Title	Novel Bioseparation Processes for Large Molecular Weight and Oligomeric Proteins by the Control of Their Interactions with Reverse Micelles
Author(s)	塩盛, 弘一郎
Citation	大阪大学, 1999, 博士論文
Version Type	VoR
URL	https://doi.org/10.11501/3155684
rights	
Note	

Novel Bioseparation Processes for Large Molecular Weight and Oligomeric Proteins by the Control of Their Interactions with Reverse Micelles

KOICHIRO SHIOMORI

1999

Novel Bioseparation Processes for Large Molecular Weight and Oligomeric Proteins by the Control of Their Interactions with Reverse Micelles

(逆ミセルとの相互作用制御による高分子量および多量体タンパク質の新規なバイオセパレーションプロセス)

KOICHIRO SHIOMORI

塩盛　弘一郎

1999

CONTENTS Page

GENERAL INTRODUCTION

As a rapid growth of biotechnology, useful and functional bioproducts such as enzymes, proteins, antibiotics and other chemicals can be easily produced on a large mass scale and in short process time. These rapidly expanding knowledge and results have been applied for a variety of fields such as pharmaceutical, medical, agricultural and genetic engineering etc.. In these various fields, biochemical engineering has played an important role in both upstream (bio-production) and downstream (bio-separation) processes. Further, novel bio-processes in production and separation utilizing various biological functions of biomembrane, biocatalysis and microorganism have attracted much attention from a viewpoint of energy saving processes with high selectivity, high efficiency and high functions. Among of these systems reverse micelles is one of the attractive simple model system for biomembrane, since it has been suggested that the reverse micellar structure may exist in the transfer of biomaterials from inner to outer parts of phospholipid bilayer membrane in cell wall (Seelig, 1984).

Reverse micelles are self-organized aggregates of surfactant in organic solvents and provide micro water pools in the aggregates dispersed in the organic phase. Sodium bis(2-ethylhexyl)sulfosuccinate (AOT) forms stable reverse micelles in the wide range of surfactant concentration and has been most frequently used in the field. The AOT reverse micelles have a dynamic structure, that is, collision and fusion between micelles, and redispersion of large micelles fused are always occurring (Fletcher *et al*., 1987). Physico-chemical structure and property of reverse micelles and inner micro water pools have been investigated using several methods such as small angle X-ray

scattering, dynamic light scattering, percolation phenomena and reaction kinetics in reverse micellar systems. The water content, *Wo*, which is defined as a molar ratio of water to the surfactant in the micellar organic phase and controls the diameter of the water pool of the micelles, has often been used to discuss these characteristics of reverse micelles.

Reverse micelles are able to solubilize a variety of biomolecules, such as proteins, DNA, amino acids and cells, into their micro water pools. They have been used as media for extraction of proteins from fermentation broth and culture media, enzymatic reaction in organic phase, and preparation of functional materials. The solubilization of these biomolecules into reverse micelles are carried out by phase transfer, injection and powder methods. The phase transfer method is carried out by contacting the organic phase forming reverse micelles with the bulk aqueous phase, and this is a typical liquid-liquid extraction system, and most frequently used in extraction and separation process of proteins from an aqueous phase. The injection method is carried out in such a way that a small volume of the aqueous solution containing desired materials is injected into the organic phase forming the reverse micelles and solubilized completely these materials, and this is most frequently used for the enzymatic reaction in the organic phase. The powder method is carried out by direct addition and solubilization of the solid materials into the organic phase forming reverse micelles.

The reverse micellar extraction has been applied for various proteins and the effects of operation parameters, such as pH and the concentrations of salts and surfactants, on the extraction have been widely investigated (Leodidis and Hatton, 1989; Leser and Luisi, 1990). The pH and salt concentration in the aqueous phase are dominant factors for the protein extraction and these are most frequently used to control the extraction. The protein-micelle and protein-

interface interactions play an important role in the extraction. The following interactions are considered as controlling factors of the extraction; i.e. electrostatic interaction between electric charge of hydrophilic group of ionic surfactant and that on protein surface, hydrophobic interaction between hydrophobic part of proteins and the micellar interface or hydrophobic chain of surfactant, and steric interaction between proteins as a guest molecule and reverse micelles as a host space. By controlling these interactions, it has become feasible to perform selective separation of proteins using reverse micelles. Separation processes for model solution containing some proteins have been reported (Göklen and Hatton, 1985; Kuboi *et al*., 1990a). Further some semi-pilot-scale separation processes from fermentation broth have been demonstrated (Rahaman *et al.*, 1988). Further a few studies have been also made for the affinity extraction to get a high selectivity for the target protein. (Chen and Jen, 1994; Kelley *et al*., 1993; Yamada *et al*., 1994a)

However, some serious problems in the protein extraction using reverse micelles are still left to be solved, such as low extraction efficiency of large molecular weight and oligomeric proteins (Lesser and Luisi, 1990), difficulty of back-extraction from the micelles (Marcozzi *et al.*, 1991) and especially denaturation and inactivation of proteins during the extraction process(Kuboi *et al.*, 1990a, 1990b). As a result, reverse micellar extraction of those proteins has been little applied for actual protein separation processes both in laboratory and industry. These problems are considered to be caused by strong interaction between proteins and micelles. Effects of the interactions on the protein extraction described above have been discussed for each protein individually. Yamada *et al.* studied systematically the extraction behavior of various proteins at their isoelectric points and found the relationship between extraction efficiency of proteins and their hydrophobic properties using the extraction

model based on steric interaction and collision between proteins and micelles. However, there is still little understanding about the problems in reverse micellar protein extractions caused by their interactions between proteins and reverse micelles.

Since reverse micelles can provide a novel reaction field of enzymes in organic solution, bio-production processes using enzymes solubilized in reverse micelles have been extensively investigated (Khmelnitsky *et al*., 1989; Chen and Chang, 1993; , Han and Rhee, 1989; Holmberg, 1994). The enzyme in reverse micelles has often shown a novel and unique reaction and functions which can not be observed in the usual aqueous environment. The enzyme activity in reverse micelles has been reported to be influenced by several factors, especially the water content, W_O, which controls the diameter of the water pool of the micelles (Khmelnitsky *et al*.,1989, Flecher *et al*., 1985, Han *et al*., 1990, Kuboi *et al*., 1992). Some reaction and kinetic models have been proposed to explain the characteristics of enzymatic reaction in reverse micelles (Tsai and Chiang, 1991, Flecher *et al*., 1985, Prazeres *et al*., 1993, Miyake *et al*., 1993, 1994). The interaction of enzymes, especially interface adsorptive enzymes such as lipase and lipoxygenase, with reverse micellar interface and surfactant is considered to play an important role on its activity and stability. The information on the interactions between enzymes and micelles or the interface will be obtained by the detailed analysis of their activity and stability in reverse micelles. Only the size effect has, however, been mainly discussed.

By increasing temperature or volume fraction of water in the reverse micellar solution, drastic change of solution properties such as electric conductivity and viscosity is observed. This is called percolation phenomena. Electric conductivity measurements have been used to assess reverse micelles formation and to probe the structural changes occurring in such systems (Jada

et al.,1989, 1990; Alexandridis *et al*., 1995). A sharp increase in electrical conductivity of micellar solution well demonstrates the inter-micellar interactions. It is generally accepted that conductivity percolation in reverse micellar solution with a spherical droplet structure is a result of reverse micellar droplet clustering (Alexandridis *et al*., 1995). The cluster formation of micelles increases the electrical conductivity after the percolation threshold indicating the starting point of the reverse micellar droplet clustering. The percolation threshold can be varied by several additives. The solubilization of proteins favors the percolation process with an increase in the conductivity at lower or higher water content and temperature, suggesting stronger or weaker attractive interactions between micelles in the presence of proteins (Huruguen et al.,1991; Holovko and Badiadi, 1993). This change of the interaction between micelles is considered to involve the information on the interaction between micelles and proteins solubilized.

In this study, design and development of novel and effective bioseparation processes using AOT based reverse micellar system have been investigated based on the evaluation and the control of the interaction of proteins with the reverse micellar interface and also AOT molecules, especially electrostatic and hydrophobic interactions. Large molecular weight and oligomeric proteins higher than 30 kDa were selected mainly to clarify the problems in the bioseparation processes using AOT reverse micelles, because large proteins often show the undesirable problems and are not thoroughly investigated in the reverse micellar extraction. The frame work and flow chart of the present study are shown in **Fig. 1**.

In Part I, in order to clarify the problems in protein extraction using AOT, the extraction of various molecular weight proteins were carried out using the AOT reverse micellar system. The structural change of proteins by the

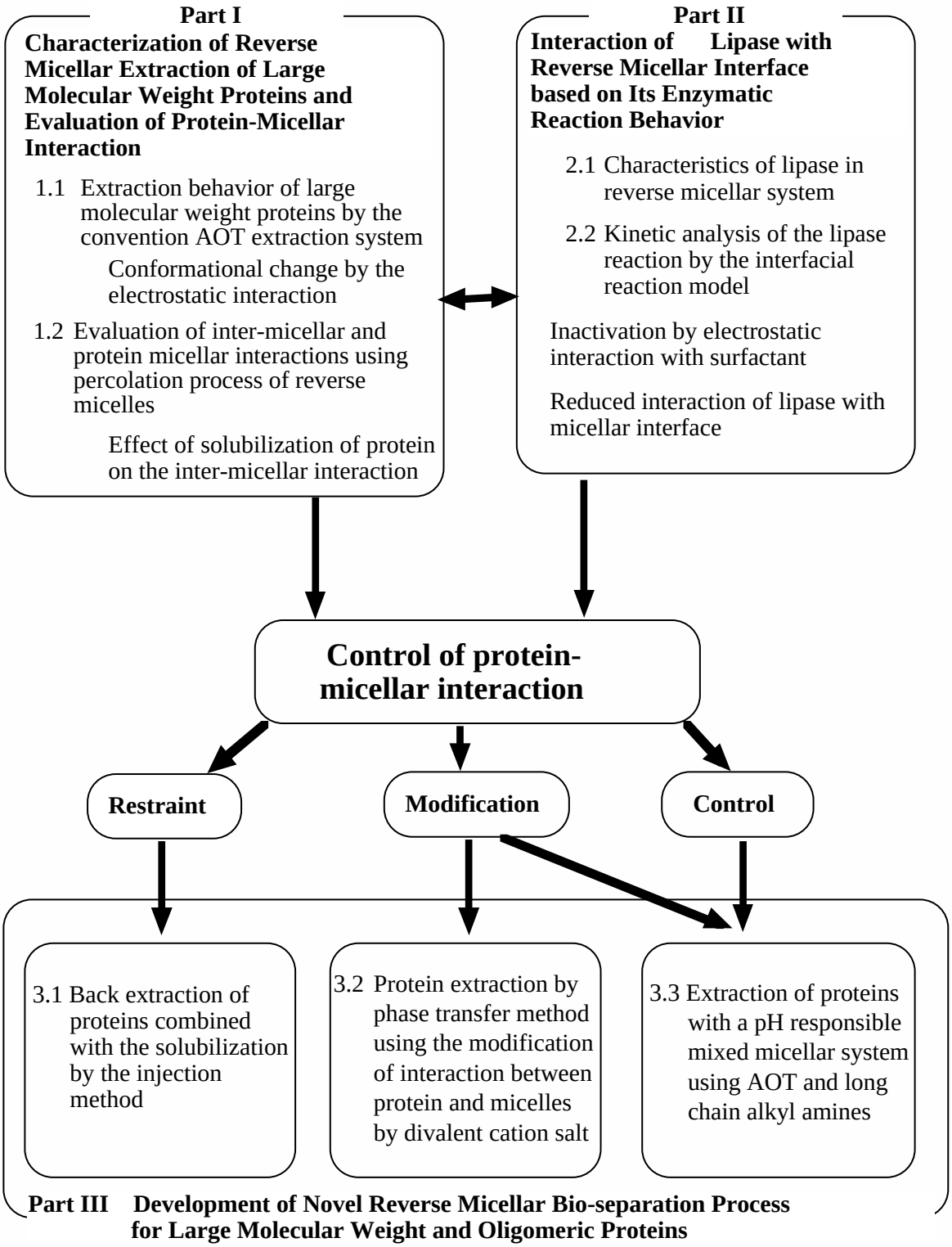


Fig. 1 The frame work and flow chart of the present study

interaction with AOT molecules were evaluated from the CD spectra of the proteins. Furthermore, the evaluation of the inter-micelles and protein-micelles interactions were investigated by the electrical conductivity change caused by the percolation of the reverse micellar solution.

In Part II, the interaction of protein with reverse micellar interface was investigated from the reaction characteristics of interface adsorptive enzyme in the reverse micellar system. Lipase catalyzed hydrolysis reaction in the AOT reverse micellar system was carried out as a model system. The activity and stability of lipase in the reverse micelles was evaluated at various conditions and the hydrolysis rate was analyzed by the kinetic analysis. The results were compared with the results in organic-aqueous two phase and O/W emulsion systems. The characteristics of lipase hydrolysis was discussed from the point of view of the interaction of lipase with the micellar interface.

In Part III, on the bases of the knowledge in Parts I and II, three novel bio-separation processes for large molecular weight proteins and small ones have been proposed using AOT based reverse micellar systems. The processes are based on the restraint, modification, and control of the interaction between proteins and the reverse micelles. Firstly, the back-extraction combined with the solubilization by the injection method was carried out with keeping electrostatic repulsion between proteins and the reverse micelles. Effects of several factors on the back-extraction were investigated. The separation of ß-galactosidase from crude extracts of *E. coli* cells was examined as an application of the present method. Secondly, divalent cations, such as Ca^{2+} or Mg^{2+}, as a salt in the aqueous phase were used to modify the electrostatic interaction between proteins and the micelles. The effect of pH on the extraction of large molecular weight and oligomeric proteins including small ones was investigated and the extraction of BSA was studied in detail as a typical example. Thirdly, mixed

systems of AOT and long chain alkyl amines were investigated to control the electrostatic AOT-amines and AOT-proteins interactions. The extraction and the back-extraction behaviors of proteins by the mixed system were studied.

The results obtained in this work are summarized in General Conclusions. Suggestions for Future Work are described as extension of the present thesis.

Part I

Characterization of Reverse Micellar Extraction of Large Molecular Weight Proteins and Evaluation of Protein-Micellar Interaction

1. INTRODUCTION

Extraction and solubilization of proteins and enzymes into reverse micelles have been widely carried out by the phase transfer and the injection methods (Luisi, 1985; Leodidis and Hatton, 1989). The phase transfer method is a typical liquid-liquid two phase extraction system and is used mainly for the separation and extraction processes of proteins and enzymes. By the injection method, it is possible to solubilize limited amounts but various many kinds of proteins and enzymes into reverse micelles and thus frequently used for the enzymatic reaction in organic phase. (Luisi, 1985; Leodidis and Hatton, 1989).

The interactions of proteins with reverse micelles and also with surfactants play an important role both in the protein extraction and the enzymatic reaction using reverse micelles. These interactions are considered to be electrostatic, hydrophobic and steric interactions. It is, however, not easy to evaluate these interactions systematically, because more than one of these interactions often act in various processes. Furthermore, complicated responses of proteins under the stresses caused by these combined interactions make difficult to understand the mechanisms of the extraction and the enzymology of reverse micellar systems.

The evaluation of the interactions between proteins and reverse micelles is, therefore, necessary to understand the extraction mechanism and the enzymatic reaction of reverse micellar systems. Recently, Yamada *et al.*

systematically investigated and evaluated the effect of the hydrophobic interaction on the extraction of proteins at their isoelectric points (Yamada *et al*.,1994b, Yamada, 1995a). They obtained the relationship between the hydrophobic properties of the proteins and the extraction characteristics. The electrostatic interaction is often utilized in the reverse micellar extraction by ionic surfactants. However, systematic studies are lacking in the evaluation and the control of the electrostatic interaction between proteins and reverse micelles. Many problems in the extraction and the reaction processes using ionic reverse micelles may be mainly caused by the electrostatic interaction at least in the initial stage of the interaction.

In this part, in order to clarify problems and to cultivate the potential in the protein extraction with the AOT reverse micelles, the extraction of various molecular weight proteins, such as ß-galactosidase, catalase, bovine serum albumin (BSA), hemoglobin, α-amylase, lipase, ovalbumin, carbonic anhydrase, α-chymotrypsinogen and lysozyme, was carried out using the conventional AOT extraction system. The results in the extraction were discussed from the viewpoint of electrostatic interaction and the structural change of proteins caused by the interaction with AOT detected by the measurement of CD spectrum. The back-extraction of large molecular weight proteins extracted by the phase transfer method was examined from the viewpoint of these interactions.

Furthermore, the interaction between proteins and reverse micelles was also investigated from the effect of the solubilization of proteins on the interaction between micelles. The effects of solubilization of both proteins and water soluble polymers on the percolation characteristics of reverse micellar systems (giant micelle cluster formation) were examined by the measurement of electric conductivity change of the reverse micellar solution.

2. EXPERIMENTAL

2.1. Materials

Sodium bis(2-ethylhexyl) sulfosuccinate (AOT) was purchased from Nacalai Tesque Co. and Tokyo Kasei Co. and was used without further purification. AOT was dissolved in isooctane as solvent at 200 mM.

The following proteins were used in this study. Lysozyme from egg white, catalase from bovine liver were obtained from Wako Pure Chemical Co. Ltd. Cytochrome-c from bovine heart, α-chymotrypsinogen from bovine pancreas, α-amylase from *Bacillus licheniformis*, ovalbumin from chicken egg white, lipase from *C. cylindracea*, bovine serum albumin and hemoglobin from

Table 1-1 Properties of proteins used.

Protein	M.W. [KDa]	pI [–]	HFS [kJ/mol]	LH [-]
Cytochrome-c	12.4	10.1	-98.4	0.07
Lysozyme (LYS)	14.4	11.1	-39.3	0
α-Chymotrypsinogen (CHY)	25.7	9.7	-85	-
Carbonic anhydrase (CAB)	30	5.9	-118	-
Ovalbumin (OVA)	42.8	4.6	-93.4	0.13
C.C. Lipase (LIP)	53	5.3	4.8	
α-Amylase (AMY)	58.5	6.3		
Hemoglobin (Hb)	65	6.9	-208	1.4
Bovine serum albumin (BSA)	66	4.9	-231	1.8
Catalase (CAT)	240	5.5	-75.7	-
ß-Galactosidase (ß-GAL)	464	5.0	-447	

bovine, ß-galactosidase from *E. coli* were purchased from Sigma Chemical Co. Ltd. All proteins purchased were used without further purification. Properties of proteins used are summarized in **Table 1-1** together with molecular weight (M.W.), isoelectric point (pI), surface hydrophobicity (HFS) and local hydrophobicity (LH) of each protein (Kuboi *et al*., 1991, 1993).

Acetic acid-sodium acetate (pH 3.6-5.6), Tris-HCl (pH 7.2-9) and glycine-NaOH (pH 8.5-11) buffer solutions were used. Concentration of these buffers was 10 mM in all cases. All proteins were dissolved in the buffer solution at desired concentration. Polyethylene glycol (PEG) 6 k, 20 k, 50 k (M. W. = 4 k, 20 k and 60 k Da, respectively) were obtained from Wako. Dextran (Dex) 60 k - 90 k and l00 k- 200 k were also obtained from Wako, and T 500 (M W.= 500 kDa was obtained from Pharmacia. KCl, NaCl, and $CaCl_2$ of guaranteed reagent were used as salt in the aqueous phase.

2.2. Reverse Micellar Extraction of Proteins

The extraction of proteins was carried out by the phase transfer method. Proteins were dissolved at 1 mg/ml in a buffer solution at given pH containing 0.1 M of salt. Same volumes of the solutions of protein and isooctane containing AOT were placed into screw capped tube and then mixed well by a magnetic stirrer for 30 min. After mixing, the solution was centrifuged at 5,000 rpm for 15 min. Both the organic and aqueous phases were collected, respectively, and the protein concentrations in each phase were measured. Back-extraction of proteins was carried out by contacting the reverse micellar solution containing proteins with a new aqueous phase as the same procedure in the extraction.

2.3 Measurements

The concentration of all proteins including hem proteins in the organic

and the aqueous phases was determined from the adsorption at 280 nm. Water content in the micellar organic phase was determined by Karl-Fisher titration.

Structural change of proteins in the aqueous phase was carried out as following method. Proteins were dissolved in a buffer solution containing 1 mM AOT and 0.1 M NaCl. After 30 min from solubilization , CD measurements of the protein were carried out with a JASCO J-700 spectropolarimeter in a range from 250 to 200 nm. The results were expressed as mean residue ellipticity (θ), which was defined as $\theta=100\theta_{obsd}/(lc)$ where θ_{obsd} was the observed ellipticity in degree, c was the concentration of residue in mole per liter, and l was the length of the light path in centimeters. The CD spectra were measured at protein concentration at 0.1 mg/L with a 1 mm-path-cell.

2.4. Conductivity Measurements

The conductivity of reverse micellar solution was measured as a function

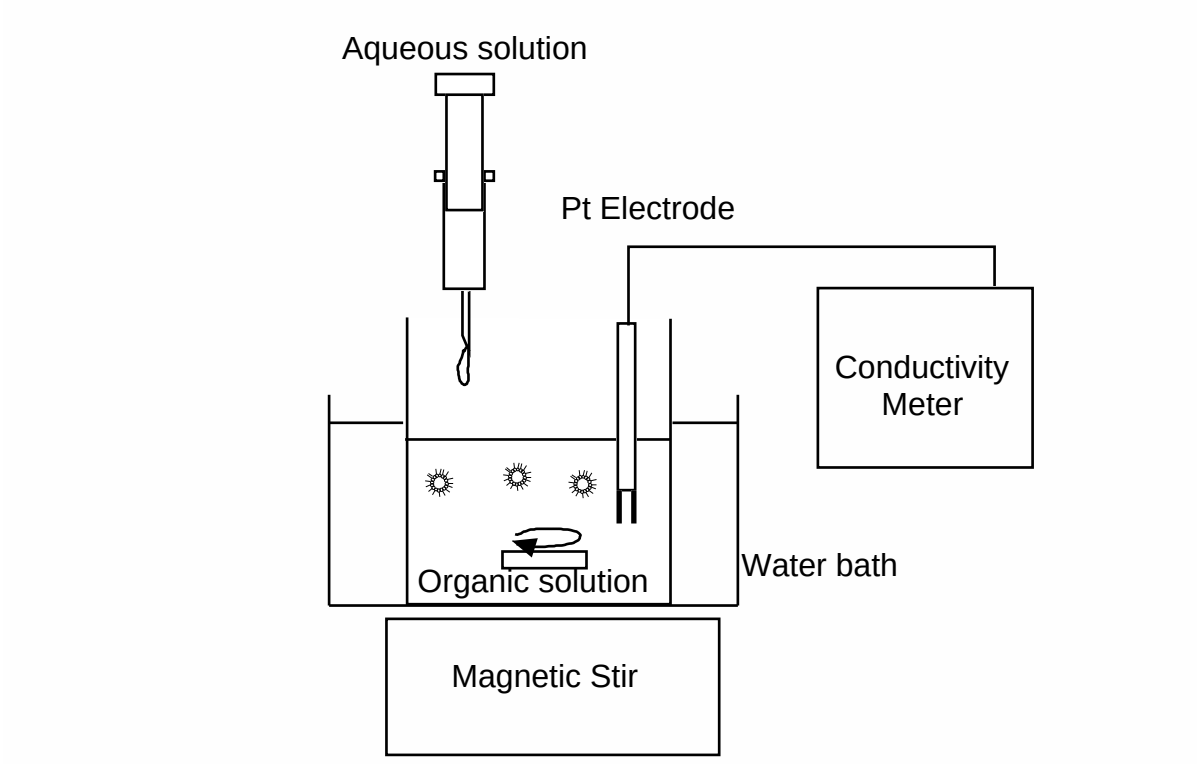


Fig.1.1. Experimental apparatus for percolation measurements

of water content and as a function of temperature at constant water content. The conductivity measurements was carried using a TOA Electronics Ltd. conductivity meter CM-8ET or CM-30S with a platinum electrode. The experimental apparatus is schematically shown in **Fig.1.1**. The electrode was inserted into the test tube containing the reverse micellar solution and the test tube was placed in a thermostated water bath. Electric conductivity measurements were performed by either a dropwise addition of an aqueous phase containing protein to 200 mM AOT /isooctane solution or a gentle increase in temperature (0.5 °C/min) of reverse micellar solution at constant water content until the percolation phenomenon observed. All reverse micellar solution samples were single-phase and optically transparent under the present conditions of the conductivity measurements.

As shown schematically in **Fig. 1.2**, individual water droplets maintain at

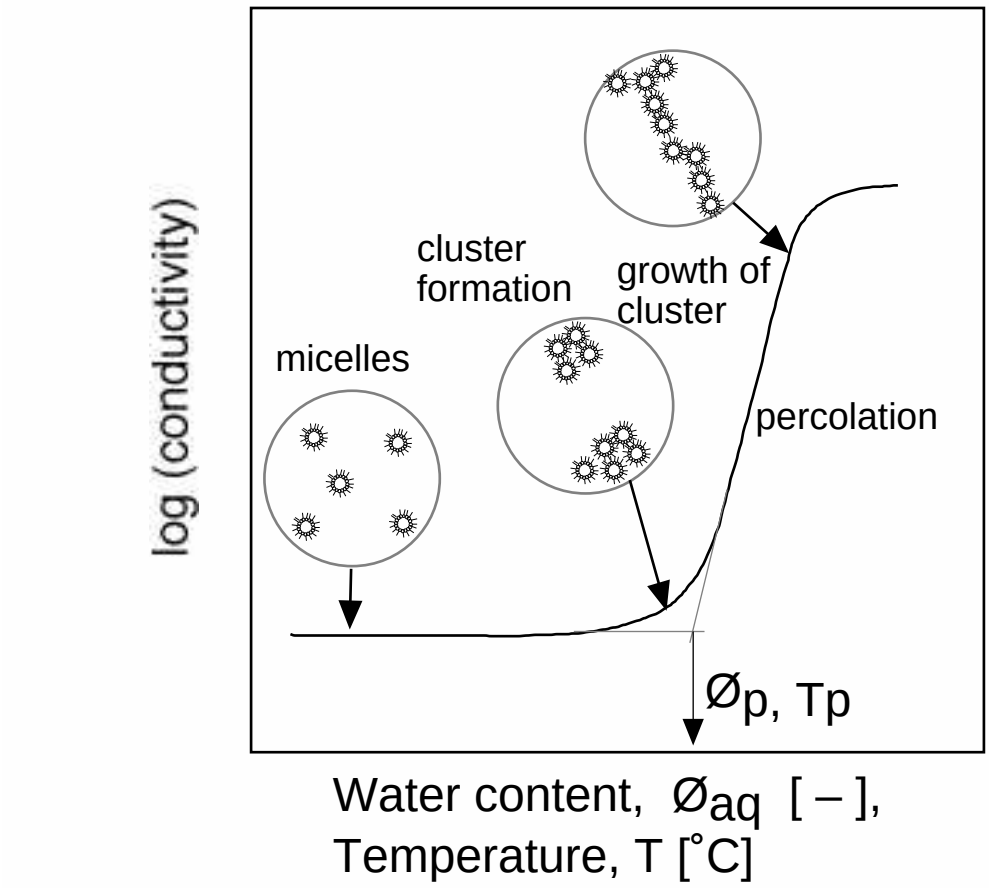


Fig.1.2. Schematic diagrams of percolation of reverse micelles dispersed in organic solution

at low conductivity, while the conductivity increase as droplets start to assemble in clusters. It is generally considered that, during percolation, the water microdroplets come in close contact and charge carriers propagate by jumping between the droplets and/or by exchange of the droplet contents when droplets coalesce and redisperse. The percolation threshold, i. e. the starting point of the sharp increase in conductivity is measured as $Ø_p$. for change of water content and T_p for change of temperature, respectively.

3. RESULTS AND DISCUSSION

3.1 Extraction behavior of large molecular weight proteins

3.1.1 Effect of pH on the extraction

The reverse micellar extraction of proteins was carried out by the phase transfer method at various pH values. An isooctane solution of AOT at 200 mM, as an organic phase, and an aqueous solution of 0.1 M NaCl or KCl which solubilized various proteins, as an aqueous phase, were used as a extraction system in this experiment. Effect of pH values on the extraction of various proteins is shown in **Fig. 1.3**. The extracted fraction of each protein into the micellar organic phase, E_f, and the removal fraction of the protein from the aqueous phase, R_f, is plotted against the equilibrium pH value.

All the used proteins except for hemoglobin and lipase could be extracted into the reverse micellar phase at a certain condition. In the extraction of proteins at pH much lower than each pI, low E_f with high R_f was observed in all cases. In these cases, large amount of aggregates of proteins was observed at the interface between the organic and the aqueous phases after extraction. CAB, ovalbumin, α-amylase and BSA were extracted in the narrow pH range somewhat higher than pI. For further higher pH range, both E_f and R_f decreased

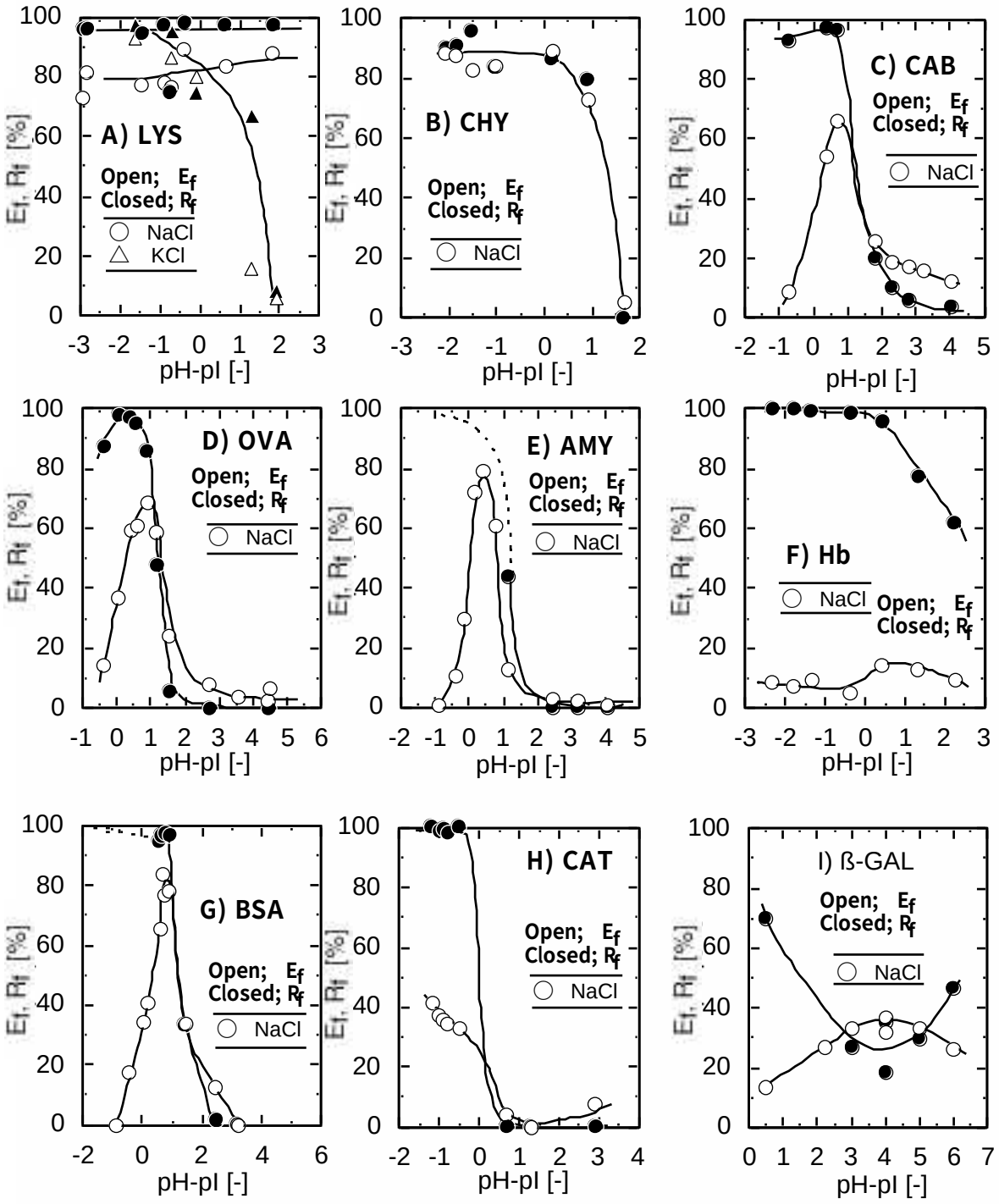


Fig. 1.3 Effect of pH on the extracted fraction and the removal fraction of proteins into the organic phase and from the aqueous phase, respectively.

with increasing pH. In the case of catalase at pH lower than its pI, R_f is higher than E_f, and both E_f and R_f decreased monotonously with an increase in pH. Hemoglobin could not be extracted and formed large amount of aggregates at all pH range examined. However, R_f decreased with an increase in pH higher than the pI of hemoglobin. This shows the interaction between AOT and hemoglobin, which causes aggregation, can be reduced by electrostatic repulsion.

In the case of lipase, which is one of the most useful enzyme in the industrial field, however, both the extraction into the organic micellar phase and the enzyme activity in the micellar phase were not observed. Especially, the extraction at pH lower than its pI resulted in large amount of aggregates after the extraction. Lipase used in this work seems to be easily denatured irreversibly by a little interaction with AOT except for the most stable condition in the micelles, this will be further discussed in the later Part. The reverse micellar extraction using the conventional AOT system by the phase transfer method is, therefore, not applicable for this lipase because of the irreversible interaction between proteins and reverse micelles.

On the other hand, low molecular weight proteins, such as lysozyme and α-chymotrypsinogen, were extracted effectively at pH lower than their pI. Both E_f and R_f decreased with increase in pH higher than the pI. This decrease is understood by the electrostatic repulsion between protein surface and surfactant head group at the micellar interface. In this case, the values of E_f were almost agreed with those of R_f. This means no formation of aggregates of the proteins during the extraction process. In the case of lysozyme using 0.1 M NaCl, E_f was high at even pH higher than its pI. *Wo* was about 34 at 0.1 M NaCl. This is considered that electrostatic repulsion between lysozyme and the micellar inner wall dose not act effectively because the distance between protein surface and the inner wall keep large enough due to large micellar size. The effect of the size

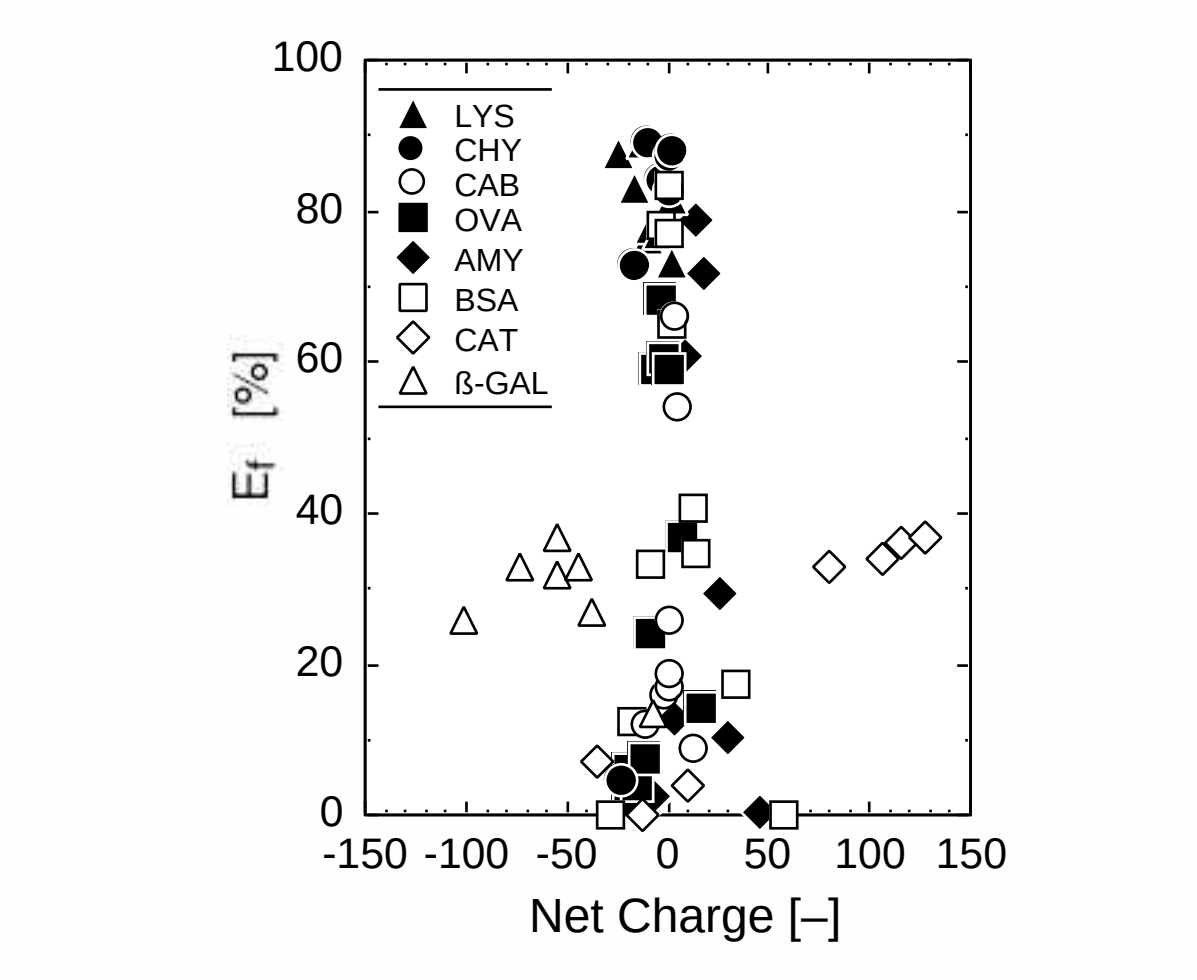


Fig. 1.4. Relationship between E_f and net charge of the protein.

of the micelles between protein and reverse micelles on electrostatic interaction have also been suggested in the previous study (Yamada *et al.*, 1994b).

