A simple complexation model and the experimental data for protein extraction using reverse micellar systems

Ali Haghtalab, Shahriar Osfouri†

Department of Chemical Engineering, Faculty of Engineering, Tarbiat Modarres University, P.O. Box 14115-143, Tehran, IR Iran.

Abstract
The reverse micelles have been used for extraction and purification of proteins and enzymes in down-stream processing. In this study a simple complexation model was developed for protein extraction using reverse micelles. We assumed that the size of protein-reverse micelle complex is a function of net charge of protein and salt concentration. The model has been applied to correlate the experimental data for reverse micellar extraction of bovine serum albumin (BSA) and lysozyme. The solutions of reverse micelles for extraction of BSA and lysozyme were composed of cetyltrimethylammonium bromide (CTAB), a cationic surfactant, and sodium bis(2-ethylhexyl) phosphate (NaDEHP), an anionic surfactant, respectively. Moreover, the effects of surfactant concentration, pH of aqueous phase, and salt concentration were investigated. In comparison with experiment the results of the model for both systems are in very good agreement.
Keywords: *Extraction, Modeling, Reverse micelle, Bioseparation, BSA, Lysozyme*

INTRODUCTION

Reverse micelles are aggregates of surfactant molecules containing the microscopic polar cores of solubilized water, called water pools. These structures are thermodynamically stable, and have ability to solubilize many different hydrophilic components such as proteins, enzymes and amino acids within their water pools. Luisi and co-workers were the first group that they used reverse micelles for extraction, separation and purification of proteins (Luisi *et al*., 1979; Meier *et al*., 1984 and Luisi *et al*., 1988). Hatton and co-workers used the reverse micelles for extraction of proteins in large scale (Goklen and Hatton, 1985 and Hatton, 1989) and they carried out this process for the selective separation of proteins from the broth mixtures (Goklen and Hatton, 1987; Comarinha and Aires-Barros, 1990; Aires-Barros and Cabral, 1991). In addition, the reverse micelles are suitable for hosting enzymatic reactions; especially those systems involve difficulty to solubilize substrates or require low water content (Luisi *et al*., 1988; Khmelnitsky *et al*., 1992; Oldfield, 1994 and Aires-Barros *et al*., 1997).

The thermodynamic modeling of revere micellar systems allows one to correlate and predict the phase equilibria in such systems and to design down stream bio-processes. Although the reverse micellar systems were recognized for separation and purification of biomolecules for more than two decades; however due to complexity and misunderstanding of interactions among various species in such solutions, the thermodynamic models are scarce in compare to the ordinary vapor-liquid or liquid-liquid equilibria systems.

So far several thermodynamic models have been presented for partitioning of protein between organic reverse micellar solution and aqueous phase. Based on the classification presented (Haghtalab and Osfouri, 2003), there are three categories for thermodynamic models: Gibbs free energy models, mass action models, and adsorption models. In the first category, the authors tended to express the partitioning of protein by using Gibbs free energy of protein partitioning and microstructure parameters of reverse micelles (Bratko *et al*., 1988 and Bruno *et al*., 1990). The thermodynamic models in the second group were presented based on mass action principle (Woll and Hatton,

Correspondence to: **Ali Haghtalab, PhD**
Tel: +98 21 8011001(3313), Fax: +98 21 8006544
E-mail: haghtala@modares.ac.ir

†Present address: Shahriar Osfouri, Department of Chemical Engineering, Faculty of Enigineering, Persian Gulf University, Boushehr, I.R. Iran.

1989; Rabie and Vera 1998; Ashrafizadeh and Khoshkbarchi, 1998). In these models, the partitioning of protein was attributed to the ion exchange reaction between protein and surfactant molecules or pseudo-chemical complexation reaction between them. In the last group, the reverse micelles were considered as active sites that are able to adsorb protein molecules (Brandani, 1994). Recently, this method was improved using a combination of adsorption approach with vacancy solution theory (Haghtalab and Osfouri, 2003).

