ANALYTICAL SCIENCES OCTOBER 1998, VOL. 14

Reviews

Aqueous Micellar Two-Phase Systems for Protein Separation

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The extractive technique for protein purification based on two-phase separation in aqueous micellar solutions (aqueous micellar two-phase system (AMTPS)) is reviewed. The micellar solution of a nonionic surfactant, such as polyoxyethylene alkyl ether, which is most frequently used for protein extraction, separates into two phases upon heating above its cloud point. The two phases consist of a surfactant-depleted phase (aqueous phase) and a surfactant-rich phase. Hydrophilic proteins are partitioned to the aqueous phase and hydrophobic membrane proteins are extracted into the surfactant-rich phase. Because of the methodological simplicity and rapidity, this technique has become an effective means, and thus has been widely used for the purification and characterization of proteins. In contrast to polyoxyethylene alkyl ether, micellar solutions of a zwitterionic surfactant, such as alkylammoniopropyl sulfate, separate below the critical temperature. Alkylglucosides can also separate into two phases upon adding water-soluble polymers. Recently, these two-phase systems have been exploited for protein separation. Additionally, hydrophobic affinity ligands, charged polymers, and ionic surfactants have been successfully used for controlling the extractability of proteins in AMTPS.

Keywords Surfactant, micelle, phase separation, cloud point, protein, separation, characterization

1 Introduction

In view of the purification of hydrophobic membrane proteins, attention is being paid to two-phase separation in micellar solutions of nonionic surfactants (aqueous micellar two-phase system (AMTPS)). Two-phase separation spontaneously occurs when the temperature of the micellar solution of a nonionic surfactant is increased above a certain temperature defined as the cloud point. One of the phases is a surfactant-rich phase, and the other is a surfactant-depleted phase (aqueous phase). Hydrophobic solutes, which are originally solubilized in micelles, can be extracted into a surfactant-rich phase, while hydrophilic ones are retained in the aqueous phase.

The first use of AMTPS as separation media was reported by Watanabe and Tanaka[1] in 1978 for the concentration of zinc ion as a metal chelate, in which two-phase separation was obtained from a homogeneous PONPE 7.5, polyoxyethylene alkyl ether (POEAE) surfactant, micellar solution upon heating above its cloud point. Thus, this method has also been termed "temperature-induced phase separation" or "cloud-point extraction". Thermally-induced AMTPS has been applied to the separation and preconcentration of metal chelates, organic compounds, and biological species.[2–5]

In 1981, Bordier[6] applied the thermally-induced AMTPS of Triton X-114 to the extraction of hydrophobic membrane proteins. In this method, membrane pro-

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teins are first solubilized from biomembranes into an aqueous Triton X-114 micellar solution. Then, upon increasing the temperature, the solution is rendered to separate into two phases. The proteins are extracted into a surfactant-rich phase with a degree depending on their hydrophobicity. Because of its technical simplicity, a number of membrane proteins were purified by the use of the Triton X-114-based aqueous two-phase system coupled with and without chromatographic separation. AMTPS has also been used for characterizing the hydrophilicity of proteins. Despite successful applications, there have been few reviews[7–10] focused on the separation and characterization of proteins using AMTPS.

The aim of this review is to introduce the availability of AMTPS for protein purification and characterization. Also, examples of applications and recent developments of AMTPS for proteins are described.

2 Phase Behavior of Micellar Solutions

2·1 AMTPS of POEAE surfactants

An aqueous micellar two-phase system is based on the unique phase separation phenomenon in surfactant micellar solutions.[11,12] In AMTPS, polyoxyethylene alkyl ethers (POEAEs), nonionic surfactants, are exclusively used.[7,8,13] An aqueous micellar solution of POEAE surfactants becomes turbid, and then separates into two isotropic phases upon heating above a certain temperature, defined as the cloud point. One of the phases is an aqueous phase in which surfactant micelles are depleted and the other is a surfactant-rich phase.

Table 1 lists the cloud points of several POEAE surfactants.[14-20] The cloud point depends on the balance between the alkyl and polyoxyethylene chain length of the surfactant molecule. For POEAEs having the same alkyl length, the cloud point increases with increasing polyoxyethylene chain length. In contrast, it decreases with increasing alkyl chain length. The cloud point (C) of POEAE having a linear alkyl chain has been empirically described by the equation[21]

$$C\ (^{\circ}\mathrm{C}) = 220 \log N_{\mathrm{E}} - 5.5N_{\mathrm{C}} - 55$$

where N_{E} and N_{C} are the ethylene oxide and alkyl carbon numbers, respectively. Recently, this has been improved and extended to various types of POEAE surfactants including not only linear alkyl but also branched alkyl, cyclic alkyl, linear alkylphenyl, and branched alkylpheny ethers.[20]

In these surfactants listed in Table 1, Triton X-114 (polyoxyethylene(7.5) *t*-octyl-*p*-phenyl ether) is the most frequently employed in AMTPS for proteins, because of its low cloud point, approximately 22°C[14,18], and its ability to solubilize membrane proteins. Figure 1 shows a phase diagram of a Triton X-114 micellar solution, in which the phase separation temperature is plotted as a function of the concentration of Triton X-114.[10] The curve which separates the two-phase region from the one-phase region can be referred to as a lower consolute boundary. It should be stressed that the phase diagram is more complex at a higher concentration range in which anisotropic phases exist.[22]

Table 1 Cloud points of POEAE surfactants.

Surfactant	Cloud point/°C
C_6E_3	40.5
C_6E_4	63.8
C_6E_5	75
C_8E_3	7
C_8E_4	38.5
C_8E_5	58.6
C_8E_6	72.5
$C_{10}E_4$	19.7
$C_{10}E_5$	41.6
$C_{10}E_6$	60.3
$C_{10}E_8$	84.5
$C_{12}E_5$	28.9
$C_{12}E_6$	51.0
$C_{17}E_7$	64.7
$C_{12}E_8$	77.9
$C_{14}E_5$	20
$C_{14}E_6$	42.3
$C_{14}E_7$	57.6
$C_{14}E_8$	70.5
Triton X-114 (t-$C_8\phi E_{7-8}$)	22.0[14,18]
Triton X-100 (t-$C_8\phi E_{9-10}$)	64[15], 65.1–65.6[16,17], 64[15]

Cloud point values are from ref. 20 unless otherwise sited.
C_nE_m, $CH_3(CH_2)_{n-1}(OCH_2CH_2)_mOH$.
t-$C_8\phi E$, $(CH_3)_3CCH_2C(CH_3)_2C_6H_4(OCH_2CH_2)_mOH$.

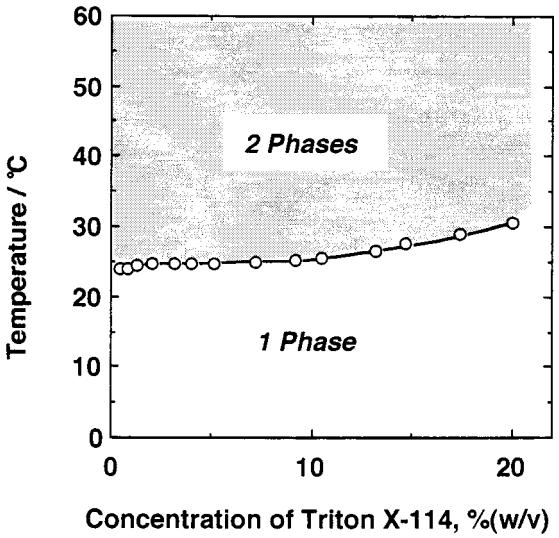

Fig. 1 Phase diagram for an aqueous solution of Triton X-114. A two-phase region is above the curve, while an one-phase region is below the curve (Reproduced with permission from ref. 10. Copyright 1995 Elsevier Science).

