PROTEIN SEPARATION
USING SURFACTANT PRECIPITATION

BY

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ABSTRACT

Surfactant precipitation applied as a surfactant mediated protein purification technique has considerable potential in protein extraction, and therefore the understanding of the interactions involved and the folding behaviour in the precipitated protein was the first aim of this thesis. The key system parameters such as buffer salt concentration, molar ratio of surfactant to protein and pH which determines the protein stability in protein-surfactant complex formation were evaluated. The surfactant:protein ratio determines saturation of protein binding sites while pH determines the strength of affinity for ionic binding which influences hydrophobic binding with surfactant monomers causing the protein to lose its conformation. The protein-surfactant binding varied for lysozyme, cytochrome c and ribonuclease A with trypsin and α-chymotrypsin, and hence the denaturation profile.

In the second aim, protein recovery from surfactant precipitation was enhanced by improving the solvent recovery method and, implementing a new and novel counterionic surfactant recovery method. The effect of a variety of recovery phases and solution conditions on lysozyme recovery was analysed in terms of their ability in maintaining protein stability, recovery yield, and activity. It was found that solvent recovery was limited by solvent polarity and protein solubility, and that the cationic surfactant, trioctylmethylammonium chloride (TOMAC), used to form nonpolar ion pairs with sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) was the most efficient method for recovering protein.

The third aim was to assess the influence of protein properties, such as charge and hydrophobicity, on protein separation. The selective extraction of a target protein from mixtures of proteins in both buffer and fermentation broth was investigated. It appears that the optimum surfactant:protein molar ratio for the extraction of the proteins from fermentation broth (lysozyme, cytochrome c and ribonuclease A; 16, 17 and 22 respectively) were similar to those in a buffer system. Lysozyme and ribonuclease A were selectively separated from a binary mixture. The extraction behaviour was well represented by surface charge distribution which is indifferent to system conditions. However, certain broth constituents induced the formation of some unfolded irreversible non-dissolvable precipitate in the recovery process.

Finally, the use of non-ionic surfactants, ionic/non-ionic mixed surfactants, and cationic surfactants were investigated in surfactant precipitation system. Non-ionic surfactant does not support direct precipitation of proteins using surfactant or recovery of protein from a protein-surfactant complex, and has no effect in a mixed ionic/non-ionic system. The application of cationic surfactant precipitation to separate trypsin inhibitor was attempted, and good recovery was obtained.

DECLARATION

The work described in this thesis was carried out in the Department of Chemical Engineering, Imperial College London, United Kingdom between November 2007 and July 2011. Except where acknowledged, the material is the original work of the author and no part of it has been submitted for a degree at any other university.

Cheng Shu Ian
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NOMENCLATURE

Å	a unit of length equivalent to 0.1 nm
A	a unit of absorbance associated with wavelengths (mAU)
Cp	protein concentration (g/L)
E	enzyme hydrolysis determination
HI	dimensionless surface hydrophobicity distribution
K_a	dissociation constant of acetic acid (M)
K_i	dissociation constant of hydrogen from a given ionisable group i
K_{LH}	solubility product constant of AOT in the presence of acid (M^2)
K_{LNa}	solubility product constant of AOT in sodium salt solution (M^2)
$K_{LysCl10}$	dissociation constant of lysozyme (M^{10})
K_{LysL10}	dissociation constant of lysozyme-ligand complex (M^{11})
k_{obs}	unfolding rate constant (s^{-1})
K_w	dissociation constant of water (M)
n_{LysL10}	amount of lysozyme-ligand complex (mole)
q	dimensionless overall surface charge
R	molar ratio of surfactant to protein

S	surface charge distribution (%)
V	volume of solutions (L)
α	parameter for electrostatic interactions between a protein molecule with a certain net surface charge and a proton
θ	signal of the circular dichroism spectra (millidegree)
Φ	average surface hydrophobicity ($kJ.mol^{-1}$)
Δ	relative difference of a certain parameter belonging to two proteins

CHAPTER 1 INTRODUCTION

1.1 DOWNSTREAM PROCESSING IN BIOTECHNOLOGY

Modern biotechnology has grown rapidly in the last decade, and improving separation technologies is critical in order to lower final product costs and enable the continued rapid development in biotechnology. Major efforts are being directed toward developing cost efficient separation of proteins considering the major role they play in the output of the Biotechnology industry (Przybycien *et al.*, 2004; Sadama and Beelaram, 1995). There has been increasing pressure on searching for more economic techniques to cope with complex large scale systems, high-value biologicals and challenging new products. There is also a need for scaleable separation methods to cope with the increasing annual production of bioproducts (Linn, 1990).

The field of bioseparations has considerable potential in reducing the manufacturing costs of industrial enzymes, as well as increasing the purity of therapeutic proteins, making downstream processes more viable. Continuous economic and environmental interest in developing industrial extraction and biotechnology purification processes have stimulated a considerable number of investigations on the separation techniques for protein purification and extraction. Lightfoot and Moscariello (2004) commented that separation mechanisms will continually improve based on increasing fundamental understanding of techniques developed back in the 1950s.

Until recently, bioseparations for therapeutic applications have been dominated by processes such as packed-bed chromatography which offers high degrees of resolution (Przybycien *et al.*, 2004), and purity is an unassailable need for therapeutic proteins. However, chromatography-dependent processes contribute to the single largest cost center in downstream processing (60-70% of the selling price). Hence, in order to substantially lower separation costs, researchers are investigating an old technique, 'Precipitation', particularly for the high volume production of industrial enzymes where such a high cost is uneconomical.

1.2 PRECIPITATION: A FUNDAMENTAL BIOSEPARATION TOOL

The primary interest in this thesis is to look into downstream processing for the purification and recovery of specific proteins from mixtures derived from biological sources. Protein precipitation is a simple procedure that has been used in the past to recover and characterize proteins for various applications. It is an attractive technique to use in Biotechnology because the solid-liquid separation required (e.g. settling, filtration, centrifugation) is well understood (Ghosh, 2004; Hilbrig and Freitag, 2003). Precipitation has advantages over other purification methods because it is rapid, simple and cheap, with good scale up potential. Crude protein purification can be achieved by precipitation alone (Temponi *et al.*, 1988), and it is employed in the fractionation of human plasma.

One early and widely used precipitation method for protein separation uses salts of various forms, eg ammonium sulphate, commonly referred to as "salting out". It is cheap, easily removed from protein solution, and does not denature proteins but exerts a stabilizing influence on them (Ghosh, 2004). Despite the history of precipitation, there is much left to understand and improve on when used in wider applications (Przybycien *et al.*, 2004). Common problems included protein loss, poor recovery efficiency and the lack of specificity in protein separation (Hilbrig and Freitag, 2003). Fractionation with salt precipitation may result in a low recovery of protein (Raweerith and Ratanabanangkoon, 2003; Saetang *et al.*, 1997), while the low selectivity of the method generally requires several sequential purification steps (Ghosh, 2004). However, a higher specificity precipitation method, such as affinity thermoprecipitation, has its overall efficiency limited by the solubility of the polymer in the aqueous phase (Vaidya *et al.*, 2001), and the method is unsuitable for temperature sensitive proteins (Mattiasson *et al.*, 1998).

Alternative techniques utilizing precipitation combined with other separation approaches gave a broader flexibility towards variation in process parameters (Hilbrig and Freitag, 2003). The aim of these alternative precipitation techniques was to retain the principle, simplicity and high concentration factors of precipitation with the goal of overcoming the disadvantages in a single and easy to perform process step. Although precipitation has achieved relative maturity, there is much left to understand when used in wider applications (Przybycien *et al.*, 2004). Bioseparation processes that improve precipitation for the purification and recovery of biomaterials are therefore important to the field of biotechnology and the pharmaceutical industry.

Several approaches to generate precipitates have appeared recently (Przybycien *et al.*, 2004). Shin and co-researchers developed a method of using surfactant, di-(2-ethylhexyl) sulfosuccinate, in protein precipitation (Shin *et al.*, 2004a). This precipitation approach has its unique specificities designed to improve protein purification. In this study, the use of surfactants as a precipitating ligand for proteins will be known as 'Surfactant Precipitation'.

1.3 SURFACTANT (SURFACE ACTIVE AGENT)

Surfactants are surface active substances; the discovery of the first surfactant dates back to 2800 B.C. by the Sumerians, while the ancient Romans used it as basic cleaning ingredient in soap made from animal fat (Putter, 2003). Surfactants are organic compounds which when dissolved or dispersed in a liquid at a low concentration, change the properties of that liquid at the surface or interface (Rosen, 1978). In 1913, Reychler, a Belgian chemist noted the detergency effects of certain synthetic surfactants. However, it was not until 1916 when World War I caused a shortage of fats and oil supplies for soap that surfactants were synthetically developed in Germany. The discovery of surfactants was driven by the need for a cleaning agent that had the functional composition of a natural surface active substance. The term 'surfactant' was created by Antara Products in 1950. Since their discovery, surfactants have attained increasing significance in research as well as in industry. Surfactants have widespread importance in consumer products, food processing, detergents, pharmaceuticals, and automotive and oil recovery industries due to their properties of solubilization, emulsification, lubrication and catalysis (Baronnet, 2003).

1.3.1 SURFACTANT PROPERTIES

Surfactants are amphiphilic products (Hjelmeland and Conn, 1986), and contain both hydrophilic and hydrophobic groups, as shown in Figure 1.1. The hydrophilic end is usually a polar or ionic group and is strongly attracted to water molecules, while the hydrophobic end (a surfactant alkyl chain) is usually made up of a long fatty acid and hydrocarbon chain (either aliphatic or aromatic) and is water insoluble.

Figure 1.1 Sketch of surfactant molecule consisting of a water soluble hydrophilic head and a water insoluble hydrophobic tail component.

Surfactants are primarily classified by the hydrophilic group which carries an ionic or electrical net charge after dissociation in aqueous solution. The categories of surfactants are anionic (negative charge), cationic (positive charge), nonionic (no net charge) and zwitterionic (both positive and negative charge) (Hjelmeland and Conn, 1986) (Figure 1.2). The anionic hydrophiles are based on carboxylate, sulfate, sulfonate, phosphate and sulfosuccinate anions. The cationic hydrophiles are the quaternary ammonium salts. The properties of ionic surfactants are strongly affected by the ionic strength and nature of the counterion (Mukerjee, 1967). The nonionic hydrophiles associate with water at the ethoxylate, alkoxylate and glucoside chain. The zwitterionic hydrophiles are some form of a betaine product, and zwitterionic surfactants are amphoteric and can be positive or negative since the ionic character of the dipolar groups depends on the pH of solution.

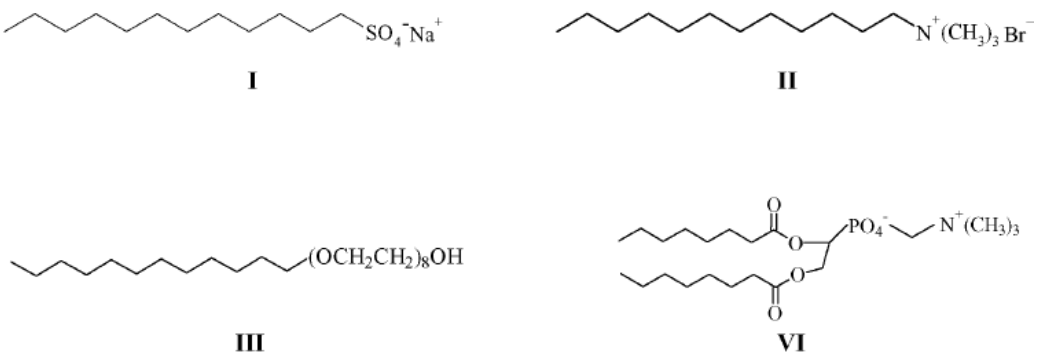

Figure 1.2 Examples of the different type of surfactants: (I) -anionic (Sodium dodecyl sulphate, SDS), (II) –cationic (Cetyltrimethylammonium bromide, CTAB), (III) –nonionic (Tetraethylene glycol dodecyl ether, $C_{12}E_4$) and (VI) –zwitterionic (Dioctanoyl phosphatidylcholine, C_8-lecithin) surfactants (Rangel-Yagui *et al.*, 2005).

The greatest amount of surfactants produced today are anionic surfactants, although the proportion of nonionic surfactants is on the increase (Chmelarova and Toth, 2000). Surfactants have evolved from being used extensively at home to being vital in industry. This shift in trends in the development of a variety of surfactants produces many other types of surfactants in large or small quantities, concurrently with the existing dominant ones.

1.3.2 SURFACTANT OPERATIONS

Surfactants change the properties of a liquid at the surface or interface (Rosen, 1978), and the process of doing so gives rise to a number of physico-chemical or chemical properties of practical interest. Surfactants preferentially adsorb at an interface and reduce the medium's surface or interfacial free energy, hence they reduce the affinity between the surface molecules (Rosen, 1978). When the surfactant concentration in the water increases, it will continually lower the surface tension of the solution until it reaches a certain point where the surface tension remains constant with further increases in the concentration of the surfactant (Mukerjee, 1967; Rosen, 1978). The corresponding surfactant concentration at this discontinuity in the plots of surface tension against surfactant concentration is known as the "critical micelle concentration" or CMC (Mukerjee, 1967). The determination of a surfactant CMC can also be made by physical properties such as osmotic pressure and detergency for all surfactants, as well as conductivity in the case of ionic surfactants. The sharp break observed in the curves of all these properties as a function of surfactant concentration is evidence of micelle formation (Figure 1.3).

At surfactant concentrations below the CMC, the individual surfactant molecules are loosely integrated with the water molecule as monomers (Hjelmeland and Conn, 1986). At this stage, the surface tension equilibrium process comprises diffusion of surfactant molecules between the bulk liquid and subsurface layer, and the transfer of surfactant molecules between the subsurface layer and the surface (Darton and Sun, 1999). The CMC is the highest monomeric surfactant concentration achievable, and therefore the highest surfactant chemical potential (Helenius *et al.*, 1979).

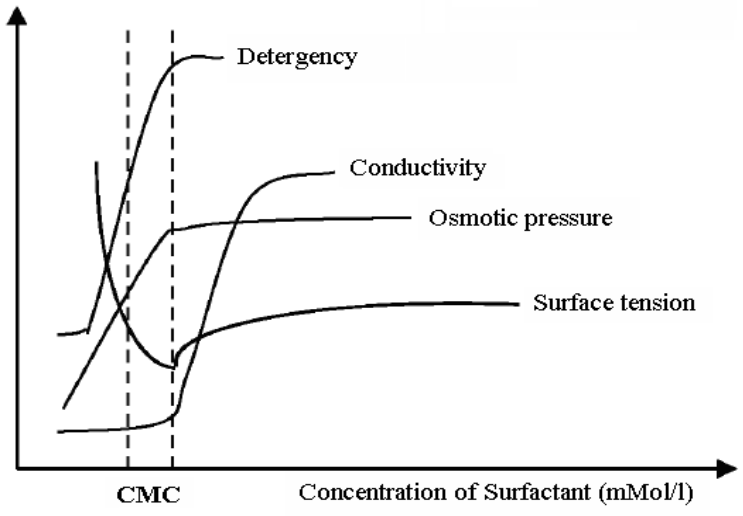

Figure 1.3 Typical variations of the physical properties of an aqueous solution of surfactant as a function of surfactant concentration. The break in the curve of each property corresponds to the Critical Micelle Concentration (CMC) (Rangel-Yagui *et al.*, 2005).

In the CMC region, the surfactant molecules build up their own structure as micelles in the interior, and monolayers at the surface of the bulk solution (Hjelmeland and Conn, 1986). Many surfactants can assemble into aggregates spontaneously at or above the CMC (self-assembling systems), and form a 'protective coating' around the suspended material (Mittal, 1977). Micelle aggregation, diffusion and disintegration rates are relevant to surface behaviour above the CMC (Darton and Sun, 1999). Surfactant solutions may, at the same time, contain an ordered phase of micelles and a disordered phase of free surfactant molecules or ions existing in dynamic equilibrium in solution (Helenius *et al.*, 1979). The number of micelles present in solution can be calculated by knowing the aggregation number of the micelles, N. Micelles are labile entities formed by noncovalent aggregation of individual surfactant monomers, and N corresponds to the average number of surfactant monomers in each micelle of a micellar solution (Rangel-Yagui *et al.*, 2005).

These surfactant-water structures dependant on surfactant concentrations are seen in Figure 1.4. The surfactant concentration has a major effect on the molecular structures formed by surfactants. Apart from their concentrations, the CMC of certain surfactants depends on the physical and chemical conditions such as temperature, pressure, pH and salt concentration (Fresta *et al.*, 2002). The occurrence of the CMC and micellization results from a delicate

balance of intermolecular forces between hydrophobic, steric, electrostatic, hydrogen bonding and van der Waals interactions (Rangel-Yagui *et al.*, 2005).

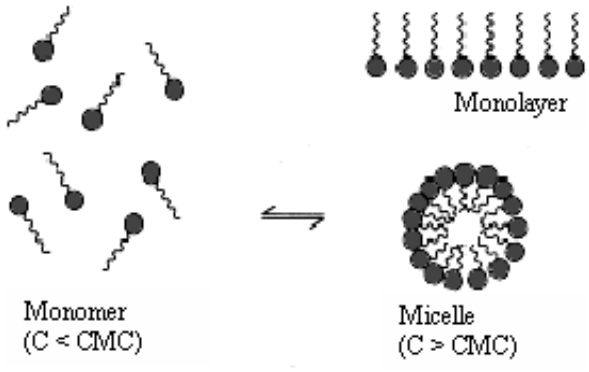

Figure 1.4 Equilibria between the surfactant monomers, surface monolayers and micelles in an aqueous solution: (●) denotes hydrophilic portion and (〰) denotes hydrophobic portion.

1.3.3 GENERAL SURFACTANT STRUCTURES IN SOLUTIONS

Interestingly, the type of solution in which the surfactants dissolve is also a factor in the surfactant-water structures. When micelles form in water, the hydrophobic tails of several surfactant molecules produce an oil-like core which is most stable as it has no contact with water, and their ionic heads form an outer shell that maintains favourable contact with water (Mittal and Lindman, 1984; Mukerjee, 1967). For nonionic surfactants with polyoxyethylene headgroups, the structure is the same except that coils of hydrated polyoxyethylene chains rather than counterions are present in the outer region (Rosen, 1978). The anisotropic water distribution property within micelle structures are observed by the water concentration decreasing from the micelle surface towards the water-excluded core (Rangel-Yagui *et al.*, 2005).

