CHAPTER II

BACKGROUND AND LITERATURE SURVEY

2.1 Proteins and Antibiotics

2.1.1 Proteins

Proteins are the most abundant organic molecules within cells, typically 30 to 70 percent of the cell's drying weight. All proteins contain the four most prevalent biological elements: carbon, hydrogen, nitrogen, and oxygen. Average weight percentages of these elements in proteins are C (50 percent), H (7 percent), O (23 percent), and N (16 percent). In addition, sulfur (up to 3 percent) contributes to the three-dimensional stabilization of almost all proteins by the formation of disulfide (S-S) bonds between sulfur atom at different locations along the polymer chains. The size of this nonrepetitive biopolymer varies considerably, with molecular weights ranging from 6,000 to over 1 million. Proteins are isolated, purified, and characterized by several different types of physical and chemical techniques. Protein separation methods are based on differences in molecular properties, which in turn are partly due to the characteristics of the constituent amino acids (Bailey and Ollis, 1986).

2.1.2 Antibiotics

Secondary metabolism products, which inhibit growth of microbial species, even at low levels, are called antibiotics. Uses for antibiotic are found in antimicrobials (human disease), antitumor agents, fungicides and pesticides for plant protection, animal disease and animal growth products, and research of more than 6,000 antibiotics are known, of which nearly 100 are produced commercially via fermentation. Antibiotic classifications are based on breadth of antimicrobial action (broad vs. narrow range), basis of activity (mechanism) source (producer strain), biosynthetic pathway, and molecular structure (Bailey and Ollis, 1986).

2.2 Microemulsions

Microemulsions are transparent, thermodynamically stable dispersions of water and oil, usually stabilized by surfactant and a cosurfactant. They contain liquid droplets smaller than 0.1 μm (Gasco, 1997). Microemulsions are stable liquid solutions that include normal micellar solutions, reverse micelles, core or droplets of water or oil, and for some systems, even bicontinuous structures, in which neither oil nor water surrounds the other. The stability of microemulsion is a consequence of ultralow interfacial tension between the oil and water phases.

2.2.1 Formation of Microemulsions

The spontaneous formation and thermodynamic stability of microemulsions are contributed to decrease the interfacial tension between water and oil. The free energy of formation of microemulsions consists of three contributions: (1) interfacial free energy, (2) energy of interaction between droplets, and (3) entropy of dispersion. Analysis of the thermodynamic factor shows that the contribution of the interaction energy between droplets is negligible and that the free energy of formation can be zero or negative if the interfacial tension is very low (in the order of 10^{-2} –10^{-3} mN/m), although not necessary negative (Ruckkenstein and Chi, 1975). Furthermore, phase behavior studies of nonionic surfactant systems as a function of temperature while ionic surfactant as a function of salinity show that the hydrophile-lipophile properties such as hydrophile-lipophile balance (HLB), of ethoxylated nonionic surfactants are highly temperature dependent. The concept of HLB temperature or phase inversion temperature (PIT) is the temperature at which the hydrophile-lipophile properties of the surfactants are balanced (Shinoda and Saito, 1968).

2.2.2 Structures of Microemulsions

Two main general structures have been proposed and are widely accepted: discrete microemulsions and bicontinuous microemulsions (Solan *et al.*, 1997). Discrete microemulsions consist of domain of one of the pseudophases (water or oil) dispersed in the other pseudophase. These structures are generally

found when the main component of one of the pseudophases (water or oil) is present in a higher proportion than the main component of the other pseudophase and little surfactant is present. The structure of this type of microemulsion resembles that of emulsions in that one phase is dispersed in another phase. In contrast to the discrete microemulsion structure, which is relatively easy to treat theoretically, the structure of bicontinuous microemulsions is more difficult to visualize and therefore its theoretical treatment is more complicated. In a bicontinuous microemulsion both the aqueous and oil phases are continuous. This continuity means that it is possible to go from one extreme of the system to the other by either an oil path or an aqueous path. The most commonly used classification for phase diagrams in oil/water/amphiphile mixture as shown in Figure 2.1 was proposed by Winsor (1984) who described three types of system:

1. Winsor Type I. At low temperature for the non-ionic and at low electrolyte concentration for the ionic, the surfactant is preferentially soluble in the water and oil-in water (O/W) microemulsions are formed. No surfactant aggregates exist in the oil phase in this region, only a small concentration of surfactant monomer.