The extraction behavior of proteins was discussed from their electric charge characteristics. The E_f values of proteins were plotted against their net charge at examined pH in **Fig. 1.4**. Net charge of proteins was calculated from the amino acid sequence of the protein and their pK values. The values of E_f of all proteins except for catalase and ß-galactosidase were obtained the largest value at around zero of net charge. As well known, an increase in negative net charge of proteins causes the repulsion between protein and the anionic AOT reverse micelles and, as a result, the extraction of proteins into reverse micelles

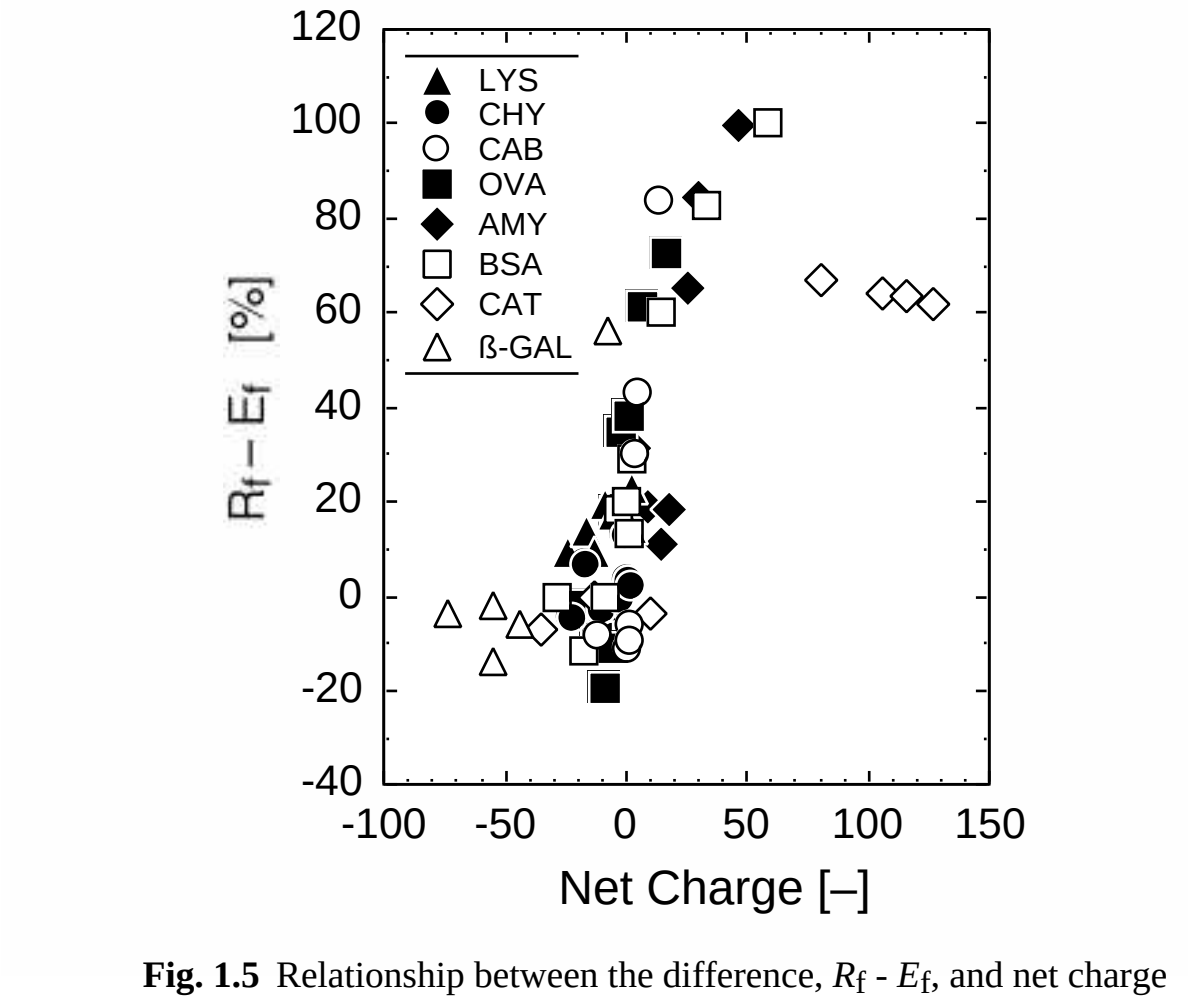


Fig. 1.5 Relationship between the difference, R_f - E_f, and net charge of the protein

decrease. On the other hand, however, an increase in positive net charge of proteins does not always lead to an increase in the values of E_f. The difference between R_f and E_f, which means a degree of the aggregate formation of the proteins, was plotted against net charge of the protein in **Fig. 1.5**. The formation of aggregates of the proteins increases with their positive net charge. Especially in the region of positive net charge higher than 10, the difference is very high and the denaturation and the formation of aggregate is furiously caused. AOT molecules corresponding to the number of positive net charge of the protein may bind to the protein molecules and strong binding of AOT onto the protein is considered to trigger the denaturation and the aggregation of the protein. Since

protein. Since the work on the extraction of small proteins has also shown that the decrease in E_f and the aggregates is formed at pH much lower than the pI (Kuboi *et al*., 1990a), it is clear that even small proteins cause aggregates in the condition that the protein have large number of positive net charges.

Though the extraction behavior of oligomeric proteins such as catalase, ß-galactosidase and hemoglobin was complicated, too strong electrostatic interaction between the proteins and AOT was undesirable for effective extraction and this seems to be a characteristic and common fact to all proteins. The complicated extraction behavior of oligomeric proteins may be due to the hydrophobic binding of AOT to the hydrophobic part between subunits and also due to the dissociation of subunits of the protein. For the extraction of oligomeric and large molecular weight proteins, therefore, it is necessary to consider and to control both electrostatic and also hydrophobic interaction between AOT and proteins.

Generally speaking, large molecular weight proteins have large number of charged amino acid residues and hence large change of net charge of the protein can be produced by a little pH change and then large number of AOT molecule may interact with the positive charge on the protein, and as a result of the binding of AOT to the protein, the tendency of the aggregate formation is often observed in large molecular weight proteins. So far the effect of net charge on the extraction behaviors of various proteins have not been discussed in detail in most of previous works. In the previous experiments reported by other investigators, where the extraction of large proteins was also carried out by the similar pH operation in that of small one, the extraction was not effectively occurred and formation of aggregate was predominantly observed, therefore the extraction of large molecular weight proteins by the conventional AOT reverse micellar system was firmly believed to be very difficult.

3.1.2 Structural change of proteins in the aqueous phase caused by electrostatic interaction with AOT

The structural change of proteins during the extraction process using reverse micelles is one of important factors. The structural change was suggested to be caused by strong electrostatic interaction with AOT. In some cases of the extraction of large molecular weight protein, large amount of aggregates of the proteins was observed after the extraction. The structural change of proteins during the extraction process is, however, difficult to be measured directly, hence the structural change of proteins in the aqueous phase containing of both 1 mM AOT and 0.1 M NaCl at various pH values was investigated using circular dichromism (CD) spectra.

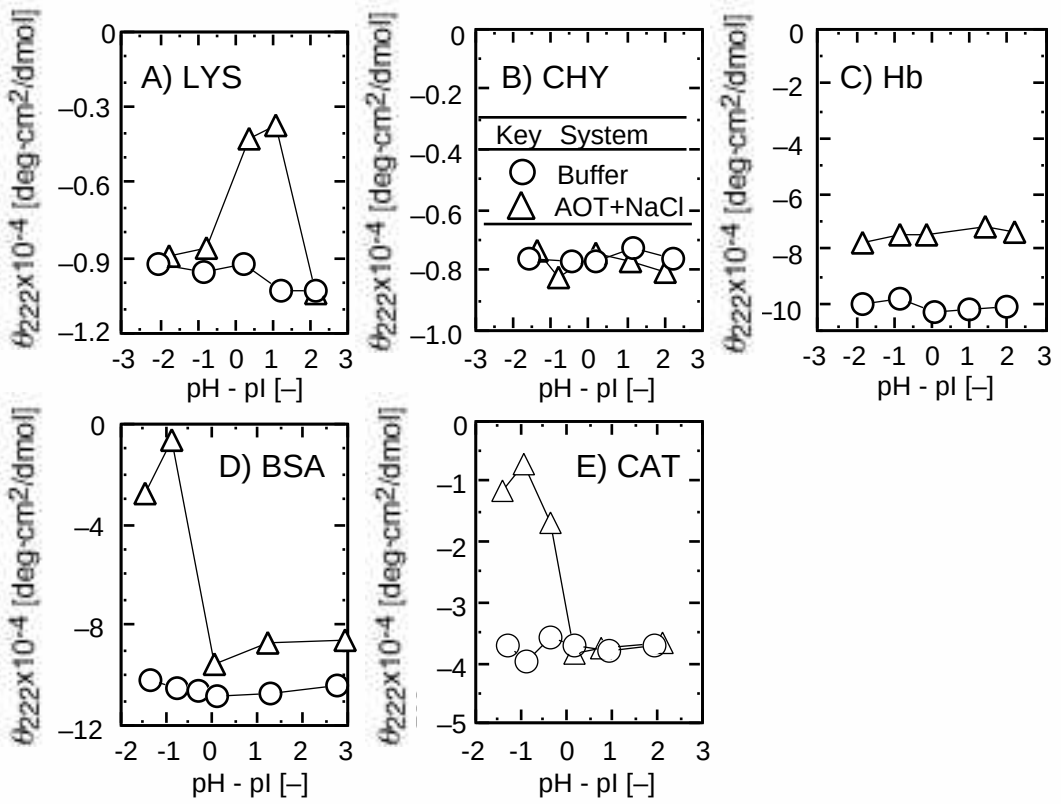


Fig. 1.6 Effect of pH on molecular elipticities at 222 or 208 nm of proteins in the aqueous solution containing 1 mM AOT and 0.1 M NaCl and in the buffer solution. A) lysozyme; B) α−chymotrypsinogen; C) hemoglobin; D) BSA; E) catalase.

The molecular ellipticity at 222 nm, θ_{222}, of protein excepted for catalase, which was used that at 208 nm, θ_{208}, in the aqueous phase with and without both AOT and NaCl was plotted against pH in **Fig. 1.6**. No conformational changes of all proteins were observed in a buffer solution at the pH range examined. The conformational change of proteins was also not observed in the buffer solution containing only 0.1 M NaCl.

In the cases of lysozyme and α-chymotrypsinogen in the buffer containing AOT and NaCl, no change of θ_{222} was observed at pH range lower than each pI. This means lysozyme and α-chymotrypsinogen are stable under the condition where proteins interact electrostatically with AOT in the presence of NaCl. On the other hand, significant structural changes of BSA and catalase were observed at pH lower than each pI. This shows that these proteins can be strongly denatured by electrostatic interaction with AOT. In the case of hemoglobin in the aqueous solution containing AOT and NaCl, θ_{222} was higher than that without AOT and the values were unaffected by pH value. Since hemoglobin has hydrophobic sites between subunits, the hydrophobic interaction with AOT may be caused strongly and induced the structural change regardless of pH value.

The characteristics of structural change of proteins agrees well with that of the aggregates formation in the extraction process. At the conditions where strong structural change of proteins was caused by electrostatic interaction, the aggregates of the protein is predominantly formed. Furthermore, the proteins, such as hemoglobin whose structural change were probably caused by hydrophobic interaction with AOT regardless of pH, will be difficult to be extracted using the conventional AOT system by the phase transfer method.

3.1.3. Back-extraction of large molecular weight proteins extracted into reverse micellar phase

The proteins extracted into the reverse micelles need to be collected from the reverse miecllar phase. The back-extraction of large proteins extracted by the phase transfer method, such as BSA, catalase and ß-galactosidase, was carried out by contacting the organic phase containing protein with a new aqueous phase with pH higher than the pI of the protein and/or high concentration of salt. This condition in the aqueous phase used for the back-extraction is the typical one in the conventional method using reverse micellar extraction with ionic surfactants. The conditions and results in the extraction and back-extraction for each protein are summarized in **Table 1.2.**

BSA could be effectively back-extracted by changing pH or salt concentration in the aqueous phase. On the other hand, catalase and ß-

Table 1.2. Extraction and back-extraction of proteins by the phase transfer method

Enzyme	Extraction				Back-extraction				
	pH	Salt [M]	E_f [%]	Wo [–]	pH	Salt [M]	E_b a) [%]	RSA [%]	Wo [–]
BSA	5.5	NaCl 0.5	83	21	7.9	NaCl 0.5	100		23.3
					9.0		100		24.2
					5.5	KCl 0.5	0		13.2
						1.0	100		8.9
						2.0	100		7.4
Catalase	4.6	NaCl 0.1	36	20.9	8.3	NaCl 0.1	34	0	18.4
ß-Galactosidase	8.0	NaCl 0.1	39	45.4	7.2	KCl 0.1	52	20	19.7

a) Based on the extracted concentration in forward extraction.

galactosidase were difficult to be back-extracted and the activity of the enzyme back-extracted were very low.

According to the results on the conformational change of the protein caused by the interaction with AOT and salt in the aqueous solution, the structural change of BSA in the aqueous phase containing AOT was relatively small at the same pH where the extraction was carried out by the phase transfer method. On the other hand, that of catalase was found to be very large. Catalase extracted into the micellar phase seems to change its structure and to be irreversibly denatured by the interaction with AOT in the extraction process. Hence catalase was difficult to be back-extracted. On the other hand, BSA extracted may be caused only slight conformational change, and thus back-extraction of BSA was possible.

The structural change of proteins by the stress of the interaction with AOT must be considered in the extraction of proteins, especially large and oligomeric proteins, using AOT reverse micellar system. The structural change of proteins in the extraction step is closely related to the degree of the back-extraction. Even if the protein is extracted by the phase transfer method, the strongly denatured proteins in the extraction step may be unable to be back-extracted from the micellar phase with keeping original biological functions.

3.2 Evaluation of inter-micellar and protein-micellar interactions using percolation process of reverse micelles

3.2.1 Characterization of AOT reverse micelles using percolation in electric conductivity

The percolation of the AOT reverse micellar solution was observed by the measurement of electric conductivity as a function of either volume of the added aqueous phase to the micellar solution at constant temperature or temperature of

the micellar solution at constant water content. The percolation is caused by infinite cluster formation of micelle droplets and reflects the easiness in the micellar-micellar interaction.

The typical result of the variation of the electric conductivity of the AOT reverse micellar solution of various alkane solvents as a function of the volume fraction of the aqueous phase, $\emptyset_{aq}$, is shown in **Fig. 1.7**. The conductivity seems to increase very sharply when the water content exceeds the respective threshold value indicating percolation phenomena. The percolation threshold, $\emptyset_p$ of the reverse micellar solution seems to decrease with the chain length of alkane solvent. The AOT reverse micellar system with branched isooctane is more stable than that with *n*-octane. As the length of alkane solvent increases, it becomes more difficult for solvent molecule to enter in the interfacial film, which is made of closely packed AOT aliphatic chains. When the alkane molecules become smaller, however, they can penetrate the interfacial film, which is made of AOT chains diluted by the solvent molecules (Lamg and Jada,

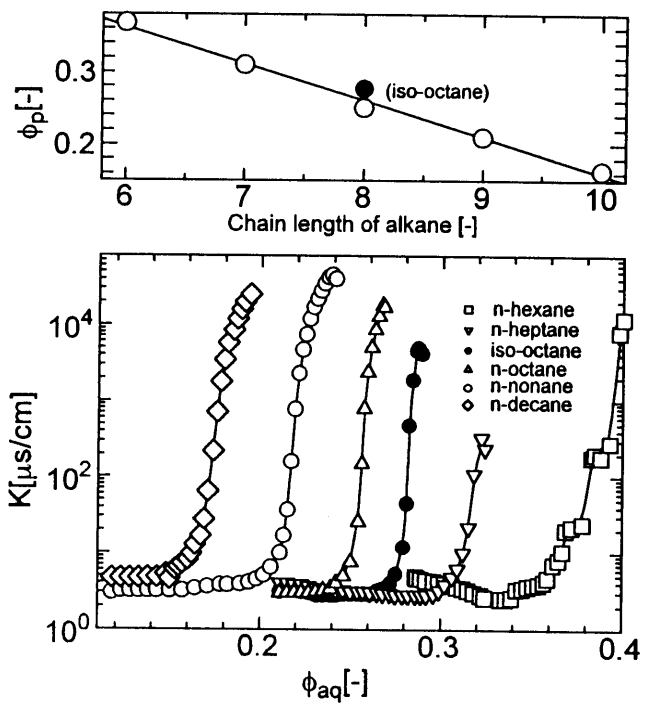


Fig. 1.7. Variation of the conductivity as a function of the water volume fraction in the various alkane solutions of AOT

1988). This has been attributed to the reduction in solvent penetration into the interface with increasing the solvent molecular weight, which may result in greater inter-micellar interactions to decrease percolation threshold (Cassin *et al*., 1994).

The effect of temperature on the percolation by changing the aqueous phase volume fraction is shown in **Fig. 1.8**. $\varnothing_p$ was strongly affected by temperature and decreased with increasing temperature.

The variation of the electric conductivity of the AOT reverse micellar solution of isooctane as a function of the temperature, *T*, at constant water content is shown in **Fig. 1.9**. The conductivity increased very sharply when the temperature exceeds the respective threshold value indicating percolation phenomena. The percolation threshold temperature, *T*p of the reverse micellar solution decrease with increasing water content of the micellar solution. This results agreed well with that in Fig. 1.8. Inter-micellar interactions are strongly

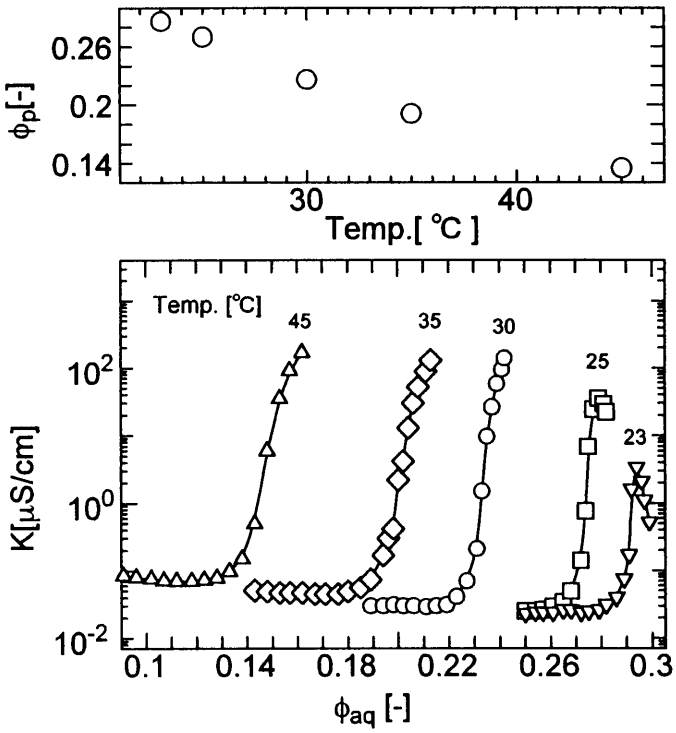


Fig. 1.8. Variation of the conductivity as a function of the water volume fraction in AOT isooctane solution at various temperatures

temperature dependent. An increase of temperature induced destabilization of the reverse micelles. An increase in the temperature would increase the probability of AOT ionization, the range of ion diffusion and the frequency of collision of the AOT reverse micelles (Alexandridis *et al.*, 1995).

The percolation of the reverse micellar solution can be easily monitored by the measurement of electric conductivity as a function of volume of the micro water phase in the micellar solution and temperature of the solution and the inter-micellar interaction is possible to evaluate from the percolation

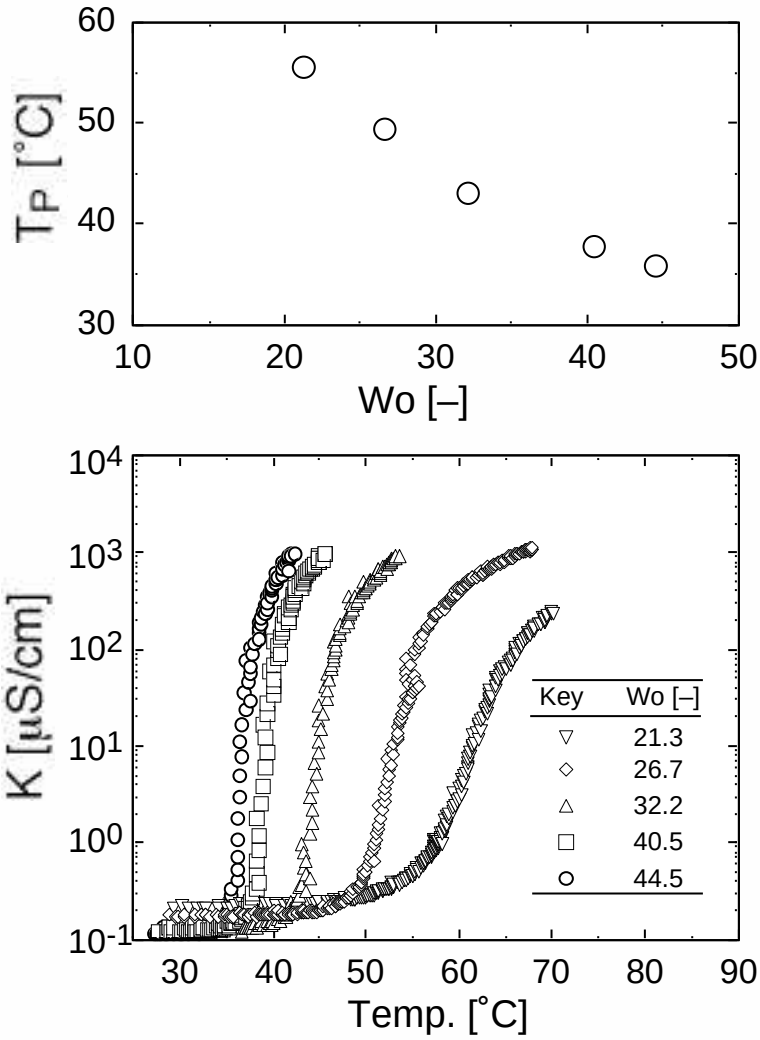


Fig. 1.9 The variation of the electric conductivity of the AOT reverse micellar solution of isooctane as a function of the temperature at constant water content

threshold. Further, the inter-micellar interaction characteristics involves the information of the interface state of the reverses micelles, such as the interaction between solutes in the water pool and the interface of the reverse micelles, stabilization and destabilization of the interface by the interaction.

3.2.2 Effect of proteins solubilized by the injection method on the percolation

The various proteins were solubilized by the injection method and their effects on the percolation behavior were investigated. The interaction between the reverse micelles and proteins solubilized was estimated and discussed from the effect of protein solubilization on the percolation. The variation of the electric conductivity of the AOT reverse micellar solution is plotted against the volume fraction of water containing catalase in the organic phase, $Ø_{aq}$ in **Fig. 1.10.** With increasing water content, the conductivities are at first insensitive to the water content. However, the conductivities increase very sharply, about three

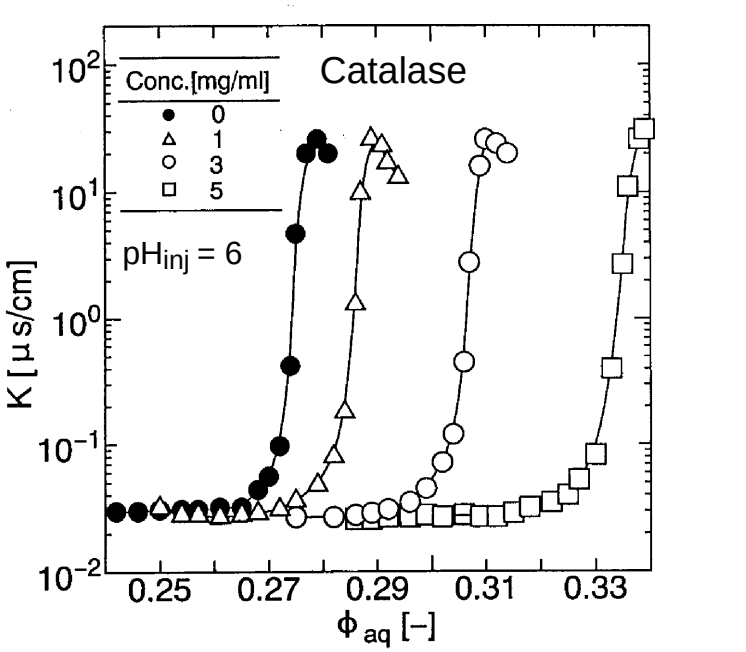


Fig. 1.10. The variation of the electric conductivity as a function of the volume fraction of water containing catalase for the AOT reverse micellar solution

orders of magnitude, when the water contents exceed respective threshold values indicating percolation phenomena. At this condition, the percolation process is clearly reflecting the protein-micellar interaction.

An increase in the concentration of the catalase shifted the percolation threshold(Ø) to a higher value of $Ø_{aq}$. The difference of $Ø_{aq}$ with and without the protein increased with the concentration of catalase. This dependency was the same as those at other pH values in this experiment.

The results of conductivity measurement in the solubilization of cytochrome c is shown in **Fig. 1.11**. In contrast to the result in catalase, for the reverse micellar solution with cytochrome c, the percolation threshold has been shifted to a lower value of $Ø_{aq}$ than for the protein free system. This behavior can be explained either by the presence of the strong dipole moment of cytochrome c inducing dipole-dipole interactions between droplets or by the hydrophobic character of the protein favoring the interconnection between the micro phase or by the charge head-group area of the surfactant. Cytochrome c in reverse micelles has been studied by several authors (Brochett *et al.*,1988;

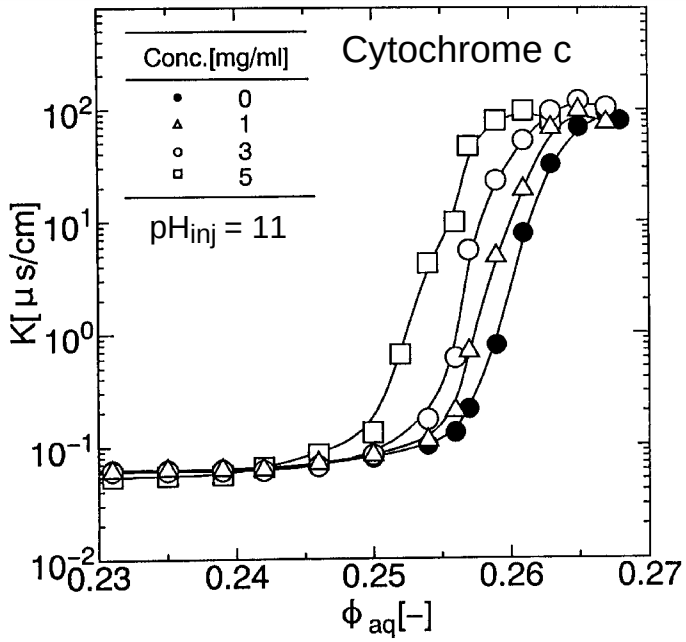


Fig. 1.11. Variation of the conductivity as a function of the water content containing cytochrome c at pH 11.

Larsson and Pileni, 1993; Gassin *et al*., 1994). There are also indications that cytochrome c interacts with the AOT surfactant layer.

The results of conductivity measurement in the solubilization of hemoglobin is shown in **Fig. 1.12**. At pH 6.0, the percolation threshold has been

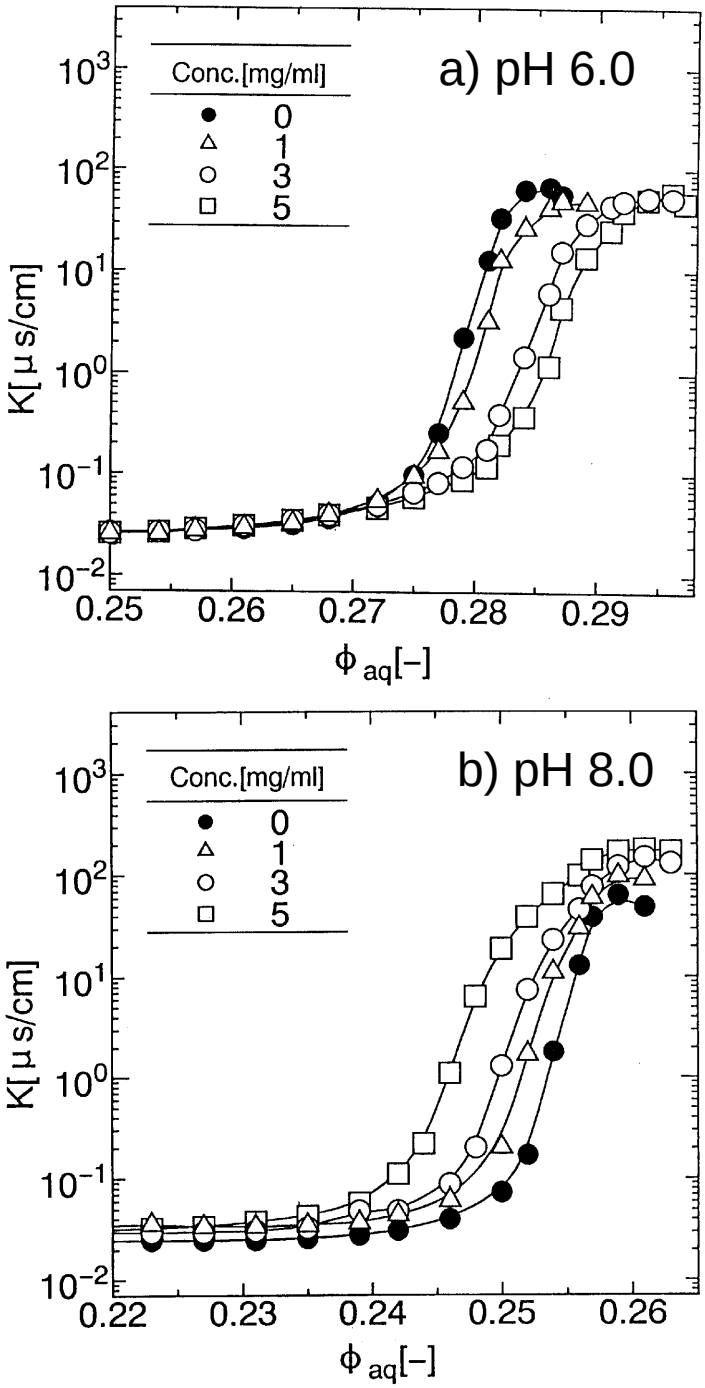


Fig.1.12. Variation of the conductivity as a function of the water content containing hemoglobin. a) pH 6.0, b)pH 8.0

been shifted to a lower value of $Ø_{aq}$ than for the protein free system. On the other hand, at pH 8.0, the percolation threshold has been shifted to a higher value of $Ø_{aq}$ than for the protein free system. The percolation behavior in the solubilization of hemoglobin was affected strongly by pH. In the range lower than pI, hemoglobin is considered to interact with interface of the micelles and cases a decrease of stability of the micelles.

Therefore, the protein-micellar (electrostatic attractive) interactions seem to decrease the stability of reverse micellar solution by decreasing electrostatic repulsive interaction between micelles. The formation of micellar clusters shows a larger hydrophobic attraction than an electrostatic repulsive force between the micelles.

The percolation threshold depends on the concentration of the proteins solubilized in the micelles. The difference, $\Delta Ø_p$ ($=Ø_{pp} - Ø_p$), shows the effect of the protein's concentration on the percolation process. Here, $Ø_{pp}$ and $Ø_p$ are the values of the percolation threshold with and without protein, respectively. $\Delta Ø_p$ is plotted against the concentration of catalase, cytochrome c and hemoglobin solubilized in the micelles under different pH conditions in **Fig. 1.13**. There is a linear correlation between $\Delta Ø_p$ and the concentration of protein. The slope, *ß,* is a measure of the effect of solubilized protein on the attractive interaction between micelles. A positive value of *ß* means the stabilization of reverse micellar solution with the solubilization of the protein. On the other hand, a negative *ß* means the destabilization of reverse micellar solution with the solubilization of the protein.

