used in this study were lysozyme (molecular weight: $M_p = 14,300$, isoelectric point: $pI = 11$) from chicken egg white, myoglobin ($M_p = 17,800$, $pI = 8.1-8.2$) from horse skeletal muscle, and hemoglobin ($M_p = 64,800$, $pI = 6.8-7.0$) from bovine blood.

Equal volumes (10cm^3) of the organic and aqueous solutions were placed in a flask and shaken in a water bath at 298K . After reaching equilibrium, the two phases were separated on a centrifuge. In some experiments, the back extraction was performed by mixing a protein-loaded organic phase with a fresh salt aqueous solution. Protein content of each phase was determined by spectrophotometry at 280nm .

Extraction ratio, E , was defined as the ratio of amount of protein extracted into the organic phase to that of the initial aqueous feed. Recovery ratio, R , was defined as the ratio of amount of protein recovered into the aqueous phase to that of protein loaded in the organic phase.

Water content of the organic phase was determined by Karl-Fischer titration and the molar ratio of the water to the surfactant, W_o , was then defined by

$$W_o = [H_2O] / C_s \quad (2)$$

2. Results and Discussion

2.1 Water solubilization

Solubilization of water in the AOT-DEHPA mixed

Fig. 2. pH dependence of water content of mixed micellar solution. $C_s = 0.05\text{M}$, $[\text{KCl}] = 0.2\text{M}$; $X_s = 0.4$ (○), 0.6 (△) —: calc.line

reverse micelle was examined in the absence of protein.

Figure 1 shows the effect of surfactant composition, X_s , on the water content in the organic phase, where the value of X_s is zero for the AOT single system and one for the DEHPA single system. With increasing X_s , the water content decreases linearly for the low solution pHs (4-4.5), whereas it increases considerably for high solution pHs (8-8.5). However, a dramatic decrease in W_o was observed at the equimolar concentration of AOT and DEHPA ($X_s = 0.5$). Beyond this point, a third middle phase containing large amounts of water was formed between the aqueous and organic phases. It is to be noted that the greater part of the surfactants added are present in the middle phase and little water is contained in the upper organic phase.

Figure 2 shows the effect of aqueous solution pH on the equilibrium water content at two X_s values. For the mixed micelles at an X_s value of 0.4, a remarkable change in the water content is observed between pH 5-7. Such pH dependence can be interpreted in terms of dissociation of DEHPA. With increasing aqueous solution pH, the DEHPA molecules start to dissociate. Consequently, a mixed reverse micelle might be formed from both AOT and dissociated DEHPA.

At an X_s value of 0.6, although the water content of the micellar solution also increases with the pH value, a remarkable decrease in W_o is observed at pH 6 (see Fig. 2). A middle phase was also observed as mentioned above. Assuming that the pK_a value of DEHPA is 5.7 (estimated from the pH dependence at $X_s = 0.4$), the concentration of anionic species of DEHPA at pH 6 was found to equal that of AOT. However, a middle phase is likely to form when the concentration of DEHPA dissociated in the organic phase is higher than that of AOT. Shioi *et al.*¹³⁾ have reported that for the sodium salt of a DEHPA/water/*n*-heptane/sodium chloride system, the middle phase is formed over a wide range of salt concentrations.

Figure 3 shows the effect of total surfactant concentration, C_s , on the water content at different X_s values for

Fig. 3. Effect of total surfactant concentration on water content of mixed micellar solution. pH = 8 - 8.5, [KCl]=0.2M; $X_s = 0$ (○), 0.20 (△), 0.33 (□), 0.43 (▽) —: calc.line

Fig. 4. Effect of salt concentration on water content of mixed micellar solution. $C_s = 0.05\text{M}$, $X_s = 0.4$, pH=8 - 8.5

a solution of pH 8-8.5. In this case, most of the DEHPA molecules are likely to be dissociated, indicating that the W_o value increases with an increase in C_s . This suggests that the sizes and/or shapes of the reverse micelles vary with the C_s value.

Here, we attempted to derive a relationship between the water content in the AOT-DEHPA mixed surfactant system and aqueous solution pH. If the concentration of DEHPA in the dissociated form, $[\text{DEHP}^-]$, is given by

$$[\text{DEHP}^-] = [\text{DEHPA}] / (1 + 10^{pK_a - pH}) \quad (3)$$

and if the concentration is higher than $[\text{AOT}]$, then the water content of the mixed micelles (when $[\text{KCl}] = 0.2\text{M}$) can be represented by

Fig. 5. Effect of surfactant composition on extraction ratio of proteins into mixed micelles. $C_{w,0} = 0.5\text{kg/m}^3$, $C_s = 0.05\text{M}$, pH = 8, $[\text{KCl}] = 0.2\text{M}$; lysozyme (○), myoglobin (△), hemoglobin (□)

$$[\text{H}_2\text{O}] = 16 ([\text{AOT}] + [\text{DEHP}^-])^{1.15} \times \exp\left(\frac{3.1 [\text{DEHP}^-]}{[\text{AOT}] + [\text{DEHP}^-]}\right) \quad (4)$$

In Figs.1-3, the calculated values are represented by solid lines and are in a reasonable agreement with the observed values.