2. Winsor Type III. As temperature is raised for non-ionic surfactant or electrolyte concentration increased for the ionic, eventually the system separates into three phases. The middle phase contains oil, water and the majority of the surfactant and is referred to as a middle- phase microemulsion. No surfactant aggregates exist in neither the aqueous nor the oil phase.

3. Winsor Type II. With further heating or electrolyte addition, the system becomes two- phase again, the surfactant is mainly in the oil phase stabilizing a water-in-oil (W/O) microemulsion; no surfactant aggregates are left in the aqueous phase, merely a smaller concentration of monomer with which the microemulsion is in equilibrium (Clint, 1992).

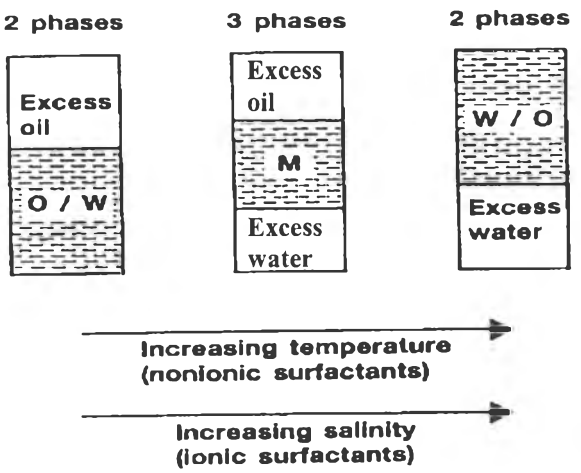

Figure 2.1 Sequence of microemulsions encountered as temperature or salinity is scanned for non-ionic or ionic surfactants, respectively. Most of the surfactant is in the shaded microemulsion phase in each case. In the three phases system the microemulsion contains both oil and water.

2.2.3 General Applications

The first marketed microemulsions were dispersion of canauba wax. They were prepared by adding soap (i.e. potassium oleate) to melted wax and following by incorporation of boiling water in small aliquots. Spontaneous formation, clear appearance, thermodynamic stability, and low viscosity are some characteristics of microemulsions that render these systems attractive and suitable for many industrial applications. However, microemulsions have important advantage, low energy input is required for their preparation (spontaneous formation) and good stability (Solans *et al.*, 1997).

Owing to their high interfacial area and ability to solubilize otherwise immiscible liquids, thermodynamically stable water-in-oil microemulsions have been used in cosmetics, pharmaceutics, food technology, agricultural sprays, coatings, environmental remediation, and chemical analysis (Das *et al.*, 1998).

One type of Winsor II is reverse micelle. Reverse micelles are thermodynamically stable nanometer-sized water droplets within an organic solvent stabilized by monolayer of surfactant molecules, and can solubilize organics such as amino acids and proteins. The extraction of proteins by using reverse micelle is

based on the net charge of the molecule. The partition coefficient is a function of solution pH and ionic strength. A protein size exclusion limit is reached based on reverse micelle size. Affinity ligands have also been used in conjunction with reverse micellar extraction. Proteins are isolated based on an affinity interaction at an adjusted pH to minimize the nonspecific extraction of other proteins.

2.3 Liquid-Liquid Extraction using Microemulsions for Proteins Separation and Purification

During the past decade there has been increasing interest in the use of microemulsions, particularly of the water droplets in oil, for various applications related to biotechnology. To a considerable degree this interest stems from the observation that many proteins can be solubilized in microemulsion based on apolar solvent such as aliphatic hydrocarbons. This process has some advantageous features in that the microemulsion phase can be a gentle solvent for extracting the protein without alerting its enzymatic or functional properties, and yet the process can be readily scaled up using conventional liquid-liquid extraction technology. In this aspect, a protein of interest cannot only be concentrated but also selectively purified from other proteins in the solution. Therefore using reverse micelles like a liquid-liquid extraction is one of the most effective methods.