3.2.3 Effect of pH on the percolation process of reverse micelles solubilized proteins

The effect of the pH of the aqueous phase with proteins on the inter

micellar interactions was investigated. The values of ß are plotted against the pH deviation of the injected solution from the isoelectric point (pI) of each protein in **Fig. 1.14**. The value of ß are affected by the pH values of the solution, that is,

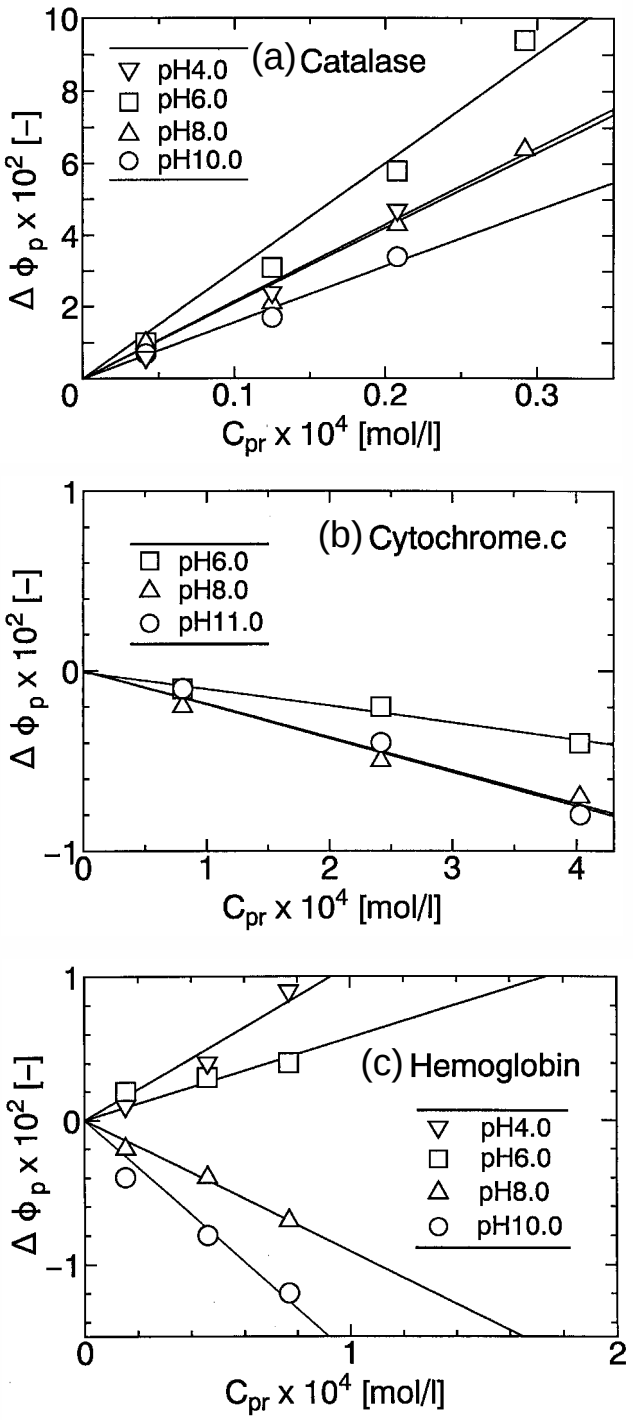


Fig. 1.13. Effect of the concentration of protein on the percolation threshold.a)catalase, b)cytochrome c, c)hemoglobin

is, by electrostatic interaction between micelles and proteins. The proteins such as BSA, catalase and C. C. lipase have high and positive values of *ß* at pH values both above and below the pI of the proteins. These proteins are defined as type A. Proteins such as cytochrome c, lysozyme and hemoglobin, however, have low and negative values of *ß*. These proteins are defined as type B. Hemoglobin and lysozyme shows either stabilization or destabilization

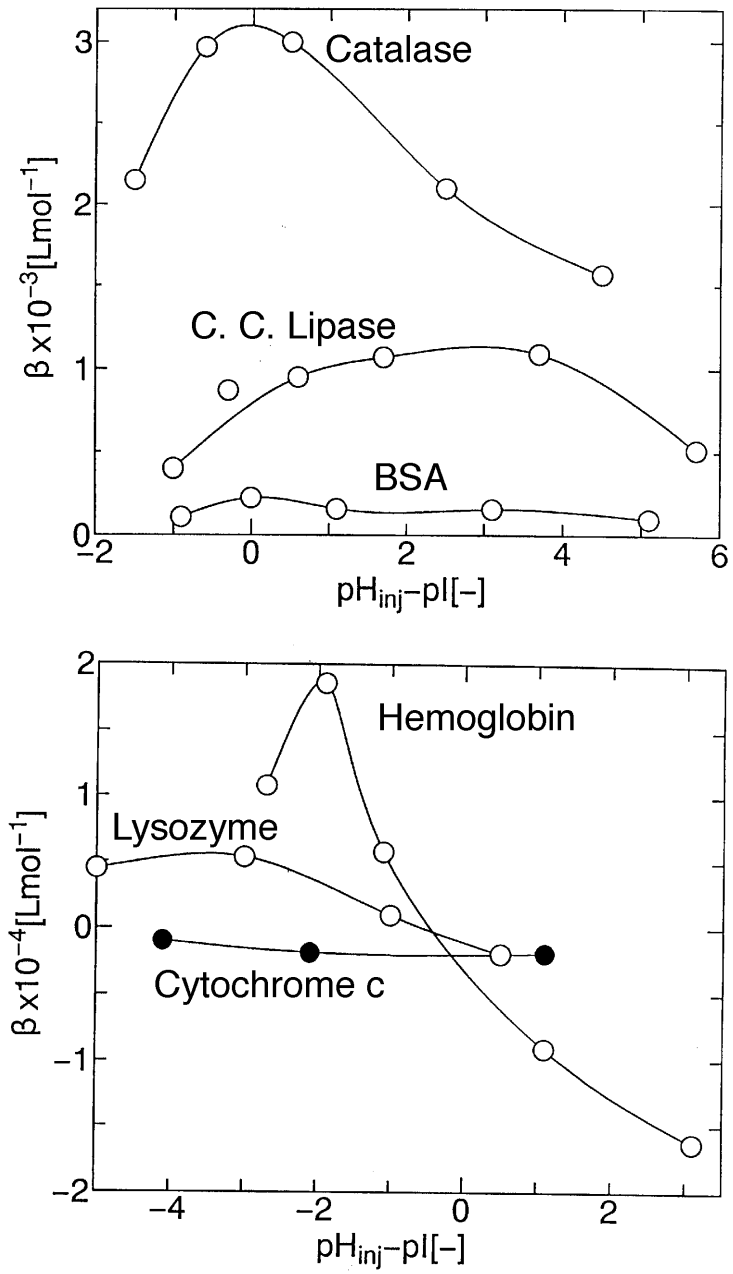


Fig. 1.14. Effect of pH on the stability value of reverse micellar solution, *ß* .

depending on the solution pH of the protein. Therefore. it is thought that the value of *ß* sensitive to the solution pH is much affected by the electrostatic interaction between the proteins and micelles.

3.2.4 Effect of polymers solubilized on the percolation process

To examine the hydrophobic interactions affecting the percolation process, polymers, with differing molecular weight and hydrophobic properties, were solubilized into the micelles and the effect of hydrophobicity on the percolation process was investigated. Polyethylene glycol(PEG) and Dextran(Dex) were examined. Both PEG and Dex have been used in aqueous two-phase systems, that are formed by two mutually incompatible components (Kuboi *et al*., 1993). PEG is more hydrophobic than Dex.

Effect of molecular weight of PEG on the variation of conductivity by changing the aqueous phase volume fraction is shown in **Fig. 1.15**. Percolation

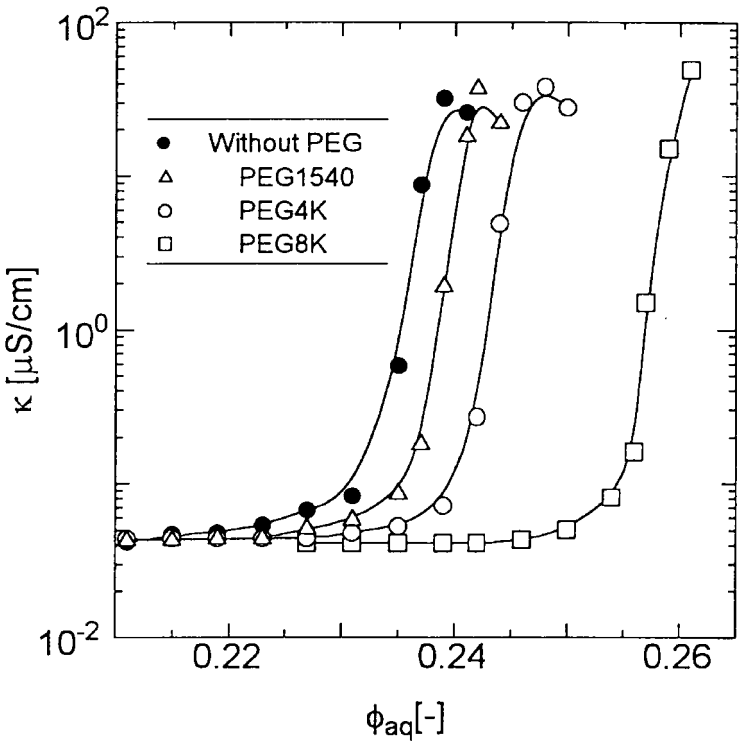


Fig. 1.15. Effect of molecular weight of PEG on the variation of conductivity of reverse micellar solution as a function of the water content. Concentration of polymers is 2 mM

phenomena was also observed in the system of water soluble polymer. The percolation threshold in the PEG system increased with molecular weight of PEG.

Effect of concentration of PEG 20K on the variation of conductivity at various volume fractions of the aqueous phase is shown in **Fig. 1.16**. As the concentration of polymers was increased, the percolation threshold was shifted to higher values of $\varnothing_{aq}$. The difference between $\varnothing_{pp}$ and $\varnothing_{p}$, $\Delta\varnothing_{p}$ (=$\varnothing_{pp}$ - $\varnothing_{p}$), is plotted against the concentration of PEG in **Fig. 1.17**. There is a linear correlation between $\Delta\varnothing_{p}$ and polymer concentration just as for proteins as shown in Fig. 1.13. However, the slope is depended on their molecular weight. In the cases of Dex, the same tendency was observed. The slopes of the straight line was obtained as *ß*. An electrostatic interaction between the polymer and the micelle is not expected.

The values of *ß* are plotted against the molecular weights in **Fig. 1.18**. It

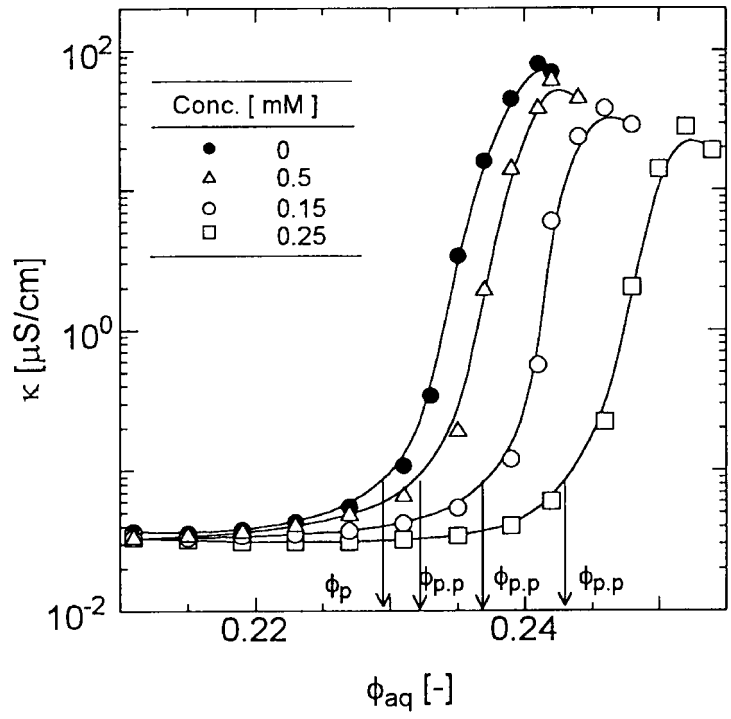


Fig. 1.16. Effect of the concentration of PEG 20K on the variation of conductivity of reverse micellar solution as a function of the water content.

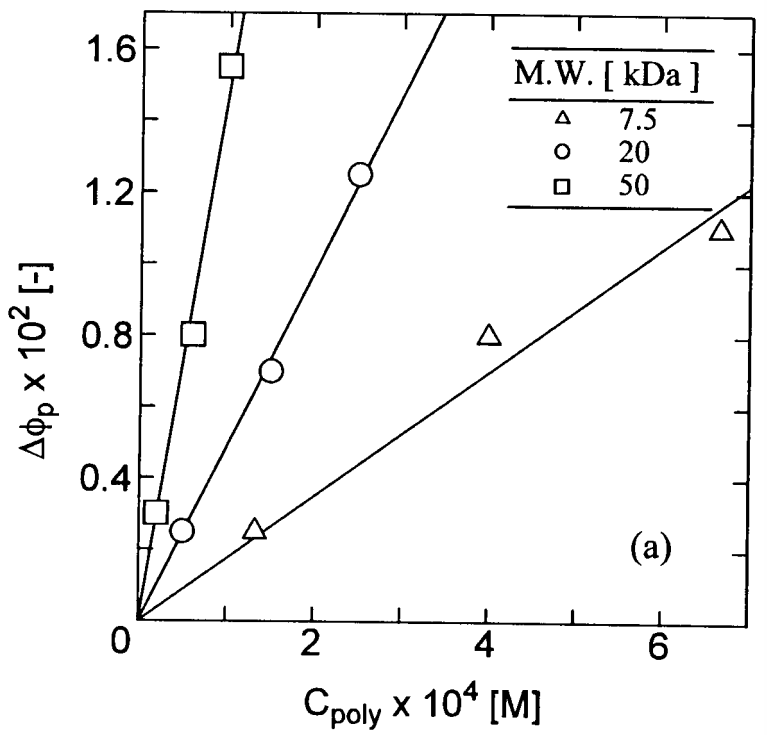


Fig. 1.17. Effect of the concentration of PEG on the percolation threshold

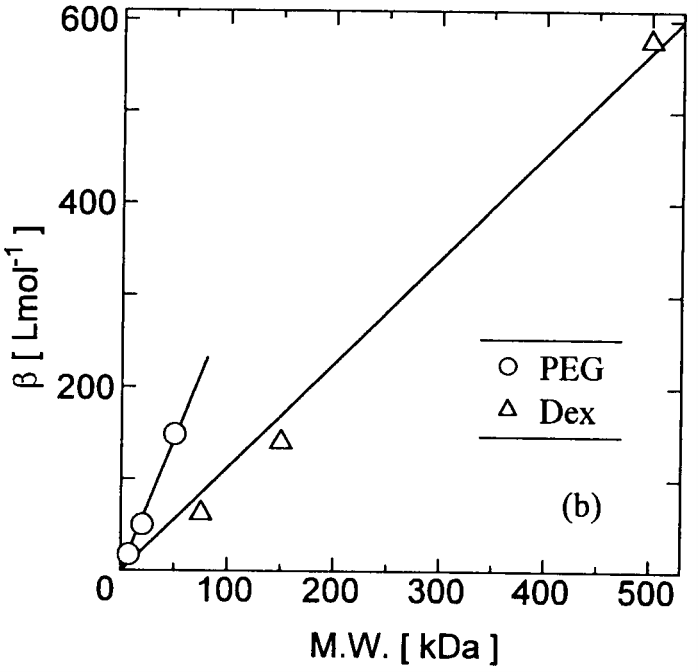


Fig. 1.18. Effect of molecular weight of non-ionic polymers on the stability of reverse micellar system, *ß*

shows that the value of ß increases linearly with increasing polymer molecular weight, but with different slopes. This means that the stability of reverse micellar solution is affected by the hydrophobicity and molecular weight of the polymer solubilized in the micelles.

3.2.5 Effect of surface hydrophobicity and molecular weight of the proteins on the stability of reverse micellar solution

Percolation threshold is affected by the molecular weight and the hydrophobicity of the polymer solubilized into the reverse micelles. The values of ß at the isoelectric point of the type A proteins were compared with the properties of the proteins. **Fig. 1.19** shows the effect of HFS and M. W. on ß. The value of ß is proportional to the HFS and M. W. of the proteins, and thus $ß_{pI}$ is expressed as;

$$ß_{pI} \propto (HFS)\ (M.\ W.) \qquad (1.1)$$

From this result, when the proteins with positive values of ß are solubilized, the stabilities of the micelles seem to increase in proportion to the

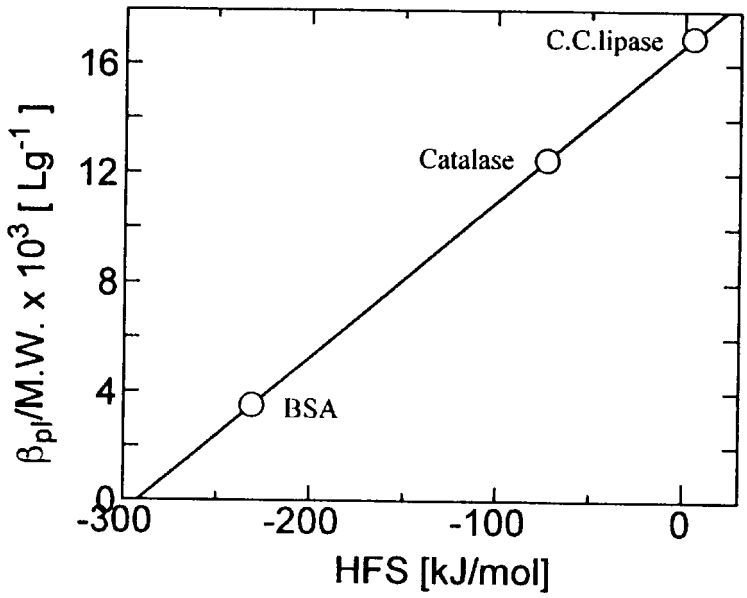


Fig.1.19. Relationship between surface hydrophobicity (HFS), molecular weight (M.W.) and reverse micellar stability, ß

protein's M. W. and HFS. For the type B proteins new parameters such as local charge density and local hydrophobicity must be studied.

4. SUMMARY

The reverse micellar extraction of large molecular weight and oligomeric proteins, which were not fully investigated previously, was carried out using conventional AOT reverse micellar extraction systems at various pH values comparing with some small proteins. Although most of proteins except for hemoglobin and C. C. lipase could be extracted at certain conditions, large amount of aggregates were also formed. It is become large molecular weight proteins have large number of electric positive charge at pH lower than each pI, and thus large number of AOT molecules interact and bind to the protein surface by electrostatic attraction force, naturally causing denaturation and aggregation of the protein. Consequently, the electrostatic interaction between AOT and the proteins must be carefully controlled in the extraction of large molecular weight proteins in particular. In other works, the reverse micellar extraction may become to be possible for various types of proteins by the control of the electrostatic interaction.

The conformational change of proteins caused by the electrostatic interaction with AOT at the extraction pH must be considered for the successful construction of the extraction process. In the case that the conformational change of proteins by the interaction with AOT is large, the denaturation and the formation of aggregate is predominantly caused, and even if proteins is extracted to the reverse micelles at that condition, the proteins will be difficult to be back-extracted. The proteins, which can be extracted with keeping its structure, can be applied for the reverse micellar extraction process regardless of

their molecular weights by the phase transfer method using conventional AOT extraction system.

Evaluations of the inter-micellar and protein-micellar interactions were possible by the measurement of electric conductivity change of the reverse micellar solution caused by the percolation as functions of the water content of the reverse micellar solution and the temperature of the solution at constant water content. The inter-micellar interaction is affected by the solvent, temperature and water content. The solubilization of proteins and water soluble polymers into the micelles affects the inter-micellar interaction. This is due to the interaction between the solute and the reverse micelles. The percolation is highly dependent on the surface property, molecular weight and concentration of solubilized proteins. The values of ß defined as the variation of percolation threshold with the concentration of proteins reflects the stability of the micellar solution or inter micellar interaction and depends on the protein's solution pH. The ß values can be utilized to evaluate inter micellar interaction in the presence of proteins. The percolation phenomenon, caused by the solubilization of nonionic polymers such as PEG and Dextran, depends on the molecular weight and hydrophobic property of the polymers, suggesting the importance of micellar-protein hydrophobic interaction. In the case of some proteins classified as type A, such as catalase, lipase and BSA, which have positive ß values at the pH both above and below pI, the value of ß at their isoelectric points ($ß_{pI}$) can be correlated with the hydrophobic factor of the solute(HFS) and the molecular weight of the proteins. Strong interaction between the micelles and proteins solubilized highly destabilize the reverse micelles.

Part II

Evaluation of Protein-Reverse Micellar Interaction based on Enzymatic Reaction at Micellar Interface

1. INTRODUCTION

Since reverse micelles can solubilize proteins keeping their activity and provide a novel reaction field for proteins in organic solvents, many studies on enzymatic reactions in reverse micellar systems and their application for micro-bioreactor systems have been reported in recent years (Khmelnitsky *et al*., 1989; Chen and Chang, 1993; , Han and Rhee, 1989; Holmberg, 1994; Yamada et al, 1995). Various proteins have been solubilized and used in reverse micellar organic phase, and the studies on lipase have been most frequently reported because various types of reactions, such as hydrolysis, esterification and ester exchange reactions, can be carried out.

The enzyme activity in reverse micelles is influenced by several factors, especially water content, W_O (Khmelnitsky *et al*.,1989, Flecher *et al*., 1985, Han *et al*., 1990, Kuboi *et al*., 1992). The optimum values of W_O for enzyme activity have been correlated with the effective diameter of the solubilized protein (Khmelnitsky *et al*., 1989). Some reaction and kinetic models have been proposed to interpret the characteristics of enzymatic reactions in reverse micelles (Tsai and Chiang, 1991, Flecher *et al*., 1985, Prazeres *et al*., 1993, Miyake *et al*., 1993, 1994). The locations of proteins and substrates are important in the study of the enzyme reaction kinetics in reverse micellar systems. A few studies have focused on the location of the substrate and

proteins (Fletcher *et al*., 1985, Miyake *et al*., 1993, 1994, Han and Rhee. 1986, Yamada, 1995). Proteins interact with the micellar interface and the surfactants constructing the reverse micelles. The interaction causes the structural change of proteins. And the solubilization of proteins into the micelles affects the stability of the reverse micelles as shown in Part I.

In this part, the interaction of protein with reverse micellar interface was investigated from the view point of the reaction behavior of lipase in the reverse micellar system. Hydrolysis of olive oil catalyzed by lipase was carried out in AOT reverse micellar systems. The effects of reaction conditions on the activity and the stability were studied and the results were analyzed by the interfacial reaction model. The results were compared with the results obtained with organic-aqueous two phase system in a Lewis-type transfer cell and with a O/W emulsion system containing the anionic surfactant. The characteristics of lipase hydrolysis in the reverse micellar system was discussed from the viewpoint of the interaction of lipase with the micellar interface.

2. EXPERIMENTAL

2.1 Reagents

Lipase from *C. cylindracea* (M. W.= 53 kDa, pI 4.3, 905 units/mg-solid) was purchased from Sigma Chemical Co., USA. Guaranteed reagents of olive oil, sodium dodecyl sulfate (SDS) and polyvinyl alcohol (PVA) from Wako Pure Chemical Co. and sodium bis(2-ethylhexyl)sulfosuccinate (AOT) from Nacalai Tesque Co. were used without further purification. Isooctane solution dissolved AOT was used as a reverse micellar solution. The buffers used were acetic acid-sodium acetate (pH 4-5.6), phosphate (pH 6-7), and Tris-HCl (pH 7.2-9), at a concentration of 100 mM.

2.2. Procedure

A small volume of buffer solution in which lipase was dissolved was injected into the reverse micellar solution, and the mixed solution was then vigorously shaken until it became transparent. Immediately after solubilization, the initial activity of the lipase in the micelles was determined. To determine the stability of the lipase in the micelles, the micellar solution containing lipase was incubated in capped sample tubes in a thermostated bath at 25°C with mild stirring. After a certain period of incubation, the residual activity of the lipase was measured.

The reverse micellar solution containing olive oil without lipase was used as the substrate micellar solution. A small volume of buffer solution without lipase was injected into the substrate micellar solution to obtain the same water content as that of the micellar solution containing lipase. The W_O value of the organic phase was defined as the molar ratio of water to AOT and was measured by Karl-Fisher titration.

The reaction was started by mixing equal volumes (10 ml) of the micellar solutions containing lipase and the substrate in a screw-cap sample tube. The reaction mixture was continuously stirred by a magnetic stirrer in thermostated bath at 25˚C. At fixed intervals, 1.0 ml samples of the mixture were collected. The concentration of fatty acids produced in the system was determined according to Lowry's method (Lowry and Tinsley, 1976). A blank test was performed by the same procedure without lipase. Lipase activity was estimated from the initial formation rates of free fatty acids.

3. RESULTS AND DISCUSSION

3.1 Characteristics of lipase in reverse micellar system

The hydrolysis rates of olive oil catalyzed by lipase immediately after solubilization into the reverse micelles were measured under various conditions. In **Fig. 2.1**, the concentration of free fatty acids formed, C_A, is plotted against the reaction time, t, at a fixed lipase concentration. The fatty acids increased linearly with the reaction time up to 1 hour in all cases. The slope increased with increasing concentrations of olive oil. The initial reaction rates, V_{in}, were calculated from the slopes of these straight lines.

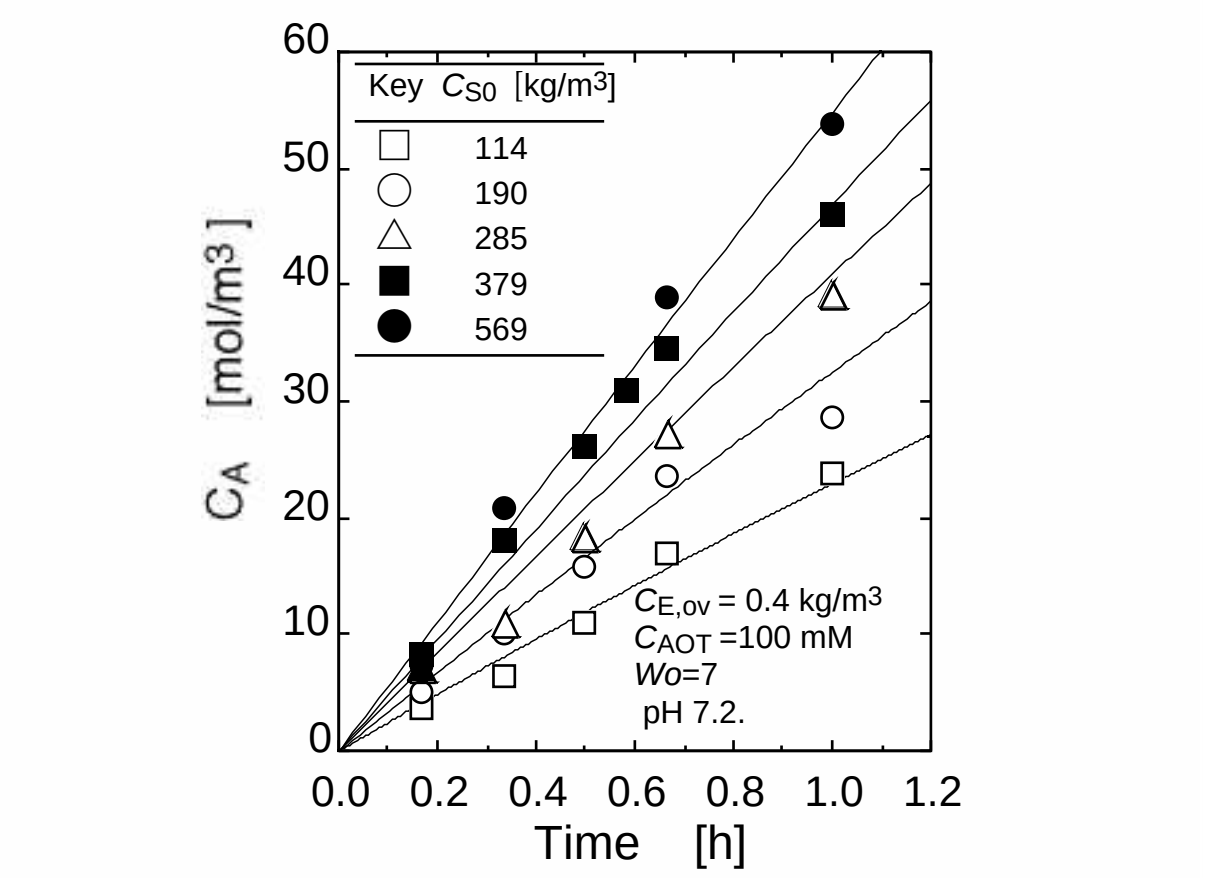


Fig. 2.1 Time-courses of free fatty acids formed at $C_{E,ov}$ = 0.4 kg/m3, C_{AOT} = 100 mM, W_O = 7 and pH 7.2.

3.1.1 Effect of pH on the lipase activity

The effect of pH on the relative activity of lipase in the reverse micelles is shown in **Fig. 2.2** and compared with the results for the two phase system carried out in a transfer cell and for the O/W emulsion system consisted from SDS and PVA. In the two phase system, lipase was active between pH 4.8 and 9, and most active at pH 7.6-7.8. In the emulsion system, the activity was influenced strongly by the pH value and was highest at around pH 7.8. The activity in the reverse micellar system was also strongly affected by the pH value. The highest activity was obtained at pH 6.3-7.3. Han and Rhee reported that lipase in AOT reverse micelles had the highest activity at pH 7.1 (Han and Rhee, 1986). Although the pH value of the highest activity in our experiment is comparable with their result, the effective pH range is somewhat different.

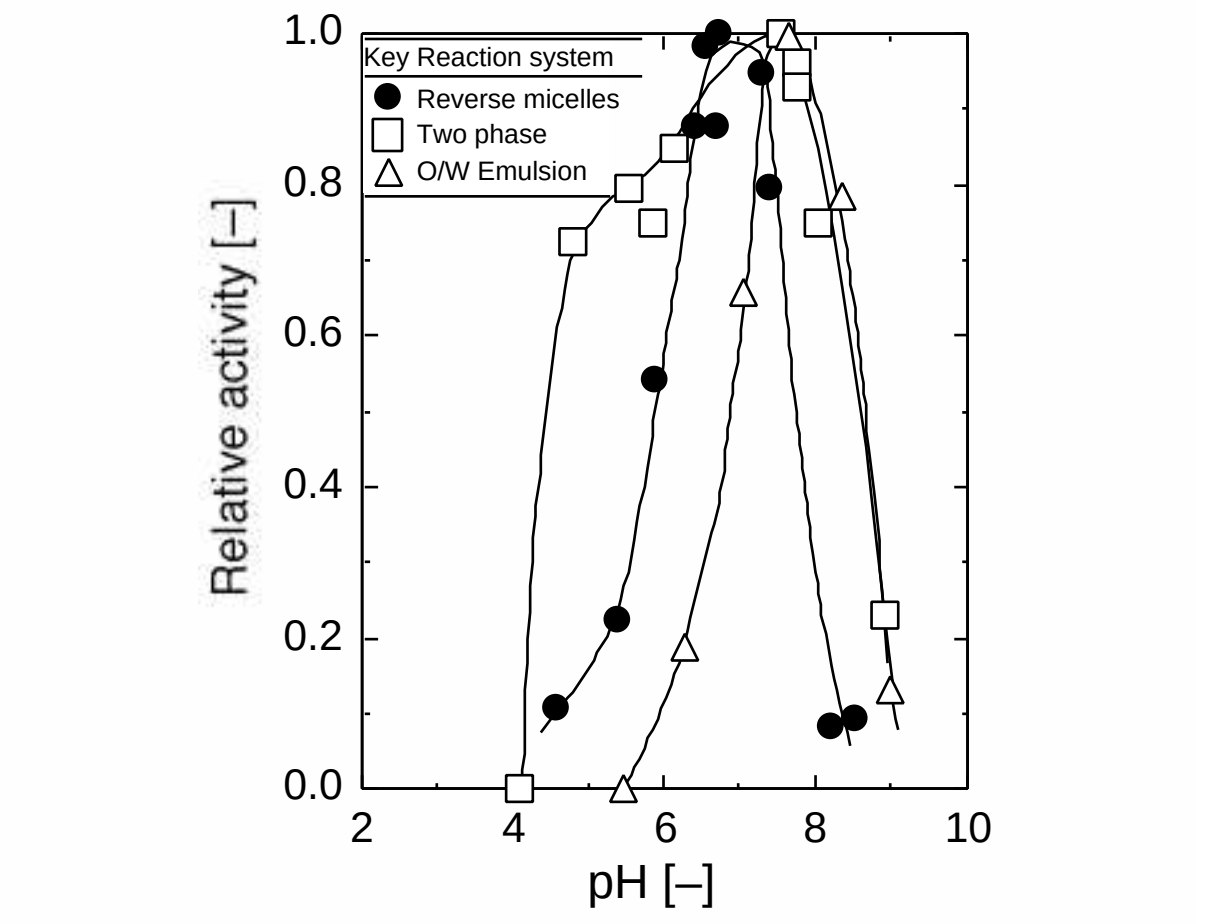


Fig. 2.2. Effect of pH on initial activity in various reaction systems

The pH value of maximum activity in the reverse micellar system was shifted to lower value compared with the transfer call and the emulsion systems. Shift in the optimal pH in reverse micelles to lower pH have also been found for other lipase (Otero *et al*., 1995). The optimal pH of enzymes in reverse micelles has been usually reported to shift to more basic region compared with that in the bulk aqueous solution. These enzymes shown the shift to basic pH are non interfacial active enzymes, such as α-chymotrypsin (Barbaric and Luisi, 1981), lysozyme (Grandi *et al*., 1981) and others (Shield *et al*., 1986). The opposite shift in the optimal pH of lipase might be due to the different localization of their enzymes in the reverse micelles. Lipase might adsorb at the interface between the micellar water pool and the organic phase. Thus, changes in the dielectric constant of the micro environment of the active site in enzyme solubilized in the micelles should produce different shift in their pK_a value of the amino acid residues in the active site of the enzyme (Castro *et al*., 1989).

At lower pHs, the activities in the reverse micellar and the emulsion systems decreased markedly compared with that in the transfer cell system which contains no anionic surfactant. The structural change of lipase by the interaction with AOT was measured in the aqueous phase containing AOT and NaCl with CD spectra. The effect of pH on molecular ellipticity at 222 nm, θ_{222}, of lipase is shown in **Fig. 2.3.** The difference of θ_{222} value with and without AOT increased with lowering pH and the value of θ_{222} with AOT was lower than that without AOT. The conformation of lipase is considered to be changed by the electrostatic interaction with AOT. This structural change is almost agreed with the pH effect on the activity in the AOT reverse micelles and also the emulsion system. As lowering pH, the lipase is considered to be denatured and inactivated by electrostatic interaction with the anionic surfactants contained in these systems.

The effects of pH on the ß value obtained from the percolation of reverse micelles containing lipase and the relative activity of lipase in the micelles is compared in **Fig. 2.4**. The pH rage of high activity of lipase is interestingly almost agreed with that of high vale of ß. When lipase shows high activity, the micellar interface is stabilized. In the pH range higher than pI, though the interaction of lipase, which has negative net charge, with the interface is considered to be reduced by the electrostatic repulsion, lipase interacts moderately with the interface and reacts effectively with substrate, at that time the stabilization of the micellar interface is occurred. In the pH range lower than pI, lipase interacts strongly with interface, and as a results the inactivation of lipase and the destabilization of micellar interface are caused.

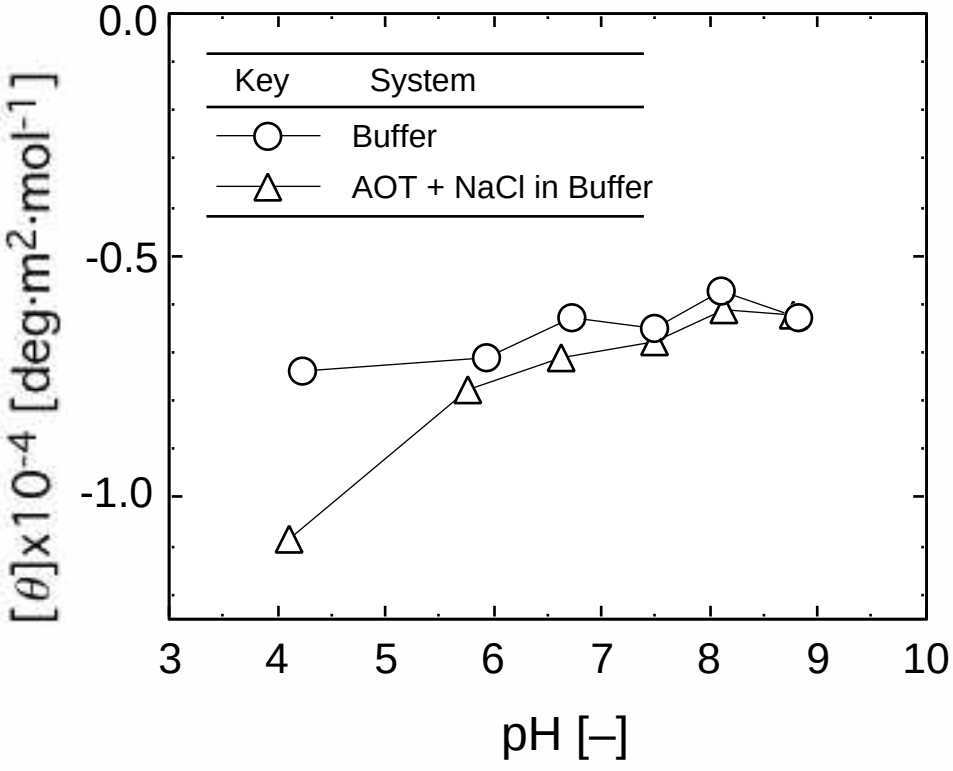


Fig. 2.3. The effect of pH on molecular ellipticity at 222 nm, θ_{222}, of lipase in the aqueous phase without and with 1 mM AOT and 0.1 M NaCl.