The phase separation is reversible, and could be due to the decrease in the solubility of micelles with increasing temperature.[23] Below the cloud point, micelles are solubilized in water due to hydrogen bonding between the solvent and the oxygen atoms in the polyoxyethylene chain. When the temperature is raised, this hydrogen bonding decreases, thus resulting in the formation of intermicellar aggregation. The micelles become macroscopically large and are deposited from the solution as a concentrated micellar phase, yet having a large amount of water.

Recently, the thermodynamics of two-phase separation in nonionic surfactant solutions has been extensively studied. Clouding phenomena could be explained by a conformational change in the polyoxyethylene chain, which results in the decrease in the polarity of an ethylene oxide unit and in the reduction of its hydrophilicity[24,25], and by large losses in the entropy of mixing of micelles and water above the cloud point.[26,27] Several theoretical models have been proposed for describing nonionic micellar solution properties including phase separation.[27-30] For example, the Gibbs free energy of a micellar solution is taken into account for predicting the solution behavior, in which the free energy is divided into three elements: the free energy of the formation of micelles, the mixing entropy of the micelles and water, and the free energy of the interaction between the micelles.[28] The Flory-Huggins theory which describes the statistical mechanical treatment of polymer solutions based on the lattice model was also successfully applied to the micellar system for predicting the cloud point.[27,29] In this application, a micelle is regarded as a polymer and a surfactant molecule as a segment which can occupy one lattice in the solution. The phase separation phenomenon was described as a function of the surfactant structure.[29] More recently, user-friendly computer programs based on molecular thermodynamic theories have been developed, allowing one to accurately predict the surfactant micellar solution behavior.[31,32]

The cloud point can be controlled by the addition of various compounds. Inorganic salts having a salting-out effect, such as chloride and sulfate, reduce the cloud point of POEAE surfactants with the effect depending on their concentration.[16,17,26,33-40] On the other hand, the cloud point is increased by the addition of nitrate or thiocyanate which are known to have a salting-in effect.[16,17,26,34-39] For example, the cloud point of Triton X-100, which is 65˚C without additives, drops to about 30˚C in the presence of 0.5 M Na_2SO_4, whereas in the presence of 2 M NaSCN it increases to 90˚C.[16] In general, shorter saturated hydrocarbons lower the cloud point but not to a large extent, while longer ones raise it.[23] Polar organic compounds, such as aliphatic alcohols, fatty acids, and phenols, depress the cloud point[41-43], whereas the addition of ionic surfactants typically results in elevating it.[19,33,44-48] Polyols, such as glucose[49], sucrose[49], or glycerol[18,50], and water-soluble polymers[51-53], such as polyethylene glycol, dextrans, or polyvinylpyrrolidone, are effective for decreasing the cloud point. The effect of the polymers depends not only on their concentrations but also on their molecular weights.[53] The phase behavior of the mixtures of POEAE surfactants and polymers have been extensively studied.[54-60]

The change in the cloud point by the addition of organic compounds should be due to the change in the nature of micelles with solubilizing them, and/or with forming mixed micelles, whereas salts and polyols should alter the cloud point upon changing the water structure around the micelles.

2·2 AMTPS of various surfactants

Other nonionic surfactants and zwitterioninc surfactants are also utilized in AMTPS for proteins.[3,5,10,61-68] Pluronics are a series of triblock copolymers of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), and are known to be a nonionic surfactant in which PEO and PPO play the role of hydrophilic and hydrophobic moieties, respectively.[69-71] Aqueous solutions of Pluronics also show thermally-induced two-phase separation. Figure 2 shows a phase diagram of Pluronic L61 and 25R2.[68] Two phases are present in the region above the respective curves.

Micellar solutions of alkylammoniopropyl sulfate or lecithin, which are zwitterionic surfactants, also separate into two phases depending on the temperature.[28,67,72] As an example, a phase diagram of 3-(nonyldimethylammonio)propyl sulfate (C_9APSO_4) and the corresponding decyl analog ($C_{10}APSO_4$) is shown in Fig. 3.[67] The phase separation temperatures are

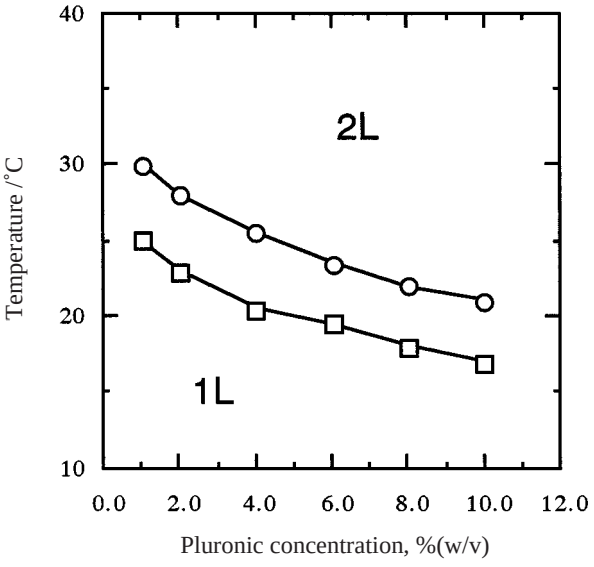

Fig. 2 Phase diagram for aqueous solutions of Pluronic L61 (□) and 25R2 (○). Above the respective curve, the solution separates into two phases (2L) while a single homogeneous solution exists below the curve (1L). All solutions are buffered with 10 mM Tris–HCl at pH 7.45 (Reproduced with permission from ref. 68. Copyright 1997 The Japan Society for Analytical Chemistry).

increased with increasing their concentrations in both surfactant solutions. In contrast to POEAE and Pluronic surfactant systems, two-phase separation is observed in the region below the respective curve (upper consolute boundary).

A dilute micellar solution of alkylglucosides (AGs) and sugar esters (SEs), which are known to be nonionic surfactants having sugar as a hydrophilic moiety, does not show the clouding phenomenon upon raising the temperature. By the addition of a water-soluble polymer, however, the solution separates into two phases, one is rich in the surfactant and another contains most of the polymer.[61,66,73-76] Thus, this can be called "polymer-induced phase separation" or "polymer-induced AMTPS". Figure 4 shows a phase diagram of *n*-octyl-*β*-D-glucoside (OG), *n*-nonyl-*β*-D-glucoside (NG), *n*-heptyl-*β*-D-thioglucoside (HTG), *n*-octyl-*β*-D-thioglucoside (OTG), and sucrose manolaurate (SML) as a function of the polyethylene glycol (PEG) concentration.[61]

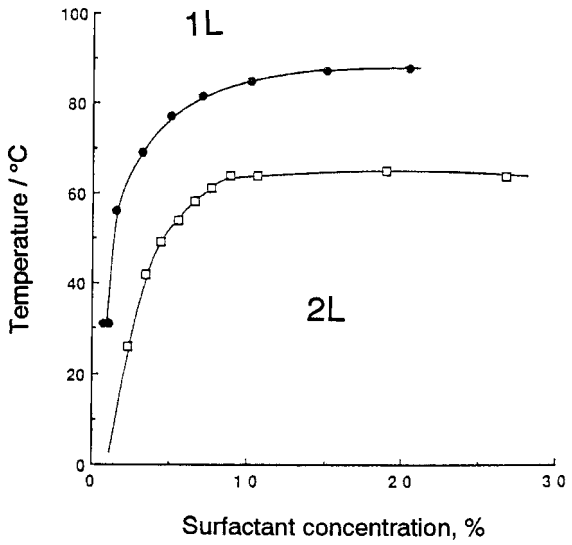

Fig. 3 Phase diagram for aqueous solutions of C_9APSO_4 (□) and $C_{10}APSO_4$ (●). 1L and 2L have the same meanings as in Fig. 2 (Reproduced with permission from ref. 67. Copyright 1991 American Chemistry Society).