2.3.1 Protein Solubilization

To extract protein molecules from an aqueous phase, an aqueous protein-containing phase is contacted with a water-in-oil microemulsion as shown in Figure 2.2. Given the right conditions, the protein then preferentially solubilzes in the microemulsion phase and after appropriate time the two phases can be separated. The attractive electrostatic interaction between the protein molecule and the reverse micelle is a driving force for solubilization of protein molecule into reverse micelle. The dependence of solubility on protein properties also makes selective separation possible.

Clearly, to utilize this approach effectively one needs an understanding of the dependence of protein solubility in the microemulsion on the

system properties. With the negative charge AOT surfactant most commonly studied thus far, electrostatic is found to play a major role in the extent of the transfer. At pH value below the isoelectric point, the protein has a net positive charge that facilitate its transfer into the microemulsion droplet, where it can interact attractively with the surfactant head groups.

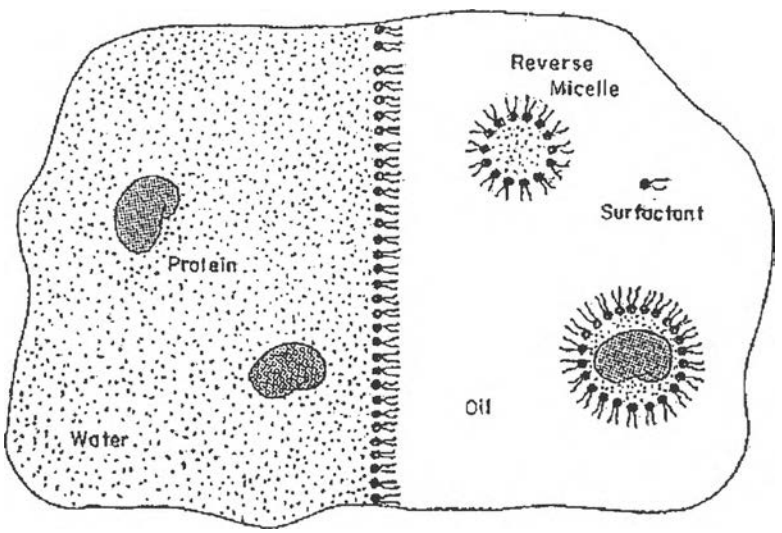

Figure 2.2 A schematic representation of the reverse micellar extraction of proteins.

2.3.2 Factors Affecting Protein Solubilization

The properties of reversed micellar solution will depend on the aqueous conditions of pH, ionic strength, and salt type, all of which affect the physicochemical state of the protein and its interaction with the aqueous solvent and the surfactant headgroups (Hatton, 1997).

2.3.2.1 Effect of pH

The pH of the solution should the solubilizatioin characteristics of a protein primarily in the way in which it modifies the charge distribution over the protein surface. Secondary factors, such as change in protein conformation as a result of pH swings, may also play a role in this regard. At pH values below its isoelectric point (pI), or point of zero net charge, the protein will take on a net positive charge, while above its pI the protein will be negatively

charged. Thus, if electrostatic interactions are the dominant factor in the solubilization process, solubilization should be possible with anionic surfactants only at pH values less than the pI of the protein, where electrostatic attraction between the protein and surfactant headgroups are favorable. At pH values above the pI, electrostatic repulsions would inhibit the protein solubilization. The reverse trends would be anticipated in the case of cationic surfactant.

2.3.2.2 Effect ionic strength

The ionic strength of the aqueous solution in contact with the reverse micellar phase affects the protein-partitioning behavior in a number of ways. The first is through the mediation of the electrostatic interaction between the protein surface and the surfactant headgroups via modification of the properties of the electrical double layers adjacent to both the charged inner micellar wall and the protein surface. An increase in ionic strength compresses the range over which electrostatic interactions can overcome the thermal motion of the solute molecules, and thus decrease the protein-surfactant interactions, inhibiting the solubilization of the protein. This electrostatic screening effect is also responsible for reducing the surfactant headgroup repulsions, leading to the formation of smaller reverse micelles. This can lead to a decrease in solubilization capacity through a size exclusion effect.

