CHAPTER II
BACKGROUND AND LITERATURE SURVEY

2.1 Proteins and Antibiotics

2.1.1 Proteins

Proteins are the most abundant organic molecules within the cell containing four most prevalent biological elements: carbon (50 percent), hydrogen (7 percent), oxygen (23 percent), and nitrogen (16 percent). In addition there is sulfur content in proteins up to 3 percent contributed tertiary structure of proteins. When protein is exposed to conditions sufficiently different from its normal biological environment, a structural change, called denaturation, typically leaves the protein unable to serve its normal function. Relatively small changes in solution temperature or pH, for example may cause denaturation. Size of this biopolymer varies considerably with molecular weight ranging from 6,000 to over 1 million. Proteins can be isolated, purified, and characterized by several different types of physical and chemical techniques based on different molecular properties (Bailey and Oillis, 1986).

2.1.2 Antibiotics

Secondary metabolism products, which inhibit growth of microbial species, even at low levels, are called antibiotics. Uses of antibiotic are found in antimicrobials (human disease), antitumor agents, fungicides and pesticides for plant protection, animal diseases and animal growth products, and research 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 Protein Separation

Separation of Protein strongly plays a role in biotechnology applications. The traditional methods for recovery of biomolecules from mixture, such as

chromatography and electrophoresis, were developed for small-scale analytical or preparative applications (Hatton, 1989). Liquid-liquid extraction offers the advantage of economy of scale but it is difficult to find a suitable solvent having desired selectivity and capacity for interesting products and gentle on sensitive protein structures. Microemulsion is a candidate extractant which can solve these problems.

2.3 Microemulsion

A microemulsion is generally considered to be nanometers droplets of one type of fluid encased in surfactant shell and dispersed throughout a second immisible fluid. It is thermodynamically stable as long as conditions around them are unchanged. On the other hand, by changing temperature, pH, or composition to the value at which the microemulsion is no longer energetically favorable, the system will be destabilized (Dungan, 1997).

2.3.1 Microemulsion Formation

The spontaneous formation and thermodynamic stability of microemulsions were contributed to decrease 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 showed that the contribution of the interaction energy between droplets was negligible and that the free energy of formation can be zero or negative if the interfacial tension is very low (of the order of $10^{-2} - 10^{-3}$ mN/m), although not necessary negative. Furthermore, phase behavior studies of nonionic surfactant systems as a function of temperature showed 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. Further studies showed that the effects produced by temperature in nonionic surfactant systems were produced by salinity in ionic surfactant systems.

2.3.2 Microemulsion Structure

Two main general structures have been proposed and are accepted: discrete microemulsions and bicountineous microemulsions (Solan, Pons, and Kunieda, 1997). Discrete microemulsions consist of the 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 restively easy to treat theoretically, the structure of bicontinuous microemulsions is more difficult to visualize and therefore its theoretical treatment is 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 1 was proposed by Winsor (1984) who described three types of system:

1. Winsor 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 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 now exist in either the aqueous or the oil phase.

3. Winsor II. With further heating or electrolyte addition, the system becomes two phases 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).
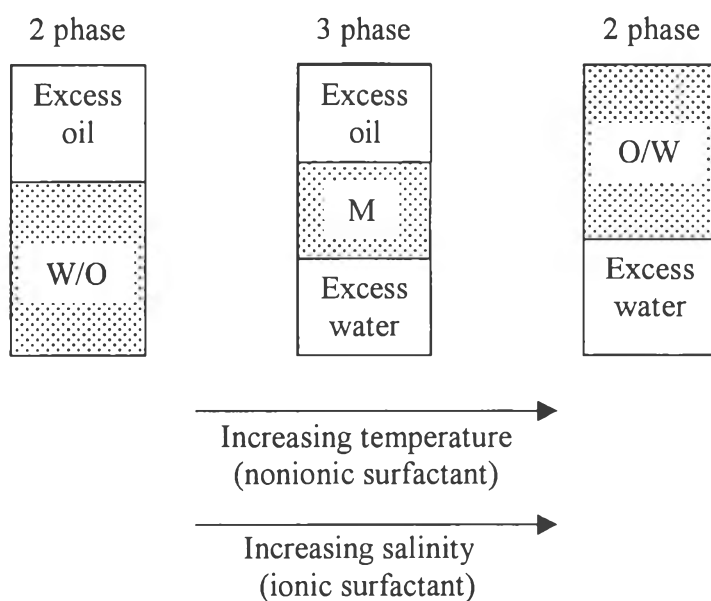
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   2 phase            3 phase            2 phase