As micelles are limited by the solubility of surfactants in water, the hydrophobic or hydrophilic sections of these groups of surfactant molecules are joined (Tanford, 1980). In Figure 1.4 micelles are represented as a spherical cluster, although they can take on other shapes with the same minimum energy configuration attained at the minimum value of surface tension (Darton and Sun, 1999). Spherical micelles growing one-dimensionally into cylindrical micelles, or two-dimensionally into bilayers or discoidal micelles, are controlled

by the surfactant heads as micelle growth requires bringing surfactant heads closer together in order to reduce the curvature of the micelle surface (Rangel-Yagui *et al.*, 2005). The size of a typical micelle is around 50 Å and is made up of about 100 surfactant molecules (Goyal and Aswal, 2001). Micellar shape and size can be tuned by changing the surfactant chemical structure as well as by altering the solution conditions such as surfactant concentration, ionic strength, temperature and pH (Quina and Hinze, 1999). In terms of dynamics, the micelle structural stability ranges from milliseconds to seconds (Shah, 1998). They break and reform at a fairly rapid rate in an aqueous surfactant solution. The hydrophobic effect associated with the nonpolar surfactant tails is the attractive driving force for micelle formation (Tanford, 1980). The main opposing repulsive forces in the process are steric and electrostatic interactions between surfactant polar heads (Rangel-Yagui *et al.*, 2005).

When surfactants aggregate in organic solvents they are referred to as an inverse/reverse micelle (Shin, 2002), although some surfactants need a cosurfactant in order to form reverse micelles (Khoshkbarchi and Vera, 1995). In a reverse micelle the heads are in the core that can encapsulate a water droplet in which biomolecules may be solubilised, while the tails maintain favourable contact with the nonpolar solution, also referred to as 'oil' (Wang *et al.*, 1995b). Reverse micelles are mostly monodispersed (Luisi *et al.*, 1987). The size of the water pool and the surface area covered by the counterions depends on the arrangement of surfactant at the interface to accommodate different guest molecules (Shin, 2002). Reverse micelles trap biomolecules in a microaqueous environment without direct contact with the organic solvent they are solubilized in (Khoshkbarchi and Vera, 1995). The reverse micelle as illustrated in Figure 1.5 looks somewhat like the reverse orientation of the micelle. There is a limited region of surfactant-water concentration and temperature where the reverse micellar aggregates are stable (Luisi *et al.*, 1987).

Figure 1.5 Simplified illustration of a reverse micelle.

Aqueous surfactant solutions take the form of microemulsions when a non-polar solvent is introduced into the system. A microemulsion is a single phase mixture of water and oil with concentrations of surfactants above the CMC (Jarudilokkul, 2000). Two immiscible liquids are brought into a clear, macroscopically homogenous phase in a mixture with surfactant. Microemulsions are a unique class of solutions with low viscosity, ultralow interfacial tension, large interfacial area, thermodynamic stability and capable of solubilising aqueous and lipophilic compounds (Paul and Moulik, 2001). Controlled addition of solutions can produce transparent dispersions comprising monodispersed droplets of water-in-oil (w/o), or oil-in-water (o/w) or colloidal dispersions. Due to various structures and components, the study and characterisation of microemulsions is difficult. A variety of techniques were used to obtain microemulsion properties; conductance-related, nuclear magnetic resonance and transmission electron microscopy, to name a few (Paul and Moulik, 2001). Despite the complexity of microemulsion structures, they are used in substantial quantities in chemical and industrial processes, from enhanced oil recovery to nanoparticle synthesis.

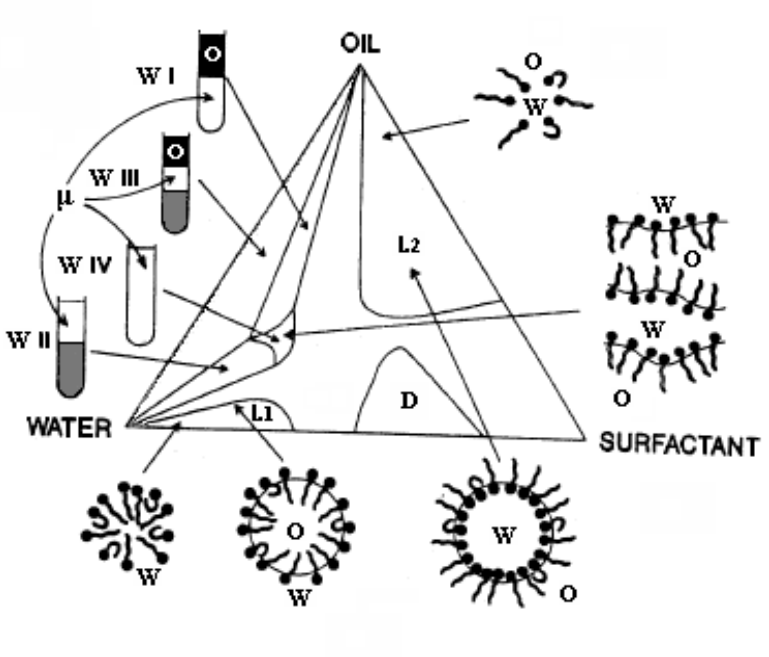

Figure 1.6 **Schematic ternary phase diagram of water-oil-surfactant mixtures representing Winsor classification and probable internal structures: L_1– single phase region of normal micelles (o/w microemulsion), L_2– reverse micelles or (w/o microemulsions), D-anisotropic lamellar liquid crystalline phase (Paul and Moulik, 2001). The microemulsion is marked by μ, oil by O and water by W.**

The miscibility of water, amphiphile and oil relies on the overall composition which is system specific (Paul and Moulik, 2001). The ternary phase diagram in Figure 1.6 describes the phase manifestations, and is useful in surfactant microheterogenous systems. The sequence of phase equilibria between phases present at low surfactant concentrations is classified as "Winsor phases" (Winsor, 1954). Winsor I has two phases, the lower surfactant aqueous phase (o/w) in equilibrium with the upper excess organic phase. Winsor II is also comprised of two phases, the upper organic phase containing surfactants (w/o) in equilibrium with the lower excess aqueous phase. Winsor III consists of three phases; the middle phase is a bicontinuous surfactant-rich phase (o/w plus w/o) in equilibrium with the upper excess oil and lower excess water phases. Winsor IV is a single phase, with homogeneously mixed surfactant-water-oil emulsions. Conversion from one phase to another can be achieved by adjusting the proportions of the constituents (Winsor, 1954). The steadily increasing numbers of researchers engaged in the study of the different surfactant solutions suggests that there is a significant future in the exploitation of their unique properties in many branches of chemistry and technology.

1.4 MOTIVATION AND AIMS

Past work mentioned above has only dealt with system parameters such as pH of the aqueous phase, ionic strength and the molar ratio of surfactant to protein to optimize the surfactant precipitation of proteins. The stability of proteins in the aqueous phase in equilibrium with surfactant at a range of submicellar concentrations, and of the final product with these parameters and interactions has not yet been discussed. **The first aim of this work was to examine the key system parameters that influence protein stability during surfactant precipitation.**

Even though the little work published claimed success in the precipitation of target proteins by direct addition of surfactant, protein recovery from the protein-surfactant precipitate has its limitations. Due to the solvent added and sensitivity of the proteins, denaturation occurred in a relatively short time in many cases. Could there be a solvent-free protein recovery method that takes advantage of the properties of surfactants? **The second aim was to improve the techniques involved in protein recovery in order to achieve higher protein extraction yields while maintaining final product bioactivity.**

When performing surfactant precipitation on a wide variety of proteins, knowledge related to the fundamental mechanisms of selectivity of extraction can save time by providing accurate predictions of extraction. **Therefore, the third aim was to investigate the key protein properties controlling the use of surfactant precipitation to separate proteins from biological mixtures.**

For surfactant precipitation to be considered a viable bioseparation method protein extraction must be carried out in a complex industrial fermentation media. Furthermore, it is necessary to explore new surfactants to examine the influence of different surfactant precipitation systems and to correlate surfactant properties and enzyme behaviour. **The final aim was to evaluate the potential and viability of surfactant precipitation for wider applications.**

1.5 THESIS OUTLINE

Chapter 2 presents a literature review on the fundamentals of protein and surfactant interactions, surfactant promoted protein denaturation, protein-surfactant processes, and an overview of surfactant-mediated purification techniques and process considerations for surfactant precipitation.

Chapter 3 describes the experimental procedures for the precipitation of lysozyme with AOT. The effect of system parameters; molar ratio of surfactant to protein and pH on protein secondary structure and chromatogram peak profiles are presented. In addition, the analytical methods (UV assay, activity assay, circular dichroism measurement, chromatography methodology and methylene blue assay) which were used to support the findings on protein-surfactant interactions versus protein stability are also detailed.

Chapter 4 describes the experimental procedures for the recovery of lysozyme from the lysozyme-AOT complex. Two methods, the use of a new solvent phase and the use of a counterionic surfactant were tested on an optimised precipitate and compared. The recovery methods were evaluated by the effect of ionic strength and pH of protein solutions together with the characteristics of different types of polar solvent and cationic surfactant.

In Chapter 5, two properties of the protein surface, charge and hydrophobicity, were investigated in a variety of proteins containing a mixture of both hydrophilic and hydrophobic groups. The molar ratios of surfactant to protein in conjunction with the surface properties required for maximum protein recovery were analysed in both buffered and non-buffered systems. Then, the protein extraction behaviour in each group of proteins was discussed. The hydrophilic group was used as a model to study the selectivity in Chapter 6.

In Chapter 6, the selective separation of three proteins having similar physical properties but a diverse range of surface properties was determined from sets of mixtures. The potential of protein extraction in a complex fermentation broth was observed. Protein selectivity was tested by least square estimates and an analysis of variance (ANOVA) approach. The influence surface properties had on selectivity and protein folding in buffer solution and in fermentation broth were examined.

Chapter 7 describes the use of some new surfactants in surfactant precipitation, which were comprised of nonionic surfactants and an ionic/nonionic mixed surfactant, to extract lysozyme. Proteins with low pIs which were not suitable for AOT precipitation were precipitated with a cationic surfactant ligand and recovered with AOT. Two proteins with such properties extracted with this method of surfactant precipitation were discussed.

Finally, the conclusions, original contributions to knowledge and suggestions for future work are summarized in Chapter 8.

CHAPTER 2 LITERATURE REVIEW

2.1 FUNDAMENTALS OF PROTEIN AND SURFACTANT INTERACTIONS

Proteins are complex macromolecules with different levels of structure. Proteins consist primarily of amino acids, each have in common a central carbon atom to which are attached a hydrogen atom, an amino group (NH_2) and a carboxyl group (COOH). Amino acids are defined by their chemical nature, hydrophobicity, charged residues and polarity. They are joined together by a peptide linkage between the carboxyl group of one amino acid with the amino group of the next. The spatial arrangement of these amino acids within the protein structure forms its conformation, and native protein refers to protein in any functional, folded conformation. Many conformations of a protein can be achieved without any peptide bond breaking and change in conformation, for example, rotation of single bonds is possible (Nelson and Cox, 2009). Multiple stable conformations of proteins are reflected in the change that take place as they bind to other molecules or catalyze reactions. A protein's conformation is stabilized largely by weak interactions, in particular, hydrophobic interactions and hydrogen bonds. The oppositely charged groups form ion pairs to provide for protein flexibility and stability (Nelson and Cox, 2009).

When water surrounds a protein molecule, the optimal arrangement of hydrogen bonds results in a highly structured system. Amino acids with hydrophobic sidechains are thermodynamically inclined to fold into the core of the protein, while hydrophilic residues are polar and located on the surface of the protein to form hydrogen bonds with water so the protein can solubilise in aqueous environments. Some proteins have hydrophobic and aromatic groups on the surface of the molecule and are more ready to interact hydrophobically. The presence of hydrogen bonds hold together the main protein secondary structures; alpha-helices and beta-pleated sheets. The alpha-helix is comprised of a polypeptide backbone tightly wound around an imaginary axis with about 3.6 residues per turn (Branden and Tooze, 1998). The structure of the beta sheet is built up from a combination of several regions of the polypeptide chain, and the strands are usually 5 to 10 residues long connected laterally by at least two or three hydrogen bonds, forming a twisted,

pleated sheet (Branden and Tooze, 1998). The tertiary structure or three-dimensional arrangement of proteins allows amino acids that are located far apart in the polypeptide sequence, and in different types of secondary structures to interact. Interacting segments of the polypeptide chains are held together by weak interactions and covalent bonds such as disulfide bridges (Nelson and Cox, 2009).

Surfactants are relatively simple amphiphilic molecules. The general characteristics of a group of surfactants such as; insolubility in an aqueous medium caused by their hydrophobic fatty acid chains, ability to form aggregate structures (micelles) from the association of their hydrophilic headgroups, and their surface activity have been discussed. We will now look at the interactions between proteins and surfactants, and also the composition and denaturing properties of surfactants in protein-surfactant systems.

2.1.1 PROTEIN-SURFACTANT INTERACTION MECHANISMS

Proteins and surfactants are different in their molecular structure, and hence their mechanisms of interaction depends on the molecular state (conformation, chemical structure, molar mass, charge) of both the protein and surfactant molecules (Semenova *et al.*, 2005). There are typically many stages for binding with ionic surfactants due to their strong affinity for proteins. The CMC is the single most prominent parameter separating the surfactant binding as either monomers or micelles, with other sub-region surrounding these interactions. The initial binding happens at a very low ratio of surfactant to protein molecules involving surfactant monomers binding via electrostatic and hydrophobic interactions (electrostatic interactions are prevalent in the binding) without inducing conformational changes (Jones, 1996). Anionic surfactants will bind to cationic protein sidechains (Arg, His, Lys), cationic surfactants to anionic sidechains (Asp, Glu), and the alkyl chains of surfactants to nearby hydrophobic patches (Andersen and Otzen, 2009).

Subsequent binding with the addition of more surfactant after exceeding the saturation point of the protein binding sites, despite still maintaining a low surfactant to protein ratio, results in the formation of surfactant clusters (micelle-like structures at sub-CMC). Surfactant clusters, unlike the bulk micelles present above the CMC, are the only form of micelle-like structures on the protein able to form, and not free micelles in solution. Uncharged surfactant does not go through cluster formation due to its low affinity for proteins. These clusters begin

to unfold the protein by association between two or more protein molecules as shared clusters, and higher sub-CMC surfactant concentrations quickly lead to individual protein-surfactant clusters (Andersen *et al.*, 2009). Protein folding is a first-order process, while protein aggregation is a second or higher order process with respect to surfactant concentration. Larger proteins have long polypeptide chains to allow shared clusters to stimulate protein association by linking up different binding sites of the protein without unnecessary aggregation, which merely causing intramolecular reorganization (Ibel *et al.*, 1994).

The formation of structures between proteins and ionic surfactants occurs with increases in protein size through electrostatic interactions at specific sites with individual surfactant molecules, and substantial binding through cooperative surfactant interactions along the unfolded polypeptide chains with surfactant clusters (Chodankar *et al.*, 2007). Cooperative binding is weaker than specific binding, and is comprised largely of hydrophobic interactions. In terms of the enthalpic change, specific binding is accompanied by an exothermic enthalpy, while nonspecific cooperative binding is endothermic (Jones *et al.*, 1973). A specific ligand binding would lead to a linear-linear relationship for single binding sites. Above the CMC, it is more difficult to observe the change in the equilibrium of protein-surfactant interactions due to the interference of free micelles resulting in a Circular Dichroism (CD) signal being too noisy to analyse. Calorimetry is unable to distinguish new binding from already saturated binding sites, and spectroscopic techniques can not pick up major rearrangements in the secondary and tertiary structures of proteins.

Kinetics has been employed to provide insight into the rate of unfolding and structural changes when micelles bind to proteins. The log of the unfolding rate constant increases linearly at sub-CMC surfactant concentrations and may decline at slightly above the CMC for cationic surfactants (Otzen, 2002; Otzen *et al.*, 2009), or plateau for anionic surfactants (Andersen and Otzen, 2009). Reduced kinetics of unfolding above the CMC does not imply that bulk micelles do not take an active part in denaturation, in fact it is a binding reaction with a high affinity for the micelle that results in the rate limiting step for subsequent conformational changes in unfolding (Otzen and Oliveberg, 2002b). It defines the concept of unfolding as an encounter between protein complexes and surfactant micelles. A decline in the unfolding kinetics could be attributed to the formation of new and weak binding sites on the protein, or the partitioning of some protein intermediates into the micelles (Viseu *et al.*, 2007), which best agreed with the structural changes noted with increasing surfactant

concentration. A protein specific explanation for the inhibited kinetics of β-lactoglobulin is that the rate constants were measured on binding to the native state protein dimer, and the β-lactoglobulin transformation from a dimer to a protein monomer with surfactant binding (Busti *et al.*, 1999; Viseu *et al.*, 2007). Micellar binding expands proteins to form a partially denatured state before a major unfolding transition following micellar change at very high surfactant concentrations, and the ensuing kinetics of unfolding are thereby dependent on the micellar properties (Otzen, 2002). The denaturation profiles are discussed from the vast structural formation of protein-surfactant complexes.

Protein-surfactant interactions and structural changes are shown in the two figures below. Figure 2.1 shows a schematic binding isotherm of surfactant molecules with a protein molecule. Regions A and B correspond to the specific and noncooperative binding regions, C the cooperative binding region, and D saturation in the region of the CMC of the surfactant. Figure 2.2 shows the stages of surfactant binding detected and quantified by steady-state fluorescence and isothermal titration calorimetric (ITC). ITC points are based on a single titration series, a negative slope in the figure signifies the decreasing heat flow contribution (endothermic) and vice versa. Low fluorescence intensity signifies highly polar solvents and the intensities increases with the hydrophobicity of the surrounding environment.

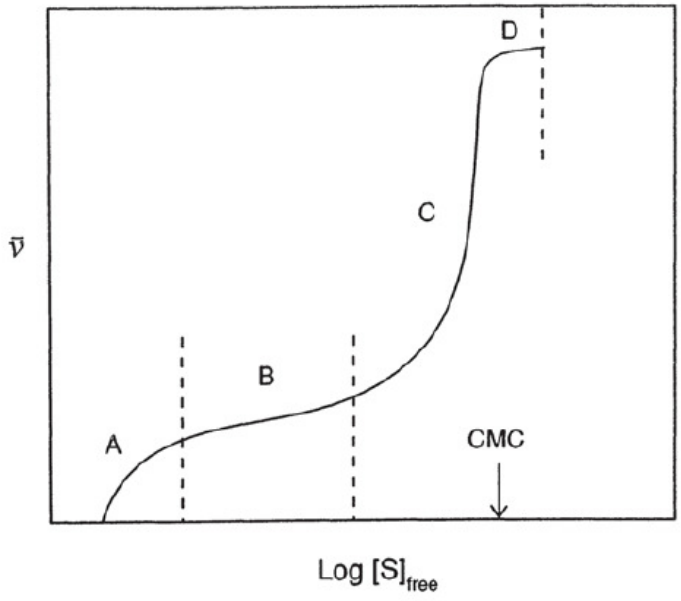

Figure 2.1 Schematic plot of the binding of surfactant ligands ($\bar{\upsilon}$=number of ligands bound per protein molecule) as a function of the logarithm

of the free surfactant concentration $[S]_{free}$ (Jones, 1996). A and B are the specific binding regions, C the cooperative binding region and D the saturated region.