A third effect of ionic strength is to salt out the protein from the micellar phase because of the increased propensity of the ionic species to migrate to the micellar water pools and to displace the protein. Finally, specific and nonspecific salt interactions with the protein or surfactant can modify the solubilization behavior, and these effects will be more pronounced at higher the salt strength.

2.3.2.3 Effect of salt type and concentration

Salt can play an important role in determining the solubilization characteristics of different proteins. Salt and buffer types are important factors in determining transfer across an organic solvent bridge between two aqueous solutions of different pH and ionic strengths. For example, for $CaCl_2$ as the salt species, the pH range over which significant solubilization cytochrome-c occurred increases, probably due to Ca^{2+} binding to the protein or to the surfactant. This would modify the electrostatic interaction between the protein and the

surfactant. The choice of buffer can also affect the solubilization characteristics of certain proteins. Use of potassium phosphate buffer to adjust pH resulted in shift of the pH solubilization when pH is adjusted by simple acid-based addition. This again point to the importance of all components in the feed solution in determining the effectiveness and selectivity of the extraction process.

2.3.2.4 Effect of surfactant concentration

An enhanced solubilization capacity of the reversed micellar phase for proteins can be achieved by increasing the surfactant concentration (Hatton, 1997). On the other hand, higher surfactant concentrations make difficult the backward transfer of proteins into a second aqueous phase (Castro *et al.*, 1988). Therefore, the optimum surfactant concentration in a double-phase transfer corresponds to the minimum limit for achieving maximum transfer into the organic phase. The selectivity of the reversed micellar phase for a given protein can thus be changed by choosing an appropriate surfactant concentration in the organic phase. For example, smaller proteins will be more easily extracted into the organic phase than proteins high molecular weight, at low surfactant concentrations.

2.3.3 Forward Extraction and Recovery (Backward Extraction) of Proteins using Microemulsions

Forward extraction is the separation of required protein or targeted protein from the filtered fermentation broth or mixed solution into reverse micelles in the oil phase. In contrast, recovery or backward extraction of the protein may be accomplished by contacting the protein containing microemulsion with a fresh aqueous solution under condition favoring the transfer of protein out of the aqueous phase. In the most cases the protein in the reverse micellar phase can be recovered by either electrostatic repulsion of the surfactant and protein by adjusting the pH or through size exclusion by increasing salt concentration in a fresh aqueous phase.

In an attempt to overcome the difficulties in separating proteins from the surfactant and the phase separation of the system, Hu and Gulari (1996) studied the extraction cytochrome-c and α-chymotrypsin into sodium salt of di-2-ethylhexylphosphoric acid (NaDEHP) reversed micellar phase by varying the pH and NaCl concentration in the aqueous phase. They found that at natural pH and

relatively low ionic strength, the proteins are extracted into the micellar phase with high yield. By contacting the micellar phase with a divalent cation (e.g. Ca^{2+}) aqueous solution, the reverse micelles are destabilized and release the protein molecules back into an aqueous solution for recovery. They used cytochrome-c from the horse heart and α-chymotrypsin from the bovine pancreas. In the forward extraction, they observed that the addition of tributyl phosphate was necessary to inhibit the formation of a middle phase or Winsor III, microemulsion system. For the effect of pH, they illustrated that the percent transfer sharply dropped with pH near the protein pI value. This is due to the weakening of the attractive interaction between the protein molecule and the negatively charged reverse micelle inner charge layer. They recommended the use of sodium bis (2-ethylhexyl) phosphate (NaDEHP) instead of bis (2-ethylhexyl) sodium sulfosuccinate (AOT) because AOT has a number of very serious limitations. Once the proteins are extracting into the AOT reverse micellar phase, it is difficult to separate the proteins from the surfactant and the phase separation of the AOT system takes a long time.