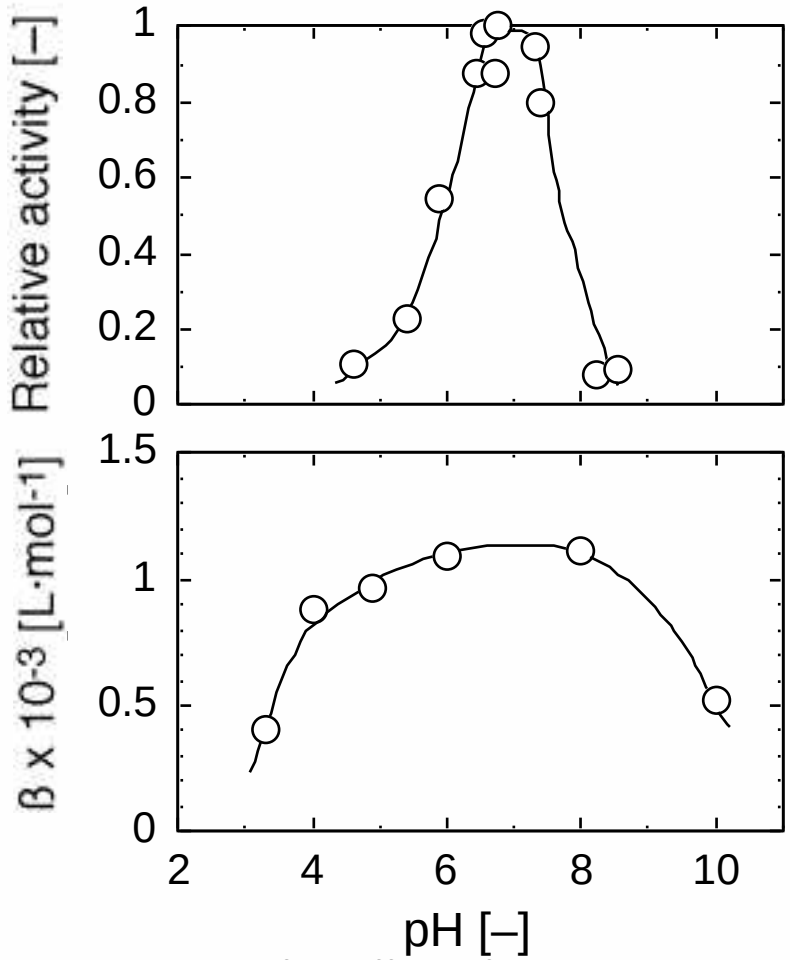


Fig. 2.4. Comparison of the effects of pH between relative activity of lipase in the reverse micelles and the values of ß obtained from percolation.

3.1.2

Effect of W_0 on the lipase activity

The effect of W_0 on the lipase activity is shown in **Fig. 2.5**. In the case of C_{AOT} = 100 mM, the maximum activities at both $C_{E,ov}$ = 0.2 and 0.4 kg/m^3 were observed at W_0 = 7, both activities being the same. Increasing the lipase concentration from $C_{E,ov}$ = 0.2 to 0.4 kg/m^3 caused an increase in the activity in the W_0 range over 10. At $C_{E,ov}$ = 0.4 kg/m^3, the activity at C_{AOT} = 75 mM had a maximum value at W_0 = 11. When the AOT concentration was increased, the W_0 dependency of the activity changed, the W_0 value giving maximum activity shifting to W_0 = 7. Han *et al.* reported that as the total lipase concentration

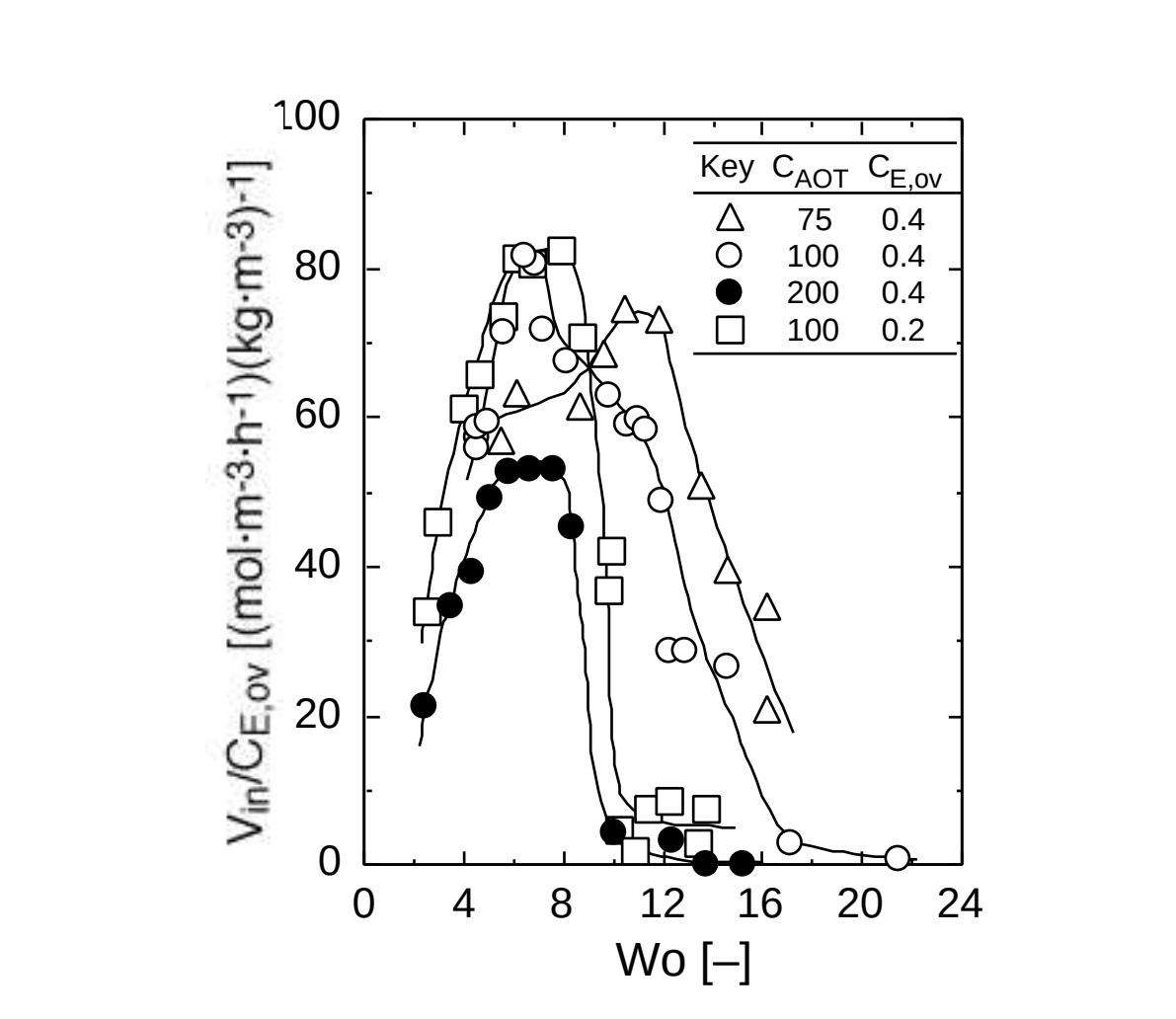


Fig. 2.5. Effect of W_O on initial activity at pH 7.2.

and the activity above the optimal W_O was steadily enhanced (Han *et al*., 1990). In this experiment, when the AOT concentration was relatively high compared with the lipase concentration, the maximum activity was observed at W_O = 7 and the activities above W_O = 10 were very low. On the other hand, when the AOT concentration was relatively low compared with the lipase concentration, the activity above W_O = 10 was enhanced.

3.1.3 Effect of lipase concentration injected into reverse micellar solution on the lipase activity

The effect of the enzyme concentration in the micellar solution on the

initial activity is shown in **Fig. 2.6**. When *Wo*=7, at C_{AOT} = 50 mM, the activity decreased rapidly with increases in the concentration of lipase. At C_{AOT} = 100 mM, the activity was independent of the lipase concentration until $C_{E,ov}$ = 0.4 kg/m^3, after which the activity decreased as the lipase concentration was increased further. On the other hand, the activity at C_{AOT} = 200 mM was almost constant regardless of the lipase concentration. The lipase activity, however, was less than that at lower AOT concentrations.

In the cases of C_{AOT} = 100 mM , the activity decreased rapidly with lipase concentration at *Wo*=5. At *Wo* = 7 and 10, the activities was independent of the lipase concentration until certain values, after which the activity decreased with further increasing the lipase concentration.

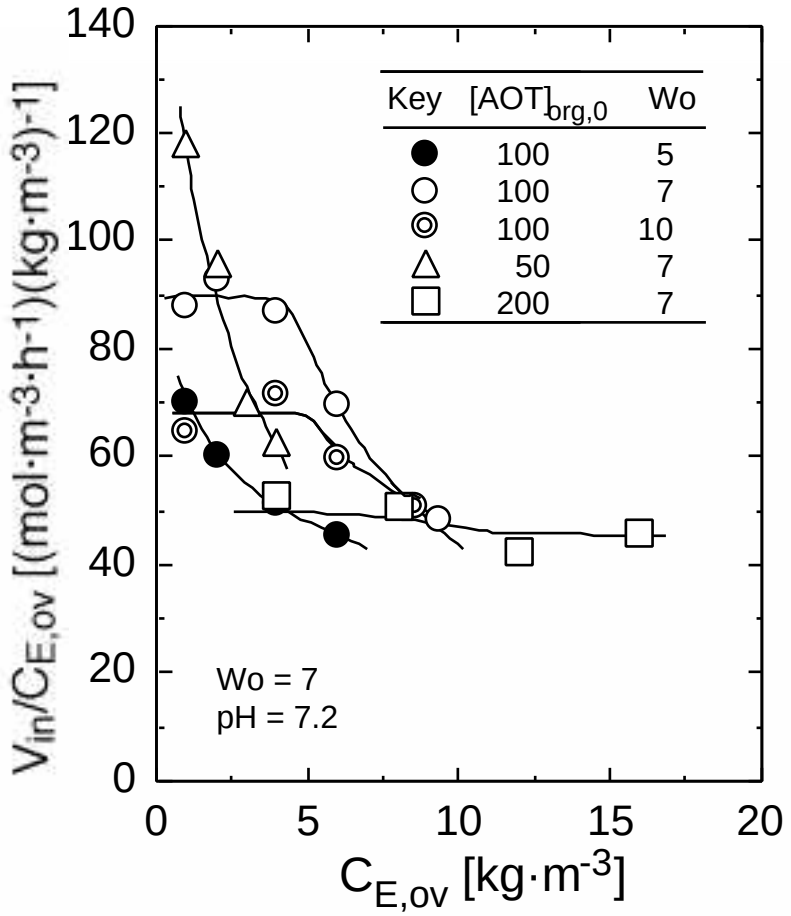


Fig. 2.6. Effect of lipase concentration on initial activity

The decrease in the activity with increasing in lipase concentration indicates that not all of lipase injected acts efficiently in the micelles. Proteins are solubilized in micelles larger than the protein size (Kuboi *et al*, 1990a). The number of micelles effective for protein solubilization depends on the concentration of AOT and the micellar size, which is correlated with the W_o value. The maximum amount of protein solubilized into the reverse micelles is determined by the concentration of micelles effective for protein solubilization (Kuboi *et al.*, 1990a, Yamada *et al*., 1994b). An increase in the concentration of lipase injected would result in a deficiency of the number of micelles effective for lipase solubilization. Excess lipase which is not solubilized completely into the micelles is considered to be inactivated. Consequently, the apparent activity decreased as the concentration of lipase injected was increased. Hence, the maximum concentration of lipase keeping constant activity is considered to be correlated with the effective micellar concentration for protein solubilization.

The complex effects of the W_o value and the concentrations of AOT and lipase on the initial activity are considered to be due to both the solubilization of lipase into the micelles with an active form and the activity behavior of the lipase in the micelles.

3.1.4 Stability of lipase in reverse micelles

The reverse micellar solution containing lipase without substrate was incubated at 25˚C, and the residual activity after incubation was measured. The effect of W_o on lipase stability is shown in **Fig. 2.7**. Lipase in the bulk buffer solution was very stable, retaining over 90% of its initial activity after 24 h. At W_o = 4.6, the lipase in the reverse micelles was as stable as that in the aqueous solution, but at higher W_o values the lipase stability decreased with increases in the W_o value. At W_o = 12, the lipase activity dropped particularly rapidly. Han and Rhee reported the existence of an optimum value of W_o for lipase stability

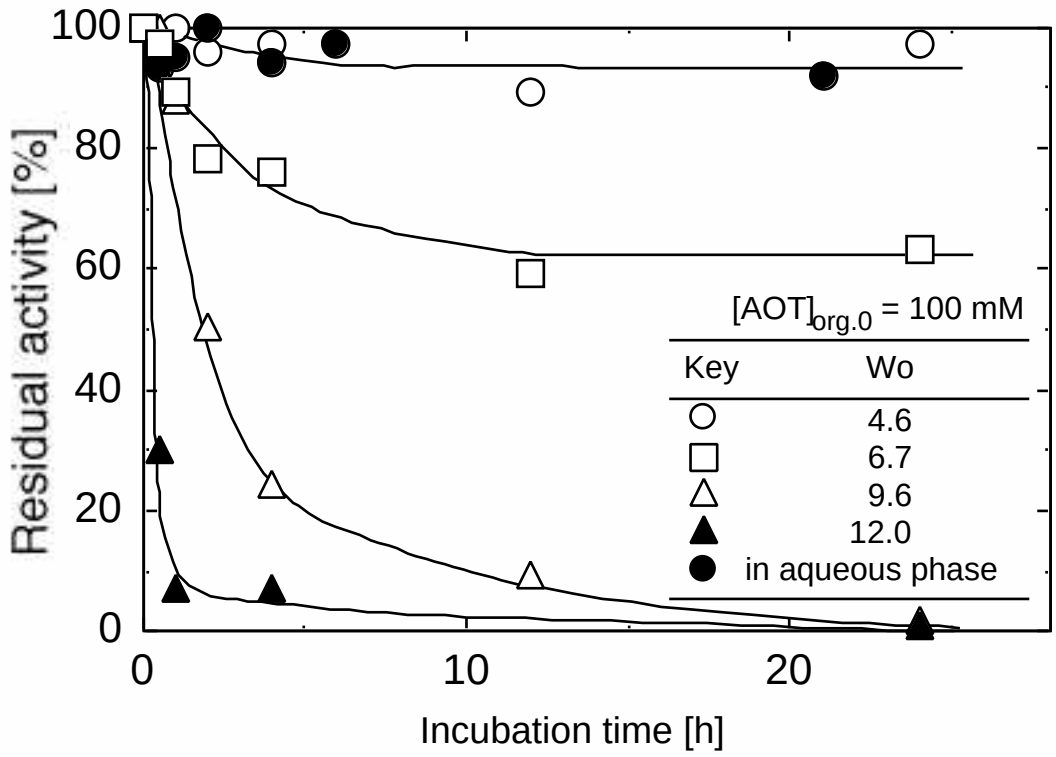


Fig. 2.7. Effect of W_O on lipase stability in reverse micelles at $C_{E,ov}$ = 0.4 kg/m^3, C_{AOT} = 100 mM and pH 7.2.

in the reverse micelles, and found that enzyme stability at W_O values higher than 9 was very low (Han and Rhee, 1986). Lipase stability thus decreases with increasing W_O, which determines the size of the micelles. It may be difficult for lipase solubilized in small micelles at a low W_O to cause structural change due to low motility in the relatively limited space of the micellar water pool.

The effect of the AOT concentration on the lipase stability in the micelles is shown in **Fig. 2.8**. The denaturation of lipase in the reverse micelles is accelerated by increasing the AOT concentration. Since an increase in the AOT concentration corresponding to the concentration of the micelle droplet causes an increase in the collisions between micelle droplets, the lipase adsorbed at the micellar interface is considered to interact with the AOT molecule due to the disorder of the micellar interface and to be denatured by the

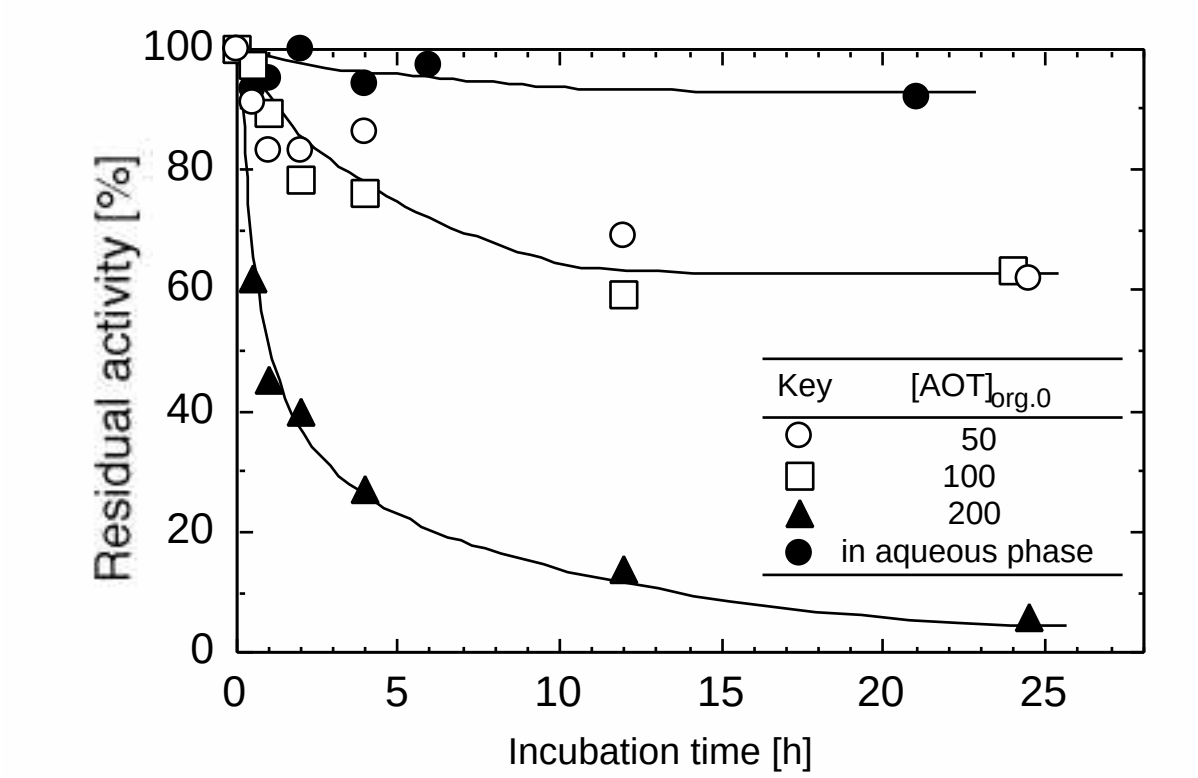


Fig. 2.8. Effect of concentration of AOT on lipase stability in reverse micelles at $C_{E,ov}$ = 0.4 kg/m^3, W_O = 7 and pH 7.2.

interaction with the AOT molecule.

The effects of the micellar conditions, especially the W_O value and the AOT concentration, on the lipase stability in the reverse micelles are somewhat different from those on the lipase activity. Though the highest activity was observed at about W_O = 7, the stability was low at this W_O value. The lipase reaction requires a higher W_O value than that suitable for high lipase stability in the micelles. Excessive high W_O value and AOT concentrations are, however, not good for either the activity or the stability of lipase. The interaction of the active site of lipase with the interface of the micelles and the steric interaction between the lipase molecule and the micelle is considered to play an important role in both the activity and stability of the lipase in the micelles.

3.2 Kinetic analysis of the lipase hydrolysis by the interfacial reaction model

3.2.1 Interfacial reaction model

Since the hydrolysis of olive oil is considered to proceed at the interface, between the organic and aqueous phases, the reaction model based on the interfacial reaction between lipase adsorbed at the interface and olive oil in the organic phase is proposed as shown in **Fig. 2.9.**

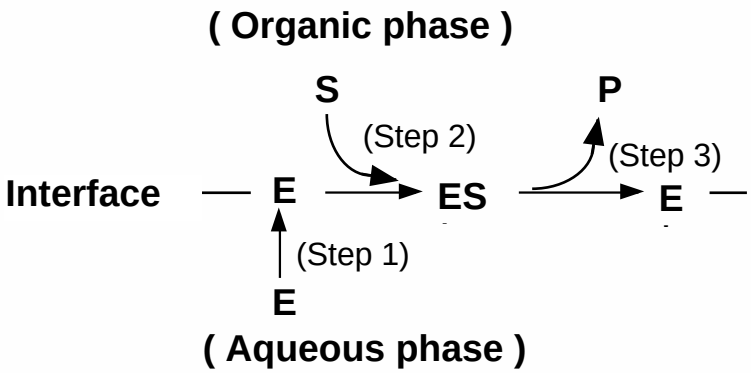
Step 1. Adsorption of lipase, E, at the interface:

$$E + \sigma = E^* \quad ; K_E \tag{2.1}$$

where σ denotes the active adsorption point on the interface and E* is the species of E adsorbed on the interface.

Step 2. Reaction between olive oil, S, in the organic phase and adsorbed lipase, E*, to form the complex ES* at the interface:

$$E^* + S = ES^* \quad ; K_M \tag{2.2}$$



$$E + \sigma \rightleftharpoons E^* \quad ; K_E \text{ (Step 1)}$$

$$E^* + S \rightleftharpoons ES^* \quad ; K_M \text{ (Step 2)}$$

$$ES^* \longrightarrow E^* + P \quad ; k_p \text{ (Step 3)}$$

Fig. 2.9. Interfacial reaction model

Step 3. Decomposition of the complex to the products, P, and enzyme, and desorption of the products into the organic phase:

$$ES^* = E^* + P^* \quad (2.3a)$$

$$E^* + P^* = E^* + P \quad (2.3b)$$

As explained later, the amount of adsorbed substrate is reasoned to be negligible compared with that of enzyme. Thus, the amount of adsorbed products, P*, is assumed to be negligible compared with that of enzyme, E*. To simplify the calculation, the reactions represented by Eqs. (2.3a) and (2.3b) can be summarized as follows:

$$ES^* = E^* + P \quad ; K_{ES} \quad (2.3)$$

The equilibrium constants for the above reactions, K_E, K_M and K_{ES}, are defined as follows;

$$K_E = \theta_E / \{C_E(1 - \theta)\} \quad (2.4)$$

$$K_M = \theta_{ES} / \{\theta_E C_S\} \quad (2.5)$$

$$K_{ES} = C_P \theta_E / \theta_{ES} \quad (2.6)$$

where θ_i and θ are the fractions of the areas occupied by the adsorbed species i and all of the adsorbed species, respectively. If the reaction rates of step 1 and 2 are very rapid compared with that of step 3, the rate of step 3 controls the interfacial reaction rate. Since both steps 1 and 2 are assumed to be at equilibrium, the fractions of the interfacial area occupied adsorbed lipase and the complex are derived from Eqs. (2.4) and (2.5) as follows:

$$\theta_E = K_E C_E / \{1 + K_E C_E + K_E K_M C_E C_S\} \quad (2.7)$$

$$\theta_{ES} = K_E K_M C_E C_S / \{1 + K_E C_E + K_E K_M C_E C_S\} \quad (2.8)$$

The total concentration of lipase, C_{E0}, is kept constant during the reaction and is given by a summation of the concentration of free lipase in the aqueous solution and adsorbed lipase and the complex at the interface.

$$C_{E0} = C_E + a \{ \theta_E / S_E + \theta_{ES} / S_{ES} \} \quad (2.9)$$

where a is a specific area [= (S_{int}/V_{aq}), S_E and S_{ES} are the interfacial are the interfacial areas occupied by unit weight of lipase and the complex. To simplify the treatment, the value of ($K_E C_E + K_E K_M C_E C_S$) is assumed to be negligible compared with 1.0. If the value of ($K_E C_E + K_E K_M C_E C_S$) were assumed to be much greater than 1.0, the formation rate of fatty acids obtained would be independent of C_{E0}, which could not interpreted the experimental results in this study. Consequently, Eqs.(2.7) and (2.8) are expressed as follows:

$$\theta_E = K_E C_E \tag{2.10}$$

$$\theta_{ES} = K_E K_M C_E C_S \tag{2.11}$$

In addition, S_{ES} is assumed to be equal to S_E. The concentration of lipase in the aqueous phase, C_E, is given as follows:

$$C_E = C_{E0} / \{1+a'K_E (1 + K_M C_S)\} \tag{2.12}$$

where a' is (a/S_E). The initial formation rate of free fatty acids per unit inter facial area, J_A, was derived by using Eqs. (2.11) and (2.12) as follows;

$$J_A = k_p \theta_{ES} \tag{2.13}$$

$$= k_P K_E K_M C_{S,org} C_{E,aq} / \{ 1 + a'K_E + a'K_E K_M C_{S,org} \} \tag{2.14}$$

$$= k_P C_{S,org} C_{E,aq} / \{K'_m + a'C_{S,org} \} \tag{2.15}$$

$$K'_m = (1 + a'K_E) / K_E K_M \tag{2.16}$$

where k_p is the desorption rate constant of the product per unit interfacial area.

The hydrolysis rates of olive oil in a two phase system using Lewis-type transfer cell were measured by changing the concentrations of lipase and olive oil and also specific area, a. The results were analyzed in detail based on this rate equation. The lipase catalyzed hydrolysis rate was confirmed to be interpreted by this reaction model. The kinetic parameters were determined from the plots based on Eq.(2.15). The analysis of hydrolysis rate using this reaction model can evaluate both the interaction of lipase with the interface and the activity at the interface. The hydrolysis rate in an O/W emulsion systems

consisted with anionic surfactant SDS and polyvinyl alcohol as dispersion stabilizer was also interpreted by this reaction model and these kinetic parameters were determined.

3.2.2 Analysis of hydrolysis reaction based on the model

The hydrolysis rate of olive oil in the reverse micellar system was analyzed by the interfacial reaction model proposed. The results under the condition that V_{in} was proportional to the enzyme concentrations were used for the kinetic analysis because the inactivation of lipase in the solubilization step into the micelles was negligible. The diameter and the interfacial area of reverse micelles were calculated according to the literature (Kuboi *et al*., 1990a). $C_{E,aq}$ in Eq. (2.15) corresponds to lipase concentration in the micellar water pool. J_A was calculated by using the values of V_{in} and a.

By taking the reciprocal of Eq. (2.15), the following relation was obtained:

$$C_{S0}/J_A = K'_m / (k_p C_{E,wp}) + \{ a' / (k_P C_{E,wp}) \} C_{S0} \qquad (2.17)$$

A plot of C_{S0}/J_A against C_{S0} is shown in **Fig. 2.10**. A straight line was obtained for each AOT concentration and W_O value, indicating that the hydrolysis rate in the reverse micellar system is also explained by Eq. (2.7).

The values of k_P and K'_m were calculated from the slope and intercept of the straight line, respectively. The value of S_E was taken from the isothermal adsorption results. The values of K_M and K_E could not be evaluated separately, because the value of a' could not change at a fixed W_O value and fixed AOT concentration in this experiments. The value of a' is independent of the AOT concentration at a fixed W_O, because the specific area of an aqueous droplet, (S_T / V_{aq}), is constant at a fixed aqueous droplet diameter regardless of the AOT concentration. The values obtained are summarized in **Table 2.1** together with

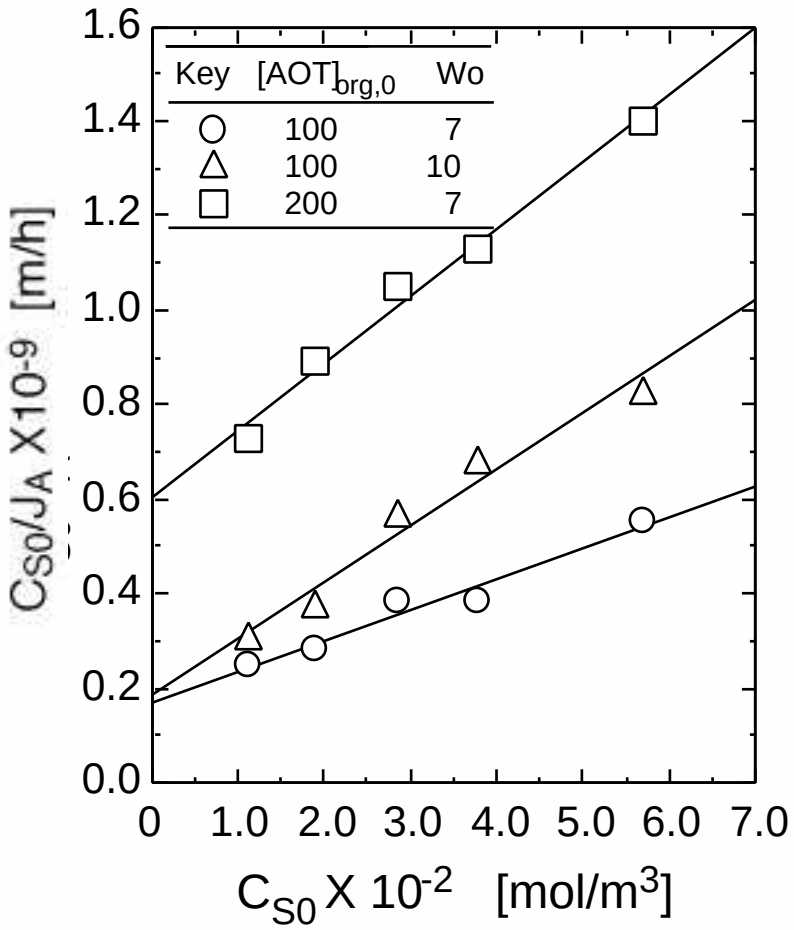


Fig. 2.10. Relationship between C_{S0}/J_A and C_{S0}.

those of two phase and O/W emulsion systems. The solid lines in Fig. 2.1 are the results calculated by Eq. (2.15) using the constants in Table 2.1; they are in good agreement with the experimental results.

At W_O = 7, the values of k_P were independent of the concentration of AOT. The value of K'_m at C_{AOT} = 200 mM was higher than that at C_{AOT} = 100 mM. The adsorption behavior of the enzyme at the interface hardly varies at a fixed water pool diameter. Hence, K_E is independent of the AOT concentration and K_M is affected by the AOT concentration. K_M at C_{AOT} = 200 mM is about 0.6 times lower then that at C_{AOT} = 100 mM from Eq. (2.16). This suggests that the reaction is inhibited by the AOT molecule and/or the distribution of the substrate in the reaction zone is affected by the micellar concentration and the

TABLE 2.1. Kinetic parameters of hydrolysis reaction

System	C_{AOT} [mM]	W_o [–]	K'_m [kg•mol/m^6]	K_E [m^3/kg]	K_M [m^3/mol]	k_P [mol/m^2•h]
Reverse micelles	100	7	8.35×10^{10}	–	–	16.7
	100	10	2.66×10^{8}	–	–	9.94
	200	7	1.41×10^{11}	–	–	15.2
O/W Emulsion[a]	–	–	–	5.3×10^{-5}	1.8×10^{-4}	54.3
Two phase	–	–	–	65.7[b]	9.6×10^{-4}	3.58×10^{-1}

[a] SDS = 0.29 wt%; PVA = 0.5 wt%; average diameter of droplet = 3.4 μm.

[b] observed from an isothermal adsorption experiment.

total interfacial area.

In the O/W emulsion system whose interface is covered with surfactant layer, a very small value of K_E and the same order of K_M compared with the two-phase system was obtained. The small K_E in the emulsion system is due to the electrostatic repulsion between lipase having negative charge (pI = 4.3) and the interface which is covered with anionic surfactant.

From a definition of K'_m and positive values of parameters, K_M must be larger than (a'/K'_m). Hence, it is possible to estimate the values of K_M and K_E in the reverse micelles as follows; K_M is larger than that of the emulsion system and strongly affected by *Wo* value and is large value at high *Wo*. Furthermore, K_E in the reverse micellar system is smaller value compared with the O/W emulsion system. Small value of K_E in the reverse micellar system suggests that the interaction between lipase and the micelles is very low. This suggestion agrees with the result in the solubilization effect of lipase on the percolation of the reverse micellar solution.

The values of k_P in the reverse micellar system are of the same order as that obtained in the emulsion system, and the k_P values in both systems are about 10^2 times that in the transfer cell. Desorption of the product, which is free fatty acid having anionic carboxylic group, from the interface may be accelerated by electrostatic repulsive force with the anionic surfactant at the interface.

4. SUMMARY

In order to study the interaction of proteins with the interface of reverse micelles, lipase-catalyzed hydrolysis of olive oil was carried out in the AOT reverse micellar system. The results were compared with organic-aqueous two

phases system in Lewis type transfer cell and with emulsion system containing SDS. The maximum activity of lipase in the reverse micellar systems was obtained between pH 6.3 and 7.3. This pH range is almost agreed with that of high ß value of percolation. The activity is decreased rapidly by lowering the reaction pH in both AOT reverse micellar and SDS emulsion systems. Lipase is readily inactivated by increasing the electrostatic interaction with anionic surfactants.

The optimum water content for initial activity of lipase is influenced by the concentrations of both AOT and lipase. The complex effects of the W_O value and the concentrations of AOT and lipase on the activity are considered to be due to both the solubilization of lipase into the micelles with an active form and the activity behavior of the lipase in the micelles. The lipase in the reverse micelles is stable at W_O = 4.6. The stability of the lipase in the micelles decreases with increase in the value of W_O and in the concentration of AOT. The structural change of lipase is considered to be protected by the repulsion from the micellar interface around the protein in the smaller micelles. A decrease in stability of the micellar interface by increasing the micellar size and in the micellar-micellar collisions may enhance the inactivation of lipase by the hydrophobic interaction of AOT.

The hydrolysis rate in the reverse micellar system was interpreted by the interfacial reaction model based on the interfacial reaction between lipase adsorbed at the interface and the substrate in the organic phase. At the pH range for high activity, the interaction between lipase and the micellar interface is reduced by the electrostatic repulsion with anionic surfactant adsorbed at the interface. This reduced interaction suggested from the stability of lipase and the stabilization of lipase in the reverse micelles by the electrostatic repulsion is well agreed with the knowledge from the percolation behavior of the micellar

solution solubilized lipase in the previous section.

The utilization of enzymatic reaction of lipase in the reverse micellar system is demonstrated to be used effectively for the evaluation and control of the electrostatic and hydrophobic interactions of lipase with the micellar interface and the surfactant molecule. The interaction between enzymes and the micelles is an important factor in both the activity and the stability of enzyme in the reverse micelles and its appropriate control is necessary in the development of effective bio-separation and bio-reaction processes using reverse micellar systems.

Part III

Development of Novel Reverse Micellar Bio-separation Process for Large Molecular Weight and Oligomeric Proteins

1. INTRODUCTION

The electrostatic interaction is most effective for the reverse micellar extraction of proteins using ionic surfactants (Shioi *et al.*, 1996). However, as studied in Part I and II, the electrostatic interaction is likely to cause a structural change and inactivation of proteins. Though the interaction between proteins and reverse micelles is necessary for the extraction using the phase transfer method, large molecular weight and oligomeric proteins are likely to be denatured and inactivated by the interaction with the reverse micelles. There are several methods to control this interaction. Firstly, the injection method can be utilized to solubilize various proteins into reverse micelles regardless of pH. It is actually possible to solubilize negatively charged proteins in AOT reverse micelles keeping repulsive interaction between them. However, only a very few limited works have been made using the injection method for the extraction and separation of proteins by reverse micelles (Giovenco *et al.*, 1987; Kuboi *et al.*, 1990b).

Secondly, the electrostatic bridging effect of divalent cation can be considered. The specific effect of salt type on the reverse micellar protein extraction has been reported by a few investigators (Adachi and Harada, 1994; Nishiki *et al.*, 1993; Leser and Luisi, 1990). Only the low molecular weight proteins were studied and further its mechanism is still not clear. The study on

the adsorption of proteins onto negatively charged polymer latex suggested the electrostatic bridging effect of divalent cation between negatively charged surfaces of latex and protein (Shirahama *et al*.;1989). The divalent cation effect between protein and AOT is expected for the reverse micellar extraction of proteins.

Thirdly, the interaction between proteins and surfactant, which is essential for the extraction into the micelles, should be eliminated effectively in the back-extraction process. The control methods of the formation and the disappearance of the micellar-protein complex and the reverse micelles by the pH change and the additions of either salts or alcohols in the recovery aqueous phase have been proposed as a conventional process. However, the activity recovery of large molecular weight and oligomeric enzymes and proteins is low because of the interactions can not be eliminated effectively in the previous conventional processes. One of novel approaches for the solution of these problems is the control of the formation of reverse micelles. Some studies have been reported on the control of the formation of reverse micelles by pH (Goto *et al.*, 1990, 1995, 1997, Kinugasa *et al.*, 1994), pressure (Philips *et al.*, 1991) and temperatures (Dekker *et al.*, 1991) for developing an effective extraction process of protein.

In this Part, on the bases of these knowledge, three novel separation method of large molecular weight and oligomeric proteins using AOT based reverse micelles have been proposed.