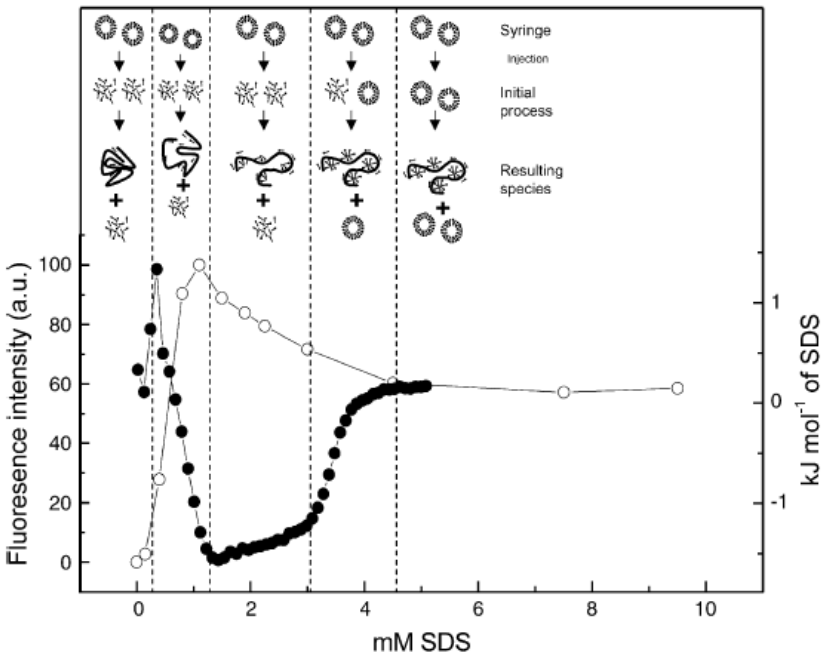

Figure 2.2 **Fluorescence intensity (○) and ITC enthalpogram (●) describing the interaction between SDS and *Humicola insolens* cutinase in relation to surfactant concentration. The dotted lines mark each region with alteration of the polarity and enthalpy of the system contributed by the SDS concentrations (Nielsen *et al.*, 2005).**

2.1.2 PROTEIN-SURFACTANT COMPLEXES

A critical determinant of the stability of a protein in any interaction is if the molecule is driven thermodynamically to rearrange or unfold by the residues in the interior, and hence to associate with neighbouring molecules. Due to the polyionic character of proteins, the intermolecular interactions with surfactants can follow a particular folding pathway via formation of protein-surfactant complexes that can determine protein aggregation to stabilize the system (Hansted *et al.*, 2011). The change in molecular structure of a protein on various scales has made the application of thermodynamic models such as those applied to interactions with surfactants more problematic (Berger, 2006; Moosavi-Movahedi, 2005).

Monomer binding at relatively low surfactant:protein ratios to oppositely charged proteins through simple charge neutralisation has a pronounced effect on reducing protein solubility. Surfactant molecules with the charged headgroups form a salt bridge with charged amino acid residues, while alkyl chains make hydrophobic contact with the tertiary structure (Jones, 1996); most surfactants bind to the protein surface. The protein-surfactant complex has a more hydrophobic surface than the original protein with which they are formed, become insoluble and precipitate. Attempts to recover the precipitate have been useful to highlight the potential of these interactions (Shin *et al.*, 2003c). More effort in understanding this non-aggregated compact protein-surfactant complex which forms in a relatively small surfactant concentration window as compared to micellar promoted protein aggregation is required in order to understand the fundamental processes involved.

The precipitate formed initially dissolves with the increasing binding of surfactants to hydrophobic residues at newly exposed sites, and electrostatic repulsion stabilizes the solution of soluble complexes. The resolubilization process encounters a phase behavioural transition via a narrow region of a viscous electrostatically swollen gel-like state between the precipitation region and the clear isotropic solution phase (Moren and Khan, 1995). The isotropic solution that follows is of low viscosity containing finite aggregates where the protein is solubilised by micelle-like aggregates (Valstar *et al.*, 1999). The qualitative difference between the complexes in the gel and in solution is that in the gel a compact protein structure interacts with adjacent protein molecules before the macroscopic network breaks up in the soluble complex into a single expanded protein that interacts with a self-assembled surfactant cluster with little protein-protein interaction. The detailed structure of the gel is at present unknown. Surfactant clusters are shown in the small angle x-ray scattering (SAXS) as small quasi-spherical micelles associated directly with only part of a protein, with the other part modelled as a disordered structure extending away from the clusters until uptake of more surfactant molecules allow one micelle per protein (Andersen *et al.*, 2009). The free energy balance of all the protein-surfactant complexes is determined by a balance between electrostatic and hydrophobic forces for the whole protein-surfactant interaction process (Stenstam *et al.*, 2001).

Structural insights into micelle complexes with proteins revealed remarkably diverse conformations. It is impossible to describe the structure of reduced proteins with their disulphide linkages broken with one model because the protein structures are less rigid,

nevertheless most suggest that the complexes have an extended conformation with surfactant molecules bound along their polypeptide chain length. The protein-surfactant structures proposed have been summarized into five main structural models (Ibel *et al.*, 1990). A "micellar complex" in which protein assembles into surfactant molecules in the form of micelles of definite size. The aggregates formed on protein by surfactants are similar to micelles in a protein-free micellar solutions. A "rod-like particle" in which a polypeptide chain forms the core of a rod with short rigid segments and surfactant bound along its length. An "α-helix-random coil" model in which the surfactant binding enhances the α-helix content of the protein and disrupts the β structures. A "flexible helix" model in which the polypeptide chain of the protein wraps around a flexible cylindrical micelle, and the structure is stabilized by hydrogen bonding between the surfactant head groups and the peptide bond NH groups. A "pearl necklace" model in which the polypeptide chain is flexible (in contrast to the rod-like model) and acts as the string of a necklace and the spherical surfactant clusters scatter along the chain (Figure 2.3). There are two possible structures for the pearl necklace model. One in which the protein wraps around the surfactant head groups of micelles of variable size and the micellar charges are sequestered by the protein (Figure 2.3a), this is known in some literature as a "decorated micelle" model (Jones, 1996). The other in which the surfactant micelles of constant size wrap around the protein hydrophobic sites because of interactions with the hydrophobic region of the clusters giving an α-helical conformation to the protein. Therefore, this has a greater effect on the surfactant chains, and exposure to the electrostatic repulsion between individual micelle drives protein denaturation (Figure 2.3b).

The extent of binding changes with surfactant concentration, and so will micellar formation. Alkyl sulphates in a surfactant tend to a spherical or a more elongated cylindrical micellar structure at very high (hundreds of mM) concentrations of surfactant (Clint, 1992; Croonen *et al.*, 1983). Short-chain alkyl surfactants are expected to need higher concentrations than the longer-chain surfactants to form cylindrical micelles. Micellar change affects further surfactant bindings, as is evident from the saturation kinetics in unfolding displayed by spherical micelles, and a steep concentration-dependent increase in the rate of unfolding displayed by cylindrical micelles (Otzen, 2002; Otzen and Oliveberg, 2002b). Cylindrical micelles denature proteins by binding preferentially to the transition state for unfolding (Otzen, 2011), thus accelerating unfolding at higher concentrations unlike the spherical micelles. The log of the unfolding kinetics largely increases with the log of the anionic micellar concentration in the micellar surfactant phase for cytochrome c (Das *et al.*, 1998), aprA-subtilisin (Narhi *et al.*, 1988) and protease Q (Han and Damodaran, 1997), just to name

a few. In contrast, cationic surfactants do not form cylindrical structures, and therefore the log-log relationship is not seen in its unfolding of protein through spherical micelles (Otzen, 2002). Under the conditions where the binding isotherm reaches a plateau or a saturation point well beyond the CMC, further binding of the surfactant does not occur on the protein, and normal micelle formation occurs as excess surfactant is added (Turro *et al.*, 1995).

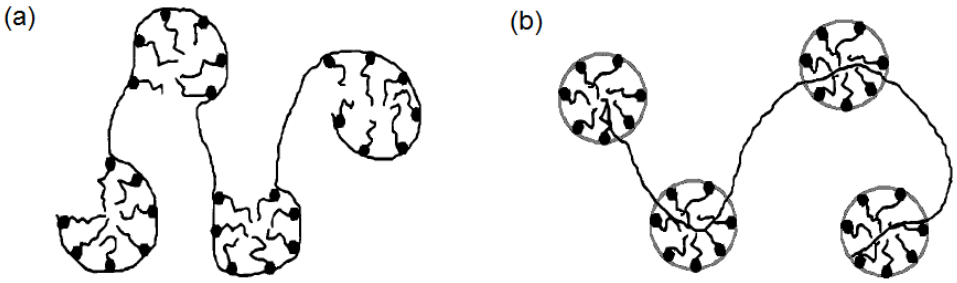

Figure 2.3 **Two possible "pearl necklace" structures of protein-surfactant complexes (Turro *et al.*, 1995): (a) the protein wraps around the micelles and decreases the mobility of the charged head groups; (b) the micelles nucleate on the protein hydrophobic sites but does not affect the mobility of the charged head groups.**

The five models proposed are reported based on different findings, and do not necessarily accommodate all types of proteins, surfactants, and analytical techniques. The detailed structure of protein-surfactant complexes depend on a whole host of factors: the protein sequence determining the nature of the binding sites and compatibility with different micellar environment; solvent conditions in conjunction with the strength of electrostatic attractions, and; the stability of micellar structures. Based on this, it is reasonable to find the same protein indulging in the formation of different types of complexes. It was seen with actyl-coenzyme-A-binding protein (ACBP) that binding with 33 SDS molecules gave a decorated micelle linked with two protein molecules, with 42 surfactant molecules it forms a larger decorated micelle bound to a single protein molecule, and finally, each unfolded and elongated protein molecule becomes associated with more than one micelle at 60 surfactant molecules possibly as a pearl necklace model (Andersen *et al.*, 2009). Cytochrome c converts from a decorated micelle at 0.02 M SDS to a pearl necklace structure at 0.5 M SDS (Xu and Keiderling, 2004).

2.2 SURFACTANT PROMOTED PROTEIN DENATURATION

The study of protein surfactant interactions has long been a topic of interest from their practical uses in cleaning products to estimating protein molecular weight (Kameyama *et al.*, 1982). The discovery that the binding of surfactants to proteins in aqueous solution were comparable with fatty acids, lipids, hormones, and drugs binding to proteins boosted advances in the area to enable applications in biological processes. Since then considerable research has examined the claims as to whether surfactants are a protein denaturant. In the last decade, more mature techniques and sophisticated analyses of protein-surfactant complexes using nuclear magnetic resonance (NMR), small-angle neutron scattering (SANS) and calorimetry, have established greater accuracy in exploring the fundamental principles with respect to protein surfactant interactions. Proteins interact very differently with monomeric and micellar surfactants. To benefit from protein surfactant interactions, it is essential to know which type of structures of surfactants (monomeric, micelles or both) are accountable for denaturation in proteins.

2.2.1 PROTEIN-SURFACTANT UNFOLDING REGIONS

Kinetic studies on a wide range of surfactant concentrations from below to above the CMC reveal different modes of denaturation by ionic and non-ionic surfactants that are closely associated with the different interactions with proteins. Manipulating the unfolding mechanism by mutation of ionic residues in the protein sidechain suggests surfactant denaturation follows simple electrostatic to hydrophobic binding, and gives denaturation profiles where surfactant sensitivity and contribution of the hydrophobic tail can be altered (Otzen *et al.*, 1999). This implies the existence and accessibility of various surfactant-mediated unfolding pathways depending on individual protein residues. Protein regions constituting the preferred sites for initial unfolding by ionic surfactants have been identified by N-terminal sequencing (Hansen *et al.*, 2009). Primary unfolding sites for α-lactalbumin and myoglobin which form well-defined fragments or stable intermediates correspond to those partially unfolded regions at low pH, or in the presence of organic solvents. The cleavage site for Tnfn3 which does not form a partially unfolded structure is rationalized from the protein's folding transition state and is more complex as well as sensitive to the choice of surfactant (Hansen *et al.*, 2009).

2.2.2 SURFACTANT MONOMERS AS STABILISING LIGANDS

Although in the early binding steps ionic surfactants bind as monomers with a high affinity for protein molecules, this is seldom related to denaturation. Instead, the coexistence of protein and small amounts of ionic surfactant relies on specific interactions with the native state; thus surfactants assume the role of a conventional ligand that stabilises proteins and protects the helical structures against denaturation (Moriyama and Takeda, 2005). The binding of a small number of monomeric molecules to exposed hydrophobic patches of dissociated proteins can serve to prevent aggregation by variables such as temperature (Waner *et al.*, 2004) and urea (Moriyama, 2003; Moriyama and Takeda, 2005), as well as reformation of the helical structures lost in denaturation (Chattopadhyay and Mazumdar, 2003; Moriyama and Takeda, 1999; Xu and Keiderling, 2004).

Measuring the protective action of alkyl sulfonate ligands (1-decanesulfonic acid, 1-dodecanesulfonic acid, 1-tetradecanesulfonic acid and 1-hexadecanesulfonic acid), researchers found that the greater the chain length, the greater the degree of binding with appropriate binding sites on the protein and the exclusion of water from the binding sites by the hydrophobic ligands, and the greater the stabilisation of the protein to unfolding (Busti *et al.*, 1999). The protein:surfactant ratio for stabilising binding to the native state differs for different proteins. It is up to 1:12 with SDS molecules for the large protein, BSA (Decker and Foster, 1966), 1:8 for S6 (Otzen *et al.*, 2008), 1:4 for α-lactalbumin (Otzen *et al.*, 2009), 1:3 for ACBP (Andersen *et al.*, 2009), 1:1 for β- lactoglobulin (Busti *et al.*, 1999), and Bet v 1 (Mogensen *et al.*, 2002), before the balance is tipped at higher stoichiometries and the unfolding becomes more favourable with more unfolded state binding sites. Nonetheless, not all proteins exhibit this stabilising binding phenomenon with ionic surfactants, myoglobin for example is destabilised with a 1:1 ratio with SDS (Andersen *et al.*, 2007).

One extraordinary role of monomeric surfactants is the activation of the enzymatic activity of *Thermomyces lanuginosus* lipase (T1L) (Mogensen *et al.*, 2005), and β-glycosidase (Dauria *et al.*, 1997). For all surfactants (ionic, nonionic and zwitterionic), low concentrations enhanced the activity of T1L. Activation of protein with ionic surfactants continued to above the CMC before an enzyme-inhibition effect causes a decline. For nonionic and zwitterionic surfactants, activation and inhibition occur below the CMC where the effect stops at a peak without declining. These activation and inhibition effects are not related to any major protein conformational change. Surfactants do not form clusters during the course of the activation

when strong monomer binding took place in the active site (Mogensen *et al.*, 2005). Activation does not necessarily incur a stabilising effect on the protein, such as the case for an uncharged monomeric surfactant (discussed in Section **2.2.5**).

2.2.3 SURFACTANT CLUSTERS IN PROTEIN DENATURATION

The Trp fluorescence method is sensitive to changes in local polarity, which is interpreted as a sign of the formation of a cluster of surfactant molecules tied to protein denaturation from surfactant binding (Andersen and Otzen, 2009; Andersen *et al.*, 2009). The role of shared clusters, rather than monomers, below the CMC initiated both partial unfolding and formation of higher order structures. Near to and above the CMC, the cooperative binding of surfactant molecules compromises the native structure and enzyme activity (Ding *et al.*, 2007; Narhi *et al.*, 1988). The unfolding process can be categorised into two stages (Nielsen *et al.*, 2005). The first stage involves an increase in hydrophobicity and the loss of protein structure. The second stage involves a considerable uptake of extra surfactant molecules (26 for ACBP (Andersen *et al.*, 2009), 24 for S6 (Otzen *et al.*, 2008), and 16 for myoglobin (Andersen *et al.*, 2007)), and the reduction in protein mobility with no advanced deterioration in the secondary structure. These stages end with the formation of bulk micelles in solution which probably contributes to additional rearrangements.

Shared clusters are intimately coupled to protein denaturation, but they are not to blame entirely. Proteins activated to bind at lower charge density experience no formation of surfactant clusters and protein denaturation, whereas proteins with less polar patches achieve cluster formation and stoichiometric binding only at the CMC (Nielsen *et al.*, 2007). A lower affinity for monomer binding and a lack of monomer binding sites seems to have a negative influence on the clustering, thereby making favourable monomer binding a prerequisite for protein unfolding by shared clusters. The same researchers also carried out surfactant binding with mixed micelles of 75% SDS (<< CMC) and 25% dodecyl maltoside, DDM (> CMC 40-fold lower than SDS). It was found that in the absence of surfactant clusters, proteins with sufficient monomer binding sites could still unfold driven by the bulk micelles, but required 4-fold more surfactant to unfold the protein than being driven by surfactant clusters. Monomer concentration does not affect the efficiency of micellar denaturation. Clearly clusters or micelles are required on an exclusive basis to denature proteins (Figure 2.4), and sub-CMC clusters are more potent denaturants than micelles.

2.2.4 SOLVENT CONDITIONS IN PROTEIN UNFOLDING

Solvent conditions are important in determining micellar structures, electrostatic effects, and activation barriers for surfactant binding, and hence surfactant-mediated protein unfolding rates. High ionic strength in surfactant solutions screen the repulsion between the ionic headgroups and encourages cylindrical micelles that contribute to a strong prevalent unfolding over that of spherical micelles (Otzen, 2002). Protein-surfactant interactions are highly dependent on the total charge of the ionisable side chains on proteins which changes with ionic strength and pH, rather than the protein pI. In a low ionic strength buffer, the unfolding of a positively charged protein with an anionic surfactant has been demonstrated to occur quickly at low pH due to the increase in the number and strength of electrostatic interactions (Stoner *et al.*, 2006). At high ionic strength (~ 250 mM), the surfactant CMC is lowered and less surfactant monomer is available for interaction with the protein; thus the unfolding rate is lower and essentially independent of pH. Temperature change (25-50˚C) does not affect the thermodynamics of unfolding as significantly as the enzymatic activity loss.

