# Effect of Diethanolamine on The Extraction Properties of Reverse Micelles with Sodium Bis(2-ethylhexyl) Sulfosuccinate

*Solvent Extraction Research and Development, Japan*, **Vol. 23, No 1, 75 – 85 (2016)**

# Effect of Diethanolamine on The Extraction Properties of Reverse Micelles with Sodium Bis(2-ethylhexyl) Sulfosuccinate

Koichiro SHIOMORI[1]*, Shinsuke SHIIBA[1], Takashi SANA[1]and Shiro KIYOYAMA[2]
[1] Department of Applied Chemistry, University of Miyazaki, Miyazaki 889-2192, Japan
[2]Department of Chemical Science and Engineering, Miyakonojo National College of Technology, Miyakonojo 885-8567, Japan
(Received February 12, 2015; Accepted April 20, 2015)

The effect of the addition of diethanolamine (DEA) on the extraction of water and bovine serum albumin (BSA) with the reverse micellar system, sodium bis(2-ethylhexyl)sulfosuccinate (AOT), was investigated using the two phase partition method. The addition of DEA in the aqueous phase affected the extraction of water in the organic reverse micellar phase which increased significantly with a decrease in pH reaching a constant value at pH values lower than 8. This behavior is in good agreement with the extraction behavior of DEA into the organic phase under changing pH. Furthermore, the increase in the water extracted in the organic phase was proportional to the DEA concentration extracted in the organic phase. The increase in water extraction with DEA can be explained by the pH change extraction model taking into consideration DEA dissociation equilibria and the extraction of DEA plus water by the formation of an ion-pair between the DEA ammonium ion and the anionic AOT molecule. The extraction of BSA is enhanced by the addition of DEA at low concentration. With an increase in the quantity of added DEA, the pH range for BSA extraction is extended to a higher pH range. BSA extraction increased with an increase in the DEA concentration over a low concentration range, but decreased with concentration over the high concentration range. The back-extraction of BSA was achieved at approximately 90% by the addition of a high concentration of DEA. The extraction and back-extraction of BSA can be controlled by the concentration of DEA.

## 1. Introduction

Reverse micelles are self-aggregates of surfactant molecules in apolar organic solvents containing micro droplets of water of nano meter size surrounded by the hydrophilic head group of a surfactant [1]. These micro water droplets can dissolve proteins and enzymes without denaturation and deactivation. Hence, reverse micellar solutions have been extensively investigated as media for the extraction of proteins and enzymes from aqueous solution [2-15]. The extraction of proteins is controlled by the interaction between reverse micelles and proteins, such as electrostatic interaction between the ionic surfactant and the charged surface of the protein, hydrophobic interaction between the interface of the reverse micelles and the hydrophobic part of the proteins, and the steric exclusion effect from the micelles [16,17]. Most previous studies were carried out in a system using sodium bis (2-ethylhexyl) sulfosuccinate (AOT) as the anionic surfactant. Extraction of proteins into AOT reverse micelles is mainly controlled by the pH and salt

concentration in the aqueous phase to control the interactions. Among these interactions, electrostatic interaction can be most effective for protein extraction. However, at the same time, the electrostatic interaction often causes a structural change and inactivation of the proteins [10].

Some studies have been reported on control of the formation of reverse micelles by pH [17-24], pressure [25] and temperature [26] and photo irradiation [27, 28] for the purpose of developing novel and effective extraction processes for proteins. Long chain alkylamines, which are hydrophobic and have low solubility in water, are used to control the formation of the reverse micelles by pH adjustment due to the dissociation of the head group of the amine which forms a cationic ammonium group which interacts with the anionic AOT head group [20-23].

In this study, the effect of water-soluble amines, especially diethanolamine, which is added to the aqueous phase and extracted into the reverse micelles, on the extraction of water and bovine serum albumin (BSA) as a model protein into the AOT reverse micellar phase has been investigated. The percolation phenomena of the AOT reverse micellar solution, which give information about the interface and interaction of the reverse micelles [17], was also examined to determine the effect of the addition of diethanolamine.