The effect of KCl concentration in the aqueous phase on the water content is shown in **Fig.4**, wherein W_o decreases with an increase in $[\text{KCl}]$. Such behavior could be responsible for the shrinking in micelle size due to lowering of repulsive interactions between the surfactant head groups, as pointed out in a previous study⁹⁾. However, formation of the middle phase leads to a very large decrease in the water content of the upper organic phase, as can be seen for $[\text{KCl}] = 0.1\text{M}$ in Fig.4. A similar result has also been reported for an AOT/water/*n*-heptane/NaCl system with $[\text{NaCl}] = 0.04\text{M}$ ⁷⁾.

2.2 Protein extraction

Figure 5 shows typical results of protein extraction using the mixed reversed micellar solutions with a variety of surfactant composition. Lysozyme is completely extracted regardless of X_s , whereas the extraction ratio of the other two proteins, myoglobin and hemoglobin, increases with increasing DEHPA concentration. This suggests that relatively large proteins, which are not entrapped in AOT single micelles, can be extracted by use of the mixed micelles. When $X_s = 0.5$, however, no protein was extracted into the organic phase owing to the appearance of the middle phase mentioned in the previous section. It appears that the proteins were partitioned in the middle phase. Presently, more fundamental research is being undertaken in our laboratory to elucidate the partitioning of proteins into the middle phase.

Further experiments were performed on the effects of total surfactant concentration, C_s , on the extraction of the three proteins at an X_s value of 0.4. Typical results are

Fig. 6. Effect of total surfactant concentration on extraction ratio of proteins. $C_{w,0} = 0.5 \text{ kg/m}^3$, $\text{pH} = 8$, $[\text{KCl}] = 0.2 \text{ M}$; lysozyme ($\bullet$, $\circ$), myoglobin ($\blacktriangle$, $\triangle$), hemoglobin ($\blacksquare$, $\square$); $X_s = 0$ (closed symbols), 0.4 (open symbols)

shown in Fig. 6, wherein the closed symbols represent the AOT single system ($X_s = 0$). With increasing C_s , the extraction ratio in the mixed micellar solution increases for every protein. Such behavior corresponds to the variation of W_o (see Fig. 3). It is to be noted that in the region of lower C_s , lysozyme is more efficiently entrapped in the reverse micelles consisting of solely AOT rather than in the mixed micelles.

Figures 7 (a)-(c) represent the effect of pH on the extraction of the three proteins at $C_s = 0.05 \text{ M}$. As Fig. 7 (a) shows, the effect of KCl concentration on the lysozyme extraction using the mixed micelles is weaker than that with AOT reverse micelles. When $X_s = 0$, the extraction ratio of lysozyme at low KCl concentration changed drastically at a pH value adjacent to its isoelectric point ($pI = 11$). In the case of the mixed reversed micellar solution, however, a large amount of lysozyme was extracted from the aqueous solution of pH above its pI . Such behavior is also observed in the case of myoglobin and hemoglobin (see Fig. 7 (b), (c)) which may be explained by the attractive interaction between head groups of the anionic surfactants and positive charges localized on the surface of the protein. The optimal pH value for protein extraction by AOT-DEHPA mixed micelles is in the range of 8-10 for myoglobin and 6-8 for hemoglobin. It should be noted that 100% of myoglobin and 80% of hemoglobin could be extracted in contrast to the maximum values of 30 and 5%, respectively, using AOT single micelles.