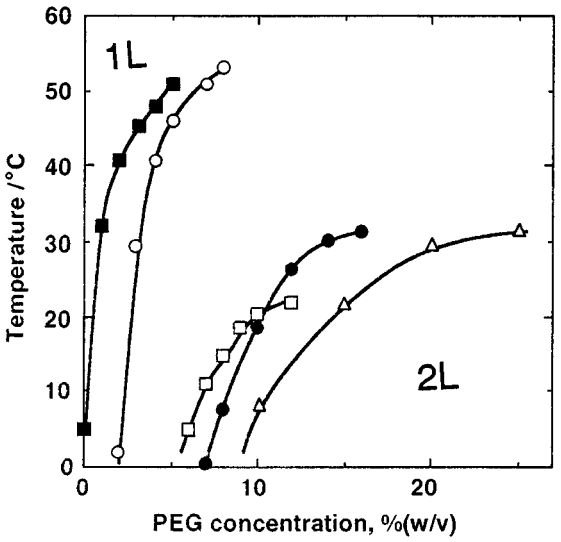

Fig. 4 Phase separation temperature in aqueous micellar solutions of OG (●), NG (○), HTG (□), OTG (■), and SML (△) as a function of the concentration of PEG (average molecular weight, 7500). A two-phase region exists below the respective curve, while a single phase region exists above the curve. Surfactant concentration, 2%(w/v) (Reproduced with permission from ref. 61. Copyright 1991 The Japan Society for Analytical Chemistry).

The phase separation temperature is increased along with an increase in the PEG concentration. In this phase diagram, the upper consolute boundary is also shown, that is, a two-phase region exits below the curves. Other water-soluble polymers, such as methylcellulose, dextran, and Ficoll, also induce phase separation in micellar solutions of OTG.[61]

In addition to the above-mentioned systems, many surfactant solutions must show the two-phase separation phenomenon under appropriate conditions.[11,12] However, applications to the separation and characterization for proteins has not yet appeared except for a few examples, which are described later.[77,78]

3 Protein Partitioning in AMTPS of POEAE Surfactants

3·1 POEAE-based AMTPS for proteins

The first application of AMTPS for the separation of proteins was made by Bordier[6] in 1981. In his report, hydrophilic proteins, serum albumin, catalase, ovalbumin, concanavalin A, myoglobin, and cytochrome *c*, were submitted in order to test partitioning in the two-phase system of Triton X-114. All of these proteins were shown to be exclusively recovered in the aqueous phase. On the other hand, hydrophobic (amphiphilic) integral membrane proteins, acetylcholinesterase, bacteriorhodopsin, and cytochrome *c* oxidase, were to be well extracted into the surfactant-rich phase. Figure 5 shows the result of the extraction of hydrophobic cytochrome b_5 and hydrophilic catalase as well as the volume fraction and recovery of Triton X-114 in the two-phase system of 2%(w/v) Triton X-114.[79] Triton X-114 was highly concentrated in a small volume of the surfactant-rich phase into which most of cytochrome b_5 was extracted. In contrast, catalase was almost recovered in the aqueous phase. From these experiments, it appeared that AMTPS can be useful for separating hydrophilic and hydrophobic proteins from each other. This is based on the fact that the surfactants can bind to and solubilize hydrophobic proteins because of their amphiphilicity, whereas they can not do so to hydrophilic ones.

With respect to the differences in the hydrophobicity/hydrophilicity of proteins, Trestappen *et al.*[80] have systematically and quantitatively tested protein partitioning employing a series of POEAE surfactants and pro-

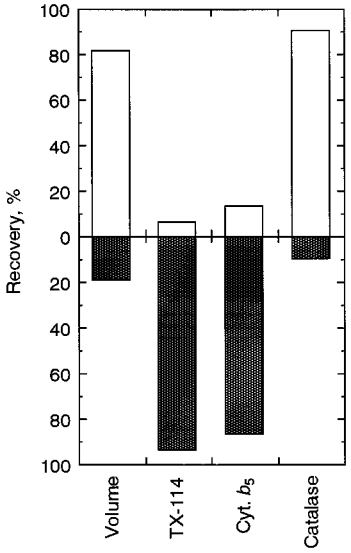

Fig. 5 Distribution of hydrophobic cytochrome b_5 (Cyt. b_5), hydrophilic catalase, and Triton X-114 into the aqueous and the surfactant-rich phases in the AMTPS of 2%(w/v) Triton X-114 containing 10 mM Tris–HCl (pH 7.4). The phase separation was conducted at 30˚C.

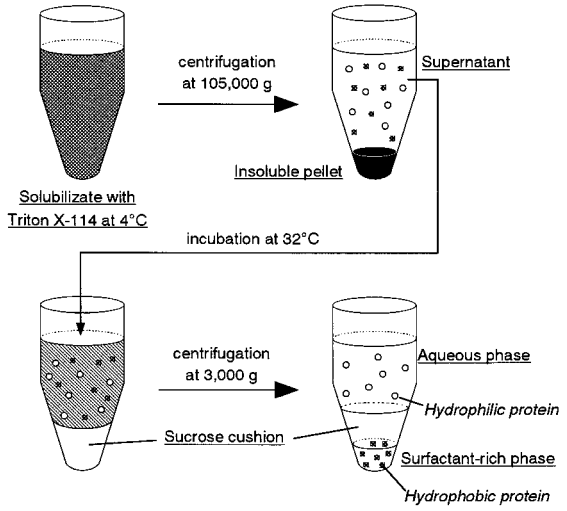

Fig. 6 Procedure for the extraction of brush border membrane proteins with the AMTPS of Triton X-114.

teins with various hydrophobicity. There was a positive correlation between the hydrophobicity and extractability of proteins into a surfactant-rich phase. They also investigated the effect of such conditions as temperature, salt, and pH on the extraction of proteins in the Triton X-114-based system using cholesterol oxidase from various sources as a model.[81] The protein as well as Triton X-114 showed a greater tendency toward the surfactant-rich phase upon increasing the temperature or adding salt. Additionally, a very close correlation between the extractability in the Triton X-114-based system and retention in the octyl-Sepharose column was observed for various types of glycosylphosphatidylinositol-alkaline phosphatase.[82] These results confirm the idea that the extraction in AMTPS is primarily controlled by a hydrophobic interaction between the proteins and surfactants.