2.2.5 ROLE OF UNCHARGED SURFACTANTS

Monomeric binding of ionic surfactants was discussed previously in terms of their role in stabilizing proteins as well as supporting denaturation, mainly due to their electrostatic interactions with proteins. Due to their lack of a charged headgroup, uncharged surfactants are not expected to have much impact below their CMC. No specific monomer binding is observed, and denaturation only sets in around the CMC. The fundamental difference with charged surfactants is that the uncharged monomers only bind and denature protein when they cooperate with micelles (Figure 2.4) (Otzen *et al.*, 2009). The unfolding rate is slow and increases with the CMC. However, when dealing with the refolding of proteins, uncharged surfactant monomers do have an effect on protein stability by acting as a molecular chaperone. Nonionic surfactant Tween binds to the hydrophobic regions of human growth hormone exposed to denaturing conditions and catalyses the correct association of folding intermediates to prevent nonspecific aggregation (Bam *et al.*, 1996; Bam *et al.*, 1998). However, there is conflicting behaviour in the monomeric binding of uncharged surfactants to unfolded protein. A destabilising effect of Tween binding retarded the refolding of interferon-γ from a chemically denatured state and increased aggregation (Webb *et al.*, 2002).

In addition, a short-chain phospholipid zwitterionic surfactant destabilised cutinase with monomeric, and combined monomeric micellar binding (Sehgal *et al.*, 2007). The destabilisation decreased with reduced monomers, and is not seen with only micelles binding to the denatured state, indicating that the monomer governs the refolding process.

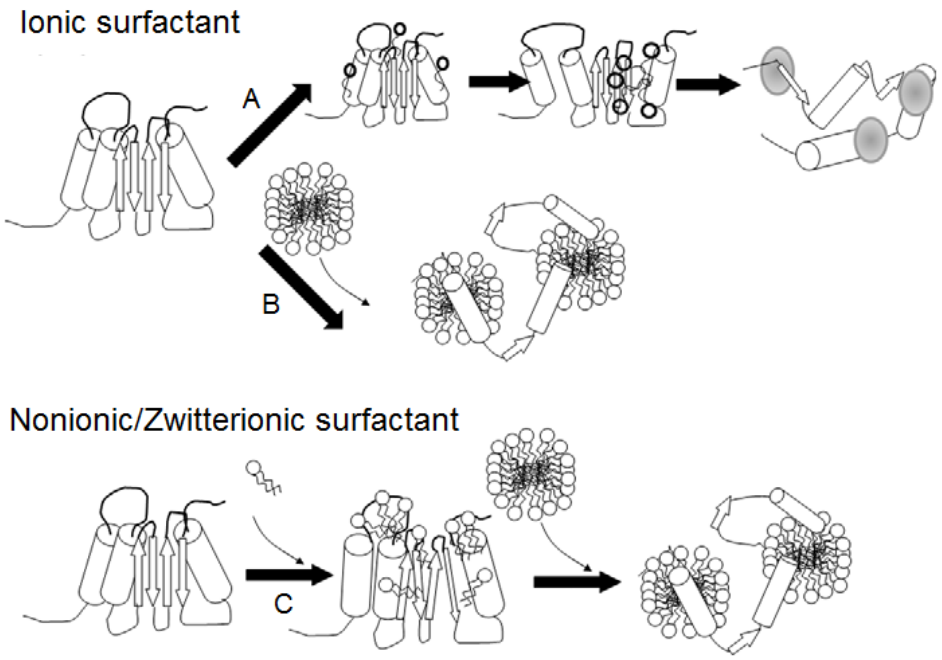

Figure 2.4 **Strategies of protein denaturation by charged and uncharged surfactant molecules (Otzen *et al.*, 2009). All phases are accompanied by the increasing uptake of surfactant molecules. Mode A is the change in protein structure from increased clustering of surfactants on the protein surface. For mode B, micelles lead to a denatured state without going through the surfactant clusters phase. For mode C, uncharged surfactant monomer binding facilitated major conformational changes above the CMC.**

2.2.6 SURFACTANT DENATURANT VERSUS CHEMICAL DENATURANT

Surfactant and chemical denaturation of proteins are both frequently used as techniques to understand specific aspects of protein folding. It is interesting to examine the common and different traits of both the denaturants in unfolding proteins. Generally, denaturation using

surfactants such as SDS, and chemical denaturants such as urea, guanidinium chloride (GnCl) and dimethyl sulfoxide (DMSO), induce unfolding by interfering with the molecular interactions of the folded form of the protein. Surfactant alkyl chain interactions with proteins exert an underlying similarity with solvation of the hydrophobic side chains in chemical denaturant (Parker *et al.*, 1995). The basic mechanism of both denaturants is to stabilise the denatured protein conformations, but surfactants have a more complex behaviour of interacting with the native compact conformations of globular proteins. SDS denatures protein at concentration as low as 5 mM (below the CMC) by binding to it directly (Andersen *et al.*, 2009), while urea and GnCl denature protein at higher concentrations (6-8 mM) as a consequence of the change they invoke in the structure of water molecules (Jones, 1996).

The high affinity of charged surfactants for proteins means that proteins unfold in multiple binding steps (Jones, 1996) with different levels of cooperativity and degrees of denaturation. The kinetics of protein conformational changes with pyrene fluorescence and stopped-flow fluorescence measurements detected a three state interaction model; activation, inhibition and destabilization which are independent processes (Mogensen *et al.*, 2005) reflecting the changes in the way surfactants bind to the protein. Monomeric surfactants have an activation effect on T1L, while in contrast chemical denaturant uniformly inactivates T1L. Kinetics are unable to provide information on the number of steps involved in denaturation with a weakly binding chemical denaturant because the log of microscopic rate constants varies linearly with denaturant concentrations, and the reaction kinetics are usually much slower than protein-surfactant binding (Fersht, 1999). A specific multiple site binding leading to the log-log relationship with chemical denaturant is seldom observed with surfactants. Although the initial increasing unfolding rate constant at low surfactant concentrations behaves like a chemical denaturant, it is many orders of magnitude greater than for a chemical denaturant.

The unfolding kinetics of a given protein, including its mutants, shows an unclear correlation between the surfactant LAS (linear alkyl benzene sulfonate) and GnCl (Otzen *et al.*, 1999). Individual sidechain mutations to adjust for the proximity of ionic residues emphasise the strong binding of the surfactant which profoundly affects the denaturation mechanism, while the weaker binding of the chemical denaturant has no effect. Chemical denaturants usually produce random coil structures (Tanford, 1968), whereas surfactant denatured states may vary. In an α/β protein, S6, the α-helices of protein were identified as the dynamic structure under attack by surfactant clusters; the site of attack of the increasing surfactant concentration range is extended to include β-sheet and helices displacement (Otzen and Oliveberg, 2002b).

In an α-helix-rich protein, cytochrome c, surfactant denaturation showed a partial decrease in helical structure, contrary to a complete loss of helical structure in GnCl denaturation (Das *et al.*, 1998). Refolding of the cytochrome c triggered by electron transfer to the folded state is faster in an SDS bound protein than in GnCl; supposedly surfactant keeps protein in a partially structured state which is more ready to refold (Chen *et al.*, 2008; Pascher *et al.*, 1996). Refolding of SDS bound S6 protein assisted by nonionic micelles, however, is much slower than in GnCl-denatured state because of mixed micelle interference with the process (Sehgal and Otzen, 2006). In a β-sheet-rich trypsin, surfactant induced significant amounts of non-native α-helix to form in the protein as part of the denaturation process (Ghosh and Banerjee, 2002). A correlation is seen from the alpha-helical preference in the secondary structure transition of β-sheet protein between surfactant unfolding, and urea unfolding in the presence of organic solvents (Ragona *et al.*, 1999).

Interactions spanning the centre of the hydrophobic core of the protein that form early in surfactant folding, is the final transition state of the protein when in GnCl unfolding (Otzen and Oliveberg, 2002a). Spherical micelles anchor α-helices to the hydrophobic core and force an expansion which weakens and denatures the protein by local unfolding (Otzen and Oliveberg, 2002b). With a cylindrical micelle, the expansion causes a global unfolding and produces an intermediate which resembles the transition-state structure for unfolding in GnCl.

2.3 REVERSIBILITY OF PROTEIN-SURFACTANT UNFOLDING

The complexation of proteins by surfactants is reversible in most cases, but reversibility is not rapid and does not occur in the presence of surfactants because of their high binding affinity. Surfactant molecules probably participate to avoid the exposure of hydrophobic portions of the protein on its unfolding with an increase in surfactant concentration. Stripping surfactant molecules from the protein has been done to leave naked unfolded protein to refold by the methods of; equilibrium dialysis across a semi-permeable membrane (Bozzi *et al.*, 2001), photoreduction through electron transfer (Chen *et al.*, 2008), dilution with nonionic micelles (Sehgal and Otzen, 2006), and complexation of surfactant with α-cyclodextrin (Otzen and Oliveberg, 2001; Yazdanparast and Khodagholi, 2006). The ability of protein to

return to its native state after the surfactant has been dialyzed out of the system depends on the extent of denaturation.

In circumstances where small numbers of surfactant molecules bind and precipitate with the native structure of protein, protein can be released without appreciable disruption of it's structure by solubilisation of the surfactant into a polar solvent (Shin *et al.*, 2003c). Another process termed "retrograde dissociation" introduces a more hydrophobic surfactant to dissociate the original surfactant from the complex by forming mixed micelles between anionic surfactant mixtures (Jones *et al.*, 1992). Retrograde dissociation is based on the greater stability of mixed micelles relative to that of a single surfactant-protein system. Molecular simulations recently developed, which go some way to studying surfactant-assisted protein refolding, have captured the "collapse-rearrangement" kinetics whereby the protein-surfactant hydrophobic interactions promote the collapse of a denatured protein and rearrangment to form a hydrophobic core as the surfactants are released (Lu *et al.*, 2007; Lu *et al.*, 2005).

2.4 PROTEIN-MIXED MICELLE INTERACTIONS

Different types of surfactants are mixed to achieve properties of the mixture which are better than those of the individual surfactant components (Rosen, 1989). Mixed micelles of ionic and nonionic surfactants in most cases follow a completely different binding strategy involving synergy between respective micelles. Attractive interactions between the surfactant components influence the; structure of mixed micelles, deviation of the surfactant CMC, the protein-surfactant interfaces, and the ionic surfactant's precipitation with protein (Clint, 1992). Charged surfactant has a much higher CMC in water compared to uncharged surfactant because of the electrostatic repulsion at the micellar interface, while mixed micelles will have a noticeably lower CMC than a relatively charged surfactant because of a reduced electrostatic repulsion from the spacing out of the individual charged surfactant molecules (Otzen, 2011).

Surfactants such as SDS versus dodecyl maltoside (DDM) mix easily and rapidly (Otzen and Oliveberg, 2001). This mixed micelle structure efficiently removes most active charged monomeric surfactants from solution and incorporates them into the micelles; nonetheless anionic and nonionic surfactants are not taken up equally into the micelles, and the

composition of micelles rarely mirrors the bulk composition. A measure of the CMC of the mole fraction of mixed surfactants enables the actual composition of the micelle phase and monomeric phase to be calculated (Rosen, 1989). Since anionic surfactant is mainly present in the micellar and not the monomeric form, interactions with proteins will be that of the bulk micelles instead of the shared clusters.

Mixed micelle systems have reported to control protein solubilisation capacity, enhancement in activity and stability of an enzyme (Chiang, 1999; Lalonde *et al.*, 1995; Russell and Britton, 2002), and improved extraction efficiencies of proteins (Rong *et al.*, 1999). The stabilization effect of mixed micelles can be explained from surfactant-surfactant interactions becoming more thermodynamically favourable than protein-surfactant interactions as a result of anionic/nonionic surfactant synergy leading to a lower CMC (Stoner *et al.*, 2006). However, it could also be due to the binding of nonionic monomers on the protein surface displacing the higher affinity anionic surfactant monomers, such as the preferential binding of a surfactant to another surfactant observed in a anionic mixed micelle system (Jones *et al.*, 1992). Anionic and nonionic mixed micelles weakened the denaturation potency of the micelles by decreasing both electrostatic and hydrophobic interactions, and forming new binding sites on the protein.

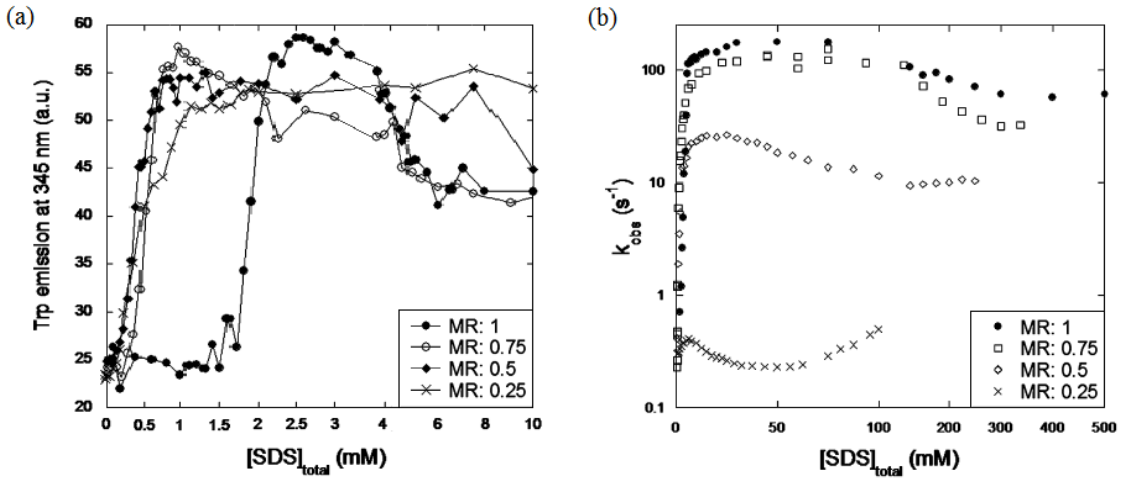

Figure 2.5 **Effect of mixed micelles on unfolding of ACBP at different mole ratios (MR) of SDS and DDM (Andersen and Otzen, 2009): (a) changes in Trp fluorenscence intensity upon titration with mixed micelles; (b) unfolding kinetics at 100% SDS, 75% SDS-25% DDM, 50% SDS-50% DDM, and 25% SDS-75% DDM.**

There is no simple relationship to summarise mixed micelles interactions with globular proteins. Mixed micelles comprising varying mole fractions of charged and uncharged surfactants follow different binding and unfolding pathways (Figure 2.5); proteins bind to the mixed micelles to different extents, and unfolding energies increase nonlinearly with the charged surfactant mole fraction (Andersen and Otzen, 2009; Nielsen *et al.*, 2007).

2.5 PROTEIN-SURFACTANT PROCESSES

Surfactants play a vital role in many processes of interest, and the inclusion of additives can broaden the phase behaviour of surfactants in solution (Shinoda and Friberg, 1986). Proteins are one class of additive that are constantly used to study the extraordinary variety of phase behaviours from the interactions between protein binding sites and the various states of surfactant; micellar, monomeric or other intermediates (Hjelmeland and Conn, 1986). Kinetic and spectroscopic studies show that the activity of protein in the presence of surfactants depends on the chemistry of surfactant head groups, and their hydrocarbon chain lengths (Blinkhor and Jones, 1973). The strong ionic surface interactions with charged groups on protein surfaces must precede before further interactions, including chain unfolding, can take place (Blinkhor and Jones, 1973), and these roles of surfactants lead to the large amount of interest in studying the nature of interactions employable in useful processes.

Reaction behaviour at surfactant interfaces is a good representation of many biological reactions (Rangel-Yagui *et al.*, 2005), and surfactant micellar catalysis has a high degree of parallel with enzyme behaviour (Price *et al.*, 2003). Catalysis can occur within surfactant micelles and reverse micelles. The reaction of solubilised protein in micelles normally occurs at the micelle-water interface, while reaction in reverse micelles occurs in the inner core (Rosen, 1978). Besides micelle-catalyzed reactions, chemical reactions sometimes occur to functional surfactants with reactive residues in the headgroups. In this case, surfactants react with the different hydrophobic and hydrophilic compounds which co-solubilise in the surfactant solutions, and in the presence of salt, leading to the formation of different reaction products (Abe *et al.*, 1983).

Surfactants play an increasingly important role in membrane biochemistry (De Grip and Lester, 1982). Surfactants are used extensively in membrane studies because similarly to lipids, they are amphiphilic molecules and behave according to some of the rules governing

lipid behaviour (Fresta *et al.*, 2002). However, surfactant systems are an interestingly simpler alternative utilized in membrane models to study biomembrane interactions. The use of surfactants acting as solubilising agents for membrane proteins has been investigated (Helenius *et al.*, 1979; Hjelmeland and Conn, 1986; Hjelmeland *et al.*, 1984; Palazzo *et al.*, 2010; Roman *et al.*, 2010). Different surfactants can be used to incorporate lipids and membrane proteins into the micelles for initial solubilisation, then for subsequent protein characterisation (Helenius *et al.*, 1979; Palazzo *et al.*, 2010). The surfactant effective for membrane solubilisation varies according to the literature. Since surfactants have the ability to bind to proteins and also act as protein denaturants (Blinkhor and Jones, 1973), during the choice of surfactant consideration must be given as to whether the native protein structure is to be maintained. In solubilisation of the membrane, the lipid around membrane proteins is exchanged for surfactant resulting in the formation of soluble lipid-protein-surfactant and protein-surfactant complexes, and mixed lipid-surfactant micelles (Helenius *et al.*, 1979; Zhou *et al.*, 2001). Surfactants are used to analyze, isolate the different protein aggregates, and characterize the individual polypeptide chains of the membrane. They have proved indispensable for structural studies, and purification of membrane proteins from mixed protein-surfactant micelles (Tanford and Reynolds, 1976).

Some important and practical applications of micelles lie in the area of separation science (Hinze and Pramauro, 1993), and various techniques in electrophoresis require surfactants. The widely used techniques of polyacrylamide gel electrophoresis (SDS-PAGE) utilise specific types of surfactant-protein interactions to identify and estimate subunit molecular weights of proteins (Nielsen *et al.*, 1978). SDS-PAGE is an analytical tool for the separation and qualitative characterisation of charged macromolecules. Restrictions of this procedure are emphasised, particularly with respect to the need for the presence of denaturing surfactants. In the absence of surfactants, no absolute molecular data can be obtained from the experiment because of the unknown in the net charge on the protein and the frictional coefficient (Nielsen *et al.*, 1978). It is known that surfactants dissociate a large number of water soluble proteins to their constituent polypeptides in the presence of a reducing agent, and these individual amino acids subunits bind to form polypeptide-surfactant complexes at the same time the polypeptides undergo a surfactant binding-induced conformational change (Nielsen *et al.*, 1978). Characterization of these complexes has been carried out, and this method of molecular weight determination is based on the understanding that the ratio of charge to frictional coefficient and the relative electrophoretic mobilities in SDS-PAGE is a unique function of the molecular weight of a group of polypeptides (Nielsen *et al.*, 1978).