## 2. Experimental

### 2.1 Reagents

The surfactant used in constructing the reverse micelles was sodium bis(2-ethylhexyl) sulfosuccinate (AOT) from Nacalai Tesque Co. Diethanolamine (DEA), tris(hydroxymethyl)aminomethane (Tris), 2-amino-2-ethyl-1,3-propanediol (AEPDO), and tris(2-aminoethyl)amine (TAEA), which were used as additives for modification of the reverse micellar system, were obtained from Tokyo Kasei Industry. Bovine serum albumin (BSA, M.W. = 66 kDa, pI = 4.9), which was used as the model protein for the reverse micellar protein extraction, was purchased from Sigma. Other chemicals were obtained from Wako Pure Chemicals.

### 2.2 Extraction of water, diethanolamine and protein

AOT was dissolved in isooctane and the solution was used as the organic phase. A buffer solution containing the water-soluble amines and NaCl was used as the aqueous phase. Acetic acid-sodium acetate (pH 3-6), 3,3-dimethylglutaric acid (DMG)-NaOH (pH 5-8), glycine-NaOH (pH 9-11) and $Na_2HPO_4$-NaOH (pH 11-12) were used as buffers at 50 mM concentration in all experiments. BSA and the water-soluble amines were dissolved in the aqueous phase. Extraction of water and BSA were carried out by the following method. Identical volumes (15 $cm^3$) of the organic and aqueous solutions were placed in a screw capped sample tube. The two phases were dispersed completely by a magnetic stirrer for 30 min. at 298 K. After mixing, the phases were separated by centrifugation at 3,500 rpm for 20 min. Back-extraction of BSA from the micellar phase was carried out by contacting the organic phase containing the protein with a new aqueous phase in the same manner as for the forward extraction.

### 2.3 Measurements

The water concentration in the micellar organic phase was determined by Karl-Fisher titration using a Kyoto Electronics MKS-1s. The concentrations of the protein in the aqueous and organic phases were measured by adsorption at 280 nm with a Hitachi UV 3200.

The concentration of DEA in the aqueous phase was measured using an ion chromatography (HIC-6A system, Shimadzu Co.) with a conductivity detector (CDD-6A, Shimadzu Co.) and a cation ion exchanging column (Shim-pack IC-C3, Shimadzu Co.). The mobile phase of the ion chromatography column consisted of an aqueous solution of 1 mM oxalic acid and 1 mM 2,6-pyridinedicarboxylic acid and flowed at 1.20 ml/min. The column and the detector cell were maintained at 313 K. The detector was operated under the following conditions; Response: slow; Gain; 10 μS/cm; Range: 512.

The conductivity of the reverse micellar solution was measured as a function of the water content with a TOA Electronics Ltd. conductivity meter CM-30S and a platinum electrode in the same manner as previously described [17]. Electrical conductivity measurements were performed by dropwise addition of distilled water to 200 mM AOT/isooctane solution containing DEA at various concentrations at 298 K until the percolation phenomenon occurred. The percolation threshold, i. e. the starting point of the sharp increase in conductivity is measured as $\phi_P$.

The diameter of the reverse micelles was measured using dynamic light scattering method (FPAR-1000, Otsuka Electronics Co.). The reverse micellar solution for the measurement was prepared by dissolving a small amount of distilled water in the 50 mM AOT solution containing DEA at the desired concentration.