3·2 Typical experimental scheme

Extraction can be conducted only by incubating a protein mixture containing Triton X-114 at a temperature above its cloud point (typically *ca*. 30˚C) after solubilization at lower temperature where Triton X-114 is miscible in water. Frequently, a sucrose cushion is used to ensure the separation of two phases. Figure 6 indicates a typical experimental scheme for the solubilization and extraction of brush border membrane proteins.[83] The membrane proteins were first solubilized with an aqueous buffered solution (pH 7.4) of 1% Triton X-114 at 4˚C. After removing a surfactant-insoluble pellet by centrifugation at 105000×*g*, the supernatant solution thus obtained was subjected to two-phase separation as follows. The solution was placed onto a cushion of 6%(w/v) sucrose containing 0.06% Triton X-114, and incubated at 32˚C for two-phase separation. After low-speed centrifugation, the surfactant-rich phase was obtained below the cushion, while the aqueous phase was retained above the cushion, as depicted in Fig. 6. When necessary, both phases were subjected to a further washing process by repeating the addition of a fresh buffer or a Triton X-114 solution and phase separation for decontamination.

3·3 Separation and characterization of proteins

Because of the methodological simplicity, AMTPS has been widely employed to process a variety of membrane proteins, enzymes, and receptors from animals, plants, and bacteria.[7–10] It has now become a good alternative to the classical separation procedures, such as fractionation with ammonium sulfate and even column chromatography. Some applications are introduced below.

The one-step purification of cytochrome *b* from cytochrome bc_1 complexes of *Paracoccus denitrificans* and *Rhodopseudomonas shaeroides* was conducted by AMTPS.[84] The cytochrome bc_1 complex from *P. denitrificans* consists of cytochrome *b*, cytochrome c_1, and iron-sulfur protein. Upon two-phase separation, cytochrome *b* was extracted into the surfactant-rich phase, while the other proteins were retained in the aqueous phase. For *R. shaeroides*, the same result was obtained.

Pyruvate oxidase was also isolated from *Escherichia coli* with a single run of the two-phase separation of Triton X-114 after preliminary heat fractionation.[85] The surfactant-rich phase containing 95% of the enzyme was collected and ultra-centrifuged for further purification. The purified enzyme was recovered as a pellet with a yield of 52% and a purification factor of 13.6. The results were superior to those obtained by the conventional method using DEAE-Sephadex chro-

matography. In the method, additionally, purification can be completed within 1 day, while the chromatographic method takes at least 3 weeks. Thus, this technique provides a highly effective means for the purification of pyruvate oxidase from *E. coli*.

Polyphenol oxidase was partially purified from grape berries[86] and from broad beans[87] with AMTPS. Upon phase separation the enzyme was retained in the aqueous phase with the same purification factor as that obtained by the previously established method including ammonium sulfate fractionation. However, the yield was higher and the time for the procedure was greatly reduced. Chlorophylls and phenolic compounds, which are interferences for the analysis of polyphenol oxidase, were also removed through the process by excluding them into the surfactant-rich phase. Additionally, the enzyme obtained by this method was almost latent, while an active form was obtained by ammonium sulfate fractionation. Thus, AMTPS is an excellent method for the isolation of polyphenol oxidase with respect to the efficiency of process, the removal of interferences, and the isolation of the latent form.

These excellent results concerning the purification of proteins by the use of AMTPS are very few. Generally, additional proteins are also extracted or retained together with the proteins of interest in some degree. For example, in the separation of rat intestinal brush border membrane proteins using AMTPS of Triton X-114, eleven proteins are reported to be extracted into a surfactant-rich phase and nine are to be retained in an aqueous phase.[83] Thus, further purification steps, such as column chromatography and electrophoresis, are required after separation with AMTPS. However, it should be stressed that AMTPS could be an effective means for purifying proteins in view of its methodological simplicity and rapidity.

Additionally, protein partitioning in AMTPS has also been exploited for the characterization of proteins.[7] First of all, one can judge the protein of interest to be an integral or not from its extractability into the surfactant-rich phase; secondly, one can monitor the change in the protein hydrophobicity due to a conformational alteration induced by pH, ligands, enzymes, and chaperones. For example, a conformational change in protein kinase C-α, induced by binding its substrate, arginine-rich peptide, has been investigated by using the AMTPS of Triton X-114.[88] The substrate-free enzyme is slightly extracted into the surfactant-rich phase, while in the presence of the substrate the extractability of the enzyme is increased. These results suggest that the binding of the substrate promotes exposure of the hydrophobic domains of the enzyme. Increased hydrophobicity induced by the substrate was also reported for pyruvate oxidase.[89] In contrast, the hydrophobicity of the insulin receptor was reported to be reduced by binding insulin.[90] A pH-induced conformational change of saposin, small lysosomal glycoproteins, has been studied in the Triton X-114-based AMTPS.[91] At neutral pH, saposins (Sap) A, B, C, and D are all partitioned into the aqueous phase. Upon decreasing the pH, Sap A, C, and D are extracted into the surfactant-rich phase, indicating exposure of their hydrophobic domains, while only Sap B remains in the aqueous phase. These results are very suggestive of their roles in lysosomes, where the pH is acidic. pH-induced conformational changes were also studied for adenovirus proteins[92], carboxypeptidase E[93], influenza virus hemagglutinin[94], clathrin[95,96], and some toxins[97-100] by using this method. Recently, the characterization of a mutant prion protein and conversion to a scrapie-like form have been studied with the AMTPS of Triton X-114.[101,102]

Some anomaly in protein partitioning has been reported.[103-108] An acetylcholine receptor, a channel-forming integral membrane protein, was first reported to be partitioned into the aqueous phase in the Triton X-114-based AMTPS.[105] This anomalous partitioning was explained by the irregular hydrophobic surface domain of the receptor, which can not interact with Triton X-114 having *t*-octylphenyl as a hydrophobic moiety, due to a steric restriction. Indeed, by the addition of linoleic acid having a linear alkyl chain to the system, the receptor was extracted into the surfactant-rich phase.

In spite of some anomalous examples, this method has now become one of the most standard procedures for monitoring hydrophobic nature of the proteins.

3·4 Theoretical aspect for protein partitioning

Few investigations have been reported concerning the mechanisms of protein partitioning in AMTPS.[62-64,109] Nikas *et al.*[109] proposed a theoretical model for protein partitioning based on an excluded-volume interaction between globular hydrophilic proteins and micelles. This model incorporates the fact that micelles grow into long and polydisperse cylindrical microstructures, and thus form an entangled-network configuration upon phase separation in aqueous micellar solutions. In the model, the partition coefficient of the protein (K_p), which is defined as the ratio of the protein concentration in the top phase ($C_{p,t}$) to that in the bottom phase ($C_{p,b}$), which is $C_{p,t}/C_{p,b}$, has been described by the equation

$$K_p=\exp\{-(\phi_t-\phi_b)(1+R_p/R_0)^2\}$$

where ϕ_t and ϕ_b are the volume fractions of the surfactant in the top and bottom phases, respectively, R_p is the hydrodynamic radius of the protein, and R_0 is the cross-sectional radius of the cylindrical micelles. From this equation, proteins are to be partitioned depending on the difference in the surfactant concentration between two phases and in the relative size of proteins to micelles. Figure 7 shows the predicted K_p curve as a function of R_p/R_0 at a fixed $\phi_t-\phi_b$ value (10%, at 21˚C) in the AMTPS of tetraoxyethylene decyl ether ($C_{10}E_4$). In this case, the top and bottom phases are surfactant-rich and aqueous phases, respectively. K_p is decreased