Common techniques in high performance liquid chromatography (HPLC) may also use surfactants to solubilise membrane proteins. Aqueous micellar media have been utilized as a mobile phase additive in HPLC (Hinze and Pramauro, 1993). The reversible self-association of proteins in HPLC was induced by variations in protein, surfactant, and lipid concentrations (Andersen *et al.*, 1986), and this research explored the possibility of using molecular sieve HPLC for preparation and characterization of monomers and well-defined oligomers of a protein. The reduced surfactant binding by active oligomers may indicate that hydrophobic interactions are involved in the self-association of native protein (Andersen *et al.*, 1986).

Interest in surfactant interactions includes concepts and results concerning enzymes in reverse micelles. A review of the physical characteristics of reverse micelles before the uptake of enzymes, and analysis of the enzyme activity and conformation are important tasks in studying the micelles, as well as for understanding the mechanism of solubilisation of biopolymers (Luisi *et al.*, 1987). The hydrocarbon micellar solutions can solubilise nucleic acids, large plasmids, and bacterial cells (Luisi *et al.*, 1987). Water inside the water pool acquires novel properties, including solubilisation power (Khoshkbarchi and Vera, 1995). Enzymes entrapped in these pools are shown capable of synthesizing apolar compounds (Laane *et al.*, 1987). Protein concentration in the hydrocarbon micellar solution can be analysed by spectroscopic methods, for example, by measurement of the optical density (Khoshkbarchi and Vera, 1995). With all these findings of surfactants in biochemistry, applications to biotechnological and basic research can also be explored.

2.5.1 EFFECT OF SURFACTANT CHARGE ON PROTEIN STABILITY

The presence of surfactants in a protein solution have effects on protein activity and stability, and this is very much dependant on one major system parameter, that is the type of surfactants (Marcozzi *et al.*, 1998). Although surfactants do not change the initial activity of catalase, different surfactants allow the protein to retain a high residual activity for different periods of time (Spreti *et al.*, 1995). Interactions between surfactants and catalase are very peculiar. Each category of the surfactants (anionic, cationic, zwitterionic and nonionic) were investigated for their behaviour in the protein's environment.

Research on protein-surfactant interactions has seen widespread use of synthetic anionic alkyl sulphates and alkylaryl sulphonates, and the common surfactant used was sodium dodecyl sulphate (SDS) (Helenius and Simons, 1972). Anionic surfactant binds extensively to most proteins, from hydrophilic proteins in aqueous solutions to hydrophobic proteins related to lipids in membranes and lipoproteins (Helenius and Simons, 1972). SDS is well-known to have a denaturing effect which causes proteins to lose their activity (Marcozzi *et al.*, 1998). Considerable experimental work has been carried out to study the effects of anionic surfactants on protein, including its denaturing effects (Jones, 1992). Figure 2.6 shows SDS efficiently denatures protein at low concentrations, and the protein is dissociated into its constituent polypeptide chains. Various models analysing the structures of these complexes indicate clearly that the surfactant headgroups bind to protein surfaces by ionic interactions, while alkyl chains bind to hydrophobic regions of the protein close to the cationic sites (Spreti *et al.*, 1995). This eventually causes denaturation when the hydrophobic binding sites buried inside the protein structure are exposed outward (Jones, 1992). Anionic surfactants deactivate and denature catalase with more cationic sites. Bacterial catalase happens to have a smaller content of cationic sites to reduce the extent of interaction and the ability of the surfactant to split the protein into subunits (Spreti *et al.*, 1995). Therefore, typically for bacterial catalase, anionic surfactants are a poor denaturant.

Cationic surfactants are generally not as potent as anionic surfactants; in fact in some cases they manage to preserve protein activity for more than twice the time compared to native protein in the absence of surfactant additives (Marcozzi *et al.*, 1998). This study indicated that the protein secondary structure was preserved for a longer period, through both stabilising and activating effects created by the cationic surfactant. Although less potent, cationic surfactant is the most used surfactant functional group in a denaturant for bacterial catalase. It could be that the efficiency of binding with negatively charged bacterial surfaces gives it a more suitable quality in bactericidal disinfectants than anionic surfactants (Marcozzi *et al.*, 1998). The contrast between anionic and cationic surfactants in protein deactivation clearly demonstrates the importance of the chemistry of the surfactant headgroup. Cationic surfactants with bulky head groups such as trimethylammonium and pyridinium groups do not significantly denature proteins because there are no strong ionic interactions with the negatively charged groups on the protein surface which contribute to chain unfolding (Blinkhor and Jones, 1973).

The stabilizing effect of zwitterionic surfactants on protein structure has also been addressed. This surfactant appears to slow down protein degradation remarkably, with protein biological activity completely preserved for weeks (Spreti *et al.*, 1995). The much longer period that zwitterionic surfactants preserve protein activity than buffer alone is shown in Figure 2.6, thus making the protein less prone to denaturation with time. The interaction between the surfactant and the intersubunit region of the protein could have stabilised the protein quaternary structure (Spreti *et al.*, 1995). Weak interactions taking place between the zwitterionic surfactant and protein will allow only certain molecular geometries to stabilise the protein to a high level (Spreti *et al.*, 1995). Here, the protein does not become activated, it is only stabilised as the specific activity is not enhanced by the surfactant additive, but preserved.

As in general non-ionic surfactants do not denature proteins (Nikas *et al.*, 1992), they are usually most effective in binding organic solutes because of their lower CMC values compared to ionic surfactants (Hinze and Pramauro, 1993). Contribution of the polar group containing polyoxyethylene residues in a non-ionic surfactant molecule is reflected in the polarisability of its solubilisation site making it a favourable solubilisation agent for a wide variety of solutes in extractions (Quina and Hinze, 1999). Non-ionic surfactant binds hydrophobically to the regions occupied by lipid in the native membrane (Helenius and Simons, 1972). Lipophilic proteins were found to bind large amounts of non-ionic surfactants, whereas hydrophilic proteins bound little or none (Helenius and Simons, 1972). Studies revealed that most hydrophilic proteins are ineffectively separated by size with aqueous two-phase non-ionic micellar systems, and some are neither able to bind monomerically nor be incorporated into existing micelles (Nikas *et al.*, 1992). Ionic bonds are not part of the nature of binding forces involved in non-ionic surfactants.

Interactions of surfactants are responsible for protein activation and stabilisation in aqueous surfactant solutions (Marcozzi *et al.*, 1998). The effect of a surfactant on the protein could be explained either with a conformational change in the environment of the active site, or with a change in the strength of subunit association (Spreti *et al.*, 1995). Protein-surfactant interactions vary markedly with the protein being hydrophobic or hydrophilic. A hydrophilic protein may bind cooperatively to ionic surfactants with a gross conformational change, while it may only bind weakly with a non-ionic surfactant without denaturation. The nature of interactions served as a basis for consideration in surfactant selection in our work.

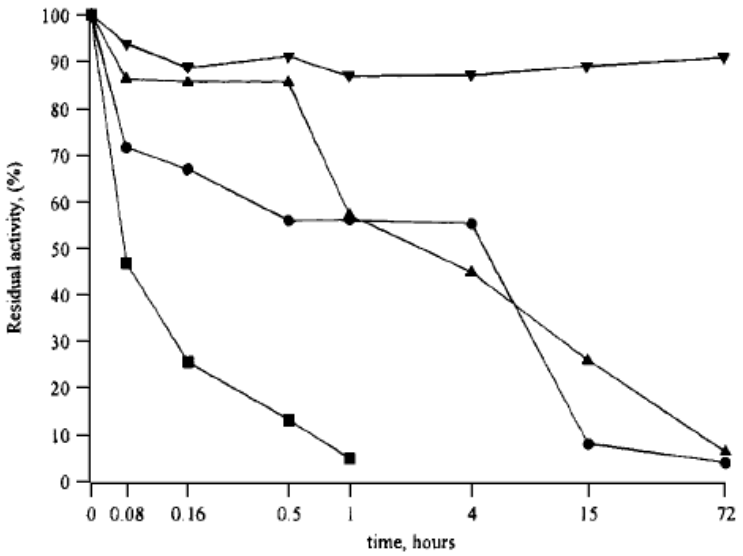

Figure 2.6 Percent residual activity versus time for non-bacterial catalase at 30˚C in the presence of (■) 2 mM SDS -anionic, (●) H_2O, (▲) 9 mM $(CTA)_2SO_4$ -cationic, (▼) 9 mM C12MeBS –zwitterionic surfactant (Spreti *et al.*, 1995).

Apart from surfactant type and structure, the catalytic properties of proteins are influenced by the concentration of surfactant. When surfactant concentrations exceed that required for protein solubilisation, there will not only be micelle-catalyzed reactions with oppositely charged proteins on the surface of the ionic micelle, but also adsorption onto the micelles and solubilisation into the micelles that result in decreased protein activity in the solution phase (Rosen, 1978). All these system parameters are often employed to alter the experimental conditions in protein-surfactant studies in order to achieve the best stabilising effect. The catalytic behaviour of proteins in the presence of surfactants was studied using the following surfactant-mediated purification techniques.

2.6 SURFACTANT-MEDIATED PURIFICATION TECHNIQUES

The use of surfactant to assist in protein purification is not a new bioseparation procedure. Many techniques using surfactant related protein purification have emerged during the last decade. The unique dual nature of surfactants enabling them to appeal simultaneously to both hydrophilic and hydrophobic proteins has stimulated research on their possible use for

extractive preconcentration and purification of biomaterials and organic compounds. The amphiphilicity of surfactant molecules allows for their separation of proteins based on their hydrophobic characteristics.

2.6.1 AQUEOUS TWO-PHASE EXTRACTION

An aqueous two-phase system is formed when two different immiscible liquids are dispersed and mixed. Aqueous two-phase extraction (ATPE) has gained a reasonable industrial maturity (Przybycien *et al.*, 2004), and has the capacity to handle high protein concentrations in purification (Nilsson *et al.*, 2002). Prior to surfactants being used, ATPE was well known to occur in polymeric systems. New developments have seen more importance in the modification of the aqueous two-phase system with surfactants. Aqueous surfactant micellar systems are successfully applied in many areas of analytical chemistry, from spectroscopy to separation science (Hinze and Pramauro, 1993).

Micellar extraction, a variant of ATPE, has been expanded with charged surfactants to impart an electrostatic component to partitioning (Przybycien *et al.*, 2004). This feature is expected to increase the range of applicability of two-phase aqueous micellar systems in the field of bioseparations. One example is two-phase aqueous non-ionic micellar systems which have been used to purify hydrophobic membrane bound proteins from hydrophilic biomolecules (Quina and Hinze, 1999). Addition of non-ionic surfactants into thermoseparating polymer systems increased the partitioning coefficient by up to 14-times its original value (Nilsson *et al.*, 2002), and surfactant selectively enhanced and optimised the separation of the target protein. The separating efficiency increased when surfactants were added indicating that the surface residues of the protein was better exposed to the phase components in the ATPE systems (Przybycien *et al.*, 2004).

Micellar extraction offers a number of advantages over conventional liquid-liquid and liquid-solid extraction in terms of lower cost and relatively non-toxic characteristics of the surfactants in comparison with organic solvents (Quina and Hinze, 1999). The system is easier to operate, and is comprised of less expensive reagents than classical polyethylene glycol (PEG)/dextran or PEG/ potassium phosphate systems (Przybycien *et al.*, 2004). Lack of a dual character caused polymer solutions to be less effective in the partitioning of proteins. Interactions between a water soluble polymer (hydrophilic) and a protein is less

sensitive to the hydrophobicity of the protein (Nikas *et al.*, 1992). It also helps to know that polymers are lyophobic colloids that require energy in their formation, are quite unstable from a thermodynamic point of view, and frequently form large aggregates (Rangel-Yagui *et al.*, 2005). Micelles, an association of colloidal molecules of surface-active substances, on the other hand are self-assembling and are thermodynamically more stable towards dissociation and aggregation.

2.6.2 CLOUD-POINT EXTRACTION

Cloud-point extractions employ the unique phase separation behaviour of surfactant micelle solutions as a means for extraction and separation (Hinze and Pramauro, 1993). The basis of cloud-point extraction was reported by Mittal and Fendler in 1982 after studying the phase phenomena exhibited by some surfactant solutions. By altering conditions such as temperature and pressure, or addition of an additive, the aqueous micellar solution separates into two isotropic phases; a surfactant-rich micellar phase and a dilute aqueous phase (Hinze and Pramauro, 1993). Any proteins binding to the micellar aggregates in solution can be extracted and concentrated in the surfactant-rich phase which can then be subjected to further fractionation and purification. The steps involved in a cloud-point extraction process are depicted in Figure 2.7, where the strength of the micelle-solute binding interactions determine the extent of extraction (Hinze and Pramauro, 1993).

Non-ionic micellar media have a unique phase separation point above the cloud point which is reversible upon cooling, and membrane proteins partition into the micelle-rich phase due to favourable hydrophobic interactions with the micelles. In another interaction, zwitterionic surfactant solutions also demonstrate temperature-dependent phase separation. In contrast to non-ionic surfactant micelles, phase separation of zwitterionic micellar systems are induced when the temperature is lowered (Hinze and Pramauro, 1993). The phase-separation behaviour of neutral (non-ionic and zwitterionic) surfactant systems is applied to the preconcentration of metal ions, separation of membrane proteins, and introduction of hydrophobic affinity ligands to extract hydrophilic proteins (Hinze and Pramauro, 1993).

Another study examined the effect of electrostatic interactions by adding an ionic (anionic or cationic) surfactant to a non-ionic surfactant solution. Since all charged proteins can be influenced by electrostatic interactions, two phase aqueous mixed (non-ionic/ionic) micellar

systems aim to attract a desired hydrophilic, water-soluble protein into the micelle-rich phase such as the use of biospecific affinity interactions in a neutral non-ionic surfactant system (Kamei *et al.*, 2002). The partitioning behaviour observed was that net positively charged proteins (lysozyme and cytochrome *c*) were being attracted electrostatically into the phase with the greater number of negatively charged micelles (Kamei *et al.*, 2002). Surfactant mixtures often give rise to enhanced performance over their individual components, and so surfactant blends are employed in a wide variety of practical applications.

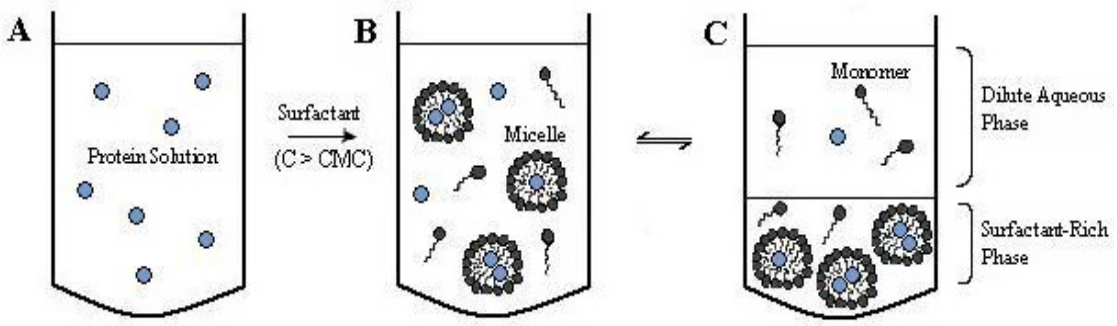

Figure 2.7 Schematic representation of a micellar-mediated phase separation (cloud-point extraction) technique: (A) Initial solution containing the hydrophobic species to be extracted; (B) Addition of micelle-forming surfactant in which hydrophobic species bind to the micellar aggregates formed; (C) Final phase-separated system after alterations to the conditions (temperature change or salt addition). Hydrophobic species are concentrated in the surfactant-rich phase and separated from the dilute aqueous phase (Hinze and Pramauro, 1993).

2.6.3 REVERSE MICELLE EXTRACTION

Surfactant-enhanced liquid-liquid extraction of proteins in reverse micellar systems has received increasing interest recently due to its ability to separate, with considerable efficiency, relatively complex protein mixtures. The concept of liquid-liquid extraction, or solvent extraction, is based on the relative solubilities of two immiscible liquids, but unlike ATPE this technique is comprised of water and an organic solvent. Water acts as a hydrophilic phase while the organic solvent forms a hydrophobic phase. Liquid-liquid extraction was studied as a separation process for bioproducts that are mostly hydrophilic, and which cannot be solubilised directly into nonpolar solvents (Khoshkbarchi and Vera,

1995). Reverse micelle extraction was first investigated in 1979 and considerable research work has been conducted since then (Shin and Vera, 2002).

Liquid-liquid extraction using reverse micellar systems involves an aqueous solution containing a target solute and electrolytes being contacted with an organic phase containing surfactants. Reverse micelle extraction is categorized as a Winsor II system (see Figure 1.6), which does not form without the electrolytes (Shin, 2002) (Figure 2.8). The solubilisation of water in a reverse micelle phase strongly depends on the nature of the solvent, the guest molecules such as ions, the temperature and the methods of forming reverse micelles (Cassin *et al.*, 1995; Luisi *et al.*, 1987; Luisi *et al.*, 1988; Shin, 2002). Similar to other micellar systems, the driving force for extraction are the electrostatic interactions between the charged surfactant head groups and the oppositely charged biomolecules (Rabie and Vera, 1997). In addition, the hydrophobic interactions between surfactant alkyl chains and the protein hydrophobic surface residues are important and govern the protein partitioning behaviour (Przybycien *et al.*, 2004).

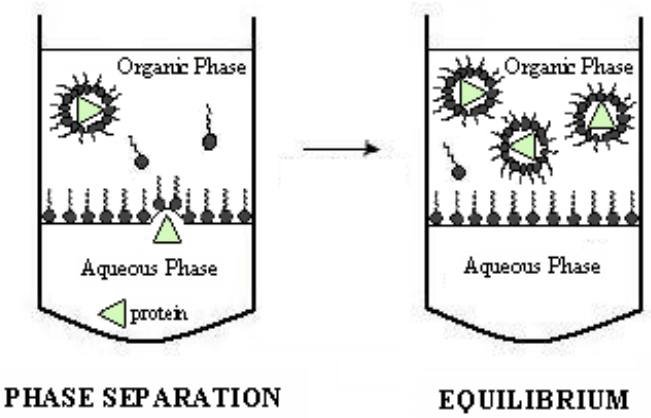

Figure 2.8 Schematic representation of the reverse micellar extraction technique.