MATERIALS AND METHODS

Most studies of reverse micellar systems have used Aerosol-OT, an anionic surfactant (Aires-Barros and Cabral, 1991; Andrews *et al*., 1994; Nishiki *et al*., 1998) and TOMAC, a cationic surfactant, to extract low molecular weight proteins (Dekker *et al*., 1986; Brandani *et al*., 1993). In this study, we used the data of partitioning of BSA (MW=68000 Da, pI=4.7) and lysozyme (14000 Da, pI 11) between aqueous and reverse micellar phases which were formed with cetyltrimethylammonium bromide (CTAB), a cationic surfactant, and sodium bis (2-ethylhexyl) phosphate (NaDEHP), an anionic surfactant, respectively. Because the extraction of proteins with ionic surfactant is mainly due to electrostatic interactions, we carried out the influence of pH, ionic strength, and surfactant concentration on the partitioning behavior of these proteins in Winsor type II system.

Lysozyme (14000 Da, pI 11) from egg white, and bis (2-ethylhexyl) phosphate, were purchased from Merck. Bovine serum albumin (68000 Da, pI 4.7) was purchased from BDH Company.

Cetyltrimethylammonium bromide (CTAB), Karl-Fischer solution with titration ratio 5-6 mg H_2O/ml Karl-Fisher, and dried methanol for titration were obtained from Fluka. The others chemicals such as solvent, cosolvent and salts were all commercially available reagents of analytical grade.

To carry out the partitioning of protein between aqueous phase and reverse micellar phase, it is necessary to prepare the reverse micellar solution. To neutralize HDEHP in isooctane, the organic phase was mixed with the same volume of aqueous phase, containing equal molar concentration of NaOH and 0.2 M NaCl. To prevent the formation of the third phase and to facilitate the reverse micelle formation, the tributyl phosphate (TBP) with 0.2 (M) concentration, was added to the organic solution. The pH of the aqueous phase was adjusted to 7.5 by adding 0.1 M NaOH and 0.1 M HCl aqueous solution. After neutralization, the upper phase was separated carefully and used as the stock solution of reverse micelles. In the case of cationic surfactant, CTAB, we add surfactant to organic solvent, Isooctane- 1-hexanol (9/1 v/v), while we add required amount of water as water pool to form reverse micellar solution.

Equal volumes of the reverse micellar and aqueous phases, containing protein, were brought into contact in the glass test tubes to solubilize protein in the reverse micelles. To accelerate the partitioning of protein between reverse micellar phase and aqueous phase, the mixture was shaken for 3 min. To reach a clear phase interface between two aqueous and reverse micellar phases, the mixture was centrifuged at 2500 rpm for 5 min. Subsequently; the test tubes were placed in the refrigerated thermostat bath at 298.15 K for 6 and 16h for lysozyme and BSA, respectively. Finally, the two equilibrium phases were separated carefully and the required analyses were performed.

The water content in the reverse micelles was measured using Karl-Fischer titrator 758 (Metrohm, Ltd., Herisau, Switzerland). The pH of the aqueous phase was monitored by a model 744-pH meter (Metrohm, Ltd). The protein concentration of aqueous solution was determined by measuring the absorbency at 280 (nm) using a UV/VIS spectrophotometer (Shimadzu 1201). The protein content in the reverse micelles was computed by mass balance calculation. The blank experiments were performed simultaneously with aqueous phase containing no protein.

Modeling

Surfactant molecules in organic solvent are in equilibrium with reverse micelles. One may assume that surfactant molecules and organic solvent are insoluble in aqueous phase. So, one may write the equilibrium as a reversible reaction:

$$m\,S \Leftrightarrow RM \tag{1}$$

where *m*, *S* and *RM* are aggregation number, surfactant molecule and reverse micelle, respectively. The equilibrium constant for this reaction is written as following:

$$K_{RM} = \frac{[RM]}{[S]^m} \tag{2}$$

where the bracket indicates the concentration of each species.