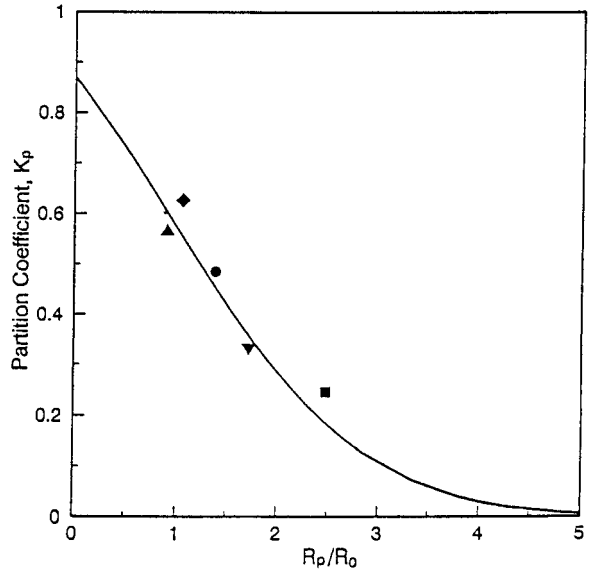

Fig. 7 Predicted protein partition coefficient (K_p) as a function of the ratio R_p/R_0 in the AMTPS of $C_{10}E_4$. R_p is the hydrodynamic radius of proteins, and R_0=21Å is the cross-sectional radius of a $C_{10}E_4$ cylindrical micelle. The difference in the volume fraction of $C_{10}E_4$ between the top (surfactant-rich) and bottom (aqueous) phases (ϕ_t–ϕ_b) is 10%. The symbols correspond to the experimentally measured K_p values of the following proteins: (▲, R_p=19Å) cytochrome *c*, (◆, R_p=22Å) soybean inhibitor, (●, R_p=29Å) ovalbumin, (▼, R_p=36Å) bovine serum albumin, and (■, R_p=52Å) catalase. (Reproduced with permission from ref. 63. Copyright 1996 John Wiley & Sons).

with an increase in the protein size, indicating the exclusion of proteins from top phase rich in micelles. The experimentally obtained K_p values for various proteins are also shown in Fig. 7. There is a good agreement in protein partitioning between theory and experiment. Similar results were obtained in the AMTPS of a zwitterionic surfactant, dioctanoyl phosphatidylcholine.[62-64] In this excellent approach, however, only the protein size is considered by the use of hydrophilic proteins. A theoretical treatment of protein partitioning including other interactions, such as hydrophobic and electrostatic, is desired.

3·5 Modification of POEAE-based AMTPS

In the separation of proteins with AMTPS, Triton X-114 has been most frequently used because of its relatively low cloud point, 22˚C. Generally, a micellar solution of Triton X-114 containing proteins is warmed to 25 – 30˚C in order to induce two-phase separation. For thermolabile proteins, however, this temperature still seems to be high to prevent denaturation. Several investigations have been reported to be successful in lowering the cloud point for protein extraction.[14,18,40,110,111] For example, a combined use of Triton X-114 with Triton X-45 having a lower cloud point than Triton X-114 was tested.[14] By changing the proportion of Triton X-45 the cloud point of the mixture could be adjusted anywhere between 0 and 22˚C. There is a linear relationship between the cloud point (*C*) and the weight percentage (*w*) of Triton X-45, described as

$$C(^{\circ}\mathrm{C}) = 21.4 - 1.23w$$

The partitioning properties of the integral membrane enzyme, phosphatidylinositol kinase, in Triton X-114/Triton X-45 were similar to those in Triton X-114 alone.

Nonionic surfactants, such as Triton X-100 and Nonidet P-40 having higher cloud points, can not be exploited in AMTPS for proteins, since many proteins would thermally denature. However, by the addition of salts, the cloud point can be lowered to physiologically acceptable temperatures. The salt-induced phase separation of Triton X-100 and Nonidet P-40 have been applied to the separation of proteins.[40,110,111] Interestingly, in the ammonium sulfate-induced AMTPS of Triton X-100, hydrophilic proteins as well as hydrophobic ones were extracted into the surfactant-rich phase.[40] The extractability of the proteins was dependent on the salt concentration. This means that the methods can be used to fractionate both membrane and non-membrane proteins from cell lysates, as the classical precipitation methods. In the extraction process, the proteins would be dehydrated by ammonium sulfate, however, it is noted that the extraction of hydrophilic proteins is not likely to be due to precipitation, because the concentrations of the salt are far too low to precipitate the proteins.[40]

Polyols, such as sugars[49] and glycerol[18,50], and water-soluble polymers[51-53] are also effective for allowing low-temperature phase separation of POEAE surfactants. Werck-Reichhart *et al.*[18,112] exploited glycerol for reducing the cloud point of Triton X-114 in the extraction of cytochrome b_5 and cytochrome P450 from plant microsomal membranes. In the presence of 30%(v/v) glycerol, since the cloud point of Triton X-114 is lowered below 4˚C, solubilization and phase separation can be conducted in a single step at 4˚C.[18] After high-speed centrifugation, the thus-obtained supernatant was spontaneously separated into two phases. The upper was the surfactant-rich phase in which more than 90% of detectable cytochromes were recovered, and the lower was the glycerol-rich aqueous phase. Since glycerol is well known to be a stabilizing agent for proteins, this technique may be useful for purifying labile membrane proteins. As described later, water-soluble polymers could be exploited not only for lowering the cloud points of POEAEs, but also for inducing phase separation of alkylglucosides and for controlling the extractability of proteins.

These additives, which can lower the cloud points of POEAE surfactants, have extended the range of the surfactants available in AMTPS for proteins regardless of their cloud points.

3·6 APTPS including POEAE surfactants

In protein purification, aqueous two-phase systems have already been utilized as effective and rapid separation techniques, which is based on phase separation in a mixed solution of polymer and salt, or two different polymers, such as polyethylene glycol and dextran.[113-118] Additionally, charged and/or affinity ligand-modified polymers have been introduced for enhancing the selectivity. Thus, many purification procedures have been developed using the aqueous polymer two-phase system (APTPS). In APTPS, however, target proteins are limited to hydrophilic ones, except for the few examples described in this section.

In APTPS, POEAE surfactants have already been employed for treating integral membrane proteins.[119-122] Albertsson *et al*.[120] have applied the aqueous two-phase system of dextran (Dx) and polyethylene glycol (PEG) to the isolation of chlorophyll-protein complexes from thylakoid membranes by introducing Triton X-100 into the system. In the absence of a surfactant, the membrane components are generally found in the lower Dx-rich phase and/or at the interface between the two phases. Increasing the concentration of Triton X-100 which is partitioned into the upper PEG-rich phase, thylakoid materials tend to be solubilized and to be extracted into the upper PEG-rich phase depending on their hydrophobicity. This approach has also been utilized to separate some other membrane proteins, phospholipase A1 from *E. coli*[122], monoamine oxidase from beef liver mitochondria[121], and cytochrome P450 and cytochrome b_5 from the yeast *Brettanomyces anomalus*[119], and so on, not only in the two-phase systems, but also in the aqueous polymer three- and four-phase systems.