  ┌─────────┐       ┌─────────┐       ┌─────────┐
  │ Excess  │       │ Excess  │       │         │
  │  oil    │       │  oil    │       │   O/W   │
  ├─────────┤       ├─────────┤       │         │
  │         │       │    M    │       ├─────────┤
  │  W/O    │       ├─────────┤       │ Excess  │
  │         │       │ Excess  │       │  water  │
  └─────────┘       │  water  │       └─────────┘
                    └─────────┘
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Increasing temperature
(nonionic surfactant)

Increasing salinity
(ionic surfactant)

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

2.3.3 Applications

Due to excellent properties, microemulsions are used in various applications. In cosmetics and foods, flavors, preservatives, and nutrients that are poorly soluble in water can be incorporated in water-based cosmetics and foods by solubilizing within surfactant aggregates. Microemulsion can be used to control drug releasing which is necessary for anticancer therapy in case of drug efficacy improvement and reduction of unwanted toxic side effects. Extraction of protein, the focus of this study can also be done efficiently by reverse micelles (Garcia-Celma, 1997).

2.4 Transfer of Protein into Reverse Micelles

Reverse micelles can be an effective extractant for recovery, purification, and concentration of proteins using liquid-liquid extraction techniques as shown in Figure 2.
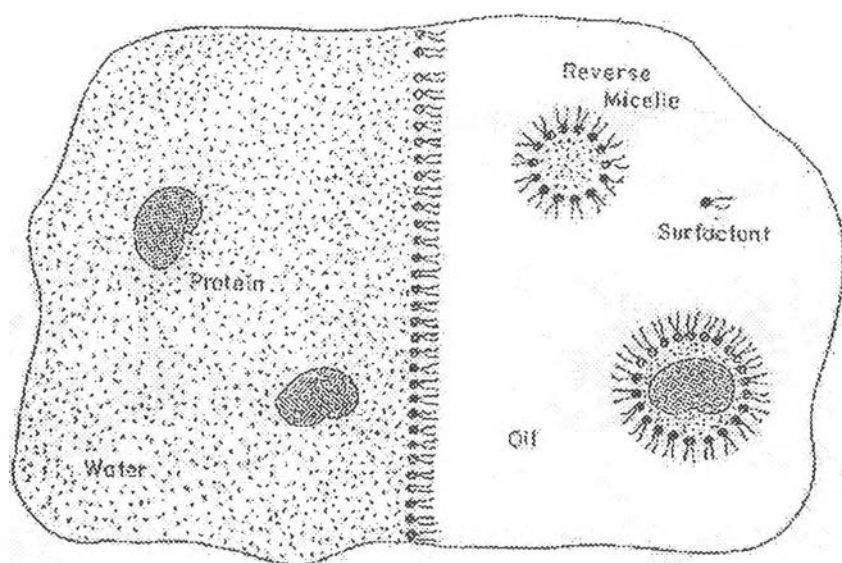

Figure 2.2 A schematic representation of the reverse micellar extraction of proteins (Hatton, 1989).

Electrostatic interaction between the charged polar heads of the surfactant and opposite charged groups of the protein is mostly accepted to be the driving force in liquid-liquid extraction of protein using reverse micelles (Hatton, 1989). However, other forces may also play an important role in the solubilization mechanism such as hydrophobic interaction (Pires and Cabral, 1993).