Similar to the work of Woll and Hatton ((1989), we

assume that partitioning of protein between two phases is due to a complexation reaction between protein molecule and reverse micelles:

$$P + nRM \Leftrightarrow PRM \quad (3)$$

where *PRM* is protein–reverse micelle complex. The equilibrium constant for this complexation reaction is presented as:

$$K_{PRM} = \frac{[PRM]}{[P][RM]^n} \quad (4)$$

The size of a protein molecule causes to restructure the solution, so the reverse micelles may fuse and rearrange themselves. This rearrangement is the result of interactions between charged proteins and polar head groups of surfactant. Thus, "*n*" indicates the number of initial reverse micelles, which rearrange to form a bigger reverse micelle after loading of protein. In the second step, to take into account the electrostatic interactions and size exclusion effect, we assume that "*n*" is in linear proportion to the charge of protein and salt concentration without any cross effect:

$$n = n_0 + \alpha Z + \beta[Salt] \quad (5)$$

where n_0 is an indication of the degree of rearrangement of empty reverse micelles needed to solubilize a molecule of protein with zero net charge and salt-free solution, Z is the net charge number of the protein, which varies in respect to pH of the aqueous solution. α and β are the two adjustable parameters.

The overall percent extraction of protein in respect to aqueous solution is calculated as:

$$E = \frac{[P_0^{aq}] - [P^{aq}]}{[P_0^{aq}]} \times 100 \quad (6)$$

where $[P_0^{aq}]$ and $[P^{aq}]$ are the initial and equilibrium protein concentration in aqueous phase, respectively. The mass balance for protein is written as following:

$$[P_0^{aq}] = [P^{aq}] + r[P^{rm}] \quad (7)$$

where *r* is the volumetric ratio of reverse micellar phase to aqueous phase V^{rm}/V^{aq}. Since the protein solubilization in reverse micellar phase is due to protein entrapment in empty reverse micelles to produce protein-reverse micelle complex, thus, the molar concentration of protein in reverse micellar phase,$[P^{rm}]$, is the same as the concentration of protein-reverse micellar complex, [*PRM*]. By combining eqs. (2), (4), (6), and (7) result in,

$$E = \frac{r}{\frac{1}{K_{PRM}(K_{RM}[S]^m)^n} + r} \times 100 \quad (8)$$

where this equation presents the final percent extraction of protein, so one can observe that we have two equilibrium constants as adjustable parameters with two unknown numbers "*m*" and "*n*". Thus, for correlating of the experimental data one should adjust two equilibrium constants, "*m*", and the three other parameters, n_0, α , and β as presented in eq. (5).

RESULTS

The adjustable parameters of the model were evaluated by least-squares fitting procedure utilizing the Marquardt optimization program (Chandler, 1985) for minimizing the following objective function:

$$OF = \sum_{i=1}^{N} (E_i^{Exp} - E_i^{Cal})^2 \quad (9)$$

where *N* is the number of data points and the superscripts *"Exp"* and *"Cal"* are denoted as experimental data and calculated values, respectively. Table 1 shows the results of optimization of eq. (9). So, as one can see the six parameters are optimized globally for the whole range of surfactant concentration, pH of aqueous solution, and salt concentration. The net charges of proteins against pH were calculated using protein titration curve that is presented in figure 1 (Haghtalab *et al*., 2003).

Figures 2-a and 2-b show the extraction percent of lysozyme and BSA, respectively, with respect to the pH of initial aqueous phase. The solid lines are the results of the model and the symbol shows the experimental data. The effect of salt concentration on the

Table 1. The values of optimized parameters for extraction of BSA and lysozyme using the surfactant of CTAB and NaDEHP, respectively.