A quantitative evaluation of the local surface hydrophobicity of proteins has been conducted by using APTPS containing Triton X-405.[123,124] When Triton X-405 is added to the Dx-PEG system, the proteins having hydrophobic sites interact with Triton X-405 and tend to partition into the PEG-rich phase. Thus, the local hydrophobicity of the protein can be quantified from the change in the partition coefficient of the protein with increase in the Triton X-405 concentration.

4 Partitioning of Proteins in AMTPS of Various Surfactants

Even in the presence of sugars or glycerol, a considerable loss in enzyme activity was often observed during solubilization or phase separation when Triton X-114 was used.[18] The decrease in the enzyme activity is probably due to the strong hydrophobic nature of Triton X-114 and to the presence of highly concentrated surfactant aggregates in the surfactant-rich phase. This problem should be derived from the limitation of the surfactants employable in AMTPS. Although many applications of AMTPS for proteins have been reported, the surfactants used in AMTPS are exclusively limited to polyoxyethylene alkyl ether type, such as Triton X-114. In the purification of membrane proteins, the most appropriate surfactant should be chosen for the protein with respect to the selectivity and denaturing character. Thus, the possibilities of other surfactants should be explored for use in AMTPS. From this point of view, some recent studies concerning AMTPS using various types of surfactants are discussed in this section.

4·1 Alkylglucosides

Alkylglucosides are known to be mild nonionic surfactants for solubilizing membrane proteins.[125-128] It has been reported that mixtures of *n*-octyl-β-D-glucoside (OG) and phosphatidylcholine (PC) are also separated into two phases, depending on their concentration ratio.[77,129] The two-phase region appears in the transition state of the vesicle to the mixed micelle. In this two-phase system, the *Torpedo californica* nicotinic acetylcholine receptor, the membrane protein, was found to partition into the surfactant-rich phase.[77]

n-Octyl-β-D-thioglucoside (OTG) was added into the AMTPS of tetraoxyethylene decyl ether ($C_{10}E_4$) in the partitioning of the bacterial photoreceptor proteins.[130] The cloud point of $C_{10}E_4$ is 20˚C, which is lower than

Table 2 Volume fraction of aqueous (AP) and surfactant-rich phase (SRP), extraction yield of proteins, and their concentration factor in surfactant-rich phase

Composition of solution	Volume fraction, %		Extraction yield, %				Concentration factor	
	AP	SRP	BR	Cyt. b_5	POD	Cyt. *c*	BR	Cyt. b_5
2% (w/v) OTG 2% (w/v) PEG	95.6	4.4	64	94	4	3	13.1	21.3
2% (w/v) OTG 2% (w/v) Dx	92.7	7.3	52	87	6	3	7.1	11.9
2% (w/v) NG 2% (w/v) PEG	97.5	2.5	52	80	6	2	20.9	31.6

All data obtained from ref. 61. OTG, *n*-octyl-β-D-thioglucoside; PEG, polyethylene glycol (average molecular weight, 7500); Dx, dextran (average molecular weight, 500000); NG, *n*-nonyl-β-D-glucoside; BR, bacteriorhodopsin, Cyt. b_5, cytochrome b_5; POD, peroxidase from horseradish; Cyt. *c*, cytochrome *c*.

that of Triton X-114, thus preventing the degradation of the pigment and the loss of the reaction center activity. The presence of OTG at a low concentration (slightly above its critical micellar concentration) did not alter the cloud point. The reaction center was extracted into the surfactant-rich phase in the pure $C_{10}E_4$ two-phase system because of its hydrophobicity. However, it was partitioned into the aqueous phase in the presence of OTG. This means that the selectivity in the extraction of proteins could be controlled by adding a second surfactant into the POEAE-based AMTPS or using AMTPS of other types of surfactants.

It is accepted that water-soluble polymers cause phase separation into two phases when mixed with a micellar solution of AG (Fig. 4). The polymer-induced AMTPS have also been utilized for protein extraction.[61] Bacteriorhodopsin, cytochrome b_5, peroxidase, and cytochrome *c* were used for testing the extractability in the polymer-induced AMTPS of AGs. Table 2 lists the extraction yields and the concentration factors. It was elucidated that two hydrophobic proteins, bacteriorhodopsin and cytochrome b_5, were well extracted into the surfactant-rich phase with a concentration factor of 7 to 32, while hydrophilic ones, peroxidase and cytochrome *c*, were not.

As shown in Fig. 4, the phase diagram of aqueous mixtures of AG and water-soluble polymer indicates an upper consolute boundary, thus extraction can be conducted at lower temperatures. This is very advantageous for preventing the denaturation of thermolabile proteins. Figure 8 shows the change in the absorbance of bacteriorhodopsin in the Triton X-114-rich phase at 30˚C and in the OTG-rich phase at 0˚C.[61] Decreasing the absorbance means denaturation of the protein. The absorbance in Triton X-114 decreased faster than in OTG, indicating that bacteriorhodopsin is more stable in the OTG-rich phase. This would be due to the differences in the hydrophobicity of the two surfactants and in the temperature for two-phase separation.

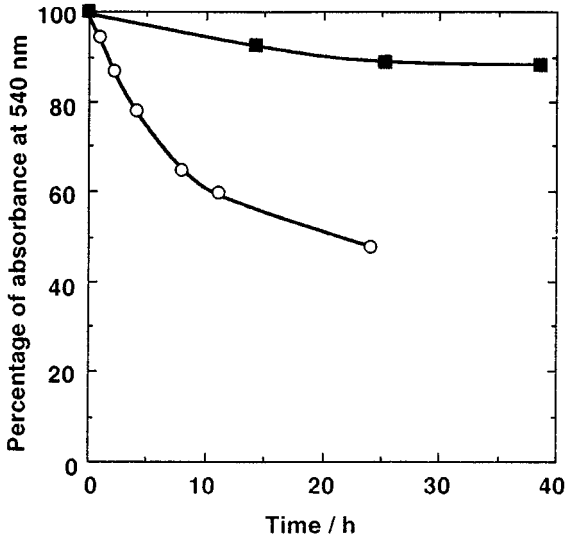

Fig. 8 Stability of bacteriorhodopsin in the Triton X-114-rich phase at 30˚C (○) and OTG-rich phase at 0˚C (■) (Reproduced with permission from ref. 61. Copyright 1994 The Japan Society for Analytical Chemistry).

One of the advantages in the AMTPS of AGs is a relatively high critical micellar concentration (cmc) value of AGs. The cmc values of OG and OTG are 25[131] and 9 mM[132], respectively, while that of Triton X-114 is 0.2 mM.[133] Thus, AGs can be removed more easily from the solution of the proteins by dialysis than Triton X-114. Additionally, AGs have a high optical transparency in the ultraviolet region, which enables the absorbance detection of proteins at 270 nm as well as the fluorescence detection, while Triton X-114 possessing an aromatic ring shows considerable absorbance in the ultraviolet region.