2.4.1 Factors Affecting Protein Solubilization

The distribution of proteins between a micellar organic phase and an aqueous solution is largely determined by the conditions in the aqueous bulk phase, namely pH, ionic strength, and salt type. The parameters related to the organic phase also influence the partition of a protein, such as the type and concentration of surfactant, presence of cosurfactant, and solvent type. Changes in temperature also

affect the solubilization of biomolecules in reverse micellar systems (Pires, Aires-Baros, and Cabral, 1996).

2.4.1.1 Effect of pH

The pH of the solution should affect 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 and solubilization should be possible with anionic surfactants. On the other hand, protein will be negatively charged while pH is greater than PI of protein, therefore cationic surfactants are suitable (Hatton, 1989).

2.4.1.2 Effect of ionic strength

Ionic strength of aqueous solution affects protein-partitioning behavior in many 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. Increasing in ionic strength reduces protein-surfactant interaction and surfactant headgroups repulsion leading smaller reverse micelles and decreasing protein solubilization through a size exclusion effect. Ionic species also migrate to the micelle water pool and displace proteins (Hatton, 1989).

2.4.1.3 Effect of salt

Salt and buffer type are important factors in determining the transfer across an organic solvent bridge between two aqueous solutions of different pH and ionic strength (Hatton, 1989).

2.4.1.4 Effect of solvent

Critical concentrations are the boundary concentrations dividing microemulsion Winsor I/III and Winsor III/II that are changed with solvent type. For alkane type solvents, the smaller the solvent molecular volume, the lower the critical cosurfactant concentration, the lesser cosurfactant is needed. Numerous studies of microemulsion have proposed that solvent penetration into surfactant hydrocarbon chain layer increases with decreasing solvent molecular volume. This penetration

can increase the interfacial mixing entropy and therefore, stabilize the interfacial layer.

2.4.1.5 Effect of surfactant

Ligand of specified surfactant affects the affinity of protein partition which is a result from selectivity of protein on that surfactant reverse micelle. Beside the effect of surfactant type, concentration is also important in capacity of reverse micellar water phase which is increasing with surfactant concentration (Hatton, 1989).

2.4.1.6 Effect of cosurfactant

A cosurfactant is a low molecular weight amphiphilic molecule presented within the interfacial layer of micelles resulting in low CMC of the surfactants (Harwell, 1991). Cosurfactants alone do not form stable aggregates in apolar solvents but they can be incorporated into reverse micellar structures to modify the interfacial properties of reverse micelles resulting in a more organized aggregate structure, increasing water capacity, and facilitating activity of the enzyme in reverse micelle (Freeman *et al.*, 1998).

2.5 Extraction of Protein Using Microemulsion

The possibility of using water-in-oil microemulsions to extract protein molecules from an aqueous phase has been extensively studied by many research groups. In this approach, an aqueous phase containing protein is contacted with a water-in-oil microemulsion and, under the appropriate conditions, the protein is then preferentially solubilized or transferred into the microemulsion phase. There have been numerous studies on extraction of various types of proteins using microemulsions, particularly the bis (2-ethylhexyl) sodium sulfosuccinate or AOT systems.

Cabral and Aires-Barros (1993) studied transfer of α-chymotrypsin into microemulsion droplet using bis (2-ethylhexyl) sodium sulfosuccinate (AOT) as a surfactant. They observed that, with the negative charges of AOT headgroups electrostatics were found to play a major role in the extent of transfer. At pH value below the isoelectric point, the protein had a net positive charge that facilitated its

transfer into the microemulsion droplet, where it can interact attractively with AOT headgroups.

Effect of surfactant concentration and water content in the microemulsion phase on amount of transfer of α-chymotrypsin into AOT reverse micelles was examined by Kelly, Rahaman, and Hatton (1991). They showed that amount of solubilized proteins increase significantly as the water content rise above a particular amount. There was also a certain ratio of water to surfactant for each specific surfactant concentration which is suitable for the partition of α-chymotrypsin in AOT reverse micelles.