Firstly, in order to suppress the unfavorable interaction between proteins and the micelles during the extraction process, the electrostatically and sterically repulsive back-extraction combined with the solubilization by injection method with keeping electrostatic repulsion between proteins and AOT have been proposed. Since the interaction between proteins and ionic surfactants is regulated by pH values and salt concentrations, these effects both in the

injection and back-extraction steps was investigated in detail. Furthermore, the separation of ß-galactosidase from crude extracts of *E. coli* cells was carried out as an application of the present method.

Secondly, modification of reverse micellar interface was attempted by using divalent cation, such as Ca^{2+} or Mg^{2+}, as a salt in the aqueous phase in the conventional AOT extraction system. The effect of pH on the extraction of various molecular weight proteins was investigated and the extraction behavior of BSA as a typical example was studied.

Thirdly, the mixed system of AOT and long chain alkyl amines such as tri-*n*-octylamine and di(2-ethylhexyl) amine was used as a reverse micellar solution to control the electrostatic interactions both of AOT-amines and AOT-proteins. The formation of the reverse micelles in the mixed micellar system was studied from the extraction of water to the organic micellar solution at various pH values. On the basis of the results in the previous section, extraction of large molecular weight proteins, such as BSA and hemoglobin, was carried out using the mixed micellar solution, in which Ca^{2+} ion was added to mediate the interaction between AOT and proteins. Then the back-extraction was carried out by pH change alone causing destruction of the micelles by AOT-amine interaction. Furthermore the extraction and back-extraction of small protein, lysozyme, was also carried out using this mixed micellar system.

2. EXPERIMENTAL

2.1. Materials

AOT from Nacalai Tesque Co. was used without further purification. AOT was dissolved in isooctane at desired concentration.Tri-*n*-octylamine and di(2-ethylhexyl) amine, which were used as additives for the modification of the

reverse micellar system, were obtained from Koei Chemical and Wako Pure Chemical, respectively. Long chain alkyl amines were solubilized in the AOT isooctane solution. Cytochrome-c (CYT) from bovine heart, α−chymotrypsinogen (CHYN) and trypsin (TRYP) from bovine pancreas, lipase (LIP) from *Candida cylindracea* , and alcohol dehydrogenase (ADH) from bakers yeast was purchased from Sigma Chemical Co.Lysozyme (LYS) from egg white was obtained from Wako Pure Chemical Industries Co. Acetic acid-sodium acetate (pH 3-6), dimethyl gulutamate-NaOH (pH 5-8), $Na_2B_4O_7$-HCl or NaOH (pH 8-11), Tris-HCl (pH 7.2-9), glycine-NaOH (pH 8.5-11) and Na_2HPO_4-NaOH (pH 11-12) was used as buffer.

2.2. Back-extraction of proteins solubilized by the injection method.

Proteins were solubilized into 200 mM AOT/isooctane solution by the injection method; the feed buffer solution (0.72 ml) dissolving protein (1-5 mg/ml) was injected into 10 ml of AOT/isooctane solution, and shaken vigorously until a clear solution was obtained. Initial *Wo* of the prepared reverse micellar solution, Wo_{inj}, was kept at 20 in all experiments in order to keep constant *Wo* values in both solubilization and back-extraction steps. No salts were added in the injected feed solution to prevent non-specific hydrophobic interaction between proteins and AOT and to keep a large value of Wo_{inj}. In the freshly prepared reverse micellar solution for all proteins used, aggregation or turbidity of micellar solution was not observed for at least one day.

Back-extraction of the protein from the reverse micelles was carried out by contacting 5ml of the reverse micellar solution containing proteins with 5 ml of buffer solution with KCl for 3 h, and then the solution was centrifuged at 5,000 rpm for 15 minutes. The pH value in the feed solution injected into reverse micelles, pH_{inj}, and the pH value, pH_{aq}, and the KCl concentration in

reverse micelles, pH_{inj}, and the pH value, pH_{aq}, and the KCl concentration in the aqueous solution used for the back-extraction were varied in the experiments.

2.3 Separation of ß-galactosidase from *E. coli* cells by the back-extraction combined with the injection solubilization

The strains of *E. coli* ML308, which constitutively produces ß-galactosidase, was grown in the modified M9 medium containing 20 g/l glycerol at 310 K (Gray *et al*., 1973). Cells were harvested by centrifugation at the end of the logarithmic growth phase and suspended in 10 mM Tris-HCl(pH=7.2) buffer, and disrupted by ultrasonic wave irradiation. The suspension was centrifuged at 16,000 rpm for 20 min, and the supernatant was used as the extracts for the separation experiments. The extract from cells (0.216 ml) was solubilized in 200 mM AOT/isooctane solution (3 ml) at Wo=20 and the resulting micellar solution was contacted with 3 ml of Tris-HCl buffer, pH 7.2, containing 0.1 M KCl. After back extraction, activity of the enzyme and concentration of total soluble proteins in the aqueous phase were measured.

2.4. Extraction of proteins by the phase transfer method

Extraction of water and proteins was carried out by the following method. Same volumes of the organic and the aqueous solutions were placed in the screw capped sample tube. The two phases were dispersed completely by a magnetic stirrer for 30 min. at 298 K. After the mixing, the solution was separated into two phases by the centrifugation at 3,500 rpm for 30 min. Back-extraction of proteins from the micellar phase was carried out by contacting the organic phase extracted protein with a new aqueous phase as the same manner for the forward extraction.

2.6. Measurement

The concentration of all proteins including heme-proteins in the organic and the aqueous phases was determined by spectrophotometry at 280 nm with Hitachi UV 3200. Water content in the organic phase, *Wo*, was defined as molar ratio of water to AOT in the feed organic phase, and measured by the Karl-Fisher titration. The concentration of Ca^{2+} in the aqueous phase before and after extraction was determined by chelatometric titration. Circular dichromism (CD) spectra measurements were carried out with a Jasco spectropolarimeter (Model J-720) at room temperature.

3. RESULTS AND DISCUSSION

3.1. Back-extraction of proteins combined with the solubilization by the injection method

3.1.1. Effect of pH on the back-extraction of proteins

All proteins were easily and completely solubilized into the AOT reverse micellar solution without any precipitate at *Wo*=20 and various pH_{inj} from 4 through 10. The transparent organic solutions were used for the following back-extraction. The procedure of this method is shown schematically in **Fig. 3.1**. The

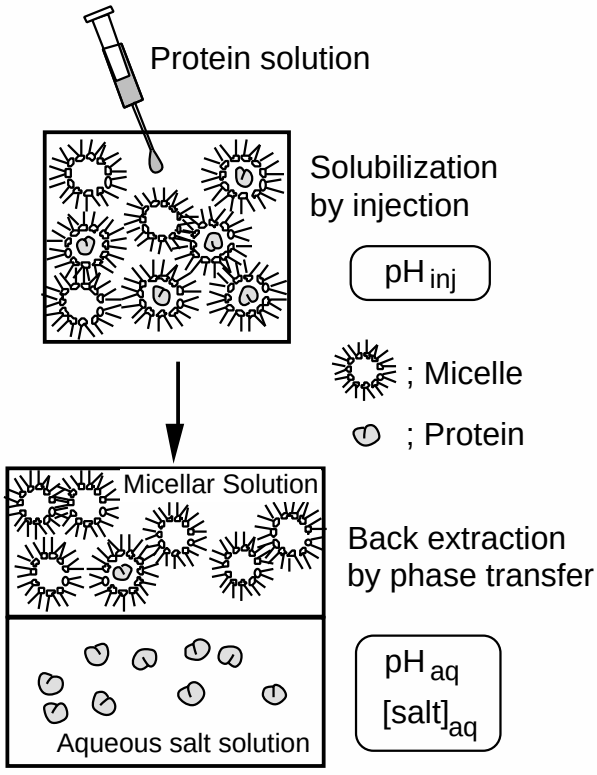


Fig. 3.1. Schematic representation of the back-extraction combined with the solubilization by the injection method.

of this method is shown schematically in **Fig. 3.1**. The back-extraction of proteins solubilized into reverse micelles at various pH_{inj} was carried out at the same pH_{aq} as pH_{inj} (pH_{aq}=pH_{inj}) and 0.1 M KCl. The results of the back-extraction and relative activity of the enzyme back-extracted are shown in **Figs. 3.2** and **3.3**, respectively. The fraction of the protein back-extracted to the aqueous phase, E_b, and relative residual activity of the enzyme, *RSA*, are plotted against the pH deviation from pI of the protein, respectively. All large molecular weight proteins solubilized at pH higher than the pI of the proteins were back-extracted effectively to the new aqueous phase with the same pH as shown in Fig. 3.2 A and B. The activity of enzyme back-extracted to the aqueous phase is very high at pH higher than their pI. In the pH range at or below the pI, the proteins were difficult to be back-extracted to the aqueous phase.

Proteins which are difficult to be extracted and back-extracted by the phase transfer method in Part I, such as catalase, ß-galactosidase, and lipase, is successfully back-extracted with high activity yield at pH higher than their pI. Both E_b and *RSA* of catalase above (pH-pI)=3 decreased with pH, this is probably due to the instability of catalase in alkaline pH. In fact, catalase in the bulk aqueous solution under similar conditions rapidly lost its activity. E_b of lipase increased with an increase in the pH value. *RSA* of lipase back-extracted was, however, strongly affected by pH values. No inactivation of lipase was observed at around 1.5 of (pH-pI) during solubilization and following back-extraction processes. This pH dependency of *RSA* is very similar with the pH profile of lipase activity in the reverse micelles. Furthermore, hemoglobin, which cold absolutely not be extracted by the phase transfer method, was also back-extracted at pH above pI.

On the other hand, back-extraction of small proteins, shown in Fig. 3.2 C, also increased with pH, however, E_b attained was comparatively low compared

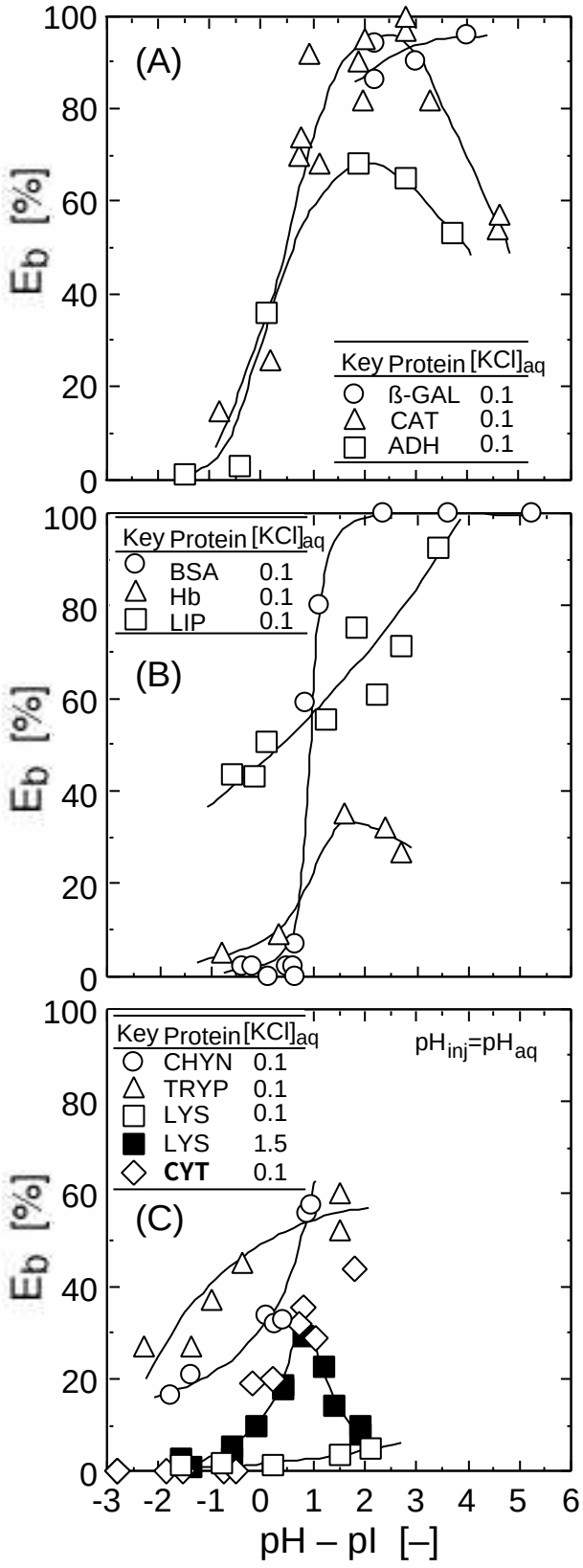


Fig. 3.2 Effect of pH on the back-extraction of proteins solubilized by the injection method with keeping $pH_{aq}=pH_{inj}$.

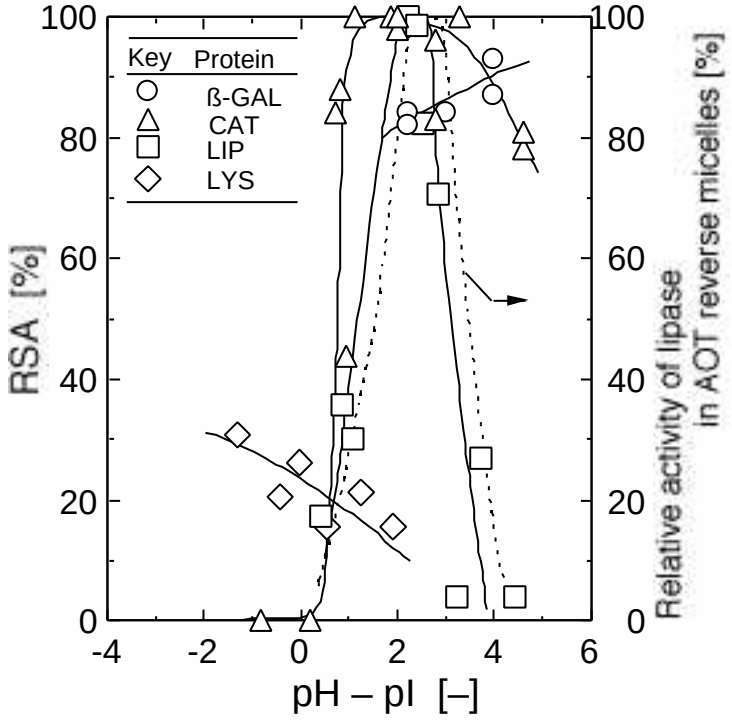


Fig. 3.3 Effect of pH on the relative residual activity of enzyme back-extracted under the same condition in Fig. 3.2.

with that of large molecular weight one and their dependencies of each small protein were different. Trypsin and α-chymotrypsinogen were back-extracted even at pH lower than their pI, and E_b increased monotonously with pH. Cytochrome c was not back-extracted in the pH range lower than the pI and E_b increased with pH. Lysozyme could not be back-extracted effectively at 0.1 M KCl. By increasing in KCl concentration up to 1.5 M, lysozyme could be back-extracted at pH higher than the pI. In spite of low E_b, *RSA* of lysozyme was relatively high at pH lower than the pI. This means lysozyme is hard to be inactivated by electrostatic interaction with AOT. *RSA*, however, decreased with increasing pH. This decrease in *RSA* is considered to be due to instability of lysozyme in high pH range (Kinugasa *et al.*, 1992).

Usually, large molecular weight proteins are difficult to be solubilized

into the reverse micelles and tend to be precipitated in the phase transfer method. By using the present method, all proteins could be solubilized into the reverse micelles to form a transparent clear solution without any precipitate, and then could be back-extracted to the aqueous phase at pH range higher than the pI of protein. The above results on effective back-extraction of large molecular weight proteins can be rationally explained by the electrostatic repulsion between the solubilized protein and the anionic surfactant, AOT, and also the steric exclusion from the micelles. If proteins carry their negative charge evenly on their surface at pH above the pI, proteins may be forced to be in the center of the micro water pool in the micelles and thus are easily back-extracted by the steric exclusion effect from the reverse micelles and the electrostatic repulsion between protein and AOT. In contrast, small proteins could not be back-extracted completely at pH over than the pI. This is considered that the electrostatic repulsion between small protein and the micelles dose not work effectively because of the micellar size at *Wo*=20 is too large compared with that of small protein and the protein has enough distance from the negatively charged inner micellar wall. The back-extraction of small proteins requires high salt concentration to make small reverse micelles.

3.1.2 Effects of pH_{inj} and pH_{aq} on the back-extraction

The effects of pH both on the back-extraction step and on the solubilization steps were further studied separately. Catalase solubilized at pH_{inj}=4.9 or 8.2 was back-extracted at various pH_{aq} values as shown in **Fig. 3.4**. When catalase was solubilized at pH_{inj}=8.2, catalase was back-extracted completely at pH_{aq} higher than pI and catalase activity back-extracted was almost as high as the injected one (*RSA*≈90%). At pH_{aq} lower than pI, E_b was lower than that obtained with pH_{aq} higher than pI and catalase activity was

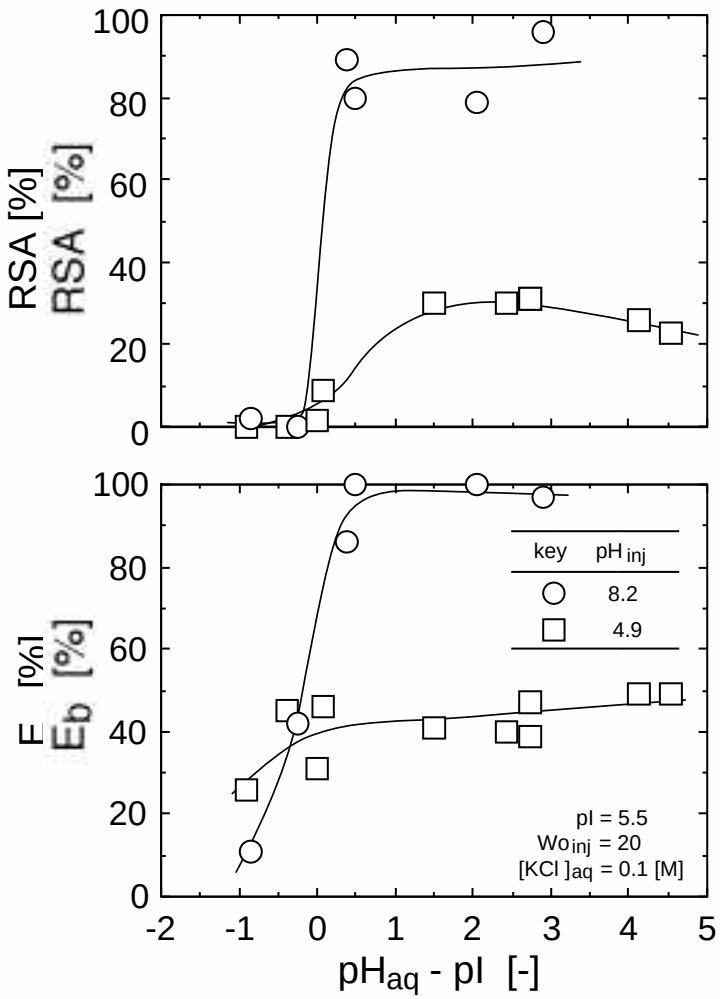


Fig. 3.4. Effect of pH_{aq} on the back-extraction of catalase solubilized at pH_{inj}=4.9 or 8.5.

completely lost. It is clear that catalase is back-extracted by an increase in the electrostatic repulsion between catalase and AOT with rising of the pH_{aq} value.

When catalase was solubilized at pH_{inj}=4.9, catalase was difficult to be back-extracted even at pH_{aq} higher than the pI, and E_b and *RSA* were lower than those at pH_{inj}=8.2. This indicates that catalase solubilized at pH_{inj}=4.9 (pH_{inj}<pI) is irreversibly denatured in the solubilization step or in the reverse micelles in the face of electrostatic interaction with AOT. It also indicates why the extracted proteins have often been denatured and difficult to be back-extracted when using the phase transfer method. In the case of the phase transfer

method, extraction of catalase is achieved at pH less than the pI, and thus catalase is considered to be inactivated by electrostatic interaction in the extraction step.

In order to clarify the effect of pH_{inj}, the protein solubilized at various pH_{inj} was back-extracted under fixed condition of pH_{aq}. The results of the back-extraction and residual activity of the enzyme back-extracted are shown in **Figs. 3.5** and **3.6**, respectively. At pH_{inj} higher than the pI, back-extraction of all large molecular weight proteins was efficient and the activity loss was small. The

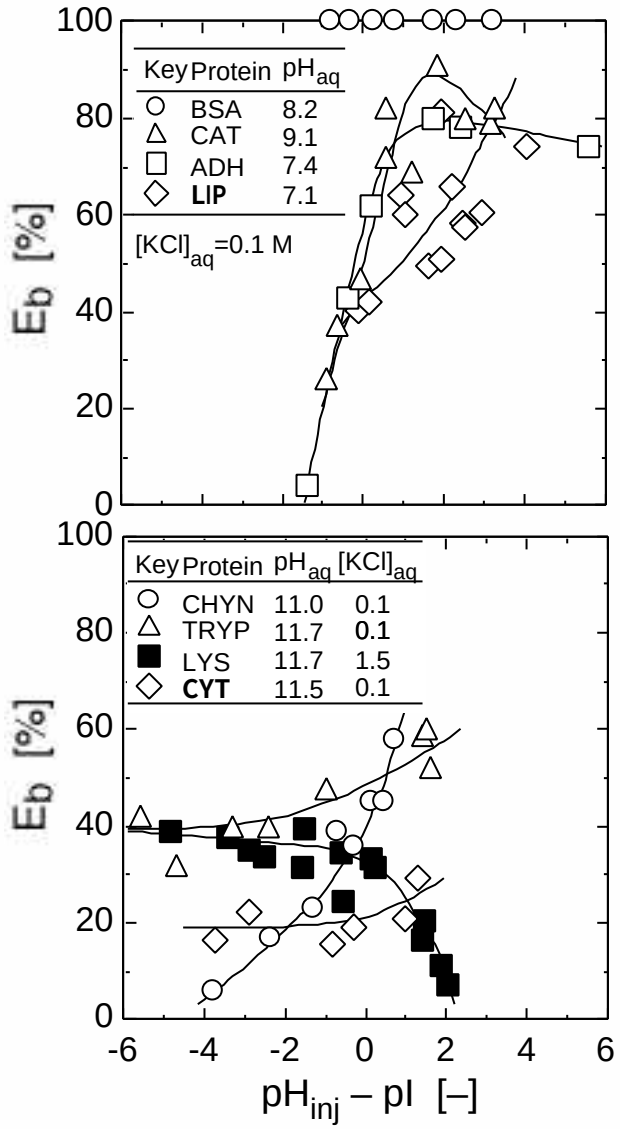


Fig.3.5 Effect of pH_{inj} on the back-extraction of various proteins under fixed condition of pH_{aq}.

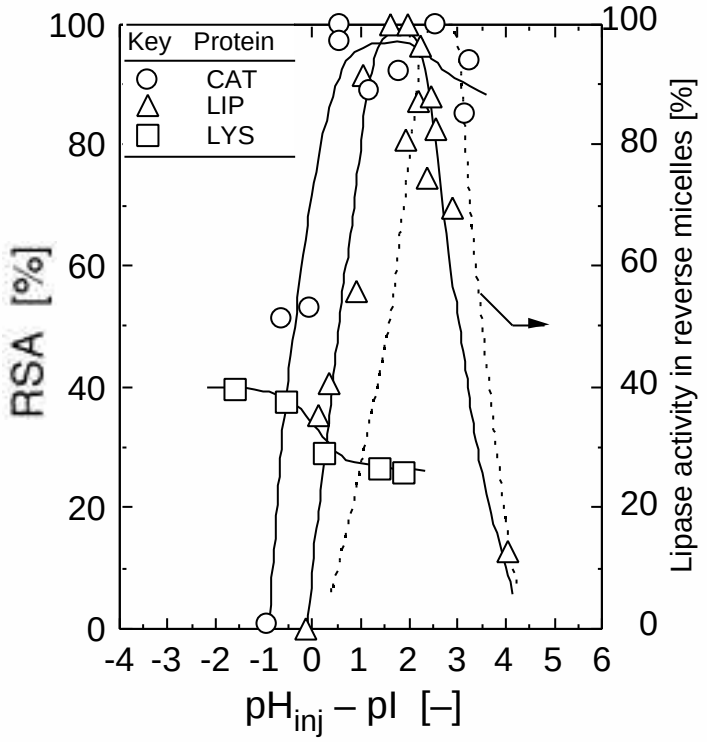


Fig. 3.6 Effect of pH_{inj} on the relative residual activity of enzyme back-extracted under the same condition in Fig. 3.5.

The electrostatic interaction between the proteins and the micelles is reduced by rising pH_{inj}, and then proteins retain its native structure in the solubilization step and in the water pool of the micelles. E_b of the proteins except for BSA decreased with decreasing pH_{inj}. These proteins is considered to be denatured irreversibly by the electrostatic interaction in the solubilization at pH_{inj} below their pI. In contrast, BSA was completely back-extracted to the aqueous phase independent of the pH_{inj}. This suggests that BSA solubilized even at pH_{inj} lower than the pI is not denatured both in the solubilization step and standing condition in the micelles, probably because that it is a monomeric stable protein.

In the cases of small proteins except for α-chymotrypsinogen, the large decrease in E_b was not observed at pH_{inj} less than their pI. It shows that these proteins does not cause irreversibly and strongly the structural change by the electrostatic interaction with the reverse micelles. In practice, *RSA* of lysozyme

solubilized at pH_{inj} lower than pI was relatively high. These small proteins are expected to be extracted successfully by the phase transfer method and have been actually reported (Kinugasa *et al*., 1992). The above effect of pH_{inj} on the back-extraction may also depend on the nature of proteins.

3.1.3. Effect of salt concentration in the aqueous phase on the back-extraction

The back-extraction of protein from micelles, while maintaining $pH_{inj}=pH_{aq}$, was carried out at various concentrations of KCl in the aqueous phase. The effects of KCl concentration on the back-extraction of proteins at pH higher than their pI are shown in **Fig.3.7**. Back-extraction of large molecular weight protein increased or was constant with increasing in KCl concentration. On the other hand, back-extraction of low molecular weight proteins tend to increase by increasing in KCl concentration. Smaller size of the micelles is considered to be need for effective back-extraction of small proteins because of the electrostatic repulsion between AOT head group and small protein in the large micelles may hardly work due to the large distance from the micellar inner wall and protein surface.

RSA of enzyme back-extracted decreased with KCl concentration as shown in **Fig. 3.8**. ß-Galactosidase was strongly denatured in the high KCl concentrations. This is probably due to the large increase in the surface hydrophobicity of ß-galactosidase with the addition of KCl as previously found (Kuboi *et al*., 1993). Lysozyme activity also decreased with KCl concentration. High salt concentration in the aqueous phase used for the back-extraction is not suitable for high activity yield.

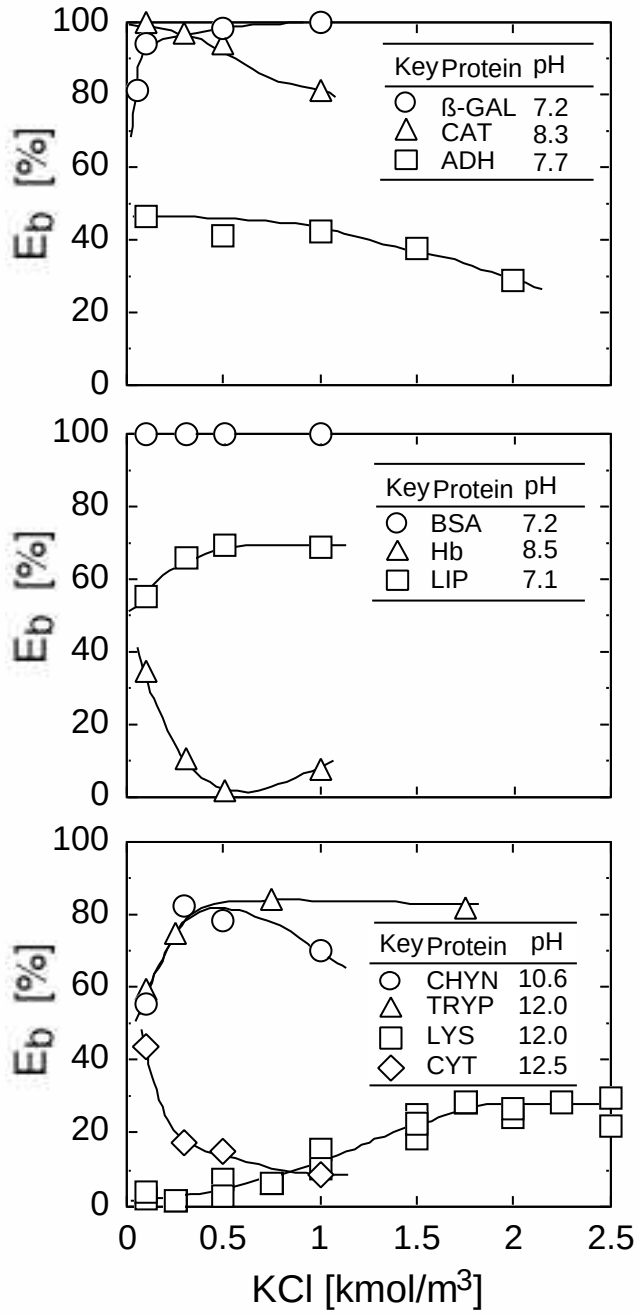


Fig.3.7 The effect of KCl concentration on the back-extraction of proteins at pH higher than pI.

3.1.4. Effect of incubation time of protein in reverse micelles on the back-extraction

The protein solubilized into reverse micelles was incubated at 298 K, and after standing for certain fixed periods the back-extraction was performed. The effect of incubation time on the E_b and *RSA* of the back-extracted proteins is

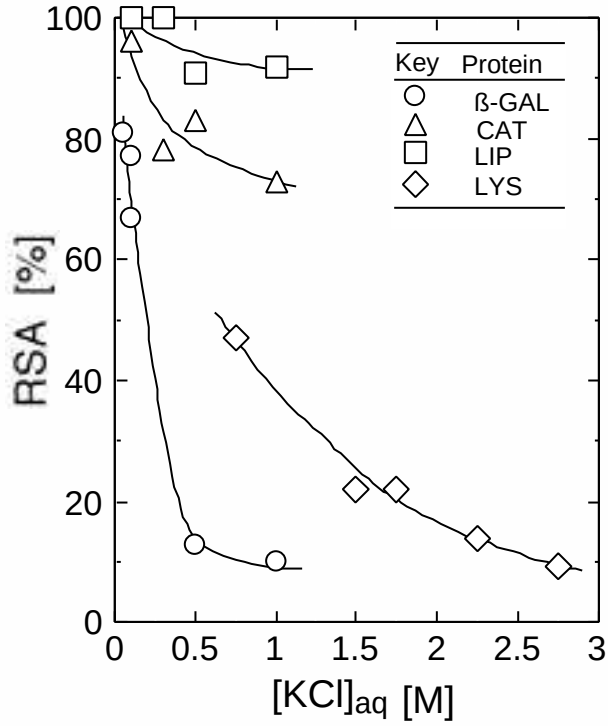


Fig. 3.8 Effect of KCl concentration on the relative residual activity of enzyme back-extracted under the same condition in Fig. 3.7.

expected to reflect the stability of the protein in the reverse micelles. The results of catalase and BSA are shown in **Fig. 3.9**. The values of E_b and *RSA* were plotted against incubation time, together with the activity of catalase in the bulk aqueous solution. In the bulk aqueous phase, catalase was very stable and a drop in activity was not observed for 16 h. In the cases of catalase and BSA solubilized at pH_{inj} 7.4 and 8.3, respectively, these pH values were higher than pI, and E_b and *RSA* values were approximately constant for 16 h. This shows that the protein solubilized under the conditions with keeping of the electrostatic repulsion in reverse micelles is quite stable and maintains the native structure for a long time.

When catalase and BSA were solubilized at pH_{inj} 5.6 and 4.1, respectively, these pH values were approximately equal or lower than their pI. E_b decreased slightly with incubation time. *RSA* of catalase, however, is

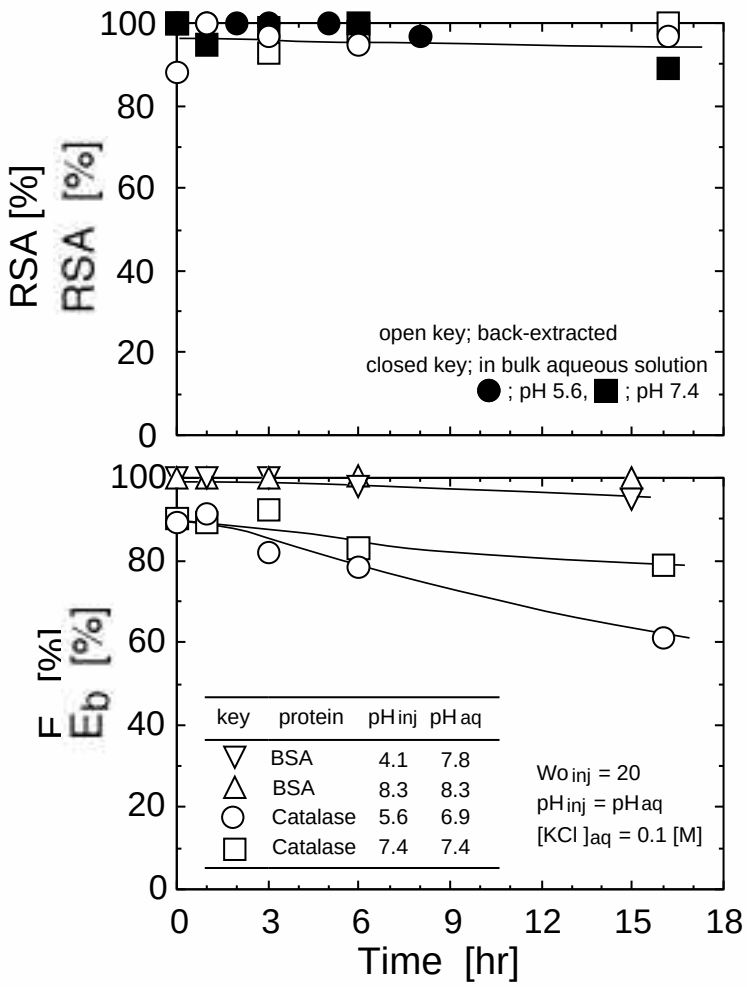


Fig. 3.9. Effect of incubation time after solubilization on the back-extraction of catalase and BSA.

constant and the back-extracted catalase in the aqueous phase is not inactivated. Since only native catalase can be back-extracted, catalase denatured in the micellar water pool is considered not to be back-extracted from the micellar phase. The increase in electrostatic interaction between the proteins and the micellar inner wall causes denaturation of the proteins.

3.1.5 Separation of ß-galactosidase from cell extracts by back-extraction combined with the injection method.

The separation of ß-galactosidase from crude extracts of *E. coli* cells was

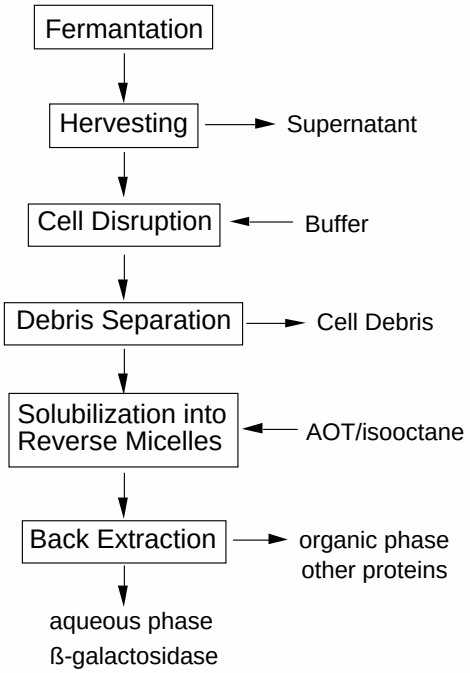


Fig.3.10 Procedure for the separation of ß-galactosidase from cells using the back-extraction combined with the solubilization by the injection method.