4·2 Zwitterionic surfactants

It has been demonstrated that two-phase separation using aqueous solutions of zwitterionic surfactants (3-(nonyldimethylammonio)propyl sulfate (C_9APSO_4), the corresponding decyl analog ($C_{10}APSO_4$), and dioctanoyl phosphatidylcholine (C_8-lecithin)) could be useful for the extraction of proteins.[62-64,67] In the AMTPS of C_9APSO_4, bacteriorhodopsin was extracted into the C_9APSO_4-rich phase with a yield of 90%, whereas hydrophilic cytochrome *c* was exclusively retained in the aqueous phase.[67] On the other hand, in the $C_{10}APSO_4$ system biological compounds such as steroidal hormones and vitamin E were effectively extracted and preconcentrated prior to HPLC analysis.[67] As mentioned in the earlier section, an aqueous solution of C_8-lecithin was exploited as a model system for the theoretical treatment of protein partitioning between two phases.[62-64]

Aqueous solutions of the zwitterionic surfactants also exhibit an upper consolute boundary as well as AG systems (Fig. 3). Thus, two-phase separation can be obtained without any heating, which prevents thermally denaturation of proteins.

4·3 Triblock copolymer surfactants

Recently, triblock copolymer surfactants, a series of Pluronic, consisting of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO), were used in the aqueous two-phase system for proteins[68] and peptides.[134] The extractability of hydrophobic and hydrophilic proteins was tested by using both Pluronic L61 (L61) and Pluronic 25R2 (25R2)-based AMTPSs.[68] The extraction of proteins was based on phase separation upon heating aqueous solutions of Pluronics above their cloud points as in the case of Triton X-114 (Fig. 2). Figure 9 shows the results of the extraction of cytochrome b_5 and cytochrome *c* in the AMTPSs of L61 and 25R2. Hydrophilic cytochrome *c* was not extracted into the surfactant-rich phase in both systems. On the other hand, hydrophobic cytochrome b_5 was extracted in the L61 system, while not in the 25R2 system. The difference in the extractability of cytochrome

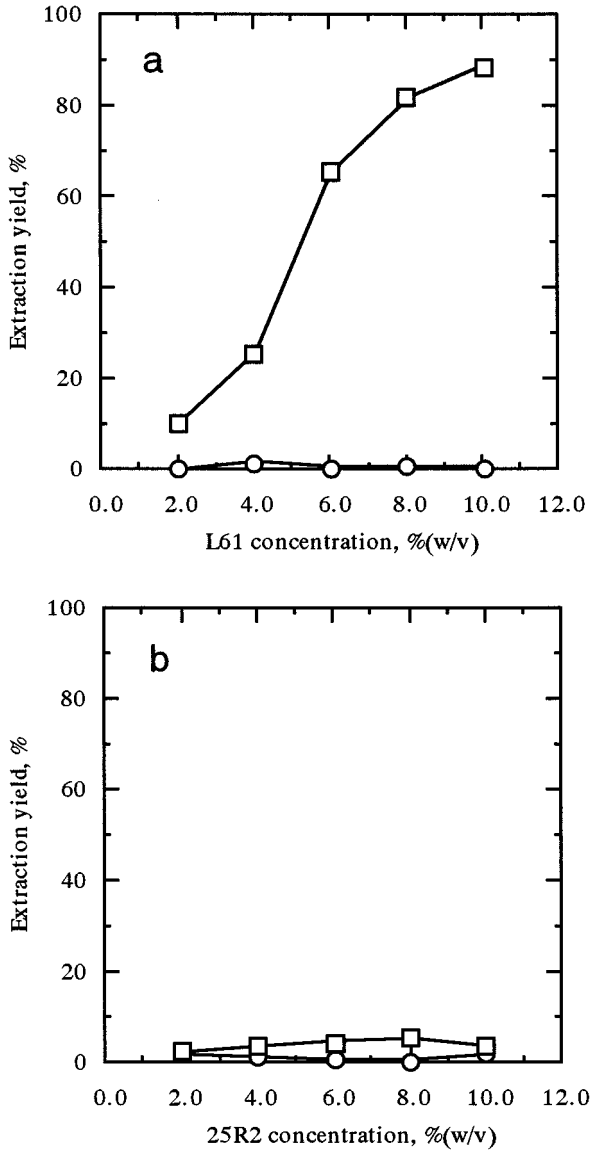

Fig. 9 Extraction of cytochrome b_5 (□) and cytochrome c (○) in the AMTPS of (a) Pluronic L61 and (b) Pluronic 25R2 (Reproduced with permission from ref. 68. Copyright 1997 The Japan Society for Analytical Chemistry).

b_5 between the two systems would be due to the micellar structure of the Pluronics in the surfactant-rich phase. Pluronic L61 having the structure of PEO-PPO-PEO forms spherical micelles and grows into a rodlike or a layered structure with an increase in the temperature and/or concentration as well as conventional nonionic surfactants.[135,136] Pluronic 25R2, PPO-PEO-PPO type, also aggregates to form spherical, rodlike, and layered micelles, depending on its concentration and temperature. Further, the micelles of PPO-PEO-PPO type can be connected to each other by a PEO chain, in which two PPO ends in one Pluronic molecule incorporate into two micelles, thus resulting in the formation of a micellar network.[137-139] Since the network structure may cause a strong exclusion of the proteins from the surfactant-rich phase, cytochrome b_5 was retained in the aqueous phase in the 25R2 system despite its hydrophobicity.

In another application, the partitioning of hydrophobic amino acids, such as phenylalanine and tryptophan, and their oligopeptides in the aqueous two-phase system of Pluronic P108 and dextran was demonstrated.[134] The hydrophobic solutes were more partitioned to the Pluronic-rich phase when the number of hydrophobic amino acids or the temperature was increased. This was explained as being due to the increased hydrophobicity of the solutes and Pluronic. These results suggested that the hydrophobic interaction of solutes with Pluronics plays an important role in the determination of partitioning in this two-phase system.

4·4 Mixture of cationic and anionic surfactants

Aqueous mixed solutions of cationic and anionic surfactants are known to form a precipitate above the cmc, and to form homogeneous mixed micellar solutions at much higher concentration.[78,140-144] At the concentration range between the precipitate-forming and the homogenated regions, and at a narrow mole ratio region, it was shown that the solutions separate into two immiscible aqueous phases, in which one is rich and the other poor in surfactants.[78,141,143] When a homogeneous mixed micellar solution of sodium dodecyl sulfate and dodecyl triethylammonium bromide was diluted with an equal volume of albumin solution, phase separation occurred and albumin was unequally distributed into the two phases, suggesting it to be a new AMTPS.[78] When being diluted with water, the upper (surfactant-rich) phase separates into two phases again, and thus multiple partitioning can be conducted. In this type of AMTPS, since most of the surfactants can be precipitated out from the solution by further dilution, a protein solution free from surfactants can be easily obtained.

5 Enhanced Selectivity for Proteins in AMTPS

Most of the applications of AMTPS to the separation and characterization of the proteins stated above are based on a difference in the hydrophobicity of the proteins. Thus, AMTPS have been limited to use for the separation of hydrophobic proteins from hydrophilic ones, and for assessing the hydrophobic nature of proteins. Recently, a new type of AMTPSs has been developed by using the affinity or charged ligands to modify the selectivity of proteins.[65,66,145,146] By using these modified AMTPSs, the separation of proteins can be conducted more effectively, thus suggesting the extended use of AMTPS for protein purification.