## 3. Results and Discussion

### 3.1 Effect of water-soluble amines on the water extraction by AOT reverse micelles

The extraction properties of water in the organic phase of the AOT reverse micellar extraction system were investigated in the presence of various amines in the aqueous phase as shown in Figure 1. When no water-soluble amine was added, the extraction of the water into the AOT reversed micelle is not influenced by the pH value. When DEA or Tris was added to the aqueous phase, the amounts of water extracted into the organic phase increased with a decrease in the pH value, and reached constant values. With a decrease in the pH value, DEA and Tris change into their cationic ammonium ion forms. It is considered that AOT with an anionic head group will interact with its cationic ammonium ion by electrostatic attraction and the extraction of water by AOT reverse micelles increased. This difference in the pH effect on water extraction by the addition of DEA and Tris will be caused by the difference in their $pK_a$ values ($pK_a$ = 8.90 for DEA and 8.30 for Tris). On the other hand, when TAEA was added, the amounts of water extracted into the organic phase decreased sharply with decreasing pH value, and a very low water content resulted. This dependency of TAEA is quite similar to the

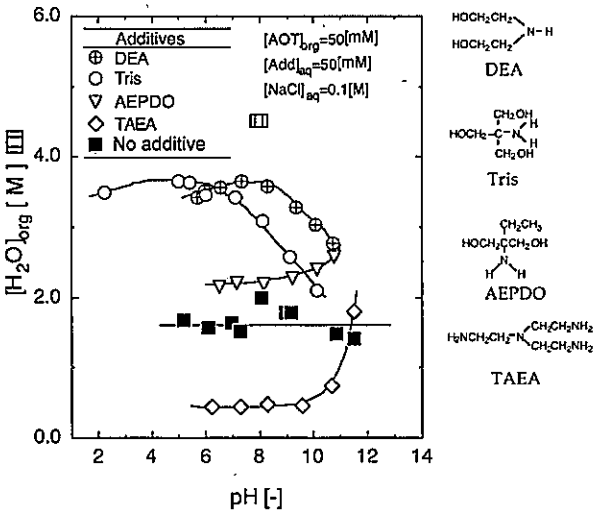


Figure 1. Effect of pH on water extraction by the AOT reverse micellar system with and without the addition of various water-soluble amines.

addition of long chain alkyl amines to the AOT reverse micellar system [22]. The complex between AOT and TAEA formed by electrostatic interaction is hydrophobic because TAEA has no hydroxide group in the molecule and is relatively hydrophobic compared with those of DEA and Tris. AEPDO slightly affected water extraction. The difference in molecular structure between AEPD and Tris is the substitution of one OH group of Tris by a methyl group. It is clear that small differences in the hydrophilic and hydrophobic balances of the water-soluble amines, which act as AOT counter ions, affect the formation and properties of the reverse micelles.

The addition of water-soluble amines in the aqueous phase is expected to control extraction and the physicochemical properties of AOT reverse micelles by electrostatic interaction depending on the hydrophobic-hydrophilic properties and the $pK_a$ values of the additives. In particular DEA shows a significant increase in water extraction at around neutral pH. Hence DEA is considered to be an effective additive for protein extraction. Therefore the effect of DEA on the AOT reverse micellar extraction system was investigated in detail.

**3.2 Effect of DEA on the extraction of water**

Diethanolamine increased water extraction significantly with decreasing pH value. The effect of pH on the extraction of water and DEA carried out at various initial DEA and AOT concentrations is shown in Figures 2 and 3, respectively. The concentrations of water, $[H_2O]_{org}$, and DEA, $[DEA]_{org}$, extracted into the organic phase and also the values of *Wo* ($=[H_2O]_{org}/[AOT]_{org,0}$) increased with a decrease in the pH value,

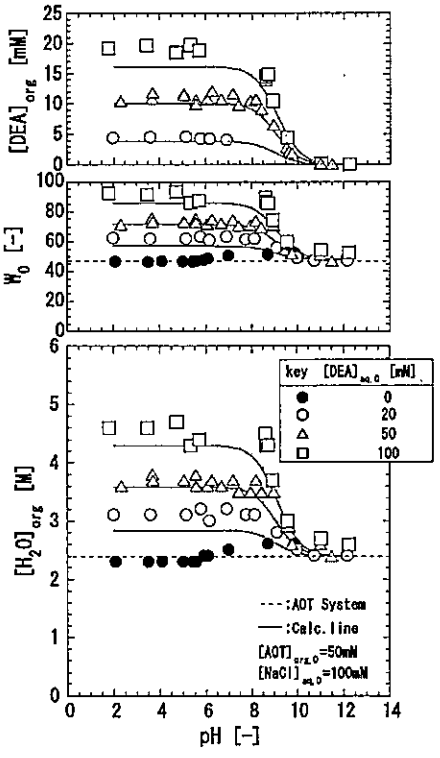


Figure 2. Effect of pH on the extraction of water and DEA in the organic phase and the *Wo* value at various initial DEA concentrations.