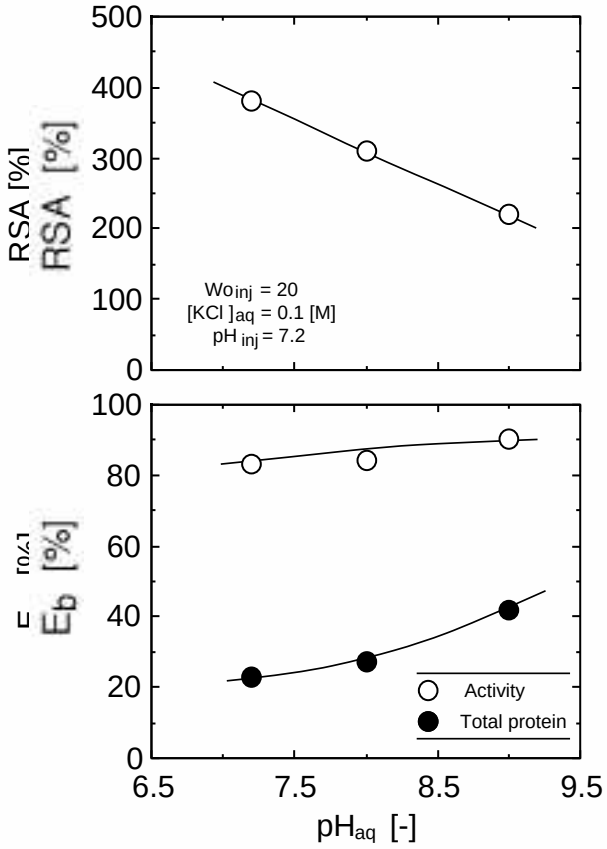


Fig.3. 11 Effect of pH_{aq} on the back-extraction of ß-galactosidase from crude extracts solubilized in the reverse micelles

carried out as an application of the present method. The separation procedure is shown in **Fig.3.10.** The extracts containing ß-galactosidase, obtained by disruption of *E.coli* cells and centrifugation, was solubilized into the reverse micellar organic solution at *Wo*=20, and then the micellar solution was contacted with an aqueous phase containing 0.1 M KCl at various pH_{aq}. The effect of pH_{aq} on E_b based on total enzyme activity and total soluble protein are shown in **Fig.3. 11** together with *RSA* of the back-extracted enzyme. About 80% of ß-galactosidase solubilized in the micelles was back-extracted to the aqueous phase almost independently of the pH_{aq} value. In contrast, the recovery of total soluble protein increased with pH_{aq}. As a result, *RSA* decreased with increase in pH_{aq}. By such manipulations of pH_{aq}, contaminant proteins in crude extracts, where the pI may be higher than that of ß-galactosidase, were back-extracted by the electrostatic repulsion with AOT. Reducing of the back-extraction of contaminant proteins at lower pH_{aq} enabled ß-galactosidase to be purified four-fold over the crude cell extracts.

3.2 Protein extraction by phase transfer method using the modification of interaction between protein and micelles by divalent cation salt

3.2.1 Effect of Ca^{2+} ion on the extraction of large molecular weight proteins in the conventional AOT reverse micellar extraction system

The salt type in the aqueous phase have been reported to affect the efficiency of extraction by the phase transfer method (Marcozzi *et al.*, 1991**;** Nishiki *et al.*, 1993). In order to modify the electrostatic interaction between proteins and the micelles, $CaCl_2$ was used as a salt in the aqueous phase instead of NaCl or KCl which are usually used in the conventional extraction system using AOT. Extraction of large molecular weight proteins with some small proteins were carried out in the AOT reverse micellar extraction system by the

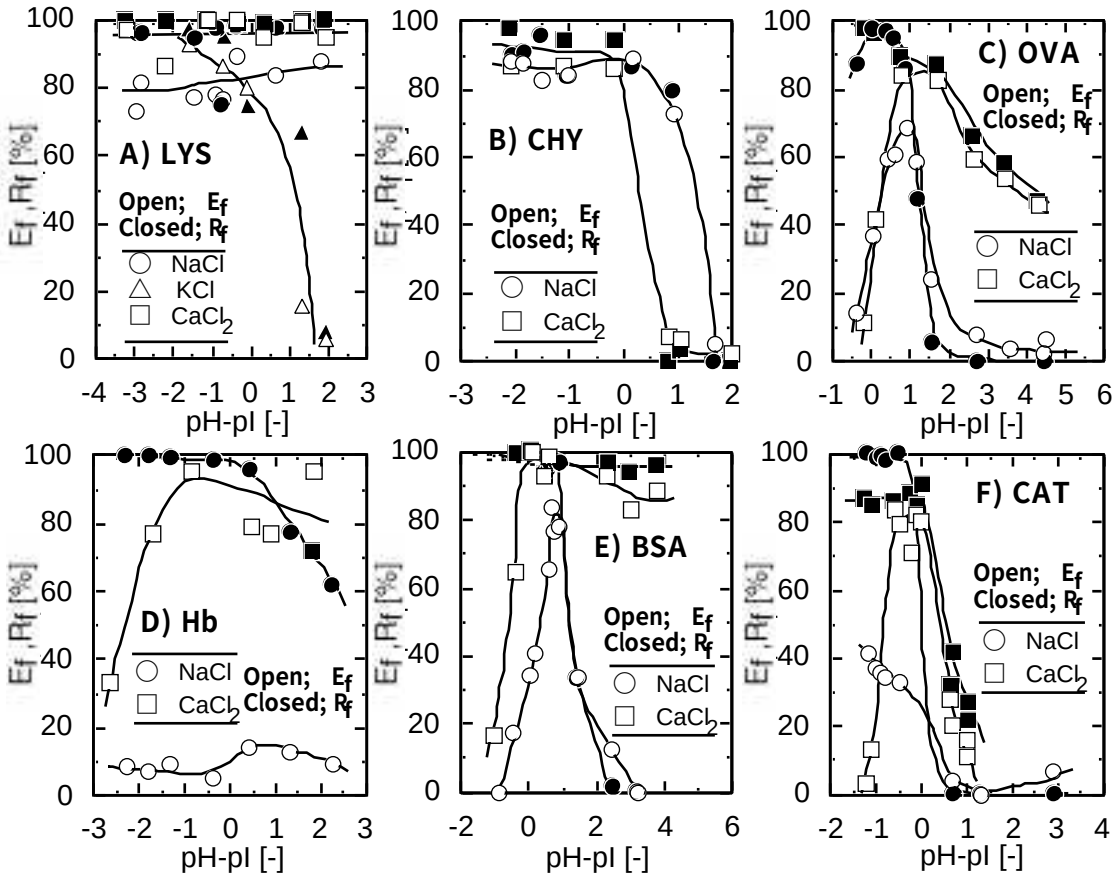


Fig. 3.12 Effect of pH on the extraction of proteins by the AOT reverse micellar system using $CaCl_2$ as a salt in the aqueous phase.

using AOT. Extraction of large molecular weight proteins with some small proteins were carried out in the AOT reverse micellar extraction system by the phase transfer method using $CaCl_2$ as a salt in the aqueous phase at various pH values. Effect of pH on the extraction of proteins is shown in **Fig. 3.12** together with those using NaCl or KCl. The positive effect of Ca^{2+} ion on the extraction was observed on some proteins, such as BSA, hemoglobin, catalase, ovalbumin and also small lysozyme and the extraction of these proteins was observed and enhanced at pH range over than each pI. BSA and also hemoglobin were extracted effectively at pH higher than their pI. However, since UV spectra of heme part of hemoglobin extracted into the micellar organic phase was changed, the structural change of hemoglobin in the organic phase was suggested. In the case of ovalbumin, E_f at pH higher than the pI was high compared with that of

compared with that of NaCl. Ca^{2+} ion is considered to reduce the electrostatic repulsion between protein surface and the micelles and mediates the interaction between negatively charged protein surface and anionic head group of AOT. The degree of the influence of $CaCl_2$ is dependent on the kinds and concentration of proteins and salts.

Since the salt effect of $CaCl_2$ was remarkably observed, the extraction behavior of BSA was studied in detail as a case study of the successful modification of the electrostatic interaction by divalent salts in the following part.

3.2.2 Effect of salt type on the pH effect for the extraction of BSA by the conventional AOT reverse micelles

Extraction of BSA was carried out at various pH values and a salt concentration of 0.1 M and AOT concentration of 200 mM. The fractions of extraction, precipitate and residual BSA in the aqueous phase and *Wo* value at each pH are shown in **Fig. 3.13**. When NaCl or KCl was used as salt, BSA was extracted to the micellar phase at narrow pH range, maximum of extraction was achieved at pH 5.0 for KCl and pH 5.5 for NaCl, respectively. In the pH range lower than isoelectric point of BSA (pI=4.9), large amount of precipitates was observed, and in a part of the experiments the BSA concentration was difficult to measure due to turbidity in the aqueous phase. The amount of precipitates decreased with increase in the pH value. In the pH range higher than the pH of maximum extraction, BSA was remained in the aqueous phase after extraction. This is considered that no BSA interacts with AOT due to the electrostatic repulsion at these pH range.

On the other hand, when $CaCl_2$ or $MgCl_2$ was used as a salt, BSA was extracted with increasing in the pH value. In the case of $CaCl_2$, E_f was highest at

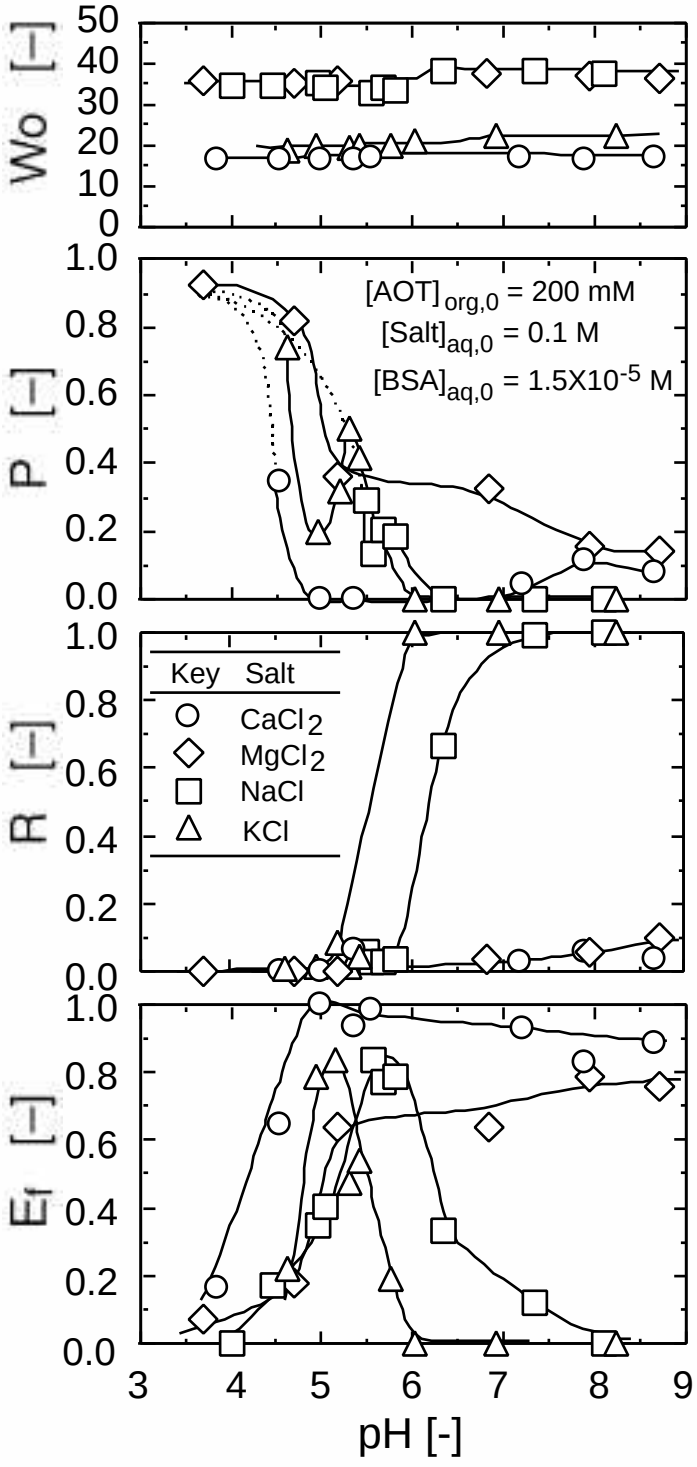


Fig. 3.13 Effect of pH on the BSA extraction using various types of salts.

pH 5.0-5.5, and then slightly decreased in the further higher pH range. In the case of $MgCl_2$, E_f increased only slightly with increasing pH value. This effective extraction at the pH range higher than the pI is particularly interesting.

Because protein extraction using AOT reverse micelles at pH higher than the pI is usually difficult to occur due to electrostatic repulsion between AOT head group and protein surface.

In the study of the adsorption of BSA to a negatively charged polymer latex (Shirahama *et al.*, 1989), it has been suggested that divalent cation have the ability to bridge between negatively charged BSA surface and latex surface. It is, therefore, expected that protein extraction using $CaCl_2$ or $MgCl_2$ at pH higher than pI may be also caused by the bridging mediated by Ca^{2+} or Mg^{2+} between negatively charged protein surface and anionic head group of AOT molecule. Those salt type effect on the protein extraction has been suggested by a few investigators (Adachi and Harada, 1994; Nishiki *et al.*, 1993; Leser and Luisi, 1990) but its effect and mechanism is still not well discussed and understood yet. The definitive effect of divalent cation such as Ca^{2+} and Mg^{2+} shown here will be expected for a new modification method on the protein extraction using AOT reverse micelles by the addition of multivalent ion reagents to the extraction system.

In all cases, large amount of precipitates were observed in the pH range lower than pI. BSA is considered to be denatured and precipitated by the strong electrostatic interaction with AOT molecules in the pH range lower than pI.

3.2.3 Effect of salt concentration on the BSA extraction

The effect of the concentration of salt on the extraction behavior at 200 mM of AOT is shown in **Fig. 3.14**. In the low salt concentration range regardless of salt type, E_f increased with an increase in the salt concentration, following a decrease of P. No soluble BSA was remained in the aqueous phase. When KCl or NaCl were used, a sharp decrease in E_f accompanying a increase in R was observed in the salt concentration range higher than 0.3 or 1.0 M,

respectively. In the case of KCl, a temporary increase in P was observed simultaneously with the decrease in E_f. In the both cases of $CaCl_2$ and $MgCl_2$, E_f decreased slightly with an increase in the salt concentration.

The change of salt concentration affects both of size and hydrophobicity

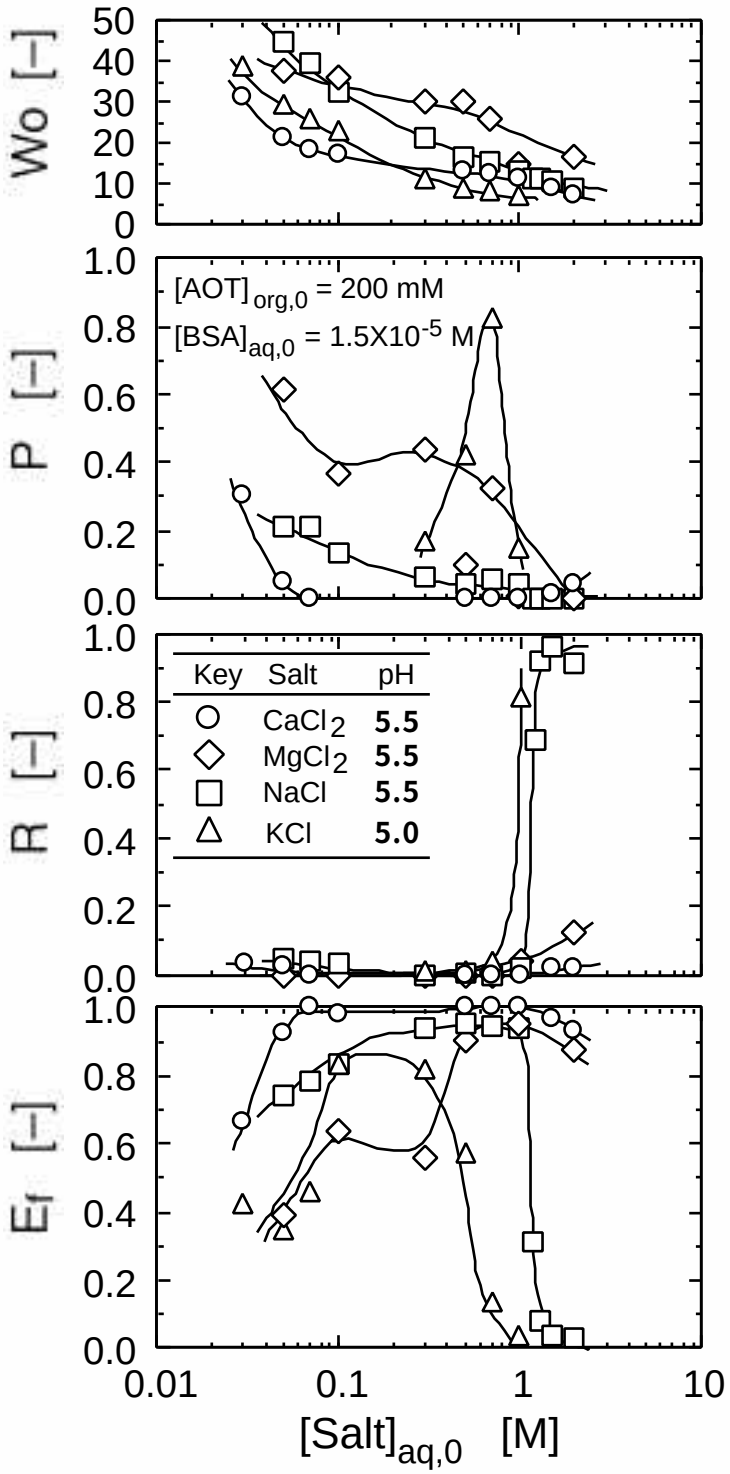


Fig. 3.14 Effect of the concentration of salt on the extraction behavior of BSA.

of reverse micelles (Kuboi *et al*., 1990b; 1992a) and hydrophobicity of proteins (Kuboi *et al*., 1991). Hydrophobicity of both the water pool of the micelles and proteins increase with an increase in the salt concentration. Since the hydrophobicity of AOT reverse micelles is, however, almost constant at the salt concentration lower than 1 M (Kuboi *et al.*, 1992b), the increase in E_f by an increase in salt concentration at the range of low concentration is considered to be due to the increase in the hydrophobicity of BSA. The decrease in E_f at high concentration of KCl and NaCl may be due to the increasing in size exclusion effect from the micelles.

3.2.4 Effect of AOT concentration on the BSA extraction

The effect of AOT concentration on the extraction behavior at 0.1 M of salt is shown in **Fig. 3.15**. In the very low AOT concentration range, no extraction of BSA was occurred and almost all BSA molecules were remained in the aqueous phase. With an increase in AOT concentration, *P* increased, following a decrease in *R*. The region, where almost all BSA fed to the extraction system were precipitated at the interface, was observed. These concentration ranges depend on salt type. With a further increase in AOT concentration, BSA started to be extracted into the organic phase, and the amount of precipitate decreased. The above AOT concentration at which BSA extraction observed was dependent on salt type, for examples about 20 mM for $CaCl_2$, 80 mM for NaCl, and 100 mM for $MgCl_2$ and KCl.

The amount of water extracted to the organic phase is very low until AOT concentration of 10^{-2} M, even if AOT concentration is higher than CMC of AOT, 4.9 mM (De and Maitra, 1995). The *Wo* value increased with an increase in the AOT concentration, and reached to a constant value, which agreed approximately with the *Wo* value without BSA, *Wo*=34 for NaCl, 21 for KCl, 13

for $CaCl_2$ and 34 for $MgCl_2$.

This effect of AOT concentration on the extraction behavior of BSA is

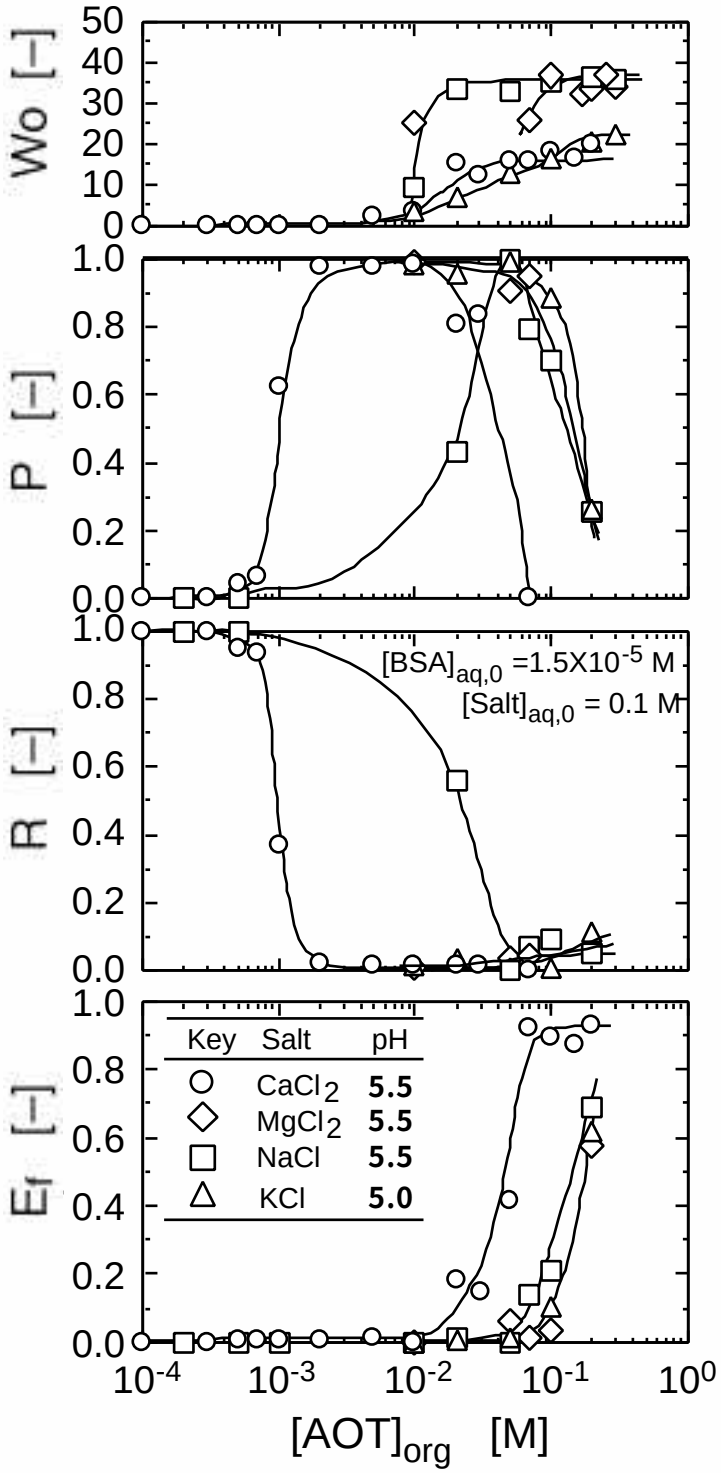


Fig. 3.15 Effect of the concentration of AOT on the extraction behavior of BSA.

similar to that of cytochrome c (Adachi and Harada, 1993). The AOT concentration to cause the precipitation and the extraction of BSA, however, differs from that of cytochrome-c. BSA extraction is required a larger AOT concentration than that of cytochrome c.

3.2.5 Effect of feed BSA concentration on the extraction

Extraction of BSA was carried out at various feed BSA concentration and fixed AOT concentration. The BSA concentration extracted into the organic phase using NaCl or $CaCl_2$ as a salt is plotted against the feed BSA concentration in **Fig. 3.16** together with the *Wo* value.

In the case of NaCl, the concentration of BSA extracted into the organic phase increased with increasing feed BSA concentration. With further increase in the feed concentration, the concentration of BSA in the organic phase decreased slightly. *Wo* value also slightly decreased with a decrease in the BSA concentration in the organic phase.

In the case of $CaCl_2$, BSA was almost quantitatively extracted to the organic phase below a certain concentration of feed BSA for each AOT concentration. With further increase in the feed BSA concentration, the BSA concentration extracted into the organic phase decreased remarkably, and the precipitate was simultaneously formed at the interface, and further BSA was not remained in the aqueous phase after extraction. The maximum concentration of BSA extracted exists for each AOT concentration.

The *Wo* values are independent of the feed BSA concentration until maximum extraction. The decrease in *Wo* at high concentration of feed BSA corresponds to the decrease in BSA concentration extracted into the organic phase. This decrease in *Wo* is considered that AOT is removed from the organic phase by the complexation between AOT and BSA followed by precipitation.

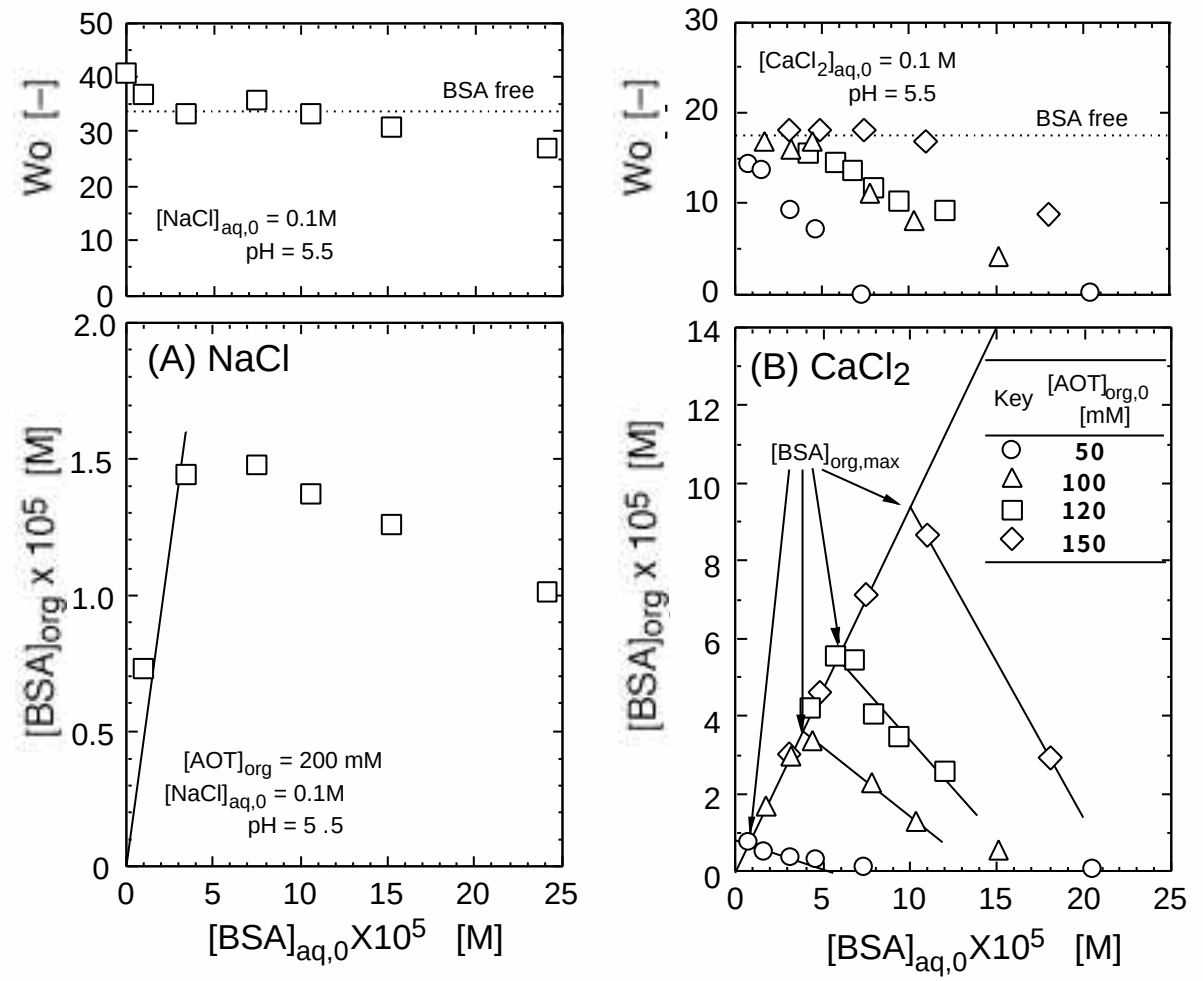


Fig. 3.16 Effect of the feed concentration of BSA on the extraction; (A) NaCl , (B) $CaCl_2$ in the aqueous phase.

Similar tendency in *Wo* was also observed in other extraction conditions, where a large amount of precipitation was formed, such as the results at pH range lower than pI shown in Fig.3.13. and at the AOT concentration range from CMC to 10^{-2} M in Fig. 1.15. A degree of the decrease in *Wo* is larger than that using NaCl. This suggests that interaction between AOT and BSA is enhanced by the presence of Ca^{2+} ion, which cause the bridging between AOT and BSA.

In the reverse micellar extraction of lysozyme and chymotrypsin using NaCl as a salt in the aqueous phase, it has been reported that the *Wo* value increases with a increase in the protein concentration extracted to the micellar phase (Ichikawa *et al*., 1992; Nishiki *et al*., 1993). In the case of extraction of

lysozyme using $BaCl_2$, as with our results, *Wo* value is also unaffected by the protein concentration extracted in the organic phase (Nishiki *et al*., 1993). These results suggest the characteristic effect of divalent cation such as Ca^{2+}, Mg^{2+} and Ba^{2+} on the extraction of proteins.

3.2.6 Quantitative relationship between BSA and AOT molecules relating to the extraction

From the results of the effects of the feed BSA concentration, the maximum amount of BSA extracted was found to be corresponding to the initial AOT concentration. The relationship between the initial AOT concentration, $[AOT]_{org,0}$, and the maximum concentration of BSA extracted, $[BSA]_{org,max}$, where $CaCl_2$ was used as a salt in the aqueous phase was shown in **Fig. 3.17**. A linear relationship with different values of slope and intercept depending on the $CaCl_2$ concentration exists between $[BSA]_{org,max}$ and $[AOT]_{org,0}$. Similar relationship has been reported for the extractions of lysozyme and ribonuclease-a (Matzke *et al*., 1992). Although the maximum concentration of BSA extracted is only one-tenth comparing with those of lysozyme and ribonuclease-a. This is considered to be due to large molecular weight of BSA.

If almost all the molecules of AOT in the initial organic phase is assumed to participate in the extraction at the point of the maximum amount of BSA extracted, an average number of AOT molecules to extract one BSA molecule is evaluated to be 1000 and 800 for $CaCl_2$ of 0.1 M and 0.5 M from the reciprocal of slope of straight line in Fig. 3.17, respectively.

Protein is considered to be solubilized in the reverse micelles which size is larger than that of the protein, as shown in the previous works (Kuboi *et al*., 1990b; Yamada *et al*., 1994b). $C_{m,eff}$ is the effective concentration of micelles larger than the size of protein, can be calculated from the size distribution of

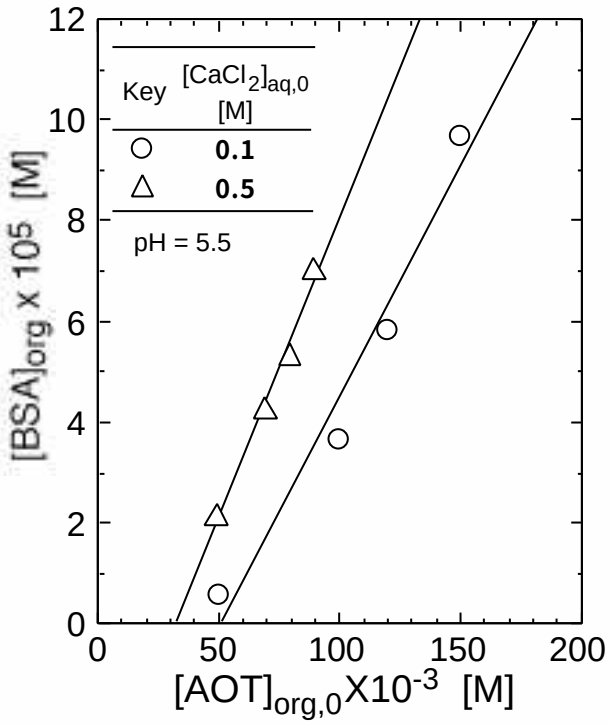


Fig. 3.17 Relationship between the initial AOT concentration, $[AOT]_{org,0}$, and the maximum concentration of BSA extracted, $[BSA]_{org,max}$, where $CaCl_2$ was used as a salt in the aqueous phase.

micelles and protein size. The size of BSA has been measured by several methods, such as 3.5 nm by gel chromatography (Horiike *et al*., 1983), 3.1 nm by dynamic light scattering (Takeda *et al*., 1992), and a major radius of 7 nm and miner radius of 2 nm on the basis of sedimentation experiments (Sequire *et al*., 1968). The size of BSA is affected by the solubilization condition, such as pH, ionic strength, and the presence of surfactants. The size of BSA was reported to increase up to 6.0 nm with increase in sodium dodecyl sulfate as anionic surfactant (Takeda *et al*., 1992).

It is difficult to define the size of BSA in the present extraction conditions. If the size of BSA is in between 3 nm and 7 nm, $C_{m,eff}$ can be calculated to be 4 - $5x10^{-4}$ M for NaCl at 200 mM AOT and *Wo*=34, and $2x10^{-6}$ - $2x10^{-3}$ M for $CaCl_2$ at 150 mM AOT and *Wo*=18 in the cases in Figs. 2.4 and 2.5. The average size of micelles is estimated from *Wo* values in the absence of BSA

(Kuboi *et al*., 1990b). In most cases, $C_{m,eff}$ is larger than $[BSA]_{org,max}$, which is $1.6x10^{-5}$ M at 200 mM AOT and 0.1 M NaCl and $9.8x10^{-5}$ M at 150 mM AOT and 0.1 M $CaCl_2$, and hence the extraction of BSA also can be understood the extraction model proposed previously (Kuboi *et al*., 1990b; Yamada *et al*., 1994b).

3.2.7 Back-extraction of BSA from reverse micellar phase

BSA extracted to the organic phase is back-extracted to a new aqueous phase. The conditions and results of the extraction and the back-extraction are summarized in **Table 3.1**. Back-extraction was carried out by the changes of pH and salt type and its concentration in the aqueous phase used for back-extraction according to the effects of pH and salts on the forward extraction. BSA extracted with NaCl solution is almost quantitatively back-extracted in the condition of high pH and high salt concentration. And further, BSA extracted with $CaCl_2$ is also back-extracted by using the high KCl concentration in the aqueous phase. This indicates that interaction between BSA and the micelles is

Table 3.1 Conditions and results of the extraction and the back-extraction of BSA

Extraction				Back-extraction			
pH [-]	Salt	[Salt] [M]	E_f [%]	pH [-]	Salt	[Salt] [M]	E_b [%]
5.5	NaCl	0.5	80-86	7.9	NaCl	0.5	100
				9.0	NaCl	0.5	100
				5.5	KCl	0.5	0
						1.0	100
						2.0	100
5.5	$CaCl_2$	0.1	91	5.5	KCl	0.5	0
						1.0	96
						2.0	96

$[AOT]_{aq.0}$= 0.20M, $[BSA]_{aq.0}$ = 1 mg/ml

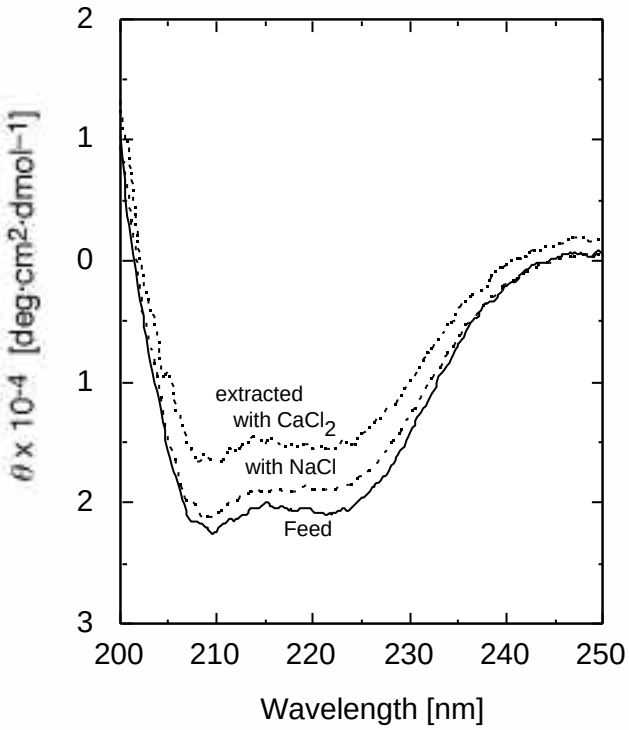


Fig. 3.18 CD spectra of back-extracted BSA which was extracted using $CaCl_2$ or NaCl.

reversible, and the strong denaturation which results in the difficulty of back-extraction is not caused.