In the lower pH region, a reduction in the extraction efficiency is found for the three proteins used in the present study. This may be due not only to the formation of aggregates from the proteins and surfactant molecules, as reported in the AOT single system⁹⁾, but also to a decrease in the water content of the organic phase as shown in Fig. 2. However, the experimental results for myoglobin and

Fig. 7. pH dependence of extraction ratio of proteins. (a) lysozyme, (b) myoglobin, (c) hemoglobin. $C_{w,0} = 0.5 \text{ kg/m}^3$, $C_s = 0.05 \text{ M}$; $X_s = 0$ (closed symbols), 0.4 (open symbols); $[\text{KCl}] = 0.2 \text{ M}$ ($\bullet$, $\circ$), 1.0 M ($\blacktriangle$, $\triangle$)

Table 1. Effects of pH and KCl concentration of strip liquor on protein recovery ratio

[KCl] [M] pH	0.2 10.4	0.2 11.8	0.2 12.6	0.5 10.4	1.0 10.4
Myoglobin	0.170	0.713	0.722	0.172	0.135
Hemoglobin	0.267	0.777	0.943	0.261	0.251

Organic phase : $C_s = 0.05\text{M}$, $X_s = 0.4$
Aqueous feed : $C_{w,0} = 0.5\text{kg/m}^3$, $[\text{KCl}] = 0.2\text{M}$,
pH = 8.4(for myoglobin), 6.3(for hemoglobin)

Table 2. Protein recovery ratio with addition of alcohols

Alcohol	Ethanol	<i>n</i> -Butanol	<i>n</i> -Hexanol	<i>n</i> -Octanol
Myoglobin	0.332	0.465	0.815	0.797
Hemoglobin	0.307	0.846	0.911	0.933

Organic phase : $C_s = 0.05\text{M}$, $X_s = 0.4$
Aqueous feed : $C_{w,0} = 0.5\text{kg/m}^3$, $[\text{KCl}] = 0.2\text{M}$,
pH = 8.4(for myoglobin), 6.3(for hemoglobin)
Strip liquor : $[\text{KCl}] = 0.2\text{M}$, pH = 10.4,
 $C_a = 2\text{vol}\%$ (based on whole volume)

hemoglobin can not be explained well by the lowering of W_o . The reason why extraction ratios in the mixed surfactant system were significantly higher than in the single AOT system is unknown. As discussed in the previous section, DEHPA does not dissociate for an aqueous solution at pH 4-4.5. Thus, the molecules have little effect on micelle formation as well as on electrostatic interaction. Nevertheless, it seems that DEHPA takes part in extracting proteins even at low pH.

2.3 Protein recovery

Protein entrapped in reverse micelles can be recovered by bringing the organic phase into contact with an aqueous strip liquor having a pH higher than the *pI* of the protein. In the present study, the recovery of myoglobin and hemoglobin from the mixed micellar solutions was examined by use of alkaline solution. Typical results are given in **Table 1**. Recovery ratios for the two proteins are found to increase with increasing pH value of the strip liquor. This can be interpreted in terms of the repulsive interaction between the protein having negative net charges and the surfactant head groups with aqueous pH. However, the protein recovery ratio was independent of the KCl concentration, unlike that of AOT single micelle system¹⁰.

Although the protein release from reverse micelles can be achieved with a relatively high efficiency by bringing the organic phase into contact with such alkaline solutions, the denaturation of these proteins will occur in higher pH solutions. Thus, back transfer must be carried out under gentle pH conditions.

With this view, the effect of addition of alcohols to the mixed micellar solution was studied and the results are shown in **Table 2**. The recovery ratios of the proteins, *R*, increase along with chain length of the alcohols, reaching a constant value for *n*-hexanol. For *n*-hexanol, both myoglobin and hemoglobin were recovered with high yields, 80 and 90%, respectively. It has been reported that

Fig. 8. Effect of *n*-hexanol content on water content of mixed micellar solution. $C_s = 0.05\text{M}$, $X_s = 0.4$, pH = 8.5, $[\text{KCl}] = 0.2\text{M}$

Fig. 9. Effect of *n*-hexanol content on extraction and recovery of proteins. $C_{w,0} = 0.5\text{kg/m}^3$, $C_s = 0.05\text{M}$, $X_s = 0.4$, $[\text{KCl}] = 0.2\text{M}$. Extraction ratio: myoglobin (○) at pH 8.4, hemoglobin (△) at pH 6.3; recovery ratio: myoglobin (●) at pH 10.4, hemoglobin (▲) at pH 10.4. ▽: Extraction ratio of hemoglobin by micellar solution after using for back extraction

the addition of alcohols such as ethanol¹⁾ and isopropanol³⁾ in a micellar solution is available for protein recovery. However, such short chain alcohols will cause contamination of the strip liquor as they are highly miscible with water. Considering efficiency of the protein back-extraction and solubility of alcohols in the aqueous phase, *n*-hexanol or *n*-octanol are rather suitable as additives for reforming the structure of reverse micelles.

Figure 8 shows a plot of the water content of the mixed micellar solution against the amount of *n*-hexanol added. The W_o value decreases sharply with increasing alcohol content, C_a . This suggests that alcohol addition

causes a change in the size or shape of the mixed micelles and thus leads to enhancement of protein release.