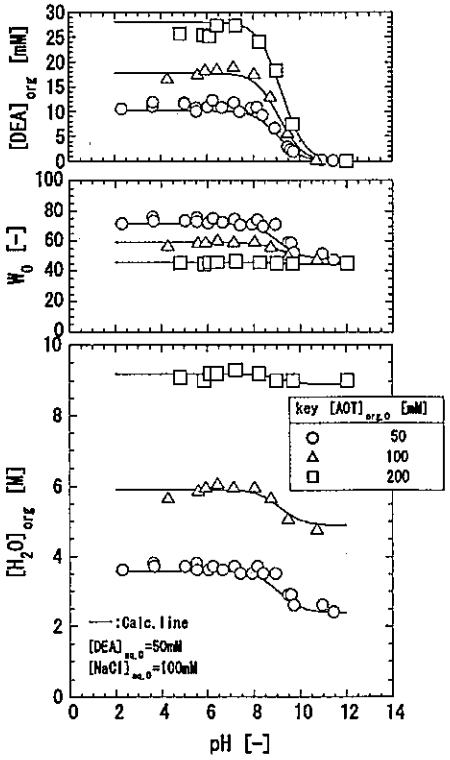


Figure 3. Effect of pH on the extraction of water and DEA in the organic phase and the *Wo* value at various AOT concentrations.

and reached constant values that increased with an increase in the initial DEA concentration in the aqueous phase. These results correspondent well with the formation of the ammonium ion of DEA with changing pH. The cationic ammonium ion of DEA is extracted into the reverse micelles by electrostatic interaction with the anionic sulfonic group of AOT. At high pH where no dissociation of DEA occurred, the *Wo* values are independent of the AOT concentration as shown in Figure 3. This phenomenon is the same as that reported for AOT reversed micelles [16].

The swelling of the reverse micelles and an increasing in water extraction are induced as a result of the extraction of DEA into the reverse micelles. The water increment in the organic phase by the addition of DEA, $\Delta[H_2O]_{org}$, was obtained from the difference of the water concentration between the extraction system with and without DEA and plotted against the concentration of DEA extracted in the organic phase as shown in Figure 4. $\Delta[H_2O]_{org}$ showed a straight line dependency on the AOT concentration. The extraction of DEA causes a linear increase in the amount of water in the reverse micelles. This suggests that DEA induces a structural change in the reverse micelles. The water concentration in the organic phase can be calculated from the concentration of DEA in the organic phase using this relationship.

Based on the above considerations, the model for the extraction of DEA into the reverse micelles is proposed as shown in Figure 5. A further relation is obtained from the dissociation equilibria and the extraction of DEA and the mass balances of DEA and AOT.

The DEA reacts with protons as shown in the following equilibrium reaction.

$$DEA + H^+ \rightleftarrows DEA^+H \qquad : 1/K_a \tag{1}$$

$$1/K_a = [DEA^+H]/([DEA][H^+]) \tag{2}$$

AOT will dissociate completely into its ion form at the interface.

$$AOT \rightarrow Na^+ + OT^- \tag{3}$$

Here, the concentration of $OT^-$ is equal to that of AOT, $[OT^-] = [AOT]$.