In order to study structural change of BSA during the extraction process, CD spectrum of BSA back-extracted to the aqueous phase from the organic phase was examined. BSA, which was extracted into the organic phase at pH 6.0 with NaCl or $CaCl_2$, respectively, was back-extracted at NaCl of 0.5 M and pH 6.0. CD spectra of BSA back-extracted was shown in **Fig. 3.18**. In the case of the extraction with NaCl, the CD spectra of BSA back-extracted almost agrees with that of feed BSA. No structural change of BSA is caused in the extraction process using NaCl. In the case of the extraction with $CaCl_2$, CD spectra change of BSA back-extracted is somewhat larger than that with NaCl. Since effective extraction is achieved using $CaCl_2$ in the aqueous phase, structural change may be caused by strong interaction between BSA and surfactant.

3.3 Extraction of proteins with a pH responsible mixed micellar system using AOT and long chain alkyl amines

A mixed reverse micellar systems containing both AOT and long chain alkyl amine was investigated to develop the effective extraction process of proteins. The potential of the mixed system was confirmed by the extraction behaviors of proteins and water.

3.3.1. Extraction behavior of water

3.3.1.1 Effect of pH

The mixed systems, AOT solutions of 50 mM with or without long chain alkyl amine of 50 mM in isooctane were used as the micellar solution, and were contacted with the aqueous phase containing NaCl of 0.1 M at various pH

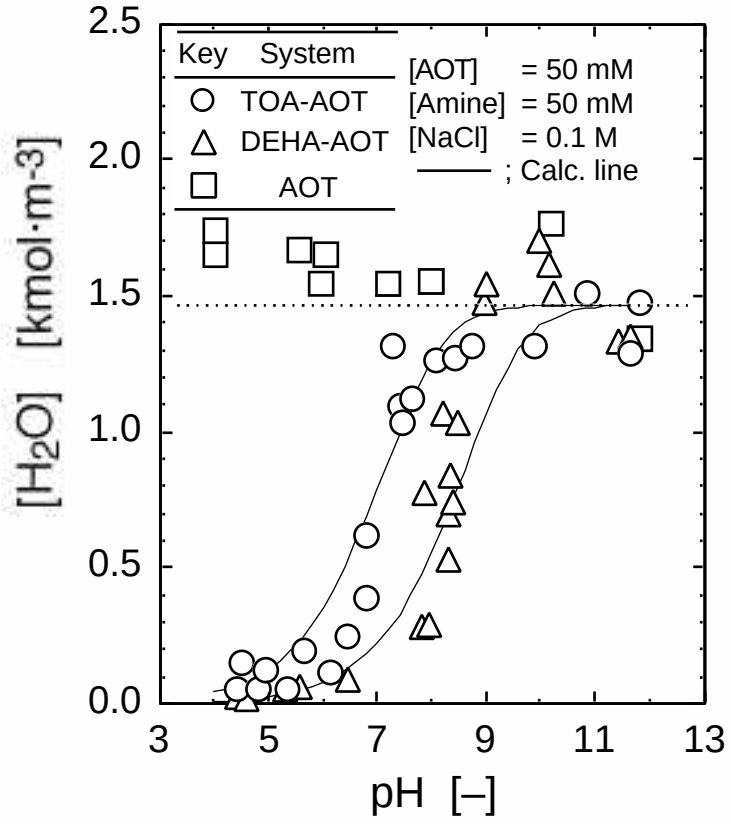


Fig. 3.19. Effect of pH on the water extraction in the AOT, TOA-AOT, DEHA-AOT systems.

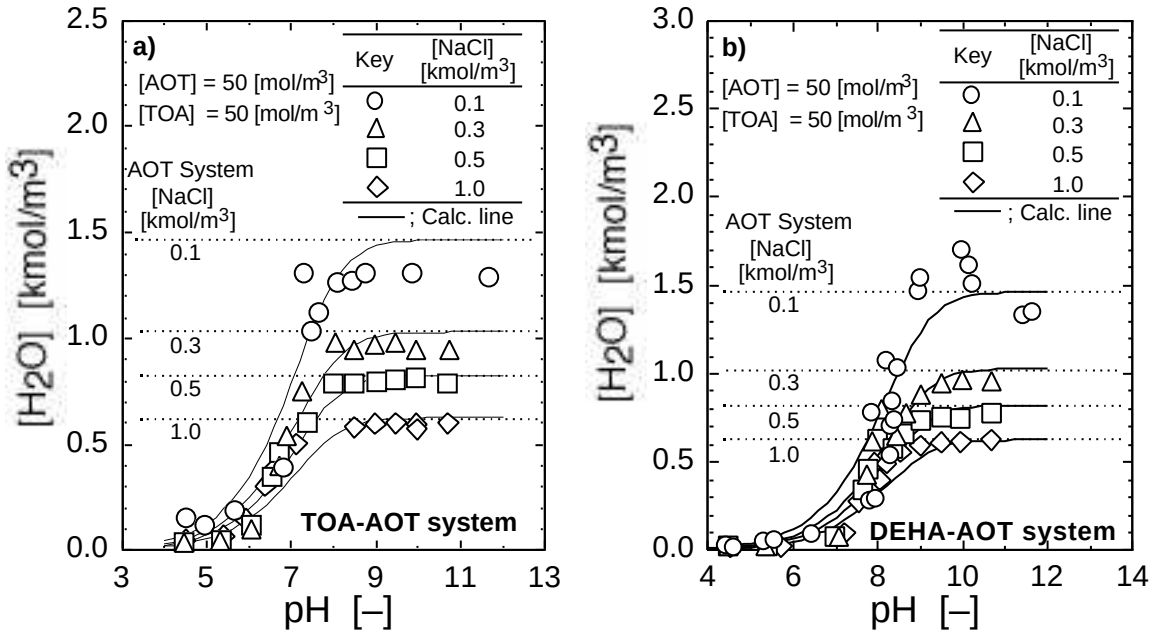


Fig. 3.20 Effect of NaCl concentration on the pH dependence of the water extraction in the mixed micellar systems. a) TOA-AOT system, b) DEHA-AOT system

values. The effect of pH on water extraction in the AOT, TOA-AOT and DEHA-AOT systems is shown in **Fig. 3.19**. Extraction of water in the AOT system is unaffected by the pH values and constant.

In mixed micellar systems with long chain alkyl amines, the water extracted into the organic phase decreases with pH lower than a specific pH, and is negligibly small in even lower pH range. The pH values which begin to decrease the water extraction are dependent on the kind of the amines. On the other hand, water extraction of both mixed systems at high pH range is not affected by pH, and is the same degree with that of the AOT system.

3.3.1.2 Effect of salt in the aqueous phase

The effect of salt concentration on water extraction was investigated at the same concentrations of both AOT and the amines at 50 mM. Effect of pH on the water extraction at various NaCl concentrations is shown in **Fig. 3.20**. Water extraction in the AOT system increases with a decrease in the NaCl concentration, as well known by previous works (Kuboi *et al.*, 1990a).

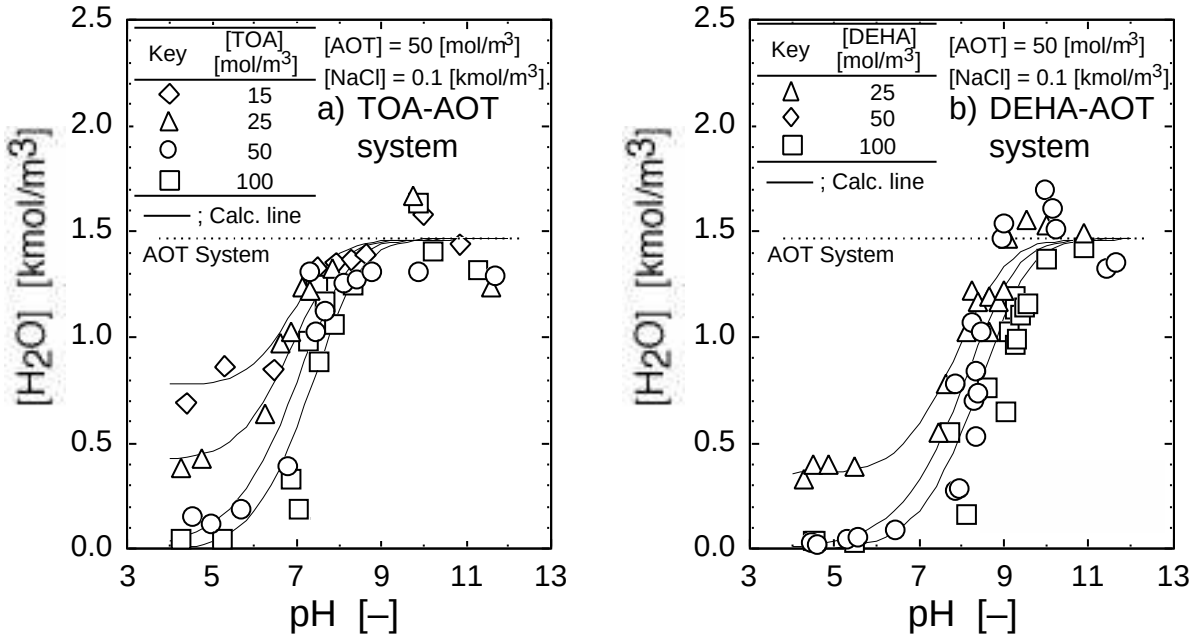


Fig. 3.21 Effect of the concentration of long chain alkyl amines on the pH dependence of the water extraction in the mixed micellar systems. a) TOA-AOT system, b) DEHA-AOT system

In the TOA-AOT and DEHA-AOT systems, water extraction in the high pH range also increases with decreasing salt concentration, and is almost the same degree as that of the AOT system. The water extraction decreases to a negligibly small concentration in the low pH range from the water concentration in the high pH range. Hence, the variation of the water concentration by pH change increases with decreasing salt concentration. In the low salt concentration, the water content in the organic phase can be drastically altered by changing the pH by only 2.

3.3.1.3.Effect of the concentration of long chain alkyl amines

The effect of the concentration of long chain alkyl amines added to the system was examined. Long chain alkyl amines were dissolved in 50 mM AOT solution at various concentrations. The mixed solutions were contacted with the aqueous phase containing 0.1 M NaCl at various pH values. The results are shown in **Fig. 3.21**. The water extractions at all concentrations of the amines decrease with pH. In the low pH range, the concentration of water in the organic

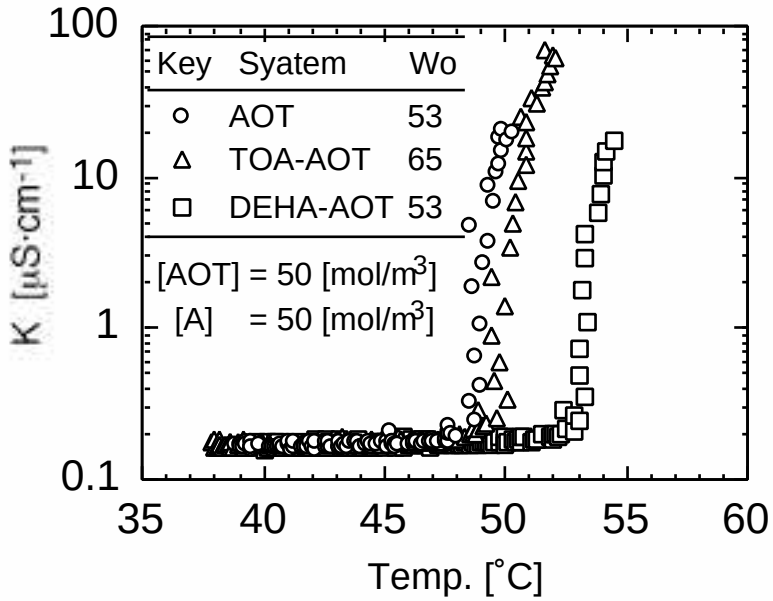


Fig. 3.22 The variation of electrical conductivity of the micellar solution equilibrated with the aqueous phase containing 0.05 M NaCl and at pH 10.7. Comparison between the mixed and the AOT micellar systems.

phase increases with decreasing amine concentration, and is negligibly small at an amine concentration almost equal or higher than the concentration of AOT.

3.3.1.4. Effect of the amine addition on the percolation of the reverse micellar solution

The electric conductivity change of the mixed micellar system was measured as a function of temperature. The solution was contacted and equilibrated with a aqueous phase at pH 11 and 0.1 M NaCl. The micelles in the mixed system is formed in this condition and large amount of water is extracted in the organic phase. The results are shown in **Fig. 3.22**. The percolation was also observed in the mixed micellar system. The percolation is influenced by the addition of amines. Though W_O values in the mixed systems are almost equal or higher than that of the AOT system, the percolation threshold temperature, T_P, in the mixed system is higher than that of the AOT system. The addition of the long chain amines decreases the inter-micellar interaction and increases the stability of the micelles. This is considered that the amine, which is like as small

solvent molecules, can penetrate the interfacial film and dilute the AOT chains at the interface as discussed in the effect of chain length of alkane solvent on the percolation in Part I. Though the addition of the amines is not affected on the extraction of water, it makes clear that the addition of the amines influences the inter-micellar interaction and the amines may partly penetrate the micellar interface.

3.3.1.5. Model of the control of micelles formation

The extraction of large amount of water into the organic phase is caused as a result of the formation of reverse micelles. The variation in water extraction is considered to occure because of the change of the formation of the reverse micelles. In the mixed micellar systems containing both AOT and long chain alkyl amines, water extraction is controlled by pH in the aqueous phase. This shows that formation of the micelles can be controlled by pH. The control mechanism of the micellar formation in the mixed micellar systems of AOT and the amines is illustrated in **Fig. 3.23**. The amines undergo protonation and form

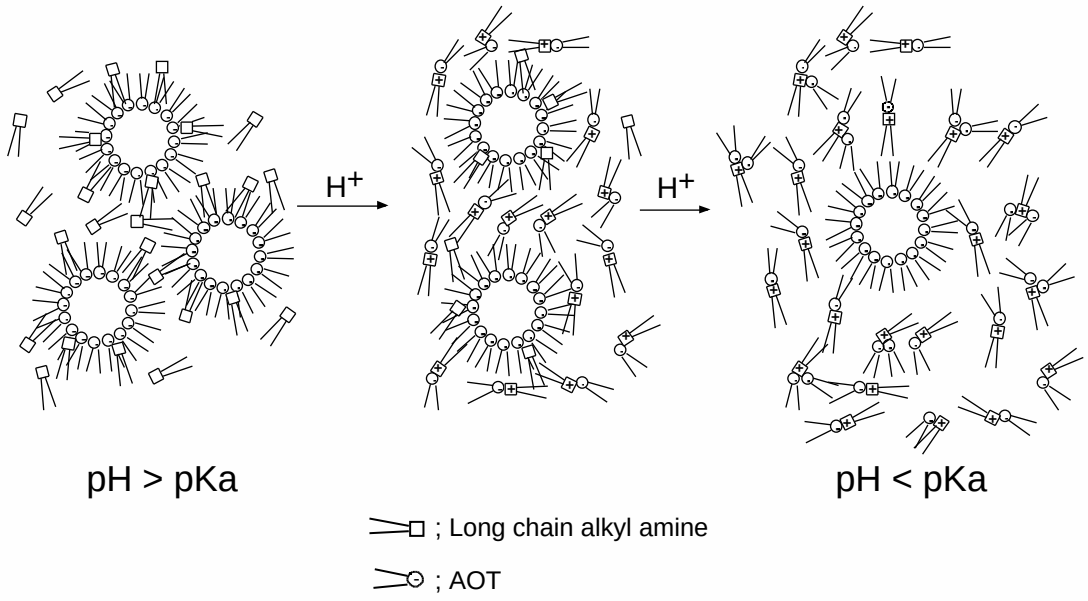


Fig. 3.23 Schematic representation of the control of the micellar formation by pH in the mixed micellar system using long chain alkyl amine and AOT

form cationic ammonium ion in acidic pH range. This ammonium ion reacts with AOT by electrostatic interaction and an intermolecular ion complex is formed. It is considered that the complex has less surface activities and no ability to form a reverse micelles, and consequently water is not extracted into the organic phase. The difference in pH dependence on the water extraction between TOA and DEHA systems is considered to be due to their basicities. Formation of the reverse micelle in the mixed system of AOT and long chain alkyl amines can be controlled by the concentration and basicities of the amine added. This large degree of freedom for design of the micellar system is one of the advantages of the mixed micellar systems proposed. Control of the micellar formation by the intermolecular interaction uptake of AOT from the micelles is not used in other previous studies.

Here, a relationship between the water content in the mixed micellar solution and pH in the aqueous phase is attempted to be derived by the following model.

The amines, B, reacts with protons as shown in following equilibrium reaction.

$$B + H^+ \rightleftarrows AH^+ \qquad : 1/K_a \qquad (3.1)$$

$$1/K_a = [BH^+]/([B][H^+]) \qquad (3.2)$$

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

$$AOT \rightarrow Na^+ + OT^- \qquad (3.3)$$

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

The ammonium ion formed will react with OT^- as follows;

$$OT^- + BH^+ \rightleftarrows OT^-\text{-}BH^+ \qquad : K_1 \qquad (3.4)$$

$$K_1 = [OT^-\text{-}BH^+]/([AOT][BH^+]) \qquad (3.5)$$

This reaction is considered to be the same as the formation of an insoluble ion-complex between anionic and cationic surfactants in aqueous solution (Fujimoto, 1981). A large decrease in water extraction in the acidic pH range

and a concentration of amines lower than that of AOT is inexplicable by only the reaction presented by Eq. (3.4). Hence, the complex, OT^--BH^+, is assumed to react further with OT^- as follows;

$$OT^-\text{-}BH^+ + (n\text{-}1)\ OT^- \rightleftarrows (OT^-)_n\text{-}BH^+ \ : K_n \qquad (3.6)$$

$$K_n = [(OT^-)_n\text{-}BH^+]/([OT^-\text{-}BH^+]^{(n-1)}[AOT])$$

$$= [(OT^-)_n\text{-}BH^+]/(K_1[AOT]^{(n-1)}[BH^+]) \qquad (3.7)$$

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

$$[AOT]_0 = [AOT] + [OT^-\text{-}BH^+] + n\,[(OT^-)_n\text{-}BH^+] \qquad (3.8)$$

$$[B]_0 = [B] + [BH^+] + [OT^-\text{-}BH^+] + [(OT^-)_n\text{-}BH^+] \qquad (3.9)$$

Assuming that the equilibrium of Eq. (3.4) lies so far to the right, $[BH^+]$ is very low compared with those of other species and negligible. Furthermore, to simplify the treatment, it is assumed that OT^--BH^+ reacts with one molecule of OT^-, n=2. The following equation is obtained from Eqs. (3.2), (3.5), (3.7), (3.8) and (3.9).

$$K_a([AOT]_0 - [AOT]) / (K_1[H^+])$$

$$= K_2([AOT]^3 + 2[AOT]^2[B]_0 - [AOT]^2[AOT]_0)$$

$$+ ([AOT]^2 + [AOT][B]_0 - [AOT][AOT]_0)) \qquad (3.10)$$

Extraction of water by AOT reverse micelles is commonly expressed using *Wo* value, which is constant regardless of AOT concentration at a fixed salt concentration.

$$Wo = [H_2O]/[AOT] \qquad (3.11)$$

If the complexes formed have no ability to form reverse micelles and to extract water, and water is only extracted by remaining AOT reverse micelles, the concentration of AOT forming the reverse micelles in the experimental results can be estimate by Eq (3.11) and *Wo* value in the AOT system.

The parameters, K_2 and K_a/K_1, are determined from Eq. (3.10) using

Table 3.2. Parameters of reaction model for extraction of water in the mixed micellar system of AOT and long chain alkyl amine

System	K_a/K_1 [-]	K_2 [m^3/mol]
TOA-AOT	$1x10^{-8}$	5.3x10
DEHA-AOT	$8x10^{-10}$	8.4x10

experimental results. The values of K_a and K_1 can not be evaluated separately in this experiment. The parameters determined are listed in **Table 3.2**. The values of K_2 in the TOA-AOT and DEHA-AOT systems are the same order, and the value of K_a/K_1 in the TOA-AOT system is about 10 times that in the DEHA-AOT system. The pK_a values (= - log K_a) of TOA measured by the $pH_{0.5}$ method are about one unit smaller than that of DEHA (Eyal *et al.*, 1991), so the K_a/K_1 difference between TOA and DEHA is considered to be mainly due to their K_a. The pK_a values of amines in organic solvents is, however, influenced by the measurement conditions (Eyal *et al.*, 1991; Eyal and Canari, 1995; Grinstead and Davis, 1968). Hence, the effect of amine structure on the parameters can not be discussed in detail.

The concentration of AOT formed reverse micelles at any pH value is calculated using the parameters and Eqs. (3.10), and then the extracted water concentration is obtained from Eq. (3.11). In Figs. 3.19-3.21, the calculated results are presented by solid lines, and almost agree with the experimental results except at high NaCl concentration in Fig. 3.20. The parameters obtained at 0.1 M of NaCl are used for calculation, and the effect of salt concentration on the parameters is not considered. The value of K_2 sensitively affects the water concentration at acidic pH range, and the pH profile of water extraction is influenced by both K_a/K_1 and K_2 in the same order. The difference between the calculated and experimental results at high NaCl concentration shows the effects

of ionic strength on the reactivity of amines and the interaction between AOT and ammonium salt. However, the effect of ionic strength on the parameters obtained from the water extraction is considered to be relatively small. The effects of the micellar interface and the salt concentration on K_a of amines and the molecular interaction between AOT and amines are not clear.

3.3.2. Extraction behavior of proteins by the mixed micellar systems

Since the mixed micellar systems consists of both AOT and long chain alkyl amine, it is able to control the micellar formation by pH change, and it is expected to be able to control protein extraction based on micellar formation. The extractions of BSA and hemoglobin, which is a model protein of large and oligomeric one, were examined using the salt effect of Ca^{2+} with the mixed micellar systems. Further, the extraction of lysozyme was carried out using with the mixed micellar systems.

3.3.2.1 Forward extraction.

The effect of pH on the extraction of BSA at 0.1 M $CaCl_2$ is shown in **Fig. 3.24**. The fraction extracted into the micellar phase, E_f, and the removal fraction from the aqueous phase, R_f, are plotted against pH. In the case of the AOT system, the extraction was not occurred and high R_f and large amount of aggregation was observed at pH lower than 4.5. BSA was disintegrated its structure and precipitated by the electrostatic interaction with AOT at pH lower than the pI. E_f increased with pH and decreased in further higher pH range.

In the cases of the mixed micellar systems, E_f increases with pH, and further increases in pH results in the decrease in E_f. In the case of $CaCl_2$ used as a salt, the micellar formation in the mixed micelles is possible to controlled by pH and the protein extraction is also able to control by pH. When E_f was low at acidic pH range, R_f is also low and almost all BSA existed in the aqueous phase

acidic pH range, R_f is also low and almost all BSA existed in the aqueous phase after the extraction. The formation of aggregates observed in the AOT system at pH lower than the pI is suppressed in the mixed micellar systems. Since AOT form a complex with cationic long chain alkyl ammonium salt, the interaction

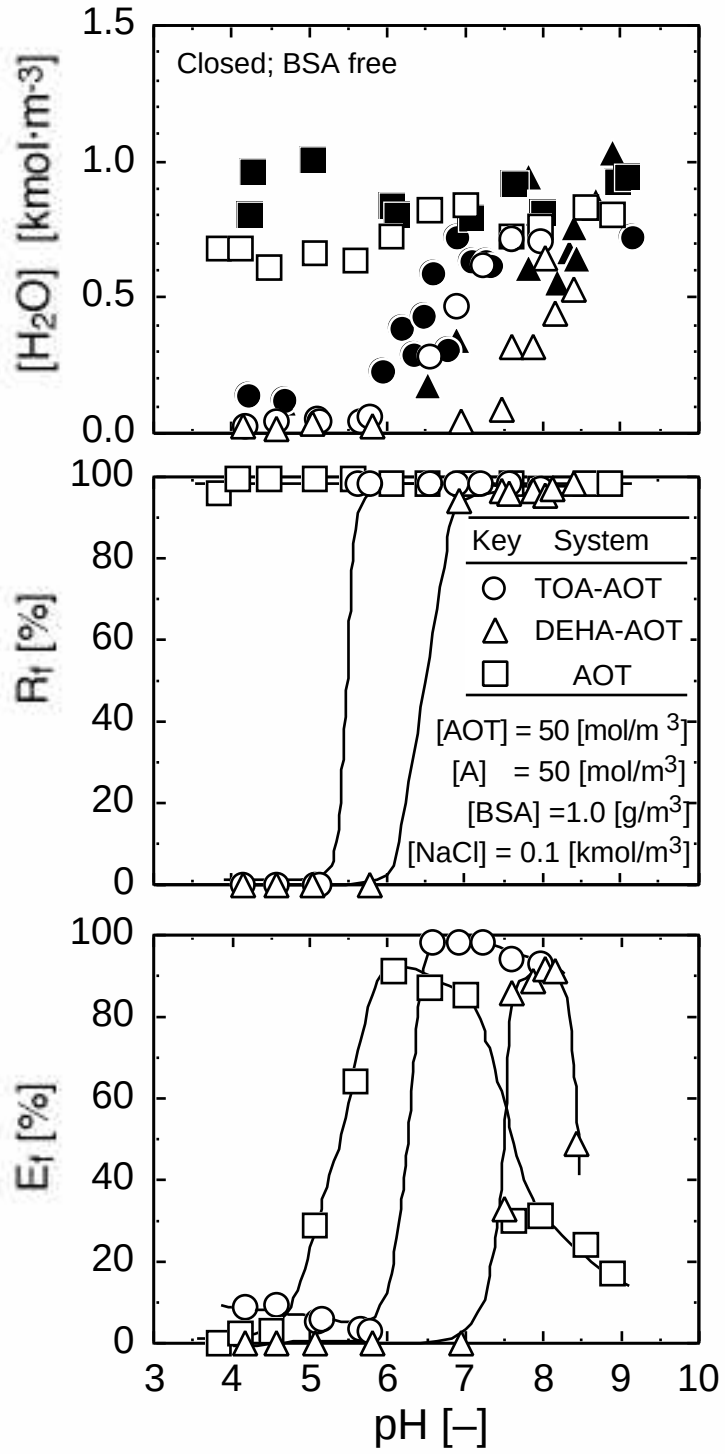


Fig. 3.24 Effect of pH on the extraction behavior of BSA in the AOT, TOA-AOT, DEHA-AOT systems

between BSA and AOT can not be observed. The pH range for effective extraction in the mixed micellar systems is somewhat different from that in the AOT system. The interaction between BSA and AOT producing the extraction is considered to be affected by the presence of the amines.

Hemoglobin solubilized in the aqueous solution containing 0.1 M $CaCl_2$ at pH 9 was extracted with both the DEHA-AOT mixed and the AOT micellar solutions which consisted of both DEHA of 100 mM and AOT of 50 mM and AOT of 50 mM in isooctane, respectively. The results are listed in **Table 3.3**. The extracted fraction into the organic phase,E_f, was 56% for the DEHA-AOT system and 43% for the AOT system, respectively.

Extraction of lysozyme was carried out at 0.1 M NaCl by the AOT, TOA-AOT and DEHA-AOT systems. Effect of pH on the extraction of lysozyme is shown in **Fig. 3.25**. In the AOT system, lysozyme extraction effectively

Table 3.3 The conditions and results of the extraction and back-extraction of proteins using the AOT and the mixed micellar systems.

Protein	System (conc., [mol/m^3])	Extraction				Back extraction				
		pH	Salt	[Salt] [kmol/m^3]	E_f [%]	pH	Salt	[Salt] [kmol/m^3]	E_b [%]	RSA [%]
Lysozyme	AOT (50)	9.6	NaCl	0.1	99	11.9	NaCl	1.5	33	-
	DEHA-AOT (50-50)	8.5	NaCl	0.1	97	4.2 5.8	NaCl	0.1	93 73	- -
	DEHA-AOT (75-50)	9.5	NaCl	0.1	95	6.0 6.1 7.0 8.2	NaCl	0.1	94 99 99 29	94 100 100 -
BSA	AOT (50)	5.5	$CaCl_2$	0.1	91	5.5	KCl	1.0	96	-
	TOA-AOT (50-50)	6.9	$CaCl_2$	0.1	98	4.7	$CaCl_2$	0.1	100	-
	DEHA-AOT (50-50)	8.1	$CaCl_2$	0.1	91	4.7	$CaCl_2$	0.1	92	-
Hemoglobin	AOT (50)	9.0	$CaCl_2$	0.1	42	9.47	NaCl	0.1	9	-
	DEHA-AOT (100-50)	9.2	$CaCl_2$	0.1	56	5.4 6.3	$CaCl_2$ $CaCl_2$	0.1 0.1	64 62	-

proceeds at wide pH range around neutral pH. In the pH range lower than 5, however, E_f decreases and R_f is very high with large amount of aggregates. Lysozyme is considered to be denatured by strong electrostatic interaction with AOT.

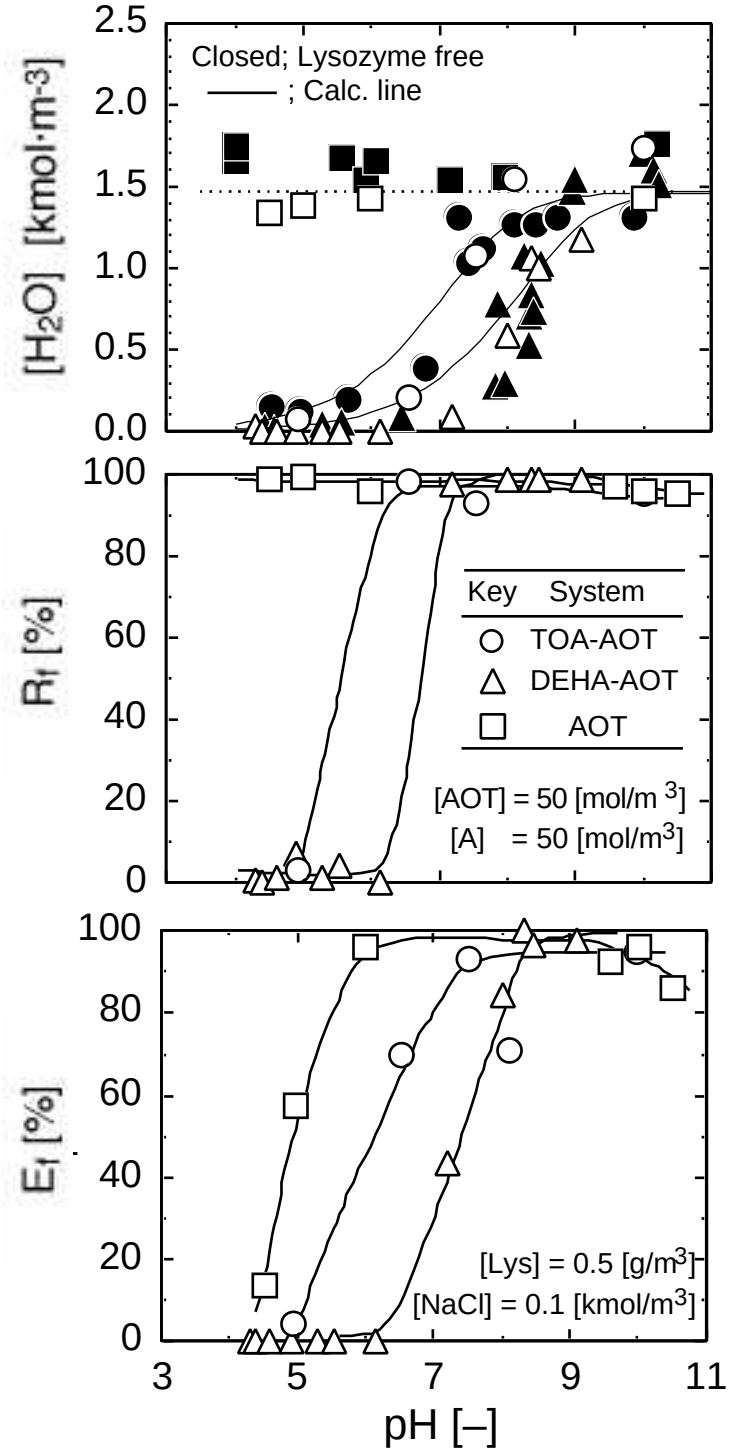


Fig. 3.25 Effect of pH on the extraction behavior of lysozyme in the AOT, TOA-AOT, DEHA-AOT systems

E_f in the TOA-AOT and DEHA-AOT mixed micellar systems also decreases with pH lower than a certain pH. The E_f values are, however, higher than that in the AOT systems. In this case, R_f increases with pH. The decrease in E_f and the increase in R_f well correspond to the decrease in the water extraction. As shown in the extraction of BSA by the mixed micellar systems, the aggregation of lysozyme is also suppressed in the mixed micellar system.

The extraction of proteins can be controlled by the control of the formation of the micelles by pH. Furthermore, in the acidic pH range where the micelles does not form, the interaction between proteins and AOT and the undesirable formation of aggregation is not caused.

3.3.2.2 Back-extraction of proteins from the micellar phase

BSA, hemoglobin and lysozyme extracted with the mixed micellar system were back-extracted by contacting with a new aqueous phase at more acidic pH where the reverse micelles wass not formed. The condition and the results in the forward and back-extraction are also shown in Table 3.3 together with those in the AOT system**.**

In the cases of the mixed micellar systems, lysozyme, BSA and also hemoglobin can be effectively back-extracted to the aqueous phase only by changing pH. Residual activity of lysozyme back-extracted to the aqueous phase is high. Since the reverse micelles in the mixed system can not form at acidic pH, proteins entrapped in the micelles is released to the aqueous phase as a result of the destruction of the micelles. Hemoglobin interacts with AOT by the hydrophobic interaction even under the electrostatic repulsive condition. Thus, in the AOT systems, hemoglobin is difficult to be back-extracted even at pH higher than its pI. In the mixed micellar systems, however, the molecules of AOT bind to the protein hydrophobic surface may be removed by the complexation between AOT and cationic spices of the amine. The effective

back-extraction in the mixed micellar system is considered to be achieved by both destruction of the reverse micelles and dissolution of the interaction between AOT and proteins which are caused by the formation of the complex between AOT and the cationic ammonium salt of the added amine at acidic pH.

In contrast, in the AOT systems, since lysozyme is basic protein and has very high pI (pI 11.1), back-extraction of lysozyme requires a aqueous phase at very high pH range and high salt concentration. Back-extraction of BSA is also carried out at high salt concentration. The extraction process in such a condition is likely to cause denaturation and inactivation of proteins. Further, a desalting process is needed in the following process.

The present mixed micellar system is able to control forward and back-extractions by only pH change. Interestingly, the relation of pH change between forward- and back-extraction in the mixed micellar systems is quite opposite to those in the AOT system, that is, forward extraction of proteins in both the mixed and the AOT systems is carried out at pH lower than pI of protein, and then back-extraction in the AOT system is usually carried out at pH higher than pI, on the other hand, that in the mixed systems is carried out at pH lower than that in forward extraction where the reverse micelles are not formed.

4. SUMMARY

On the bases of the control of the interaction between proteins and the AOT micelles, three novel separation methods using AOT based reverse micelles for large to small molecular weight and oligomeric proteins were investigated.

Firstly, in order to suppress the interaction during the extraction process, the back-extraction combined with the solubilization by injection method with

keeping electrostatic repulsion between proteins and AOT was carried out at various conditions. The back-extraction of the protein is affected by the pH value and the salt concentration in the feed solution and in the aqueous solution used for the back-extraction. Large proteins are back-extracted with high activity yield by both solubilization and back-extraction at pH higher than pI. The back-extraction of large molecular weight proteins is considered to be driven effectively by the electrostatic repulsion from the micellar interface and the steric exclusion from the micelles, which are strongly given to large molecular weight proteins due to its large molecular size. The back-extraction of low molecular weight proteins is required to serve strongly the size exclusion effect by increasing the salt concentration. The condition of high KCl concentration in the back-extraction step tends to enhance the inactivation and the precipitation of the enzymes and proteins. An increasing in hydrophobic interaction between proteins and AOT by increasing in the salt concentration caused the structural change and inactivation of proteins as well as those of the electrostatic interaction. The separation of ß-galactosidase from crude extracts of *E. coli* cells was achieved by the present method. Although there remain problems such as the limited amount and relatively low selectivity, the present method is used effectively for the separation of large molecular weight and oligomeric proteins which is easily inactivated by the interaction with AOT.