If the spent organic phase is recycled in the extraction process, the reduction in W_o from alcohol addition will result in a decrease in the forward extraction ratio as an unfavorable effect. Thus, both the extraction and stripping of myoglobin and hemoglobin were examined in the presence of *n*-hexanol for the mixed reverse micellar system.

Figure 9 shows plots of E (solid lines) and R (dashed lines) versus C_a . The extraction ratio of the two proteins decreases with an increase in the alcohol content as a result of the lowering of the water content. As for the yield of protein recovered from the stripping subsequent to the extraction, the maximum is 72% for myoglobin and 60% for hemoglobin at 1 vol% of *n*-hexanol.

Furthermore, Fig.9 shows the extraction ratio* of hemoglobin using the spent micellar solution that has been used for extraction and stripping operations. It was found that there is no appreciable difference between the fresh and spent micellar solutions in the presence of *n*-hexanol. This suggests that the addition of higher alcohols to the mixed micellar solutions is useful for protein extraction, though it remains a problem with regards to denaturation.

Conclusion

It was found that a mixture of surfactants, AOT and DEHPA, in isooctane could form mixed reverse micelles over a range of surfactant ratios. The characteristics of water solubilization and protein extraction by AOT-DEHPA mixed micelles were examined by bringing the organic into contact with aqueous solutions. The amount of water solubilized into the mixed micelles can be controlled by pH and KCl concentration in the aqueous phase as well as by the composition and total concentration of these surfactants in the organic phase. At a high concentration of charged species of DEHPA and a low salt concentration, however, a middle phase was formed between the aqueous and organic phases.

The mixed micelles could effectively extract proteins having relatively large molecular weights, such as hemo-

globin. Moreover, it was found that the addition of alcohols into the organic phase is useful for the stripping of proteins from the mixed micelles into alkaline recovering solution under conditions of gentle pH. It is illustrated that both the extraction and recovery of hemoglobin and myoglobin can be successfully achieved by adding *n*-hexanol to the mixed micellar solution.

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Nomenclature

C_a	= alcohol concentration	[vol%]
C_s	= total concentration of surfactant	[kmol/m ³]
C_w	= protein concentration in aqueous phase	[kg/m ³]
E	= extraction ratio of protein	[-]
R	= recovery ratio of protein	[-]
W_o	= molar ratio of water to surfactant	[-]
X_s	= molar fraction of DEHPA in surfactant	[-]
[]	= molar concentration in bracket	[kmol/m ³]

Literature Cited

- 1) Aires-Barros, M.R. and J. M. S. Cabral: *Biotechnol. Bioeng.*, **38**, 1302-1307 (1991).
- 2) Ayala, G.A., S. Kamat, E. J. Beckman and A. J. Russell: *Biotechnol. Bioeng.*, **39**, 806-814 (1992).
- 3) Carlson, A. and R. Nagarajan: *Biotechnol. Prog.*, **8**, 85-90 (1992).
- 4) Dekker, M., R.Hilhorst and C. Laane: *Anal.Biochem.*, **178**, 217-226 (1989).
- 5) Goto, M., K.Kondo and F. Nakashio: *J. Chem. Eng.Japan*, **23**, 513-515 (1990).
- 6) Goto, M., K. Kondo and F. Nakashio: "Solvent Extraction 1990," Part B, ed. by T. Sekine, p.1845-1850, Elsevier, Amsterdam (1992).
- 7) Harada, M., N. Shinbara, M. Adachi and Y. Miyake: *J.Chem. Eng. Japan*, **23**, 50-57 (1990).
- 8) Hatton, T.A.: "Surfactant-Based Separations", ed. by J. F. Scamehorn and J. H. Harwell, p.55-90, Marcell Dekker, New York, (1989).
- 9) Kinugasa, T., S. Tanahashi and H. Takeuchi: *Ind. Eng. Chem. Res.*, **30**, 2470-2476 (1991).
- 10) Kinugasa, T., K. Watanabe and H. Takeuchi: *Ind. Eng. Chem. Res.*, **31**, 1827-1829 (1992).
- 11) Kuboi, R., Y. Yamada, Y. Mori and I. Komasa: *Kagaku Kogaku Ronbunshu*, **17**, 607-613 (1991).
- 12) Luisi, P. L., M. Giomini, M. P. Pileni and B. H. Robinson: *Biochim. Biophys. Acta*, **947**, 209-246 (1988).
- 13) Shioi, A., M. Harada and K. Matsumoto: *J. Phys.Chem.*, **95**, 7495-7502 (1991).