Reverse micelles of ionic surfactants in organic solvents are suitable for the purification of extracellular enzymes (Krei and Hustedt, 1992). By far the most favourable reversed micellar medium in the studies carried out contained the anionic surfactant sodium di-(2-ethylhexyl) sulfosuccinate (AOT) and isooctane or hexane as the oil phase (Laane *et al.*, 1987). Ono *et al.* (1996) noted that no reverse micelles can be formed by a single surfactant without a cosurfactant except for AOT. For a product enzyme with a relatively low isoelectric point to remain within its stable pH range, reverse micelles of cationic surfactants are used (Krei and Hustedt, 1992). The earliest study used trioctylmethyl ammonium chloride (TOMAC) to

solubilise α-chymotrypsin and pepsin (Luisi *et al.*, 1979). Despite being the most commonly used cationic surfactant in the literature, TOMAC reverse micelles failed to work with lysozyme (Wolbert *et al.*, 1989). Dioctyldimethyl ammonium chloride (DODMAC) reverse micellar system extracted biomolecules such as amino acids; aspartic acid, glutamic acid and threonine (Wang *et al.*, 1995a), as well as proteins; albumin, α-chymotrypsin and lysozyme (Rabie *et al.*, 1998). DODMAC required the cosurfactant decanol to form reverse micelles (Shin, 2002). Other quaternary ammonium salts include cetyltrimethyl ammonium bromide (CTAB), cetylpyridinium bromide (CPB) and N-benzyl-N-dodeceyl-N-bis (2-hydroxyethyl) ammonium chloride (BDBAC) (Krei and Hustedt, 1992).

For non-ionic surfactants, poly(oxyethylene)sorbitan Trioleate (Tween 85) has been used. Proteins in a bulk aqueous phase are difficult to solubilise in reverse micelles of non-ionic surfactant alone because there are no strong interactions between the micelles and the protein (Jarudilokkul, 2000). Among the variables being determined for the surfactants in reverse micelles were pH, ionic strength, co-ion and counterion of the surfactant, cosurfactant concentration, protein size and effect of the solvent on the reverse micellar extraction.

Shin looked into various ways of improving reverse micellar extraction (Shin and Vera, 2002; Shin and Vera, 2004; Shin *et al.*, 2003a; Shin *et al.*, 2003d). Reverse micelle systems have showed considerable potential in separating specific proteins from filtered fermentation broths (Jarudilokkul *et al.*, 2000a), and have been used for higher molecular weight polypeptides such as monoclonal antibodies (MAbs) (George and Stuckey, 2010). Although the technology has been around for a long time, there are still limitations in using reverse micelle extraction. A common mechanism of protein loss in reverse micelle extraction is found in the formation of a water insoluble protein-surfactant complex at the aqueous-organic interface (Dekker, 1990) due to the solubilisation limit of protein in the reverse micellar phase (Shin and Vera, 2002). The precipitate was undesirable as the protein was thought to be denatured, and attempts were made to avoid its formation by changing the technique (Jauregi and Varley, 1998) and using new surfactants (Ono *et al.*, 1996). Effort was also expanded in recovering the complex by dissolving it in a polar organic solvent instead of an aqueous phase (Shin *et al.*, 2003b). **The white insoluble complex was reported to resolubilise in acetone without loss of enzyme activity (Shin *et al.*, 2003c). The basis of this finding resulted in a new technique being proposed of using surfactants as precipitating ligands in contrast to reverse micellar extraction, and this thesis will explore this technique in more detail.**

2.7 SELECTIVE PRECIPITATION: AN ALTERNATIVE SEPARATION APPROACH

New approaches to precipitation have been developed in an attempt to increase protein bioavailability (Przybycien *et al.*, 2004). Shin *et al.* (2004a) was investigating protein loss as a precipitate in a reverse micellar system when a new alternative, surfactant precipitation, was developed. Their studies showed that di-(2-ethylhexyl) sulfosuccinate (AOT) (Figure 2.9) can directly precipitate lysozyme from an aqueous solution without loss of protein activity (Shin *et al.*, 2003c). A unique discovery in the surfactant precipitation approach was that the presence of reverse micelles and an organic phase were not necessary for the purification of proteins (Shin *et al.*, 2003b). Comparison of the two methods concluded that surfactant precipitation simplifies the method of reverse micellar extraction while eliminating the use of an isooctane organic phase to form the reverse micellar phase, and reduces the amount of surfactant required per mole of purified protein (Shin *et al.*, 2003b). The term 'ligand' is used in this method to show that the surfactant's main function was to act as a precipitating ligand in surfactant precipitation in order to improve the efficiency of the purification process.

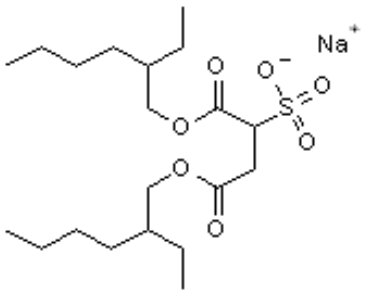

Figure 2.9 Structure of the di-(2-ethylhexyl) sulfosuccinate (AOT).

The research to date on this method uses a commercially available anionic surfactant, AOT, as the precipitation reagent, and a polar organic solvent such as acetone (Shin *et al.*, 2004b) as a recovery solvent. The technique involves the direct addition of a surfactant below it's CMC to the oppositely charged protein; upon contact an ionic complex pair is formed. Electrostatic interactions drive the complexation of the surfactant and protein (Shin *et al.*, 2004c). The protein-ligand complex is insoluble in water but can be dissolved in a polar organic solvent. The separation of proteins from the surfactant in acetone cannot be accomplished without an electrolyte being present (Fox and Foster, 1957), therefore sodium

chloride was introduced to the solvent. The insoluble compound was observed to disappear instantaneously when in contact with acetone and protein precipitated out of the solution a few minutes after the addition of salt. Analysis concluded that polar solvent dissociates the complex and recovers protein as an insoluble precipitate (Figure 2.10).

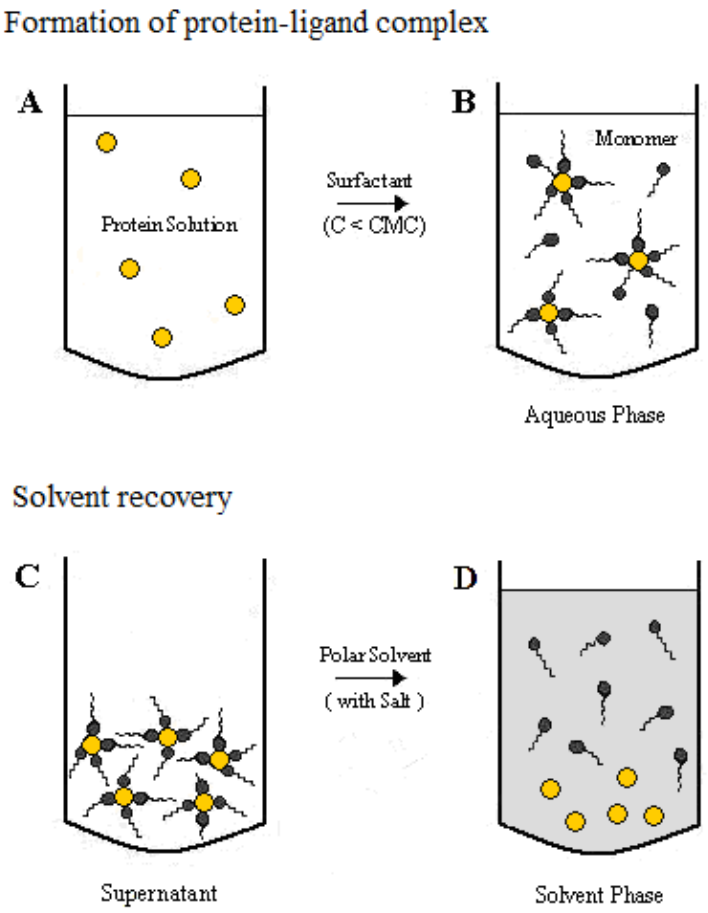

Figure 2.10 **Schematic of surfactant precipitation: (A) Initial solution containing the proteins to be extracted; (B) Addition of surfactant below the CMC where proteins bind to the surfactant monomers to form a protein-ligand complex; (C) Separation of protein-ligand complex by centrifugation; (D) Surfactant-free proteins precipitated out from the solvent phase after polar solvent and salt addition. Solid protein is recovered into a fresh aqueous phase.**

In surfactant precipitation no other extractant is required, only an initial selection of surfactant that exhibits protein precipitation behaviour, and manipulation of conditions to achieve precipitation. A typical experimental procedure for all other surfactant-mediated

techniques discussed previously is that the amount of surfactant added must be such that the final surfactant concentration in solution exceeds the CMC value to ensure the formation of micelles or reverse micelles. Surfactant precipitation is different from other existing surfactant-mediated techniques in the sense that the initial concentration of surfactant needed in the protein solution is considerably below the critical micelle concentration (CMC), and the surfactant functions as a precipitating ligand to form an insoluble protein-surfactant complex. The surfactant being used precipitated proteins at concentrations of at least 1 order of magnitude smaller than those required to partition proteins into reverse micelle phases (Shin et al., 2003c; Shin et al., 2004b).

The sequence of extraction in surfactant precipitation was believed to maintain the protein's biological activity and stability. Surfactant when used directly to precipitate lysozyme from an aqueous solution did not cause denaturation (Shin, 2002; Shin *et al.*, 2003c). Few studies were performed but each result demonstrated protein being recovered from aqueous solutions without loss of enzyme activity (Shin et al., 2003d; Shin et al., 2004a; Shin et al., 2004c). Surfactant precipitation was studied with four different anionic surfactants, but AOT showed much better removal of protein compared to sodium di-(n-octyl) phosphinate, sodium di-(n-dodecyl) phosphinate, and dioctyldimethyl ammonium chloride surfactant (Shin *et al.*, 2004c). Recovery was carried out with acetone (Shin *et al.*, 2004c).

2.7.1 SYSTEM PARAMETERS IN SURFACTANT PRECIPITATION

The role of system parameters in the aqueous phase is important for the selective precipitation of the target protein. The conditions in the initial aqueous phase affect the physiochemical state of the protein and its interaction with the surfactant head groups in the formation of a protein–ligand complex. The recovery of protein in a solvent phase can be manipulated by adjusting the salt concentration which affects the interaction of the complex with the solvent.

2.7.1.1 Effect of Molar Ratio of Surfactant to Protein

In the experiment performed by Shin, an absolute (100%) precipitation or removal of lysozyme was obtained at a molar ratio between the surfactant and protein, R, of about 10 when calculated using the mass of initial lysozyme and the mass of lysozyme remaining in

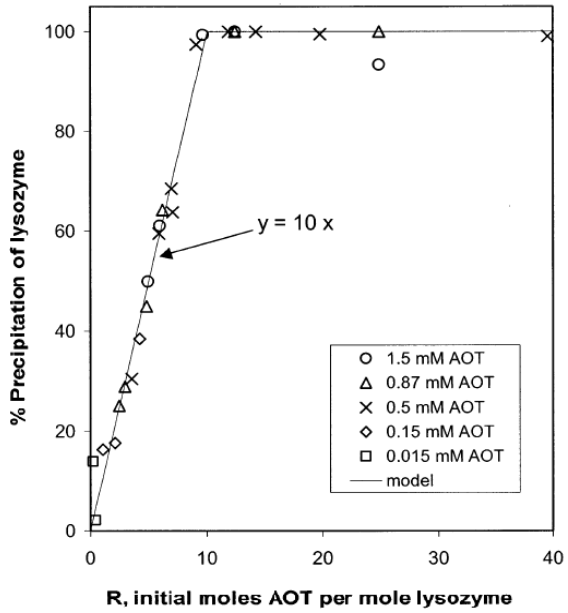

Figure 2.11 **Percent mass of lysozyme removed with AOT. From slope of line y=10x, 1mole of lysozyme was complexed with 10 mole of AOT (Shin *et al.*, 2003b).**

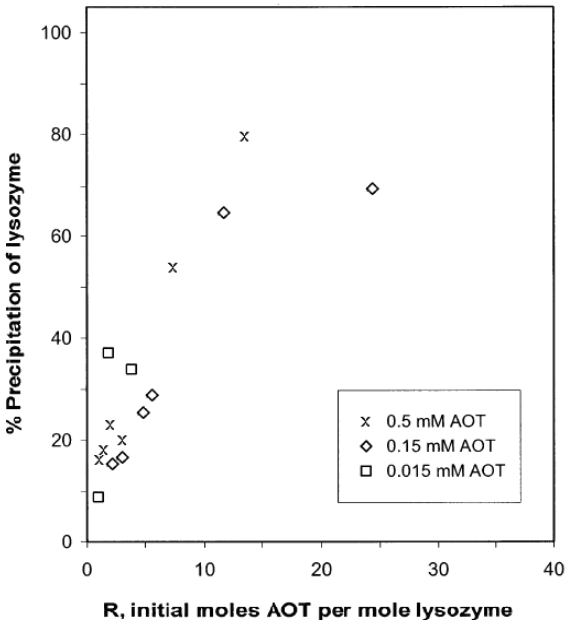

Figure 2.12 **Percent mass of lysozyme recovered from lysozyme-AOT complex using acetone. Mass of initial lysozyme is the basis of the calculation (Shin, 2002).**

the initial aqueous phase after formation of an insoluble complex (Shin, 2002). Figure 2.11 shows an increase in the percentage removal of lysozyme in accordance with the increase in AOT in the aqueous phase until complete removal was achieved. The trend was the same for all AOT concentrations tested, and therefore surfactant concentration had no significant effect on this method of extraction. At molar ratios greater than that required for protein removal, excess ligand was detected in the aqueous phase (Shin, 2002).

The precipitation efficiency when calculated using the mass of initial lysozyme and the lysozyme recovered in the final aqueous phase instead, gave an overall percent mass recovery of lysozyme of approximately 80% with the increase of R (Figure 2.12). The difference between the values of percent mass was expected to occur from the loss of lysozyme during washing (Shin, 2002). Despite protein loss, final products recovered at every molar ratio, R, retained their original biological activity (Shin, 2002).

2.7.1.2 Effect of Salt

The studies on salt addition to protein-surfactant solutions were performed at two different stages in surfactant precipitation. The effect of salt on the formation of protein-surfactant complexes was studied using sodium chloride (NaCl) and the initial protein solution. In the first stage, the addition of NaCl to the initial protein aqueous phase resulted in a decrease in the amount of protein complexed with surfactant at higher salt concentrations; highest precipitation was obtained when no salt was added (Figure 2.13). The same results were obtained with α-chymotrypsin and ribonuclease A (Shin *et al.*, 2004b).

The second stage involved salt addition to the recovery solvent. The effect of salt on protein activity recovery from an acetone phase was analysed. The salt being added was intended to neutralize the charges and dissociate protein from the surfactant (Shin *et al.*, 2003c). The most common salt used in acetone was NaCl. Sodium acetate was also evaluated (Shin *et al.*, 2004c); no activity was measured in the solvent or in the final aqueous phase when sodium acetate was not added. Higher concentrations of salt (acetate), however, resulted in more activity in the solvent being retained but reduced the recovery (Shin *et al.*, 2004c). Optimum amounts of salt act as a precipitant buffer to initiate the formation of a surfactant-free protein in acetone.

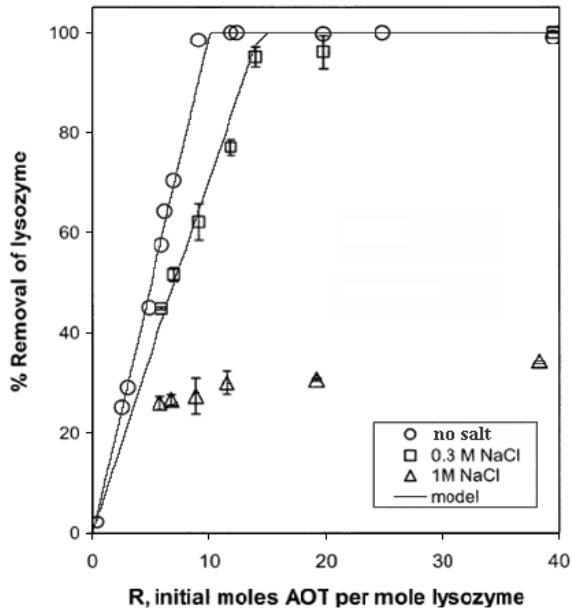

Figure 2.13 **Effect of salt on the percent removal of lysozyme precipitated with AOT (Shin, 2002).**

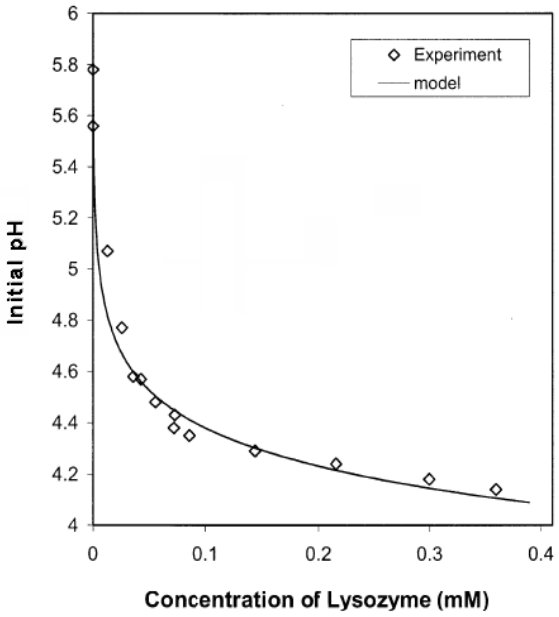

Figure 2.14 **Initial pH of the protein solution as a function of lysozyme concentration with no pH adjustment and no salt addition (Shin *et al.*, 2003b).**

2.7.1.3 Effect of pH

In Shin's work there was a variation of the initial pH in the non pH and ionic strength adjusted solutions when different amounts of protein were dissolved (Figure 2.14). After protein-ligand formation, there was a pronounced shift in the equilibrium pH (Figure 2.15) as more lysozyme was precipitated with a rise in R (Shin, 2002). To study the effect of pH on percent removal, the pH of the initial lysozyme solution was adjusted using HCl and NaOH before extraction. For an adjusted pH range of 4 to 11, the percent removal after the formation of a protein-ligand complex was maximized, as shown in Figure 2.16; below and above this pH range had a negative effect on the formation of insoluble complex, (Shin, 2002). Results show a dependence on the pH of the initial protein solution in surfactant precipitation of a non-salt buffered system.