Secondly, modification of electrostatic interaction between proteins and micelles in the conventional AOT extraction system was made by using divalent cation, such as Ca^{2+} or Mg^{2+}, as a salt in the aqueous phase . The effect of pH on the extraction of proteins solubilized in the aqueous phase containing $CaCl_2$. was investigated using the conventional AOT reverse micelles. The effect of Ca^{2+} on the extraction was observed on some proteins, such as BSA, hemoglobin, catalase, ovalbumin and also small lysozyme. The extraction of these proteins was enhanced at pH range higher than their pI using $CaCl_2$ as a

salt in the aqueous phase. It is suggested that Ca^{2+} ion mediates the interaction between negatively charged protein surface and anionic head group of AOT. The extraction behavior of BSA was investigated as a case study of the extraction mediated divalent cation. The salt effect was also observed in the case of Mg^{2+}. When KCl or NaCl was used, the extraction of BSA is occurred at narrow pH range and high AOT concentration, and decreased sharply at high salt concentration. The linear relationship between the maximum concentrations of BSA extracted and AOT feed was observed. BSA extracted to the organic phase can be back-extracted to a new aqueous phase at the high pH and the high salt concentration ranges. Molecular conformation of BSA back-extracted was, however, found to be affected to some extent by the extraction condition from the results of CD measurement. Slight structural change of BSA extracted using NaCl was observed. The extraction using $CaCl_2$ caused larger structural change than that with NaCl.

Thirdly, the mixed reverse micellar system of AOT and long chain alkyl amines, i. e. tri-*n*-octylamine and di(2-ethylhexyl) amine, was used for the protein extraction process. Extraction of water into the mixed micellar systems was affected by pH in the aqueous phase. The formation of micelles is considered to be controlled by the intermolecular complexation between AOT and the cationic ammonium salt of the added alkyl amine. The effect of pH on the water extraction in the mixed micellar system is explained by the simple model which consists three reaction; protonation of the amine, complexation between AOT and ammonium salt, and further reaction of the AOT-amine complex with AOT.

The large molecular weight proteins,such as BSA and hemoglobin, extracted into the mixed micellar systems at pH higher than pI using salt effect of Ca^{2+} can be effectively back-extracted by the destruction of the micelles at acidic pH. The pH change in this extraction process is quite opposite direction for the conventional AOT system. The extraction of the proteins into the micellar solution is also controlled by the formation of reverse micelles. The

formation of aggregate of the proteins by the electrostatic interaction with AOT was not observed in the mixed micellar system. This is considered that AOT reacts predominantly with cationic ammonium salt rather than cationic charge on protein surface. This mixed micellar systems is also effective for the extraction of small proteins such as lysozyme. Integrations of salt effects and the formation of the reverse micelles in the mixed micellar system is effective for the development of the bio-separation process using reverse micelles.

GENERAL CONCLUSIONS

Novel and effective bio-separation processes for large molecular weight and oligomeric proteins by using AOT-based reverse micellar systems were studied based on understanding and controlling the interactions between proteins and the micelles.

The reverse micellar extraction of large molecular weight and oligomeric proteins including some small ones was carried out. The appropriate control of the electrostatic interaction between protein and the micelles is necessary for successful extraction of proteins, especially for large molecular weight and oligomeric proteins. Too strong electrostatic interaction between the micelles and proteins results in the aggregation and denaturation of proteins. The conformational change of proteins caused by the electrostatic interaction with AOT in the aqueous solution is related with the aggregation behavior in the extraction step. In the case that the conformational change of proteins by the interaction with AOT is large, denaturation and formation of aggregates is predominantly observed in the extraction step, and the proteins will hardly be back-extracted if proteins is extracted to the reverse micelles at that condition.

The inter-micellar and protein-micellar interactions can be evaluated successfully by the measurement of electric conductivity caused by the percolation phenomena as a function of volume of the aqueous phase solubilized into the reverse micellar phase and the temperature of the solution at constant water content. The inter-micellar interaction is affected by the solvent, temperature and water content. The condition of the micellar interface zone reflects its percolation properties. Protein solubilized in the micelles by the injection method affects the inter-micellar interaction. The inter-micellar

interaction is also affected by charge, hydrophobic property, molecular weight and concentration of proteins solubilized. Strong interaction between the micelles and proteins solubilized is found to highly destabilize the reverse micelles.

In order to investigate the interaction of proteins with reverse micellar interface, the reaction behavior of lipase, which is interface adsorptive enzyme, in the AOT reverse micellar system was studied and compared with those in the bulk organic-aqueous two phase and oil in water emulsion systems. Lipase is very sensitive to the electrostatic interaction with anionic surfactants and denatured easily by the interaction. The pH range of high activity of lipase in the micelles almost agrees with the characteristic parameter ($ß$) quantified by percolation of the micellar solution containing lipase. The lipase in the micelles is stable for long time by reducing the interaction with the micelles and the mobility in the reverse micelles. The hydrolysis rate in the reverse micellar system is reasonably explained by the interfacial reaction model for lipase adsorbed at the interface and the substrate in the organic phase. It is clear that the interaction of lipase with the micellar interface is reduced compared with that of interface without anionic surfactant. This is due to the electrostatic repulsion between anionic surfactant and negatively charged lipase at pH higher than pI.

Based on those results, three novel separation processes using AOT based reverse micellar systems for large molecular weight and oligomeric proteins were proposed and demonstrated. As the first method, in order to depress the interaction during the extraction process, the solubilization of proteins into the micelles by the injection method was combined with the back-extraction by the phase transfer method. Large molecular weight and oligomeric proteins were back-extracted successfully with high activity yield when the pH values in both

the solubilization and the back-extraction solutions was kept higher than their pI and salt concentration was low. The interaction between the proteins and the micelles is depressed by the electrostatic repulsion. Since the back-extraction of low molecular weight proteins requires high salt concentration, the present method is suitable for large and oligomeric proteins. The separation of ß-galactosidase from crude extracts of *E. coli* cells was carried out successfully by the present method.

As the second method, in order to modify the electrostatic interaction between proteins and micelles, divalent cation, such as Ca^{2+}, was used as a salt in the aqueous phase in the conventional AOT extraction system. The extraction of some proteins, such as BSA, hemoglobin, catalase, ovalbumin and also small lysozyme was enhanced at the pH range higher than their pI using $CaCl_2$ as a salt in the aqueous phase. The extraction behavior of BSA was investigated in detail as a case study of the extraction mediated by divalent cation. The linear relationship between the maximum concentrations of BSA extracted and AOT fed was observed. Divalent cations such as Ca^{2+} and Mg^{2+} mediate the interaction between negatively charged protein surface and anionic head group of AOT.

As the third method, the mixed systems of AOT and long chain alkyl amines such as tri-*n*-octylamine and di(2-ethylhexyl) amine were used to develop the effective extraction process. Successful extraction process is possible by the control of the AOT-amine and AOT-protein electrostatic interaction. The formation of the reverse micelles was controlled by the complexation between AOT and cationic species of long chain alkyl amines. Large molecular weight proteins,such as BSA and hemoglobin, extracted into the mixed micellar systems at the pH higher than their pI using salt effect of Ca^{2+} could be back-extracted successfully by the destruction of the micelles at

acidic pH. Lysozyme could also be back-extracted with high enzymatic activity at acidic pH. Consequently, the proteins extracted with the mixed micelles can be back-extracted effectively by destruction of the micelles at acidic pH.

SUGGESTIONS FOR FUTURE WORK

In order to extend and develop further the findings obtained in this work, the following studies are recommended.

(1) Modification of interaction between protein and reveres micelles by poly ionic reagents

In this study, remarkable effect of divalent cation such as Ca^{2+} on protein extraction was observed in the conventional AOT reverse micellar system. This suggests the interaction between reverse micelles and the target biopolymer can be modified and controlled by a bridge of multivalent ionic compounds. The modification of the protein-micellar interaction can be quantified by the electrical conductivity measurement exploiting percolation. Further, ionic compounds may properly modify protein surface property by their electrostatic binding with protein surface.

(2) Functional reverse micellar system based on intermolecular interaction

The formation of reverse micelles and AOT-protein interaction are possible to be controlled by the specific intermolecular interaction between AOT molecules and long chain alkyl amines added to the micellar system. This suggests that the formation of AOT reverse micelles can be controlled by the formation of cationic molecules formed by the environmental spur factors or stimuli such as pH, temperature and light. Based on this idea, design of novel reverse micellar systems and their application are expected. Further, the possibility of stimuli responsible molecular self-assembling system and structure

control of molecular assembly by the intermolecular interaction is suggested.

(3) Bio-reaction processes using functional reverse micellar system.

Separation of bio-products, recovery and recycle use of enzymes are important subjects in the upstream and down stream processes using reverse micelles. The stimuli-responsible reverse micellar systems are especially useful for the back-extraction of enzymes form reverse micelles without inactivation. Further, the combination of the stimuli-responsible reverse micellar system with organic-organic two phase system (Yamada, 1995) will be able to develop novel functional bio-reaction processes where enzymatic reactions can be efficiently carried out with product separation and enzyme recovery.

(4) Utilization of percolation state of reverse micellar solution.

The reverse micellar solution in the percolation state has dynamic structure. In the percolation state, mass transfer across micellar interface and exchange of water in their micro water pools may be enhanced. Novel and interesting functions of the percolation state of the micellar solution are expected for novel reaction and separation media such as liquid membranes with high flux and media in the electrophoresis.

NOMENCLATURE

a	= specific area of aqueous droplet (S_T/V_{aq})	[1/m]
a'	= (a/S_E)	[kg/m^3]
C_{AOT}	= concentration of AOT	[mM]
C_S	= concentration of olive oil	[mol/m^3]
C_E	= concentration of lipase	[kg/m^3]
$C_{m,eff}$	= effective micellar concentration for protein solubilization	[mol/m^3]
dp	= avarage hydrodynamic diameter of the protein	[nm]
E_b	= fraction of back-extraction= $[Protein]_{aq}/[Protein]_{org,0}$	[–]
E_f	= extracted fraction of protein into the micellar organic phase	[%] or [-]
HFS	= surface hydrophobicity of protein	[kJ/mol]
J_A	= interfacial reaction rate	[mol/m^2·h]
K	= equilibrium constant	
K_a	= dissociation constant of salt of amine	[mol/m^3]
K_1	= equilibrium constant of reaction between AOT and amine	[m^3/mol]
K_2	= equilibrium constant reaction between complex and AOT.	[m^3/mol]
k_P	= desorption rate constant of fatty acids.	[mol/m^2•h]
P	= fraction of precipitates	[-]
RSA	= relative specific activity based on feed specific activity	
R_f	= removed fraction of protein from the aqueous phase	[%]
R	= fraction of BSA remained in the aqueous phase	[%]
S_T	= total interfacial area	[m^2]
S_E	= surface area occupied by unit weight of adsorbed lipase	[m^2/kg]
T_P	= temperature at percolation threshold	[˚C]
t	= reaction time	[h]
V_{in}	= initial velocity of fatty acid formation	[mol/m^3·h]

Wo	= $[H_2O]_{org}/[AOT]_{org,0}$	
	=water content, molar ration of H_2O to AOT	[–]
q	=mean residue ellipticity	[deg·cm^2/dmol]
[ ]	= molar concentration in bracket	[mol/m^3] or [M]
ß	= stability of reverse micellar systems	[l·mol^{-1}]
$Ø_P$	= aqueous volume fraction of percolation threshold without polymer	[–]
$Ø_{P.P}$	= aqueous volume fraction of percolation threshold with polymer	[–]
$\Delta Ø_p$	= $Ø_{P.P} – Ø_P$	[–]

<subscript>

0	= initial state
A	= acid
aq	= aqueous phase used for back-extraction
E	= enzyme
inj	= condition of solubilization by the injection method
M	= reaction intermediate
max	= maximum
org	= organic phase
ov	= overall in micellar solution
poly	= synthetic polymer
S	= substrate
wp	= micro water pool in reverse micelles

LITERATURE SITED

Adachi, M., and M. Harada; "Solubilization mechanism of cytochrome c in sodium bis(2-ethylhexyl) sulfosuccinate water/oil microemulsion," *J. Phys. Chem.*, **97**, 3631-3640 (1993)

Adachi, M., and M. Harada; "Effect of Salt Type on Partition of Cytochrome c between AOT Water-in-oil Microemulsion and Coexisting Aqueous Phase," *Separations for Biotechnology*, **3**, 8-14 (1994)

Alexandridis, P., J. F. Holzwarth and T. A. Hatton; "Thermodynamics of droplet clustering in percolating AOT water-in-oil microemulsions," *J. Phys. Chem.*, **99**, 8222-8232 (1995)

Barbaric, S. and P. L. Luisi; "Micellar Solubilization of Biopolymers in Organic Solvents. 5. Activity and Conformation of α-Chymotrypsin in Isooctane-AOT Reverse Micelles," *J. Am. Chem. Soc.*, **103**, 4239-4244 (1981)

Brochett, P., C. Petit and M. P. Pileni; "Cytochrome c in Sodium Bis(2-ethylhexyl) Sulfosuccinate Reverse Micelles: Structure and Reactivity," *J. Phys. Chem.*, **92**, 3505-3511 (1988)

Chen, J.-P. and K.-C. Chang; "Lipase-catalyzed hydrolysis of milk fat in lecithin reverse micelles. *J. Ferment. Bioeng.*, **76**, 98-104 (1993).

Chen, J.-P., and J.-T. Jen; Extraction of Concanavalin A with Affinity Reversed Micellar Systems," *Sep. Sci. Tech.*, **29**, 1115-1132 (1994)

Davis, D.M., D. McLoskey, S. J. S. Birch, P. R. Gellert, R. S. Kittlety, and R. M. Swart; "The fluorescence and circular dichroism of proteins in reverse micelles: application to the photophysics of human serum albumin and N-acetyl-L-tryptophanamide," *Biophys. Chem*, **60**, 63-77 (1996)

De, T. K., and A. Maitra; "Solution Behavior of Aerosol OT in Non-Polar Solvents," *Adv. Colloid Interface Sci.*, **59**, 95-193 (1995)

Dekker, M., K. Van't Riet, J. J. Van Der Pol, J. W. A. Baltussen, R. Hilhorst and B. H. Bijsterbosch; "Effect of temperature on the reverse micellar extraction of enzymes," *Chem. Eng. J.*, **46**, B69-B74 (1991)

Eyal, A. M., B. Hazan and R. Bloch; "Recovery and Concentration of Strong Mineral Acids from Dilute Solution Through LLX. II. Reversible Extraction with Branched-Chain Amines," *Solv. Extr. Ion Exch.*, **9**, 211-222 (1991)

Eyal, A. M. and R. Canari; "pH Dependence of Carboxylic and Mineral Acid Extraction by Amine-Based Extractants: Effects of pKa, Amine Basicity, and Diluent Properties," *Ind. Eng. Chem Res.*, **34**, 1789-1798 (1995)

Fletcher, P. D. I., A. M. Howe, B. H. Robinson; "The Kinetics of Solubulisate Exchange between Water Droplets of a Water-in-oil Microemulsion." *J. Chem. Soc. Faraday Trans. 1*, **83**, 985-1006 (1987)

Fletcher, P. D. I. and D. Parrott; Structure and Reactivity in Reverse Micelles, M. P. Pileni ed., p.303-322, Elsevier Co., Amsterdam (1989)

Fletcher P. D. I., B. H. Robinson, R. B. Freedman and C. Oldfield; "Activity in lipase in water-in-oil microemulsions," *J. Chem. Soc., Faraday Trans.* 1, **81**, 2667-2679 (1985).

Fujimoto, T.; Shin Kaimenkasseizai Nyumon, p.78-79, Sanyo Kasei Kogyo, Kyoto, Japan (1981)

Cassin, G., S. Illy and M. P. Pileni; "Chemically modified proteins solubilized in AOT reverse micelles. Influence of proteins charges on intermicellar interaction," *Chem. Phys. Lett.*, **221**, 205-212 (1994)

Giovenco, S., F. Verheggen and C. Laane; "Purification of intracellular enzymes from whole bacterial cells using reversed micelles," *Enzyme Microb. Technol.*, **9**, 470-473 (1987)

Goto, M., M. Kuroki, T. Ono and F. Nakashio; "Protein Extraction by New Reversed Micelles with Di(Tridecyl) phosphoric Acid," *Sep. Sci. Tech.*, **30**, 89-99 (1995)

Goto, M., K. Kondo and F. Nakashio; "Protein Extraction by Reversed Micelles using Dioleyl Phosphoric Acid," *J. Chem. Eng.,Japan*, **23**, 513-515 (1990)

Goto, M., T. Ono, F. Nakashio and A. A. Hatton;" Design of Surfactant Suitable for Proteins Extraction by Reversed Micelles," *Biotech. Bioeng.*, **54**, 26-32 (1997)

Göklen, K. E., and T. A. Hatton; "Protein Extraction Using Reverse Micelles," *Biotechnol. Prog.*, **1**, 69-73 (1985)

Grandi, C., R. E. Smith and P. L. Luisi; "Micellar Solubilization of Biopolymers in Organic Solvents. Activity and Conformation of Lysozyme in Isooctane Reverse Micelles," *J. Biol. Chem.*, **256**, 837-843 (1981)

Gray, P. P., P. Dunnill and M. D. Lilly; "The effect of controlled feeding of glycerol on the production of ß-galactosidase production by *Eschericia coli* in butch culture," *Biotechnol. Bioeng*., **15**, 1179-1188 (1973)

Grinstead, R. R. and J. C. Davis; "Base Strengths of Amine-Amine Hydrochloride Systems in Toluene," *J. Phys. Chem.*, **75**, 1630-1638 (1968)

Han, D., P. Walde and P. L. Luisi; "Dependence of lipase activity on water content and enzyme concentration in reverse micelles," *Biocatalysis*, **4**, 153-161 (1990).

Han, D. and J. S. Rhee; "Characteristics of lipase-catalyzed hydrolysis of olive oil in AOT-isooctane reversed micelles," *Biotechnol. Bioeng.*, **28**, 1250-1255 (1986).

Holmberg, H.; "Organic and bioorganic reaction in microemulsion," *Adv. Colloid Interface Sci.*, **51**, 137-174 (1994).

Holovko, M. and J. P. Badiadi; "Effect of an association potential on percolation. Applications to reverse micellaes containing proteins," *Chem. Phys. Letter,* **204**, 511 (1993)

Horiike, K., H. Tojo, T. Yamano and M. Nozaki; "Interpretation of the Stokes Radius of Macromolecules Determined by Gel Filtration Chromatography," *J. Biochem.*, **93**, 99-106 (1983)

Huruguen, J. P., M. Authier, J. L. Greffe and M. P. Pileni; "Percolation process induced by solubilizing cytochrome c in reverse micelles," *Langmuir;* **7**, 243-249 (1991)

Ichikawa, S., M. Imai and M. Shimizu; "Solubilizing Water Involved in Protein Extraction Using Reversed Micelles," *Biotechnol. Bioeng.*, **39**, 20-26 (1992)

Jada, A., J. Lang and R. Zana; "Relation between Electrical Percolation and Rate Constant for Exchange of Material between Droplets in Water in Oil Microemulsion," *J: Phys. Chem.,* **93**, 10-12 (1989)

Jada, A., J. Lang and R. Zana; "Ternary Water in Oil Microemulsions Made of Cationic Surfactants, Water, and Aromatic Solvents. 1. Water Solubility Studies," *J. Phys. Chem.*, **94**, 381-387 (1990)

Jones, M. N.; Biological Interfaces, p. 101, Elsevier Co., New York (1975)

Kamihira, M., H. Yanagisawa, K. Takahashi and H. Takeuchi; "Fractionation of IgG Fragments Using Reversed Micellar Extraction," *J. Ferment. Bioeng*., **77**, 80-84 (1994)

Kelley, B. D., D. I .C. Wang and T. A. Hatton; "Affinity-Based Reversed Micellar Protein Extraction: I. Principles and Protein-Ligand Systems," *Biotechnol. Bioeng.*, **42**, 1199-1208 (1993)

Khmelnitsky, Y. L., A. V. Kabanov, N. L. Klyachko, A. V. Levashov and K. Martinek; Structure and Reactivity in Reverse Micelles, M. P. Pileni ed., p.230-261, Elsevier Co., Amsterdam (1989)

Kinugasa. T., K. Watanabe and H. Takeuchi; "Activity and Conformation of Lysozyme in Reverse Micellar Extraction," *Ind. Eng. Chem. Res.*, **31**, 1827-1829 (1992)

Kinugasa, T., A. Hisamatsu, K. Watanabe and H. Takeuchi; "A Reversed Micellar System using Mixed Surfactants of Sodium bis(2-ethylhexyl) sulfosuccinate and Di(2-ethylhexyl) phosphoric acid for Extraction of Proteins," *J. Chem. Eng.,Japan*, **27**, 557-562 (1994)

Kuboi, R., K. Hashimoto and I. Komasawa; "Separation of Proteins with Reverse Micellar Liquid Membranes (in Japanese)," *Kagaku Kogaku Ronbunshu*, **16**, 335-342 (1990a)

Kuboi, R., Y. Mori and I. Komasawa; "Reverse micelle size distribution and mechanism of protein solubilization into reverse micelles (in Japanese)," *Kagaku Kogaku Ronbunshu*, **16**, 763-771 (1990b).

Kuboi, R., Y. Mori and I. Komasawa; "Denaturation and deactivation of Porcine Pancreas lipase in reverse micelle extraction (in Japanese)," *Kagaku Kogaku Ronbunshu*, **16**, 1060-1066 (1990c).

Kuboi, R., H. Tanaka and I. Komasawa; "Effect of Salt Addition on the Hydrophobicities of the System and Proteins in Aqueous Two-Phase Extraction Systems (in Japanese)," *Kagaku Kogaku Ronbunshu*, **17**, 67-74 (1991)

Kuboi, R., D.-R. Li, Y. Yamada and I. Komasawa; "Effect of Salt Addition on the Hydrophobicity of Reverse Micelles Partition System (in Japanese)," *Kagaku Kogaku Ronbunshu*, **18**, 66-71 (1992a)

Kuboi, R., Y. Yamada and I. Komasawa; "Effect of water content on the activity of lipase-hydrolysis of olive oil in reverse micelles (in Japanese)," *Kagaku Kogaku Ronbunshu*, **18**, 72-77 (1992b).

Kuboi, R., K. Yano, H. Tanaka and I. Komasawa; "Evaluation of surface hydrophobicities of proteins using hydrophobic interaction with non-ionic surfactants in aqueous two-phase partitioning systems (in Japanese)," *Kagaku Kogaku Ronbunshu*, **19**, 446-454 (1993)

Lang, J. and A Jada; "Structure and Dynamics of Water-in-Oil Droplets Stabilized by Sodium Bis(2-ethylhexyl) Sulfosuccinate," *J. Phys. Chem*., **92**, 1947-1953 (1988)

Larsson, K. M. and M. P. Pileni; "Interaction of native and modified cytochrome c with a negatively charged reverse micellar liquid interface, " *Eur. Biophys. J.*, **21**, 409 (1993)

Leodidis, E. B. and T. A. Hatton; Structure and Reactivity in Reverse Micelles, M. P. Pileni ed., p.270-302, Elsevier Co., Amsterdam (1989)

Leser, M. E. and P. L. Luisi; "Application of Reverse Micelles for the Extraction of Amino Acids and Proteins," *Chimia*, **44**, 270-282 (1990)

Lowry, R. R. and I. J. Tinsley; "Rapid calorimetric determination of fatty acid," *J. Am. Oil Chem. Soc.*, **53**, 470-472 (1976).

Luisi, P. L.; "Enzymes hosted in reverse micelles in hydrocarbon solution," *Angew. Chem. Int. Ed. Engl.*, **24**, 439-528 (1985).

Lye, G. J., J. A. Asenjo and D. L. Pyle; "Extraction of Lysozyme and Ribonuclease-a Using Reverse Micelles: Limits to Protein Solubilization," *Biotechnol. Bioeng.*, **47**: 509-519 (1995)

Marcozzi, G., N. Correa, P. L. Luisi, M. Caselli, "Protein Extraction by Reverse Micelles: A Study of the Factors Affecting the Forward and Backward Transfer of α-Chymotrypsin and Its Activity," *Biotechnol. Bioeng.*, **38,** 1239-1246 (1991)

Marzola, P., C. Pinzino and C. A. Veracini; "Spin-Labeling Study of Human Serum Albumin in Reverse Micelles," *Langmuir*, **7 ,**238-242 (1991)

Matzke, S. F., A. L. Cregh, C. A. Haynes, J. M. Prausnitz and H. W. Blanch; "Mechanisms of Protein Solubilization in Reverse Micelles," *Biotechnol. Bioeng.*, **40**, 91-102 (1992)

Miyake, Y., T. Owari, K. Matsuura and M. Teramoto; "Enzymatic reaction in water-in-oil microemulsions: I. Rate of hydrolysis of a hydrophilic substrate, acetylsalicylic acid," *J. Chem. Soc. Faraday Trans.*, **89**, 1993-1999 (1993).

Miyake, Y., T. Owari, K. Matsuura and M. Teramoto; "Enzymatic reaction in water-in-oil microemulsions: II. Rate of hydrolysis of a hydrophobic substrate, 2-naphtyl acetate," *J. Chem. Soc. Faraday Trans.*, **90**, 979-986 (1994).

Naoe, K., M. Imai and M. Shimizu; " Optimal Amphiphile Concentration for Lysozyme Extraction using Reverse Micelles," *Trans IChemE. Part C*, **74**, 163-170 (1996)

Nishiki, T., I. Sato, T. Kataoka and D. Kato; "Partitioning Behavior and Enrichment of Proteins with Reversed Micellar Extraction: I. Forward Extraction of Proteins from Aqueous to Reversed Micellar Phase," *Biotechnol. Bioeng.*, **42**, 596-600 (1993)

Philips, J. B., H Nguyen and V. T. John; "Protein Recovery from Reversed Micellar Solutions through Contact with a Pressurized Gas Phase," *Biotechnol Prog.*, **7**, 43-48 (1991)

Pires, M. J., M. R. Aires-Barros and J. M. S Cabral; "Liquid-Liquid Extraction of Proteins with Reversed Micelles," *Biothecnol. Prog.*, **12**, 290-301 (1996)

Prazeres, D. M. F., F. Lemos, F. A. P. Garcia and J. M. S. Cabral; "Modeling lipolysis in a reversed micellar system: Part I. Conventional batch reactor," *Biotechnol. Bioeng.*, **42**, 759-764 (1993).

Rahaman, R. S., J. Y. Chee, J. M. S. Cabral and T. A. Hatton; "Recovery of an Extracellular Alkaline Protease From Whole Fermentation Broth Using Reversed Micelles," *Biotechnol. Prog.*, **4**, 218-224 (1988)

Seelig, J.; Lipid Polymorphism, Reverse Micelles, and Phosphorus-31 Nuclear Magnetic Resonance, Reverse micelles, Biological and Technological Relevance of Amphiphilic Structures in Apolar Media, Luisi, P. L. and Straub B. E. (ed.), Plenum Press, New York (1984).

Sequire, P. G. P. Moser and C. T. O'Konski; The Hydrodynamic Properties of Bovine Serum Albumin Minoner and Dimer. *Biochemistry*, **7**, 4261-4272 (1968)

Shield, J. W., H. D. Ferguson, A. S. Bommarius and T. A. Hatton; "Enzymes in Reversed Micelles as Catalysts for Organic-phase Synthesis Reactions," *Ind. Eng. Chem. Fundam.*, **25**, 603-612 (1986)

Shioi, A., M. Harada, H. Takahashi and M. Adachi; "Protein Extraction in Polyoxyethylene Alkyl Ether/AOT Microemulsion Systems," *Proc. of ISEC '96*, 1381-1386 (1996)

Shirahama, H., T. Shikuma and T. Suzawa; "Participation of Electrolyte Cations in Albumin Adsorption onto Negatively Charged Polymer Latices," *Colloid Polym. Sci*., **267**, 587-594 (1989)

Takeda, K., H. Sasaoka, K. Sasa, H. Hirai, K. Hachiya and Y. Moriyama; "Size and Mobility of Sodium Dodecyl Sulfate-Bovine Serum Albumin Complex as Studied by Dynamic Light Scattering and Electrophoretic Light Scattering", *J. Colloid Interface Sci.*, **154**, 387-392 (1992)

Takeda, K., K. Harada, K. Yamaguchi and Y. Moriyama; "Conformational changes of bovine serum albumin in an aqueous solution of sodium bis(2-ethylhexyl) sulfosuccinate and in the reverse micelles of the same surfactant," *J. Colloid Interface Sci.*, **164**, 382-386 (1994)

Tsai, S.-W. and C.-L. **Chiang**; "Kinetics, mechanism, and time course analysis of lipase catalyzed hydrolysis of high concentration olive oil in AOT-isooctane reversed micelles," *Biotechnol. Bioeng.*, **38**, 206-211 (1991).

Yamada, Y., C. Kasai, R. Kuboi and I. Komasawa; "Preparation of Lecithin Reverse Micellar Systems and Application to Selective Extraction of Cholesterol Oxidase (in Japanese)," *Kagaku Kogaku Ronbunshu*, **20**, 54-60 (1994a)

Yamada, Y., R. Kuboi and I. Komasawa; "Extraction of Proteins and its

Yamada, Y., R. Kuboi and I. Komasawa; "Extraction of Proteins and its Modeling Using AOT and AOT/TDCA Mixed Reverse Micellar Systems," *Solv. Extr. Res. Dev. Japan*, **1**, 167-178 (1994b)

Yamada, Y.; "High Functional Bioprocesses for Proteins Separation and Enzymatic Reaction Using Mixed Reverse Micellar Systems," Osaka Univ. Ph. D. thesis (1995a)

Yamada, Y., R. Kuboi, I. Komasawa; "Effective Bioconversion with Continuous Product Recovery Using AOT/Lecithin Mixed Reverse Micellar Systems and Centrifugal Partition Chromatography as a Novel Bioreactor," *Biotechnol. Prog.*, **11**. 682 (1995b)

LIST OF PUBLICATIONS

Papers:

1) Hydrolysis Kinetics of Olive Oil with Lipase in a Transfer Cell. Y. Kawano, M. Kawasaki, K. Shiomori, Y. Baba and T. Hano, *J. Ferment. Bioeng.*, **77**, 283-287 (1994)

2) Activity of β-Galactosidase Solubilized in Reverse Micelles and Selective Back Extraction from Micellar Phase. K. Shiomori, Y. Kawano, R. Kuboi and I. Komasawa, *J. Chem. Eng., Japan*, **27**, 410-414 (1994)

3) Effective Purification Method of Large Molecular Weight Proteins Using Conventional AOT Reverse Micelles. K. Shiomori, Y. Kawano, R. Kuboi, I. Komasawa, *J. Chem Eng., Japan*, **28**, 803-809 (1995)

4) Hydrolysis Rate of Olive Oil by Lipase in Monodispersed O/W Emulsion System using Membrane Emulsification. K. Shiomori, T. Hayashi, Y. Baba, Y. Kawano, and T. Hano, *J. Ferment. Bioeng.*, **80**, 552-558 (1995)

5) Characteristics and Kinetics of Lipase-Catalyzed Hydrolysis of Olive Oil in a Reversed Micellar System. K. Shiomori, M. Ishimura, Y. Baba, Y. Kawano, R. Kuboi, I. Komasawa, *J. Ferment. Bioeng*., **81**, 143-147 (1996)

6) Effect of Proteins Solubilized into AOT reverse Micelles on Their Back-extraction and Percolation Processes. R. Kuboi, D.-P. Hong, I. Komasawa, K. Shiomori, Y. Kawano and S.-S. Lee, *Solv. Extr. Res. Dev. Japan*, **3**, 223-230 (1996)

7) Extraction Characteristic of Bovine Serum Albumin Using Sodium Bis (2-Ethylhexyl) Sulfosuccinate Reverse Micelles. K. Shiomori, N. Ebuchi, Y. Kawano, R. Kuboi and I. Komasawa, *J. Ferment. Bioeng*., **86**, 581-587 (1998)

8) Extraction of Proteins and Water with Sodium Bis(2-ethylhexyl) Sulfosuccinate / Long Chain Alkyl Amines Mixed Micellar System. K. Shiomori, Y. Kawano, R. Kuboi and I. Komasawa, *J. Chem Eng., Japan*, **32**, 177-183 (1999)

Related Works:

9) Hydrolysis of Olive Oil with Lipase in a "VibroMixer". Y. Kawano, S. Kiyoyama, K. Shiomori, Y. Baba, T. Hano, *J. Ferment. Bioeng.*, **78**, 293-297 (1994)

Proceedings:

1) Reverse Micellar Extraction of β-Galactosidase from E. coli. K. Shiomori, R. Kuboi and I. Komasawa, *Proc. Symp. Solv. Extr.*,111-115 (1992)

2) Extraction of BSA Using AOT Reverse Micelles. K. Shiomori, N. Ebuchi, Y. Kawano, R. Kuboi, I. Komasawa, *Proc. Symp. Solv. Extr.* , 3-4, (1995)

3) Extraction of Proteins Using Reverse Micelles and Percolation Process. R. Kuboi, D.-P. Hong, I. Komasawa, S.-S. Lee, K. Shiomori and Y. Kawano, *Proc. Symp. Solv. Extr.*, 5-6, (1995)

4) Solubilization of Proteins into Reverse Micelles and Its Effect on Inter-miceller Interaction. S.-S. Lee, D.-P. Hong, R. Kuboi, I. Komasawa, K. Shiomori, Y. Kawano, *Adv. Biosep. Eng. 1995*, 24-29, Soc. Chem. Engrs. Japan (1996)

5) Purification of Large Molecular Weight Proteins by Using the Selective Back-extraction: Method in AOT Reverse Micellar Extraction Systems. K. Shiomori, Y. Kawano, R. Kuboi, I. Komasawa, *Proc. ISEC'96*, 1483-1488 (1996)

6) Extraction Behavior of BSA to AOT Reverse Micellar System. K. Shiomori, N. Ebuchi, Y. Kawano, R. Kuboi, I. Komasawa, *Adv. Biosep. Eng. 1996-1997*, 74-79 (1998)

7) Extraction Behaviors of Large Molecular Weight Proteins into AOT Reverse Micelles. K. Shiomori, Y. Kawano, R. Kuboi and I. Komasawa, *Adv. Biosep. Eng. 1996-1997*, 103-107 (1998)

ACKNOWLEDGMENT

The author is greatly indebted to Professor Dr. Isao Komasawa and Professor Dr. Ryoichi Kuboi (Department of Chemical Science and Engineering, Graduate School of Engineering Science, Osaka University), and Professor Dr. Yoshinobu Kawano (Department of Materials Science, Miyazaki University) for their constant guidance and helpful advice throughout this work.

The author would like to express his grateful acknowledgment to Professor Dr. Yoshinobu Kawano for his helpful advice and encouragement throughout this work.

The author is also indebted to Professor Dr. Masahito Taya (Graduate School of Engineering Science, Osaka University) and Professor Dr. Ken-ichi Suga (Graduate School of Engineering, Osaka University) for their valuable comments and suggestions during the completion of this thesis.

The author wishes to thank Professor Dr. Yoshinari Baba and Mr. Yasuo Saito (Department of Materials Science, Miyazaki University), Professor Dr. Yasuo Hatate (Faculty of Engineering, Kagoshima University), Assistant Professor Dr. Takayuki Hirai (Graduate School of Engineering Science, Osaka University), Dr. Izumi Tsuboi (Nippon Lever B. V.) for their kind encouragement.

The author is also grateful to Professor Dr. Yuji Goto (Institute for Protein Research, Osaka University) and Assistant Professor Dr. Masahide Yasuda (Cooperative Research Center, Miyazaki University) for CD measurements.

Thanks are given to following colleagues for their experimental cooperation : Drs. H. Tanaka, Y. Yamada, K. Yano, H. Sato and H. Umakoshi (Graduate School of Engineering Science, Osaka University), S. Shiojiri, Messes. T. Maruki, D.-P. Hon, M. Ishimura, S. Kiyoyama (Miyakonojyo Technical Institute of College), T. Hayashi, N. Ebuchi, K. Nagatomo, M. Yoshida, S. Hiramizu, K. Kamimura, and T. Honbu, Mses. M. Kawasaki and C. Kasai and also other members at the same laboratory.

The author would like to express his thanks to his parents Hiromu Shiomori and Reiko Shiomori, and his parents-in-low Hiroshi Yamanaka and Tsuyako Yamanaka for their continuous and hearty encouragement.

Finally, the author would like to express his grateful thanks to his wife Masumi Shiomori for her continuous and hearty encouragement.

Koichiro Shiomori