	BSA-CTAB	Lysozyme-NaDEHP
K_{RM}	8.34×10^{7}	5.29×10^{5}
K_{PRM}	2.97×10^{8}	2.05×10^{2}
m	23	40
n_0	2.77×10^{-1}	3.28×10^{-2}
α	-2.68×10^{-3}	-3×10^{-3}
β	6.56×10^{-1}	2.62×10^{-2}

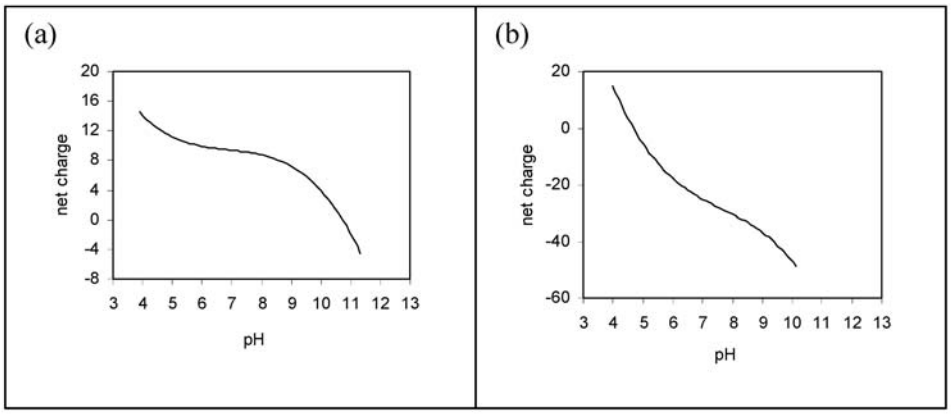

Figure 1. The titration curves of proteins: (a) lysozyme, and (b) BSA.

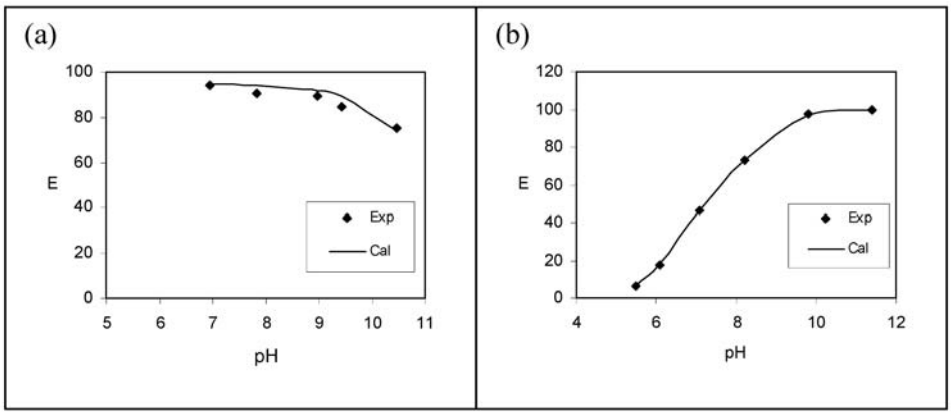

Figure 2. Effect of pH on the extraction percent of proteins: (a) lysozyme with initial reverse micellar phase, 0.1 (M) NaDEHP, 0.2 (M) TBP; initial aqueous phase, 0.5 (g/l) protein, 1 (M) NaCl; and (b) BSA with initial reverse micellar phase: 0.02 (M) CTAB; initial aqueous phase, 1 (g/l) protein, 0.1 (M) KCl.

extraction percent of proteins is shown in figure 3. The agreement of the results of the model with experimental data is very good. The experimental measurements of water in reverse micelles showed that when the NaCl concentration in aqueous phase was increased from 0.35 (M) to 2 (M), the mass percent of water in NaDEHP reverse micellar phase was decreased from 4.62% to 2.29%, respectively. The reduction of water content was observed for CTAB reverse micellar system by increasing concentration of KCl. Figure 4 shows the extraction percent of protein with respect to the surfactant concentration. Similar to the previous figures, the solid lines are the results of the model and the symbol shows the experimental data. The results show that the extraction percent was enhanced with increasing surfactant concentration. At the surfactant concentration of 40 (mM), Due to high local concentration of salt inside the reverse micelles, denaturation of lysozyme was observed. To see accuracy of the present model, the mean relative error for each system was calculated as,

$$Mean\ relative\ error = \frac{1}{N}\sum_{i=1}^{N} 100 \times \left(\frac{E_i^{Exp} - E_i^{Cal}}{E_i^{Exp}} \right) \qquad (10)$$

The mean relative errors are 2.3 and 3.1 percent for Lysozyme-NaDEHP and BSA-CTAB systems, respectively.