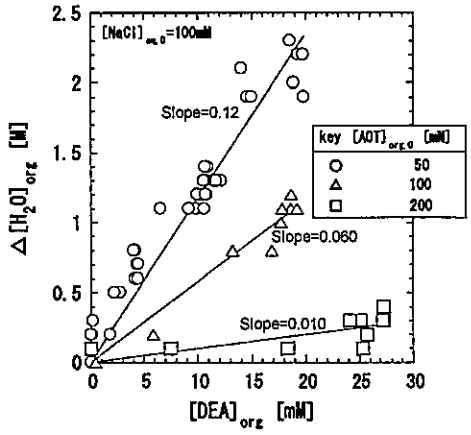


Figure 4. Relationship between the increased concentration of water in the organic phase by DEA addition and the DEA concentration in the organic phase at various AOT concentrations.

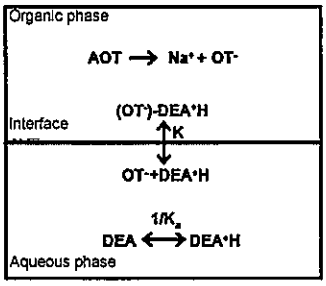


Figure 5. Reaction and extraction model of DEA with AOT.

The ammonium ion of DEA so formed will react with $OT^-$ as follows;

$$OT^- + DEA^+H \rightleftarrows (OT^-) - DEA^+H \qquad : K \tag{4}$$

$$K = [(OT^-) - DEA^+H]/([AOT][DEA^+H]) \tag{5}$$

Here, the concentration of $(OT^-) - DEA^+H$ is equal to that of DEA extracted in the organic phase;

$$[(OT^-) - DEA^+H] = [DEA]_{org} \tag{6}.$$

Mass balances of AOT and DEA are represented by the following equations, respectively.

$$[AOT]_0 = [OT^-] + [(OT^-) - DEA^+H] = [OT^-] + [DEA]_{org} \tag{7}$$

$$[DEA]_0 = [DEA] + [DEA^+H] + [(OT^-) - DEA^+H]$$
$$= [DEA] + [DEA^+H] + [DEA]_{org} \tag{8}$$

The distribution ratio of DEA between the organic and aqueous phases is expressed as the following equation from Eqs. (2) and (5).

$$D = \frac{[DEA]_{org}}{[DEA]_{aq}} = \frac{K[OT^-][DEA^+H]}{[DEA^+H]\left(1+\frac{K_a}{[H^+]}\right)} = \frac{K[OT^-]}{1+\frac{K_a}{[H^+]}} \tag{9}$$

The following equation is obtained from the rearrangement of Eq. (9).

$$D\left(1+\frac{K_a}{[H^+]}\right) = K[OT^-] \tag{10}$$

The concentration of $OT^-$ can obtain from the mass balance of AOT from Eq. (7) as follows.

$$[OT^-] = [AOT]_0 - [DEA]_{org} \tag{11}$$

All experimental results were plotted based on Eq. (10) as shown in Figure 6. All experimental data fall on a straight line passing through the origin. This shows the extraction equilibrium of DEA is expressed by the proposed reaction model. The equilibrium constant for DEA extraction, *K*, was determined to be 5.1 $m^3$/kmol from the value of the slope of the straight line. The calculated concentrations of DEA using the model and the equilibrium constant so determined are shown in Figures 2 and 3 as solid lines. The concentrations of water extracted into the organic phase were also calculated by the proportional relation with the concentrations of DEA extracted in the organic phase in Figure 4, and then the *Wo* values were calculated as shown in Figures 2 and 3. The calculated results closely agree with the experimental results.

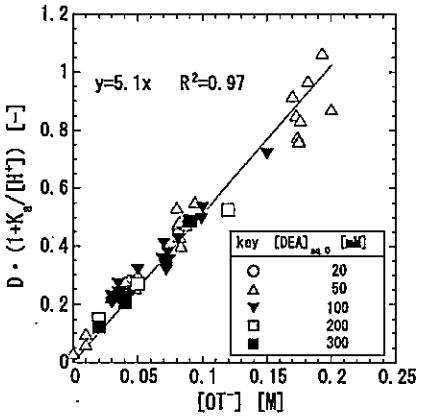


Figure 6. Relationship between $D\cdot(1+(Ka/[H^+])$ and $[OT^-]$.