5·1 AMTPS including hydrophobic affinity ligands

In the first application, alkyl-biotin and *n*-octyl-β-D-glucoside were used as an affinity ligand in C_9APSO_4-based AMTPS for the extraction of avidin and hexokinase, respectively.[65] As shown in Fig. 10, without affinity ligands, two proteins are not to be extracted

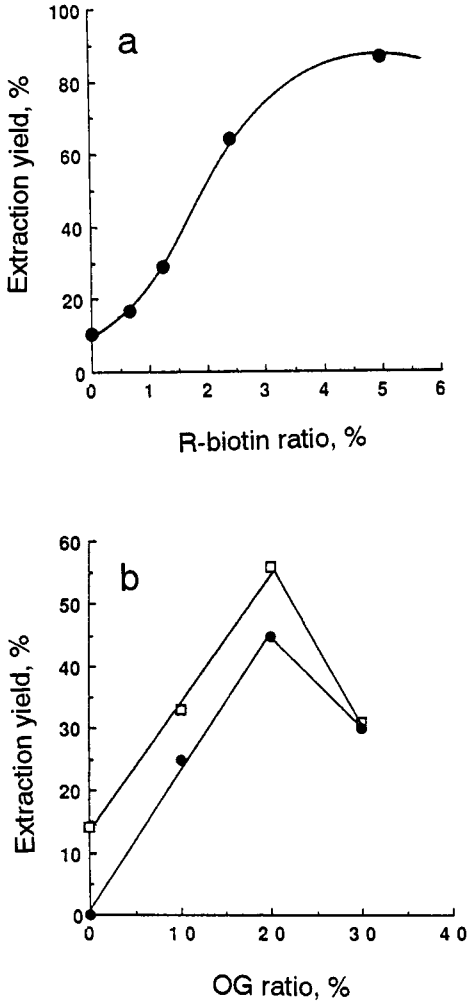

Fig. 10 Extraction of (a) avidin and (b) hexokinase (□, at pH 7.92; ●, at pH 8.44) in the AMTPS of C_9APSO_4 as a function of the concentration ratio of added affinity ligand (*i.e.* [affinity ligand]/([affinity ligand]+[C_9APSO_4])), *N*-(biotinoyl)-dipalmitoyl-L-α-phosphatidylethanolamine (R-biotin) and *n*-octyl-β-D-glucoside (OG), respectively (Reproduced with permission from ref. 65. Copyright 1995 Elsevier Science).

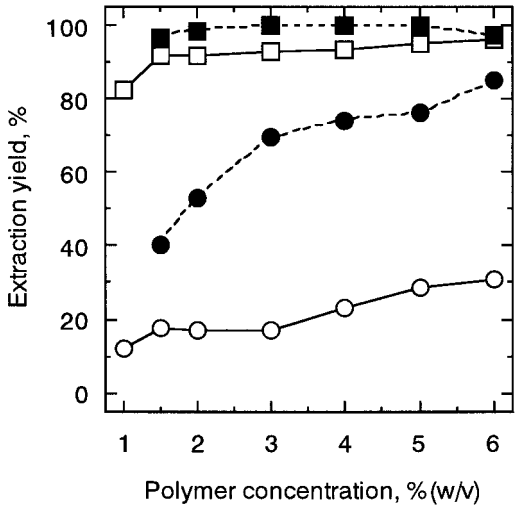

Fig. 11 Extraction of cytochrome b_5 (○, ●) and cytochrome P450 (□, ■) as a function of the water-soluble polymer concentration. The dotted lines and filled symbols indicate the use of dextran as a water-soluble polymer, while the solid lines and open symbols mean the use of diethylaminoethyl dextran (Adopted with permission from ref. 66. Copyright 1997 John Wiley & Sons).

into the surfactant-rich phase, while when increasing the concentration ratio of the ligands, the proteins tend to be extracted. In this extraction process, the ligand serves dual functions. That is, the ligand strongly binds to the protein of interest due to its high affinity, and partitions into a surfactant-rich phase with its hydrophobicity occurring from an alkyl tail in the ligand molecule. These results suggest that by using an appropriate hydrophobic affinity ligand, hydrophilic proteins can be extracted into the surfactant-rich phase.

5·2 AMTPS including charged polymers and charged surfactants

Recently, the partitioning of hydrophilic proteins in the aqueous two-phase systems of pentaoxyethylene dodecyl ether ($C_{12}E_5$) and dextran (Dx) in combination with a charged surfactant or a charged polymer has been studied.[145] The charged surfactant used is sodium dodecyl sulfate or dodecyl trimethylammonium chloride. They can form mixed micelles with $C_{12}E_5$ and distribute into the surfactant-rich phase. The charged polymer used is dextran sulfate, which is retained in the aqueous phase. In this system, five well-characterized hydrophilic proteins having different net charges were used as models. The results suggested that they could be partitioned into the surfactant-rich phase according to the electrostatic repulsion from the charged polymer in the aqueous phase or the attraction to the charged surfactant in the surfactant-rich phase. The results are largely dependent upon the protein net charge, the concentration of the charged surfactant, and the pH. Thus, by introducing a charged polymer or surfactant into AMTPS, effective separation of hydrophilic proteins based on the difference in the protein charge can be obtained.

In contrast to the above-mentioned approaches, the extractability of hydrophobic membrane proteins has been successfully controlled by introducing a charged water-soluble polymer into AMTPS of alkylglucoside.[66] In the polymer-induced AMTPS of *n*-octyl-β-D-thioglucoside, cytochrome b_5 and cytochrome P450 are well extracted into the surfactant-rich phase when nonionic dextran is used as a polymer. On the other hand, the use of cationic diethylaminoethyl-dextran (DEAE-Dx) highly reduced the extractability of cytochrome b_5, while that of cytochrome P450 remained almost unchanged, as can be seen in Fig. 11. Adding salt to DEAE-Dx-induced AMTPS of OTG causes to increase in the extraction of cytochrome b_5. Since the extraction has been conducted at pH 7.4, where cytochrome b_5 is negatively charged and cytochrome P450 almost neu-

tral, the depressed extraction of cytochrome b_5 must be due to an electrostatic interaction with DEAE-Dx in the aqueous phase.

In applications, anionic dextran sulfate (Dx-S) was demonstrated to be effective for purifying cytochrome b_5 from pig liver microsomes in Triton X-114-based AMTPS.[146] Without Dx-S, 84% of cytochrome b_5 and 55% of the total microsomal proteins were extracted into the surfactant-rich phase. In the presence of Dx-S, the extractability of the total microsomal proteins was markedly decreased, while that of cytochrome b_5 was slightly increased. After triplicate extraction, cytochrome b_5 was purified by more than 10-fold from the microsomes with a recovery of *ca*. 90% in the surfactant-rich phase. The purification factor and the recovery are comparable to those in the separation using DEAE-cellulose column, which is frequently used in the first step in the purification of cytochrome b_5.

In view of operational simplicity, charged polymer-induced AMTPS provides a good means for the separation and purification of membrane proteins.

6 Conclusions

An aqueous micellar two-phase system has been successfully employed in the purification and characterization of proteins, especially hydrophobic membrane proteins, because of its methodological simplicity and rapidity.

Recently, the successful combination of affinity ligands, charged polymers, and charged surfactants with AMTPSs has been developed, which enables us to control the extractability of proteins. The modified-AMTPSs have now become a powerful tool for the highly efficient separation of membrane proteins, and will extend the range of the applications not only to their purification, but to their characterization.

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(Received June 30, 1998)
(Accepted July 27, 1998)