DISCUSSION

A very good agreement between the experiment and calculated results was observed by comparing the experimental data of both reverse micellar systems with the results of the present model. The results have clearly shown that the solubilization process depends on the pH of aqueous solution. As shown in figure 2,

the extraction percent of lysozyme was enhanced by varying pH below its isoelectric point. However, above isoelectric point of BSA the extraction percent increases. This result confirms that the protein extraction is mainly due to the electrostatic interaction between charged amino acid residues on the protein molecule and the polar head groups of surfactant. The negative sign of α in the model may interpret as lesser fusion of reverse micelles, i.e., the electrostatic interactions between charged protein molecule and polar head groups of surfactant are intensified.

Increasing salt concentration leads to better shielding of electrostatic interactions between the surfactant head groups resulting in smaller reverse micelles. In addition, as shown in figure 3 due to increasing the size exclusion effect; the water content reductions justify the reduction of the extraction rate of proteins. On the other hand, the positive sign of β in the model shows that the reverse micelles are shrinkaged, so "*n*" should be increased for solubilization a certain protein. The result presented in figure 4 showed that another factor affecting protein solubilization was the concentration of the surfactant, so the model was able to correlate the effect of this parameter on protein partitioning.

CONCLUSION

A simple complexation model was developed for extraction of protein from aqueous phase using reverse micellar solution. The results show that the model is capable to correlate the partitioning of protein between two liquid equilibrium phases. Moreover, the model

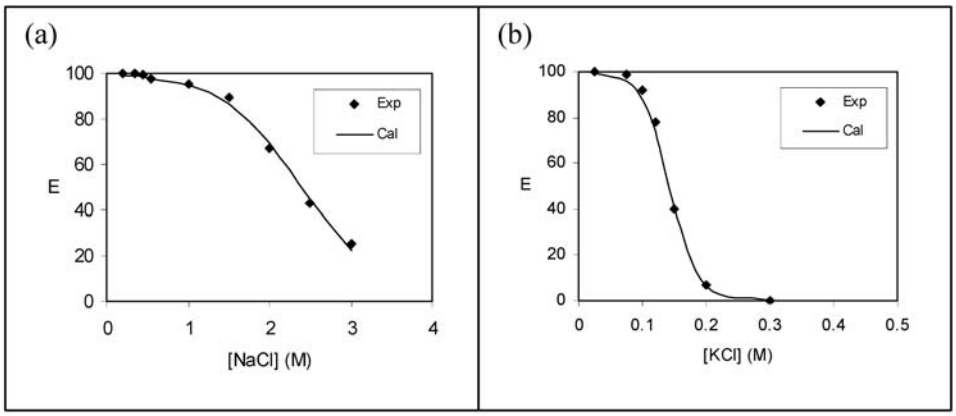

Figure 3. Effect of salt concentration on the extraction percent of proteins: (a) lysozyme with initial reverse micellar phase, 0.1 (M) NaDEHP, 0.2 (M) TBP; initial aqueous phase, 0.5 (g/l) protein, pH=7; and (b) BSA with initial reverse micellar phase: 0.02 (M) CTAB; initial aqueous phase, 1 (g/l) protein, pH=9.