**3.3 Physicochemical properties of the AOT reverse micelles containing DEA**

**3. 3. 1 Percolation behavior of the AOT reverse micelles containing DEA**

In order to clarify the micellar-micellar interaction of the AOT reversed micelles containing DEA, the percolation behavior of the micellar solution was measured by the electrical conductivity of the solution as a function of water content. The electrical conductivity of the reverse micellar solution at various DEA concentrations plotted against the volume fraction of water in the organic phase, $\phi_{aq}$, is shown in Figure 7a). At low $\phi_{aq}$ values, the conductivities were insensitive to the value of $\phi_{aq}$. However, the conductivities

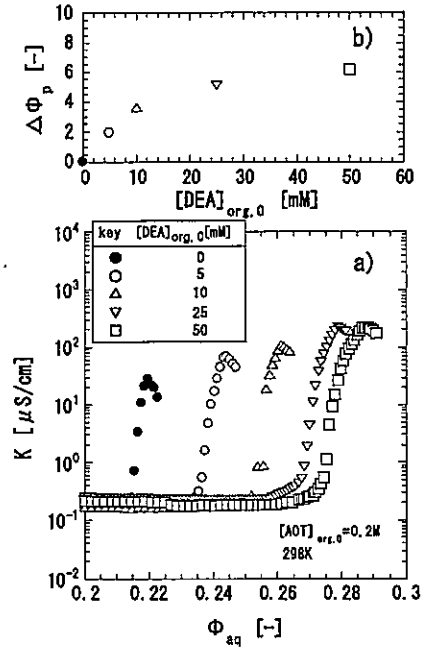


Figure 7. Percolation behavior of the AOT reverse micellar solution containing DEA: a) Variation of the conductivity as a function of the water content, b) Effect of the DEA concentration on the percolation threshold.

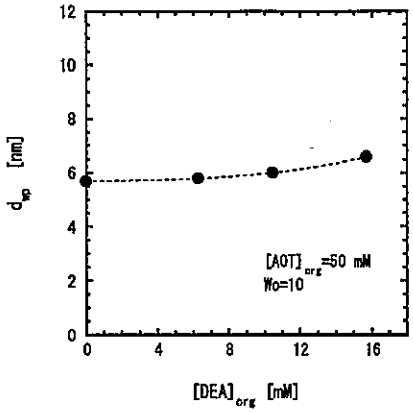


Figure 8. Effect of DEA concentration on the diameter of the AOT reverse micelles.

increased sharply when the $\phi_{aq}$ value exceed respective threshold values, $\phi_P$, indicating percolation phenomena. An increase in DEA caused an increase in the percolation threshold as shown in Figure 7b) and the maximum conductivity as shown in Figure 7a). This result indicates the stabilization of the reverse micelles by the addition of DEA. It is considered that the interaction of the anionic sulfonic group of AOT with cationic $DEA^+H$ will enhance the surface activity of AOT at the interface, thus causing stabilization of the interface and inhibition of the micellar-micellar interaction.

**3. 3. 2 Effect of DEA on the diameter of the AOT reverse micelles containing DEA**

The diameter of the AOT reverse micelles, $d_{wp}$, with and without DEA was measured by the dynamic light scattering method. The effect of DEA concentration in the organic phase on the diameter of reverse micelles is shown in Figure 8. The diameter increased only slightly with an increase in the DEA concentration in this concentration range. The effect of DEA on the size of the AOT reverse micelles was not so large compared to that on the percolation phenomena. Though the extraction of water increased on the addition of DEA, the diameter of the reverse micelles containing DEA was constant. This means that the number of the reverse micelles was increased by the addition of DEA. Because the AOT concentration is constant, the AOT existing at the interface will decrease which would cause an increase in micellar-micellar interaction and the acceleration of percolation phenomena with a decrease in percolation threshold, $\phi_P$. The $\phi_P$ value, however, increased with the DEA concentration and the addition of DEA suppressed the percolation. Hence, it is suggested that AOT interaction with DEA, which will act as an AOT counter ion, strongly stabilizes the interface of the reverse micelles.