For the proteins cytochrome-c, lysozyme, and ribonuclease-A, Jarudilokkul *et al.* (1999) showed that the back-extraction of proteins encapsulated in AOT reverse micelle could be performed by adding the counterionic surfactant, either trioctylmethylammonium chloride (TOMAC) or dodecyltrimethylammonium bromide (DTAB). This novel transfer method gave higher backward extraction yield compared to the conventional method with high salt and high pH of the aqueous stripping solution. The protein activity was maintained in the resulting aqueous solution, which in this case near the neutral pH and low salt concentration. The backward transfer mechanism was postulated to be caused by electrostatic interaction between oppositely charged surfactant molecules, which lead to collapse of the reverse micelles.

In 2000, Jarudilokkul *et al.* investigated the bis (2-ethylhexyl) sulfosuccinate (AOT) system for selective separation of cytochrome-c, lysozyme, and ribonuclease-A from a buffer solution and a filtered fermentation broth at various concentrations, pH, and temperatures. It was shown that a minimal AOT concentration existed for ≥ 90% extraction from a buffer solution, and this

concentration depended on the pH of the solution and hydrophobicity of the protein. Extraction from filtered broth resulted in a reduction of the minimal concentration for both cytochrome-c and lysozyme, while it was unchanged for ribonuclease-A. It appeared that certain broth constituents acted as cosurfactant and reduced the charge repulsion between the surfactant headgroups leading to smaller micelle. They concluded that the broth constituents were responsible for changes in the minimal AOT concentration. Finally, response surface methodology was used to optimize key system parameters in order to maximize protein extraction, and this technique minimized the number of experiments required. By using the optimized conditions, a mixture of the three proteins could be recovered from a filtered broth with high yields (70-97%) and high purity. The initial protein concentration was found not to influence protein recovery.

Adachi *et al.* (1997) illustrated that using reverse micellar phase containing trypsin inhibitor as an affinity ligand, trypsin was selectively separated with high recoveries from a mixture of several kinds of contaminating proteins by forward and backward extractions. No reduction in activity of the recovered trypsin was observed through these operations. In this work, they also investigated the method of using nonionic surfactants, tetra-oxyethylenedecylether, to the trypsin-trypsin inhibitor (TR-TRI) system as a typical protein-protein affinity interaction. They found that the activity of the reverse TR was maintained almost completely through both forward and backward extraction operations using their method.

For the same target protein (trypsin), Das (1999) investigated the probing for the local molar concentrations of water and bromide counterions present inside the water-pool of cationic reverse micelle system [cetyltri-methylammonium bromide (CTAB)/isooctane/n-hexanol/water] across water content (ω_o) in the range of 12-44. The reverse micelles have been observed to increase with the increase in the molar concentrations of water in the water-pool and with the decrease in bromide counterion concentrations present inside the water-pool.

For other proteins such as concanavalin-A, myelin, and chymotrypsin, Kelley *et al.* (1993) illustrated that the affinity cosurfactants, consisting of hydrophilic ligands derivatized with hydrophobic tails, increased the efficiency of

selective protein recoveries using reversed micelles by extending the operating range of pH and salt concentration over which an extraction can be performed. Three different affinity cosurfactant-protein pairs have been used to demonstrate the principle of this extractive technique: (i) concanavalin-A, a lectin, was extracted with the addition of octyl glucoside; (ii) natural amphiphiles, such as lecithin, were used to extract myelin to recognize and bind the phosphatidylcholine headgroup; and (iii) alkyl boronic acid were used to extract chymotrypsin. The enhancement in protein transfer correlated with the binding strength of the free ligand and protein in aqueous solution. The shift in the transfer curves, when affinity cosurfactant was added, indicated the stronger interaction between the extracted protein and the reversed micellar phase in the presence and in the absence of the affinity cosurfactant. Higher protein concentration could be achieved at the highest water transfers (lowest salt concentrations), while greater selectivity was observed at higher salt concentrations. The binding strength of the protein and the ligand headgroup of the cosurfactant determined the increase in protein transfer and the most dramatic increase in protein partition coefficients being observed for dissociation constants was in the order of $1x10^{-6}$ M.