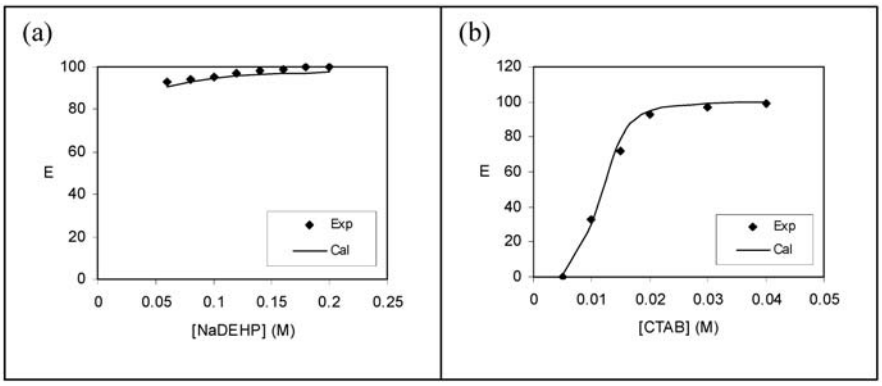

Figure 4. Effect of surfactant concentration on the extraction percent of proteins: (a) lysozyme with initial reverse micellar phase, 0.2 (M) TBP, initial aqueous phase, 0.5 (g/l) protein, pH=7, 1 (M) NaCl; and (b) BSA with initial aqueous phase, 1 (g/l) protein, pH=9, 0.1 (M) KCl.

can be used to correlate the extraction percent of protein in reverse micellar systems by varying some parameters such as surfactant concentration, pH of aqueous phase, and salt concentration. The optimization was performed globally for the data of each reverse micellar system at various variables, so the adjustable parameters are valid for the whole range of operating conditions. In addition to the accuracy of the model, the experiments showed that both reverse micellar solutions, forming with CTAB and NaDEHP, are suitable for extraction of BSA and lysozyme, respectively.

Acknowledgement

This research project has been supported by Grant no. NRCI5158 of National Research Projects and with the support of National Research Council of Islamic repub-lic of Iran.

List of symbols

BSA	Bovine serum albumin protein
CTAB	Cetyltrimethylammonium bromide
E	Extraction percent of protein from initial aqueous phase
NaDEHP	Sodium bis(2-ethylhexyl) phosphate
K_{PRM}	Equilibrium constant of reaction (3)
K_{RM}	Equilibrium constant of reaction (1)
N	Number of data points
P	Protein molecule
PRM	Protein-reverse micelle complex
RM	Reverse micelle structure
S	Surfactant molecule
TBP	Tributyl phosphate
Z	Net charge of protein
[i]	Molar concentration of species "i"
m	aggregation number, eq. (1)
n	Number of reverse micelle, eq. (3)
n_0	n at zero net charge of protein and zero salt concentration, eq (5)
V_r	Volumetric ratio of reverse micellar phase to aqueous phase

Greek letters

α	Constant in eq. (5)
β	Constant in eq. (5)

Superscript

aq	Aqueous phase
Cal	Calculated
Exp	Experimental
rm	Reverse micellar phase

Subscript

0	Initial state (concentration)

References

Aires-Barros MR, Cabral JMS. (1991) Selective separation and purification of two lipases from Chromobacterium viscosum using AOT reversed micelles. *Biotechnol. Bioeng.*, 38: 1302-1307.

Aires-Barros MR, Carvalho CML, Serralheiro MLM. , Cabral JMS. (1997) Application of factorial design to the study of the transesterification reactions using Cutinase in AOT-reversed micelles. *Enzyme Microb. Technol.*, 21: 117-123.

Andrews BA, Pyle DL , Asenjo JA. (1994) The effect of pH and ionic strength on partitioning of four proteins in reverse micelle systems. *Biotechnol. Bioeng*., 43: 1052-1058.

Ashrafizadeh SN, Khoshkbarchi MK. (1998) Modeling and experimental data for the reverse micellar extraction of proteins using a new surfactant. *Sep. Sci. Technol*., 33: 2579-2595.

Brandani V, Giacomo Gdi, Spera L. (1993) Extraction of protein α–amylase by reversed micelles. *Process Biochemistry*, 28: 411-414.