### 3. 4 Extraction of BSA

#### 3. 4. 1 Effect of pH on the extraction of BSA

The effect of pH on the extraction of BSA, water and DEA carried out at various initial DEA concentrations is shown in Figure 9. In the absence of DEA, maximum BSA extraction is obtained at around pH 6 and the extraction decreases sharply with any further increase in the pH value. In the presence of DEA, the extraction of BSA increases with the concentration of added DEA and the pH range for BSA extraction is extended to higher pH values. The electrostatic repulsion between the reverse micelles and BSA (pI = 4.9), which induces a decrease in the extraction of BSA, would be reduced by the presence of cationic DEA-ammonium ions at the interface and in the water pool of the reverse micelles. The extraction of DEA was not affected by the extraction of BSA and the *Wo* value was almost constant while the BSA concentration was significantly changed by changing the pH value. These extraction behaviors are considered as follows; there is significantly more DEA and AOT than BSA, and DEA would interact strongly with the anionic AOT head group. Hence, the extraction of BSA did not affect the DEA extraction or the *Wo* value.

#### 3. 4. 2 Effect of the initial BSA concentration on BSA extraction

BSA extraction was carried out at various initial BSA concentrations and a fixed AOT concentration. The BSA concentration extracted in the organic phase and the *Wo* values are plotted against the initial BSA concentration in Figure 10. The BSA concentration extracted in the organic phase increased with the initial

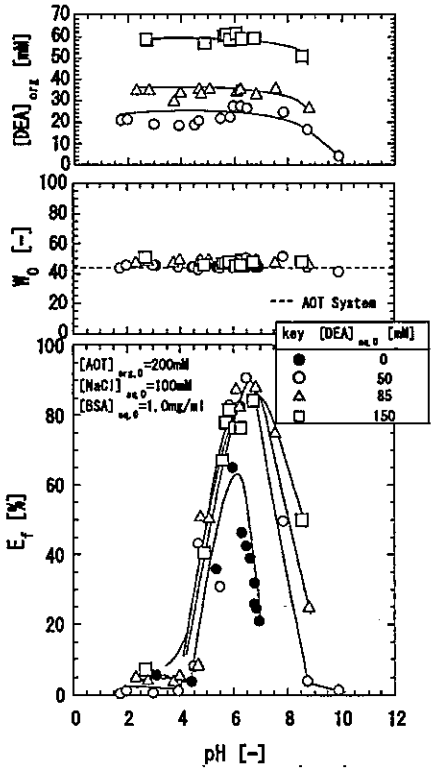


Figure 9. Effect of pH on the extraction of BSA, water and DEA carried out at various initial DEA concentrations.

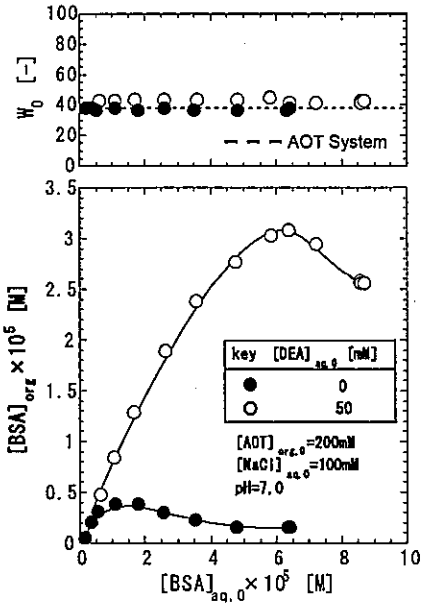


Figure 10. Effect of initial BSA concentration on the extraction of BSA and water carried out without and with DEA.