Hu and Gulari (1996) made an argument that AOT has a number of very serious limitations: once proteins were extracting into the AOT reverse micellar phase, it was difficult to separate the proteins from the surfactant, and the phase separation of the AOT system took a long time. Consequently, they used sodium bis (2-ethylhexyl) phosphate (NaDEHP) in their work instead of AOT. NaDEHP, which is commonly used as the organophosphorus extractant, has a similar hydrocarbon tail to AOT but different in chemical compositions and structure of the headgroups. The structures of the two surfactants are as follows:
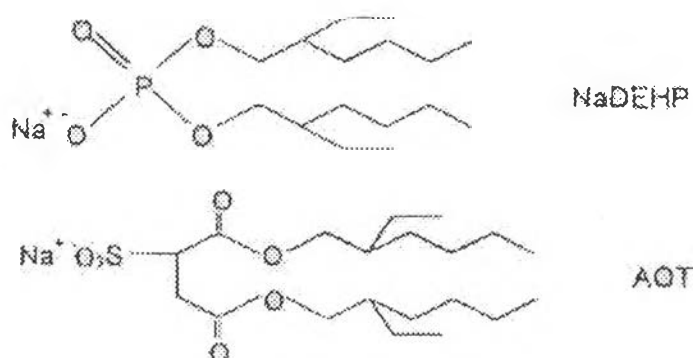

Figure 2.3 Structures of NaDEHP and AOT surfactants.

Hu and Gulari (1996) showed that extract cytochrome-c and α-chymotrysin in to 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 contracting the

micellar phase with a divalent cation (e.g. Ca^{2+}) aqueous solution, the reverse micelles were destabilized and released the protein molecules back into an aqueous solution for recovery. They used cytochrome-c from the horse heart and α-chymotrysin 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 drops 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. The backward extraction, they used 0.1 M $CaCl_2$ as the stripping solution to break the reverse micelles.

Jarudilokkul *et al.* (2000) illustrated the three systems that influenced on the selective separation of cytochrome-c, lysozyme, and ribonuclease-A from a buffer solution and a filtered fermentation broth are bis (2-ethylhexyl) sulfosuccinate (AOT) concentration, pH, and temperature. It was that a minimal AOT concentration exists for ≥ 90% extraction from a buffer solution, and this concentration depended on the pH of solution and hydrophobicity of the protein. Extraction from filtered broth resulted in a reduction of the minimal concentration for both cytochrome-c and lysozyme, but it was unchanged for ribonuclease-A. It appeared that certain broth constituents act 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. By using the optimized conditions, a mixture of the three proteins could be recovered from a filtered broth with high yields (70-97%) and highly purity. Furthermore, the initial protein concentration did not influence protein recovery.

For proteins cytochrome-c, lysozyme, and ribonuclease-A, Jarudilokkul et al. (1999) showed that the back-extraction of proteins encapsulated in AOT reverse micelle was 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 phase, 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 leaded to collapse of the reverse micelles.

Freeman *et al.* (1998) studied effect of bile salt, sodium taurocholate (NaTC), on α-chymotrypsin activity in AOT reverse micelles. They observed the increasing of enzymatic activity by the addition of bile salt. NaTC diversified the interfacial properties of the reverse micelles and increased their water capacity, resulting in a more favorable environment for enzymatic catalysis. The reaction velocity for the hydrolysis of the substrate N-glutaryl-L-phenylalanine p-nitroanilide (N-GPNA) by chymotrypsin more than double when NaTC is added to AOT reverse micelles.

Activity and stability of Lipase in AOT reverse micelles with bile salt cosurfactant were also investigated by Freeman *et al.* (2000). The study showed that both an anionic bile salt, sodium taurocholate (NaTC), and a zwitterionic bile salt, 3-[(3-cholamidyl-propyl)dimethylammonio]-1-propane sulfonate (CHAPS), increased the reaction velocity of the hydrolysis reaction and the stability of the enzyme in reverse micelles. They also concluded that the mechanisms by which bile salt affected enzymatic activity and stability in AOT reverse micelles varied from protein to protein. The effects which generally were positive (increasing activity and stability), may be dominated by the increase in size of water pool or modification of the detergent interfacial properties such as reduced polarity at the surfactant/organic interface (chymotrypsin and lipase).