2.7.1.4 Effect of Polar Solvent Recovery

The percent recovery of protein with solvent was believed to be a strong function of the processing time used in the recovery process (Shin *et al.*, 2004a) because the recovery time when a protein-surfactant precipitate was dissolved in the solvent to produce solid protein may vary. The conformational stability of cytochrome c with respect to the time in the acetone phase was monitored with HPLC (Shin *et al.*, 2004a). Protein recovered within 10 minutes showed an original peak shape and a relatively good recovery, but when left in the solvent for more than 30 minutes, a severely distorted peak shape and zero recovery was obtained. The different conformational stability achieved was the effect of acetone, not surfactant because the AOT concentration in the final aqueous solution of the recovered cytochrome c was below the detection limit of the HPLC.

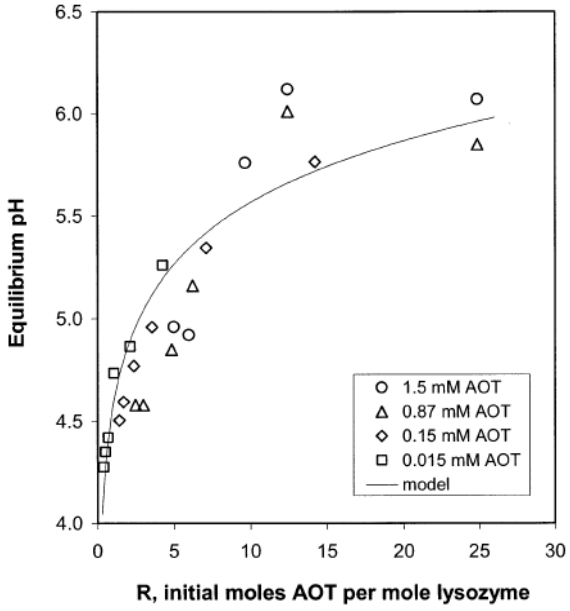

Figure 2.15 **Change in the equilibrium pH of the aqueous phase upon removal of lysozyme as an insoluble lysozyme-AOT complex: no pH adjustment and salt addition (Shin *et al.*, 2003b).**

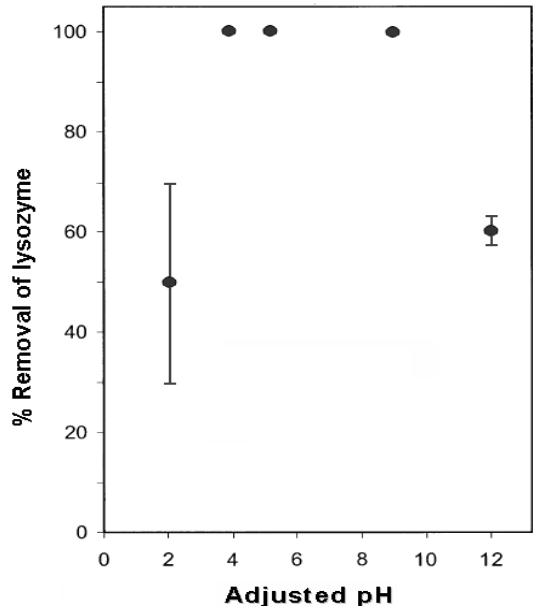

Figure 2.16 **Effect of pH on the percent lysozyme precipitated with AOT through formation of an insoluble lysozyme-AOT complex (●) at R=10 (Shin, 2002).**

2.7.1.5 Effect of Protein Characteristics

Protein characteristics have a considerable effect on the interactions with the surfactant headgroups, the hydrocarbon solvent and/or the lipophilic part of the surfactant (Jarudilokkul, 2000); these are critical in protein extractions. Among the protein characteristics related to electrostatic and hydrophobic forces are the size of the protein (MW), isoelectric point (pI) and the variation of hydrophobicity of the extracted proteins (Jarudilokkul, 2000).

For example, proteins with different pIs were purified by controlling the pH of the initial protein solution. Lysozyme (pI=11) was selectively precipitated with AOT from a mixture of proteins such as albumin (pI=5) and ovotransferin (pI=6), which are present in hen egg white (Shin *et al.*, 2003c). This enables proteins of interest to be recovered free of contaminant proteins provided their pI's are different. In separate work, surfactant precipitation managed to recover α-chymotrypsin (pI=7.8) and ribonuclease-A (pI=8.5), from a mixture of the two proteins, but not selectively separate them because of the proximity of the enzymes' pI values (Shin *et al.*, 2004b). No work was found in surfactant precipitation that has selectively separated protein with similar pI's. Literature was found describing reverse micelles using cationic surfactant CTAB to separate a set of proteins with the same pI but different MWs (BSA, α-amylase and trypsin inhibitor) by manipulation of surfactant concentration and pH (Jarudilokkul, 2000). Hence, knowledge of the influence of protein characteristics is necessary to enhance selectivity.

2.7.2 MODELLING OF SURFACTANT PRECIPITATION COMPLEX FORMATION

Shin (2002) proposed a model to explain the formation of a protein-ligand complex in surfactant precipitation. It is based on the research of lysozyme precipitated with AOT and recovered with acetone. The basis of the complex formation is that charged lysozyme reacts with the oppositely charged headgroup of the surfactant. The model was built on the following assumptions (Shin, 2002):

i. Commercially available lysozyme contains hydrochloric acid. This is supported by the pH measurement of initial lysozyme given in Figure 2.14.

ii. The positively charged lysozyme has chloride ions as counterions in the aqueous phase. According to the product details, the crystallized lysozyme used contains 5 wt % of sodium chloride (NaCl) and sodium acetate ($NaC_2H_3O_2$) besides hydrochloride acid, all which are fully ionized in lysozyme solution.

iii. The lysozyme-ligand complex precipitates with a fraction of hydrochloride from the acid in the crystallized protein, therefore explaining the increasing equilibrium pH upon addition of AOT solution in Figure 2.15.

iv. The lysozyme-ligand complex does not remove other inorganic salts from the aqueous phase according to the results of the sodium and chloride ion balance before and after the insoluble complex formation. The change in the ion concentrations was within experimental error.

v. The lysozyme-ligand complex is formed by a well-defined number of anionic ligand residues, *Z*, based on Figure 2.11. The intermediate stage where lysozyme contains less than *Z* ligands and is freely dissociated from the ligand in the aqueous phase does not affect the model.

The study incorporated net surface charge at a given pH, electrostatic interactions, solubility, charge balance and mole balance of proteins and ligands to analyse surfactant induced protein precipitation. Lysozyme-AOT complex formation was described as a function of equilibrium pH, molar ratio of AOT to lysozyme, and salt concentration. The model unravelled the extraction mechanism by detailing the interactions at every stage. It looked into the surface charge of the initial lysozyme solution, addition of ligand to the aqueous phase interaction and lysozyme-ligand precipitation, which can be represented by the overall mole balance of the process in Figure 2.17. Shin successfully determined the parameters and developed a model that was in agreement with the experimental results.

2.7.2.1 Surface Charge of Lysozyme in Aqueous Solution

The model proposed first investigated the protein's net surface charge, ***Z***, at a certain pH. Crystallized lysozyme dissolving in water will start off with the ionization of the carboxylic and amino groups, and the process is dependant on the pH of the solution. Using the net surface charge equation (2.1) combined with the charge balance equation (2.2), the pH of the initial protein solution with a known concentration of lysozyme can be calculated.

$$Z = 19 - \sum_{i=1}^{32} \frac{K_i \alpha}{H^+ + K_i \alpha} \qquad (2.1)$$

This equation was developed for lysozyme from the Linderstrøm-Lang equation (Kuramitsu and Hamaguchi, 1980). The model considers 32 ionic groups, ***n***, from lysozyme which contain a total of 33 amino acid residues; 14 acidic groups (Aspartic, Tyrosine and Glutamic acid) and 19 basic groups (Arginine, Histidine and Lysine) (Sakakibara and Hamaguchi, 1968), excluding one interior carboxylic group buried in the protein molecule which is not ionized (Kuramitsu and Hamaguchi, 1980). K_i indicates the dissociation constant of hydrogen from a given ionisable group ***i***. α is a parameter for the electrostatic interactions between a protein molecule with ***Z*** net surface charge and a proton. ***Z*** is an average of a large number of individual ionized molecules constantly giving and taking protons, therefore actual net charge can be larger or less than the average value ***Z*** (Shin, 2002). The calculation of protein charge versus the pH of the solution is shown in Figure 2.18.

$$C^0_{Lys} Z + C_{H^+} + C_{Na^+} = C_{OH^-} + C_{Cl^-} + C_{C_2H_3O_2^-} \qquad (2.2)$$

where C°_{Lys} is the initial concentration of lysozyme in an aqueous phase without ligand, and $C_{C_2H_3O_2^-}$ is the acetate ion concentration. The value of C_{OH^-} is produced using the ion product for water, K_w and the dissociation constant of acetic acid, K_a.

$$K_w = C_{H^+} C_{OH^-} \qquad (2.3)$$

K_w is taken as 10^{-14} (Smith and Martell, 1976).

$$K_a = \frac{C_{H^+} C_{C_2H_3O_2^-}}{C_{HC_2H_3O_2}} \qquad (2.4)$$

K_a is taken as 1.8×10^{-5} (Noggle, 1996).

The C_{H^+} obtained for every lysozyme concentration by solving equation 2.2 is used to get the pH values. Results of the calculation reproduced the variation of the pH plotted in Figure 2.14. It confirmed that the pH of the initial aqueous lysozyme solution without any pH adjustment obtained from the model is close to the experimental values.

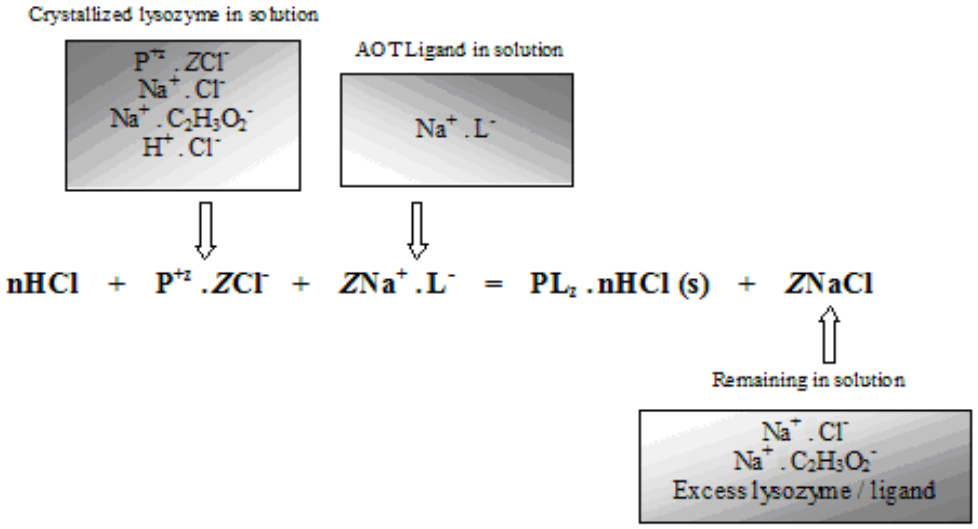

Figure 2.17 **Schematic representation of positively charged lysozyme forming an insoluble complex with AOT ligand; P^{+Z} denotes the positively charged lysozyme protein, L^- the negatively charged headgroup of the surfactant ligand, and *Z* the net surface charge at a given pH (Shin, 2002).**

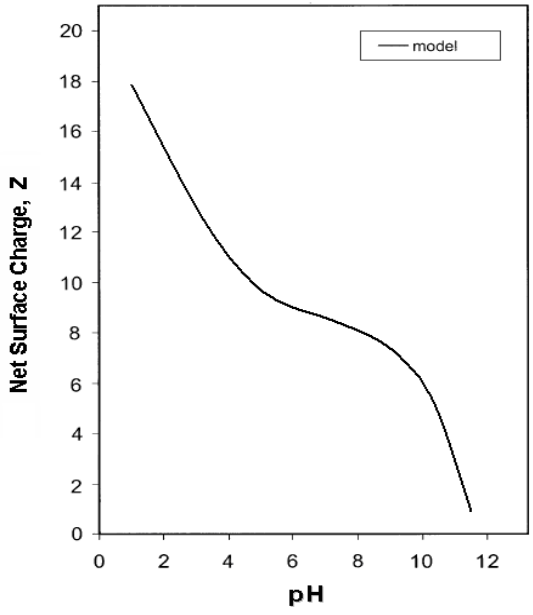

Figure 2.18 **Net surface charge of lysozyme as a function of pH using equation 2.1; α set to unity reproduced the experimental results (Kuramitsu and Hamaguchi, 1980).**

2.7.2.2 AOT Ligand in Water

The anionic surfactant, di-(2-ethylhexyl) sodium sulfosuccinate (AOT), when solubilised in water releases its negatively charged ligand, L^-. The solubility of AOT is limited in a salt solution (equation 2.5) and in an acidic solution (equation 2.6) by the common ion effect.

$$K_{LNa} = C_{Na^+} C_{L^-} \tag{2.5}$$

K_{LNa} describes the solubility product of AOT and is reported to be 1.1 x 10^{-3} (M^2) (Caryl, 1941). C_{L^-} is the concentration of sulfosuccinate group from the AOT. C_{Na+} is the concentration of sodium ion as the counterion of the ligand.

$$K_{LH} = C_{H^+} C_{L^-} \tag{2.6}$$

The acidic form of the ligand has hydrogen ions, H^+, as counterions in the presence of acid. Shin adjusted the value of K_{LH} to 9.2 x 10^{-9} (M^2) to fit the data in Figure 2.15 to model the results of the equilibrium pH in the aqueous phase after the precipitation of the lysozyme-ligand complex.

2.7.2.3 Lysozyme-Ligand Complex Formation

The dissociation constant of lysozyme, $K_{LysCl_{10}}$, by assuming Cl^- as the counterion is written in equation 2.7. When the pH is adjusted to lower than lysozyme's pI, the protein has an overall positive charge ($Z > 0$). The pH range between 4.2 and 5.8 from Figure 2.14 was inserted into equation 2.1 and calculation yields a net surface charge between +11 and +9. For simplicity of the modelling, Z was taken as an average constant +10 in the equations.

$$K_{LysCl_{10}} = \frac{C_{Lys^{+10}} (C_{Cl^-})^{10}}{C_{LysCl_{10}}} \tag{2.7}$$

$K_{LysCl_{10}}$, a model adjusted parameter of 2.5 x 10^{-23} (M^{10}), is a good fit to match the experimental data plotted in the Figure 2.13 (Shin, 2002). For the effect of salt concentration on lysozyme-ligand formation, the model was consistent and valid with the experimental

results up to a concentration of 0.3M. The model predicted zero percent removal at 1M NaCl as large amounts of lysozyme and ligand will remain associated with chloride ions at the high concentration of counterions, but experiments resulted in a 35% recovery, which seems independent of R, and this is believed to be induced by salt precipitation of lysozyme (Curtis *et al.*, 1998).

The dissociation constant is very small indicating that lysozyme is highly associated with chloride unless the AOT ligand is present in solution; the dissociated lysozyme will react with negatively charged ligands to form a lysozyme-ligand complex. The solubility product of the insoluble compound, $K_{LysL_{10}}$, is given in equation 2.8.

$$K_{\mathrm{LysL_{10}}} = (C_{\mathrm{Lys^{+10}}})(C_{\mathrm{L^-}})^{10} \qquad (2.8)$$

$K_{LysL_{10}}$ was measured from the experiment to be 4 x 10^{-55} (M^{11}) (Shin, 2002). The solubility product of lysozyme-AOT is very low suggesting that the complex should be water insoluble and precipitates upon formation. The amount of the complex formed can be found from the equations of mole balances on the lysozyme (equation 2.9) and the ligand (equation 2.10).

$$C^0_{\mathrm{Lys}} = C_{\mathrm{Lys^{+10}}} + C_{\mathrm{LysCl_{10}}} + \frac{n_{\mathrm{LysL_{10}}}}{V} \qquad (2.9)$$

$n_{LysL_{10}}$ refers to the moles of the insoluble lysozyme-ligand complex in the aqueous solution, V the total volume of the mixture, and $C_{LysCl_{10}}$ the concentration of lysozyme associated with chloride ions and unavailable to bind with the ligand.

$$C^0_{\mathrm{L}} = C_{\mathrm{L^-}} + 10 \cdot \frac{n_{\mathrm{LysL_{10}}}}{V} \qquad (2.10)$$

There is no charge balance equation at this stage because the reaction between lysozyme and the ligand goes to completion. The precipitation efficiency of lysozyme is then obtained from equation 2.11.

$$\% \text{ Removal} = \frac{n_{\mathrm{LysL}_{10}}}{C^0_{\mathrm{Lys}} V} \times 100 \qquad (2.11)$$

The percent removal of lysozyme forming an insoluble complex agrees well with Figure 2.11 when the model is tested with different AOT to protein molar ratios at a fixed value of $\boldsymbol{Z}$=10 and the experimental $\boldsymbol{K_{LysL10}}$. Although the model developed is successful in replicating most of the experimental results, it is unable to predict the precipitation efficiency as a function of pH as shown in Figure 2.16. Shin discussed the possibility of improving the current model by studying the complex change of $\boldsymbol{Z}$ with pH, as well as the unknown solubility of the lysozyme-ligand complex with a different number of ligands, suggested from $\boldsymbol{K_{LysL1}}$ to $\boldsymbol{K_{LysL18}}$.

2.8 SURFACTANT PRECIPITATION: IDENTIFICATION OF GAPS IN KNOWLEDGE

With the advancement in biotechnology, the outlook for new separation processes has evolved. Reaction behaviour at surfactant interfaces is expected to be more representative of biological reactions than reactions studied in dilute aqueous solutions (Price *et al.*, 2003), thus surfactant enhanced separations have been targeted as a lower-cost alternative. The discovery of surfactant precipitation was driven by the need for an efficient and cheap purification technique. Surfactant precipitation shows promising characteristics in view of the industrial need for cost effective bioseparation processes. The extraction technique is compatible with the first step in many existing biomaterial purification schemes. Surfactant precipitation offers a simple and inexpensive approach, and in terms of cost and time, this separation process is appealing from an economic viewpoint.