BSA concentration, reached a maximum value and then decreased. The maximum concentration of BSA in the presence of DEA was about 6 times higher than that in the absence of DEA. The extraction capacity for BSA in the AOT reverse micelles was significantly enhanced by the addition of DEA. This high extraction capacity of BSA is quite similar to the effect of $CaCl_2$ on BSA extraction as previous reported [8]. It has been considered that the $Ca^{2+}$ ion provides a bridging effect mediated by $Ca^{2+}$ between the negatively charged protein surface and the anionic head group of AOT to enhance the BSA extraction. In the case of DEA, DEA would reduce only the repulsion between the negatively charged protein surface and the anionic head group of AOT and enhance the stability of the reverse micelles through electrostatic interaction with the anionic head group of AOT.

**3. 4. 3. Back-extraction of BSA from the reverse micelles by changing the DEA concentration**

BSA extracted in the AOT reverse micelles with DEA could be back-extracted by contact with a new aqueous phase containing 0.5-1.5 M NaCl (data not shown), which is a conventional back-extraction method in the AOT reverse micellar extraction of proteins. The back-extraction of BSA was also possible by contact with a new aqueous phase containing DEA with a concentration higher than 1M. The effect of the DEA concentration on the back-extraction is shown in Figure 11, together with the effect of DEA on the extraction step. In the extraction step, the extraction of BSA increased with the initial concentration of DEA in the aqueous phase, and reached a maximum and then decreased at higher DEA concentrations. The back-extraction of BSA increased with DEA concentrations higher than 1M. The DEA concentration in the organic phase increased linearly with the DEA concentration in the aqueous phase. The *Wo* value increased at the quite low DEA concentrations in the extraction step, and decreased gradually with the concentration at high values range in both the extraction and the back-extraction steps. At high concentration values of DEA, the salting out effect would act on the reverse micelles causing a reduction in the micelle size and thus squeezing out the BSA from the water pool of the reverse micelles [8,16]. The extraction and back-extraction of BSA using the AOT reverse micelles can be achieved by changing the concentration of DEA added in the aqueous phase.

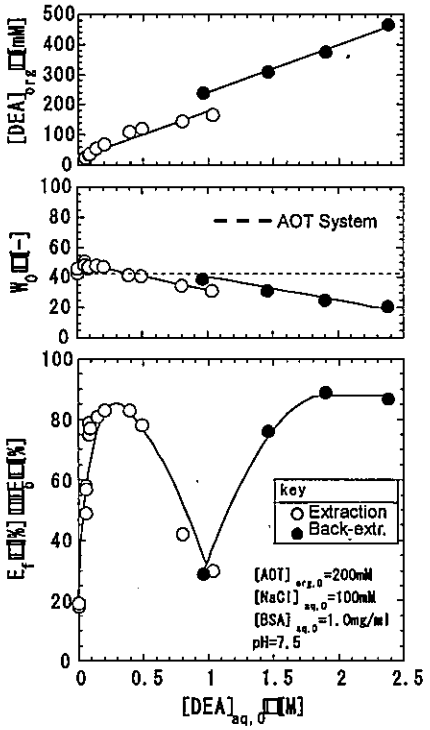


Figure 11. Effect of DEA concentration on the extraction and back-extraction of BSA and water content in the organic phase.

## 4. Conclusion

Water-soluble amines were added to the aqueous phase of the AOT reverse micellar extraction system. DEA and Tris enhanced water extraction at pH values lower than their p*K*a value. The extraction of DEA and water was affected by pH and increased with the initial DEA concentration in the aqueous phase

and the AOT concentration in the organic phase. The extraction of DEA and water into the reverse micelles was explained by the reaction model based on both the formation equilibrium and the extraction of the DEA ammonium ion. The extraction of BSA is enhanced by the addition of DEA at low concentrations. The extraction of BSA increases with the concentration of added DEA and the pH range for the extraction is extended to higher pH values. The extraction capacity of BSA was significantly enhanced by the DEA addition. BSA extracted into the reverse micelles can be back-extracted at high DEA concentration. DEA is concluded to be an effective additive in the aqueous phase to control both the extraction and back-extraction of proteins.

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