Brandani S, Brandani V, Giacomo GD. (1994) A thermodynamic model for protein partitioning in reverse micellar systems. *Chem. Eng. Sci*., 49: 3681-3686.

Bratko D, Luzar A, Chen SH. (1988) Electrostatic model for protein / reverse micelle complexation. *J. Chem. Phys*., 89: 545-550.

Bruno P, Caselli M, Luisi PL, Maestro M, Traini A. (1990) A simplified thermodynamic model for protein uptake by reverse micelles: theoretical and experimental results. *J. Phys. Chem.*, 94: 5908-5917.

Chandler JP. (1985), MARQ 2.3 A.N.S.I. Standard Fortran, Oklahoma State University, Stillwater Oklahoma.

Comarinha MLC, Aires-Barros MR. (1990) Purification of Chromobacterium viscosum lipases using reverse micelles. *Biotech. Tech.*, 4: 137-142.

Dekker M, Van't Riet K, Weijers SR, Baltussen JWA, Laane C, Bijsterbosh BH. (1986) Enzyme recovery by liquid-liquid extraction using reversed micelles. *Chem. Eng. J.*, 33: 1327-1333.

Goklen KE, Hatton TA. (1985) Protein extraction using reversed micelles. *Biotechnol. Prog.*, 1: 69-74.

Goklen KE, Hatton TA. (1987) Liquid-liquid extraction of low molecular proteins by selective solubilization in reversed micelles. *Sep. Sci. Technol*., 22: 831-841.

Haghtalab A, Mokhtarani B, Maurer G. (2003) Experimental results and thermodynamic modeling of the partitioning of lysozyme, bovine serum albumin, and α-amylase in aqueous two-phase systems of PEG and (K_2HPO_4 or Na_2SO_4). *J. Chem. Eng. Data*, 48: 1170-1177.

Haghtalab A, Osfouri Sh. (2003) Vacancy solution theory for partitioning of protein in reverse micellar systems. *Sep. Sci. Technol*., 38: 553-569.

Hatton TA. (1989) Reverse micellar extraction of proteins. In Scamehron JF, Harwell JH (eds). *Surfactant-Based Separation Process*. Marcel Dekker, New York.

Khmelnitsky YL, Gladilin AK, Roubailo VL, Martinek K, Levashov AV. (1992) A new microheterogeneous medium for enzymic reactions. *European J. of Biochemistry*, 206: 737-745.

Luisi PL, Bonner FJ, Pelligrini A, Wiget R, Wolf R. (1979) Micellar solubilization of proteins in aprotic solvents and their spectroscopic characterization. *Helv. Chim. Acta*, 62: 740-753.

Luisi PL, Giomini M, Pileni MP, Robinson BH. (1988) Reversed micelles as hosts for proteins and small molecules. *Biophys. Acta*, 947: 209-246.

Meier P, Imre E, Fleshar M, Luisi PL. (1984) Further in aggregations on the micellar solubilization of biopolymers in apolar solvents. In Mittal MK, Lindman B (eds). *Surfactant in solution*. Plenum Press, New York.

Nishiki T, Sato I, Muto A, Kataoka T. (1998) Mass transfer characterization in forward and back extraction of lysozyme by AOT- isooctane reverse micelles across a flat liquid-liquid interface. *Biochemical Eng. J*., 1: 91-97.

Oldfield C. (1994) Enzymes in water-in-oil microemultions (reversed micelles): Principles and applications. In Tombs MP (eds). *Biotechnology and Genetic Engineering Reviews*. Intercept Ltd, Andover UK.

Rabie HR, Vera JH. (1998) A simple model for reverse micellar Extraction of proteins. Sep. Sci. Technol., 33: 1181-1193.

Woll JM, Hatton TA. (1989) A simple phenomenological thermodynamic model for protein partitioning in reverse micellar systems. *Bioprocess Eng.*, 4: 193-199.