Within the surfactant-enhanced extraction systems, surfactant precipitation can be used instead of micellar extraction to recover protein from an aqueous phase with some major advantages. The direct precipitation method has a short processing time (instant formation of protein-surfactant complex), and operates under non protein denaturing conditions (Shin *et al.*, 2003b). Despite the common belief that the water pool in a reverse micelle is essential to preserve protein stability, protein recovered from a protein-surfactant complex in the aqueous phase did not lose its original activity (Ghosh, 2005; Shin et al., 2003b). Hjelmeland and

Conn (1986) claim that the main structural feature of surfactants in determining denaturation is the micelle, rather than the structure of the surfactant monomer which interacts with proteins in a fundamentally different way. Examining protein stability with regards to the interactions involved with surfactant monomers is a definite way to be certain of the effect of surfactants on the structure of proteins.

Surfactant precipitation can overcome the problems of various extraction systems. It is a convenient alternative to conventional liquid-liquid extraction that uses organic solvents because enzymes often show a low solubility, or lose their activity in organic media (Krei and Hustedt, 1992). Surfactant precipitation represents a viable approach in comparison with affinity thermoprecipitation, which has its precipitation efficiency limited by the solubility of the polymer in the aqueous phase (Vaidya *et al.*, 2001). Surfactant precipitation does not require membrane separation to recover protein in the end process, and is well suited for the majority of thermolabile proteins. Nevertheless, considerable opportunity for improvement is seen in the techniques employed for the recovery of protein from the protein-ligand complex. Existing acetone recovery techniques only allowed for a short processing time before zero recovery of protein was obtained.

This selective precipitation technique provides an opportunity to expand the use of anionic surfactants in extraction processes; as anionic surfactants are usually linked to protein denaturation (Marcozzi *et al.*, 1998), their use in protein extraction is limited to a few techniques, e.g. solvent extraction (Kamei *et al.*, 2002), reverse micellar extraction (Jarudilokkul et al., 2000a; Juang and Mathew, 2005) and cloud-point extraction (Sicilia *et al.*, 1999). The use of other surfactants in this type of application in addition to the anionics employed to date should be examined, for example, an uncharged surfactant that is mild and normally does not alter the bioactivity of the extracted materials (Hinze and Pramauro, 1993), a cationic surfactant that has no strong affinity which contributes to unfolding in protein (Blinkhor and Jones, 1973), or perhaps a mixture of these surfactants might provide possible advantages with sufficient study. Besides lacking in the type of surfactants employed, one such experiment reported that the effect of buffer salt on protein activity recovery is fairly well understood (Shin *et al.*, 2004c). Shin recommended comparative studies using other types of salts in surfactant precipitation as one way to minimize the activity lost in the solvent phase.

In biological reactions, proteins in the form of enzymes can be regarded as highly selective catalysts and they are very useful in organic synthesis, especially the synthesis of chiral compounds (Spreti *et al.*, 1995). Most of the time the use of enzymes is restricted because they lose their activity in a short time (Jones, 1992). Surfactant precipitation has proved a promising method in hindering denaturation, while at the same time retaining protein activity. However, non-micellar surfactant-mediated separation is new and not fully understood; the mechanisms of surfactant precipitation need to be thoroughly defined, and the practical uses of this system in bioprocessing must be examined. In view of its considerable potential, there is a definite need for more studies that would lead to a better definition of its strengths and drawbacks, and in time lead to an improved design of such a separation system.

2.9 OBJECTIVES

- **Protein Stability**

  - To examine the unfolding behaviour and secondary structure of native lysozyme in a solution of AOT monomers.

  - To understand the interactions involved under various protein-surfactant complex formation conditions (phosphate salt, molar ratio of surfactant to protein, and pH).

- **Protein Recovery**

  - To optimise the solvent recovery method by examining commercially viable solvents (ethanol, methanol, ethanol/acetone and ethanol/water) besides pure acetone.

  - To evaluate the use of different cationic surfactants (TOMAC, DTAB and DODMAC) to develop a new and improved method of counterionic surfactant recovery.

  - To compare the efficiency between solvent and counterionic recovery based on the effect of phosphate buffer conditions (ionic strength and pH) and activity recovery.

- **Mechanism**

  - To examine the influences surface charge and hydrophobicity have on extraction of a single protein (lysozyme, cytochrome c, ribonuclease A, trypsin and α-chymotrypsin) in solution.

  - To investigate the selective separation of sets of protein mixtures with the same pI and range of molecular weights as a function of surface properties.

- **Application**

  - To examine the selectivity of extraction and protein folding from a fermentation broth.

  - To develop a surfactant precipitation technique with TOMAC for trypsin inhibitor and lipase (low pI) inappropriate for precipitation with AOT.

  - To evaluate alternative non-ionic surfactants (Triton X-100, Tween 85, Brij 30, AOT/Triton X-100 and DTAB/Triton X-100) for precipitation and recovery.

CHAPTER 3 PROTEIN PRECIPITATION USING AN ANIONIC SURFACTANT*

3.1 INTRODUCTION

Little published research has examined the structure of native proteins in a solution of surfactant monomers; most research has focussed on native protein structure in a micellar system. Earlier work in the non micellar system was done by researchers with lysozyme and other proteins to give an overview of the method of precipitation with AOT and recovery with acetone (Shin et al., 2003b; Shin et al., 2004a; Shin et al., 2004b). These workers mainly focussed on concentration and activity recovered with regards to parameters such as the molar ratio of surfactant to protein, and pH. In this work these parameters will be studied again in more depth by investigating their effect on protein structure and on surfactant binding behaviour in the surfactant precipitation procedure. Emphasis was placed on understanding the non micellar interactions involved under various precipitation conditions, with a focus on identifying individual protein-surfactant interactions, and evaluating interactions that occur simultaneously. Up until now it has only been reported that electrostatic interactions drive the formation of the protein-surfactant complex.

Maintaining the structure and function of a protein is an absolute prerequisite for purifying proteins using a surfactant. It is important to promote surfactant-averaged protein interactions in the precipitation where the surfactant system operates at an averaged condition targeted to achieve the most desirable protein interactions so that the surfactant precipitate can be separated efficiently. Lysozyme was chosen to study this extractive system because it is an inexpensive and well-characterized protein, and can be compared with past data. Lysozyme was precipitated from an aqueous solution by the direct addition of the anionic surfactant sodium bis-(2-ethylhexyl) sulfosuccinate (AOT), and the change of lysozyme structure under different binding conditions was observed from its circular dichroism (CD) spectra and high-performance liquid chromatography (HPLC) chromatogram. This work aimed to understand the protein secondary structure framework, which is useful for developing effective

* This work presented in this chapter has been submitted for publication;
Cheng, S.I. and Stuckey, D.C. 2011. Protein precipitation using an anionic surfactant. Process Biochemistry.

surfactant-averaged protein interactions that can predict surfactant precipitation in a complex aqueous mixture containing surfactant and many other biomolecules.

3.2 MATERIALS AND METHODS

Materials: The experiments were performed using a crystallized and lyophilized lysozyme powder from chicken egg white (EC 3.2.1.17, Mucopeptide N-acetylmuramylhydrolase, pI=11.0, molecular weight of 14.3 kDa) purchased from Sigma (Missouri, USA), which contained 5-10% buffer: sodium acetate/sodium chloride/hydrochloride acid. A commercial anionic surfactant, sodium bis-(2-ethylhexyl) sulfosuccinate (AOT- 99% purity) was obtained from Fluka (Switzerland). For enzymatic and AOT measurements, *Micrococcus lysodeikticus* and methylene blue were purchased from Sigma. Potassium phosphate monobasic (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4) and sodium sulphate (Na_2SO_4), all with minimum 99% purity from Sigma, USA, were used to make a buffer when preparing protein and assay solutions. Concentrated hydrochloric acid (HCL) (37%) and sodium hydroxide (NaOH) pellets were obtained from AnalaR (VWR Ltd, UK). The high-performance liquid chromatography (HPLC) grade organic solvent, acetonitrile (ACN), chloroform, and reagent grade trifluoroacetic acid (TFA), 99.5%, were purchased from Sigma, USA. The water used throughout the experiment was distilled and deionised.

Instruments: Chemicals were weighed using an analytical balance (AL204 by Mettler-Toledo AG, Greifensee, Switzerland). A Hanna Instruments benchtop microprocessor pH/mV/ºC meter model pH 213 was used to monitor the pH (standard deviation = ±0.02). A UV-VIS scanning spectrophotometer (UV-2101PC, Shimadzu, Japan) and 1-cm quartz cuvettes were used to measure the absorption of lysozyme at 280nm and 450nm to determine the protein concentration and protein activity, respectively. A vortex mixer was used for mixing (VWR Ltd). A centrifuge (Biofuge Stratos, Heraeus Instruments) was used for solid-liquid separation. Disposable syringes (B.Braun Melsungen AG, Germany) and 0.2µm Minisart syringe driven filter units (Sartorius Stedim Biotech, Germany) were used for removal of the particulates before analyses. Circular dichroism spectral measurements were performed on a Chirascan CD spectroscopy (Applied Photophysics Ltd, Leatherhead, UK) in a 1-mm quartz cell. Chromatographic measurements were carried out with an HPLC system consisting of a system controller (LC-20AB Prominence), an autosampler unit (SIL-20A), a degasser unit (DGU-$20A_3$), and a diode array detector (SPD-M20A) (Shimadzu, USA). A

silica-based C18 HPLC column (J'Sphere ODS-M80 by YMC, Europe) of 150 x 4.6 mm, with particle diameter 4 μm and pore size 8nm was used to analyse for the proteins.

3.2.1 PREPARATION OF LYSOZYME SOLUTION

An initial solution containing 1.0 g/L lysozyme was prepared in a potassium phosphate monobasic or sodium phosphate dibasic buffer solution. The phosphate buffer was chosen because its pK_a value is a relatively weak function of temperature (Stoner *et al.*, 2006). The desired pH of the protein solution was adjusted accordingly by using HCl or NaOH. As a control solution, lysozyme was prepared in 20 mM potassium phosphate buffer and pH adjusted to 6.2.

3.2.2 PREPARATION OF AOT PHASE

The AOT solution used contained 1.5 g/L (3.4 mM) to 11 g/L (24.7 mM) of AOT in distilled water. The solubility of AOT in water was reported to be 33.8 mM at a temperature ranging from 20 to 70ºC (Caryl, 1941). Conductivity measurements were used to determine the critical micelle concentration (CMC) by a continuous dilution of a concentrated aqueous solution of surfactant, and surfactant plus protein (Ruso *et al.*, 2003). The CMC of AOT in 20 mM phosphate buffer and water is 2.5 and 4.1 mM (Linfield, 1976), respectively, at 25ºC, and with lysozyme prepared in buffer (between 0.03 to 0.2 mol%) increases to 6.7 mM.

3.3 PRECIPITATION PROCEDURES

All experiments were conducted at room temperature using 6 to 8 replicates, and average data and a statistical analysis of the experiments is reported.

3.3.1 PRECIPITATION OF LYSOZYME WITH AOT

In the surfactant precipitation experiment, aqueous protein solution was contacted with AOT directly. A volume of 1 mL AOT solution in the concentration prepared was added to 10 mL of the initial lysozyme-containing aqueous solution. At the moment of addition, the AOT concentration in the total aqueous mixture varied between 0.31 and 2.25 mM, which is below

the CMC of AOT in water, to avoid the formation of micelles in the protein solution. Upon addition of the AOT solution, an instant formation of an insoluble complex was observed. The mixture was subjected to 5 sec of vortex mixing to allow for interactions between the surfactant and protein (this mixing time was found to be sufficient for good removal efficiencies with surfactant precipitation), and the lysozyme-AOT complex precipitated out of solution. The samples were then centrifuged at 5000 rpm for 1 min to separate the precipitated lysozyme-AOT from solution, and the supernatant analysed for lysozyme. It was also found that filtration of the supernatant using 0.2 μm disposable syringe filters gave the same efficiency in removing particles from the liquid phase as centrifugation, and results of the two separation methods were statistically the same.

The soluble lysozyme samples were studied because any changes in the protein structure after the addition of AOT will be noticed here first. Incomplete protein-surfactant binding and the extent of intermolecular interactions (electrostatic and non ionic forces) between surfactant monomers and a lysozyme molecule resulting from the lysozyme-AOT precipitation process can be identified by analysing the soluble lysozyme samples from the aqueous phase. Lysozyme remaining in the aqueous phase, which was independent of the protein recovery procedure, was taken to represent the efficiency of the precipitation in this work. The recovery of protein from the precipitated complex will be discussed in more detail in the next chapter.

3.4 ANALYTICAL TECHNIQUES

Quantitative and qualitative analyses were carried out on the filtrate after the protein precipitation procedure, and are discussed below. The coefficient of variation (CV) for the precipitation process was within ±5% for lysozyme concentration and activity measurements (the error bars indicating the standard deviation of the measurements are too small to be seen in the figures plotted).

3.4.1 PROTEIN ASSAY

The protein content in the initial lysozyme solution and the aqueous phase after the formation of lysozyme-AOT complex was measured in a UV spectrophotometer using absorbance at 280 nm (A_{280nm}) (Hamaguchi and Kurono, 1963). Interference with the protein concentration

assay at A_{280nm} due to the presence of AOT in the solution was negligible (Shin *et al.*, 2004c), and blanks consisted of phosphate buffer solution used to prepare the lysozyme samples; the spectrophotometer was zeroed against the buffer blank. Samples with an absorbance above 1.5 were diluted. A standard calibration curve plotted from measuring the absorbance of properly diluted lysozyme standards containing from 0 to 1.0 g/L actual lysozyme concentrations was used to determine the protein concentration (g/L) in all the lysozyme samples (R^2 of the calibration line ~1.0).

3.4.2 LYSOZYME ACTIVITY ASSAY

The measurement of lysozyme activity was carried out using an absorbance assay with *Micrococcus lysodeikticus* cells. The enzymatic activity of lysozyme in aqueous solution was determined at 25˚C according to the method described by Davies *et al.* (1969). A substrate solution of 0.3 g/L *Micrococcus lysodeikticus* was prepared in 50 mM phosphate buffer solution at pH 6.2. The suspension of intact bacteria is cloudy and its optical density was read at 450 nm (A_{450nm}). The enzyme activity assay was performed with a UV spectrophotometer in which the change in *Micrococcus lysodeikticus* concentration was measured over time. A cell suspension volume of 3.0 mL was pipetted into the reference and sample cuvettes. Immediately after adding 100 µl of filtered lysozyme sample solution into the sample cuvette, both the cuvettes were placed into the UV cell holder and the timer started. The decrease in the turbidity of the cell suspension was monitored at 15-second intervals over a period of 3 minutes. A graph of absorbance (A_{450nm}) as a function of time was plotted, and the rate of enzyme action ($\Delta A_{450nm/min}$) in the substrate reaction mixture was measured from the linear portion of the curve (R^2 of all the linear portions were ≥ 0.99). Dilution of samples to give 200-400 units/mL of lysozyme was necessary for the activity assay, and corrections for dilution were made in the calculation of units of enzyme activity.

3.4.3 CIRCULAR DICHROISM MEASUREMENT

Circular dichroism (CD) spectra of native lysozyme and lysozyme in the recovered solution were recorded over a far-UV wavelength range of 200-240 nm with a scan at 20˚C in a thermostated cell holder. The path length was 1 mm, the step resolution 0.5 nm and the bandwidth 1 nm. The scan speed was 10 nm/min. The concentration of each sample was diluted to approximately 0.1 g/L with phosphate buffer (20 mM) prior to measurement.

Simultaneously, the buffer background was measured and subtracted from the original spectra to determine the lysozyme spectra, and data were presented as ellipticities (θ, millidegree). The observed ellipticities were converted into molar ellipticities [θ] based on a mean molecular mass per residue of 129 Da (2005). Average spectra of the replicate scans were analyzed using a deconvolution software (CDNN program version 2.1 (Böhm, 1997) which calculates the secondary structure of the peptide by comparison with a base set of 13 known protein structures. Spectra analysis enabled a better understanding of the effect of precipitation on protein structure. The ellipticity was reproducible within an error of ±2%, which was mainly attributable to signal noise and inaccuracy in the light path length.

3.4.4 CHROMATOGRAPHY ON REVERSED PHASE HPLC COLUMN

The protein sample (15μL) was injected into a YMC J'Sphere ODS-M80 HPLC C18 column equilibrated with 0.1% v/v TFA in water (mobile phase A). The column was eluted using 0.1% v/v TFA in acetonitrile (mobile phase B); retention and recovery were measured for proteins at 25ºC at pre-determined HPLC conditions. Gradients were run from 0-90% B in 15 min at a flowrate of 1 mL/min for lysozyme. Retention time (t_R) for a lysozyme sample is given in the Figure 3.5 caption. A diode array detector with variable UV wavelengths was operated at 280nm to detect the absorbance (mAU) of the protein sample, and protein recovery was estimated from the areas of the eluted peaks. A standard calibration curve plotted from measuring the areas of eluted peaks of properly diluted protein standards containing from 0 to 1.0 g/L actual lysozyme concentrations was used to determine the protein concentration (g/L) in the samples. The CV of the measurements was within ±3%. LCsolution chromatography software was used to process and store the data.

3.4.5 DETERMINATION OF AOT CONCENTRATION

The concentration of anionic surfactant in solution after precipitation was quantified using the methylene blue assay (Fuda *et al.*, 2004; Takagi *et al.*, 1975). 0.007% w/v methylene blue was prepared in 1% w/v aqueous Na_2SO_4, and 1 mL of the methylene blue solution was mixed with 5 mL of chloroform. 0.1 mL of the sample was added to the mixture and vortexed for 20 sec. Methylene blue forms a salt with AOT and dissolves in chloroform to give a coloured layer. The upper aqueous phase containing excess dye was removed, while the

chloroform phase was pipetted into a cuvette and its absorbance measured in a UV spectrophotometer at 650 nm. The colour intensity was linear for AOT between 0 to 1.0 mM with the assay detection limit of 0.01 mM, and the AOT concentration from the sample was read from a calibration curve.

3.5 RESULTS AND DISCUSSION

In this work, the molar ratio (R) between AOT and lysozyme was first tested to determine its effect on the precipitation of lysozyme from a pure lysozyme solution at 20 mM, pH 6.2. R indicates the moles of surfactant added to the initial aqueous solution per mole of protein. This optimum R was then fixed and used to evaluate the effect of pH on the precipitation efficiency. The effect of R and pH on lysozyme precipitated with AOT was quantified in terms of the concentration and activity of lysozyme recovered from the aqueous phase.
The percent precipitation of lysozyme was calculated as:

$$\text{Fraction of precipitated lysozyme}(\%) = \left(1 - \frac{C_{P^e} \cdot V_e}{C_{P^o} \cdot V_o}\right) \times 100 \qquad (3.1)$$

where Cp^o refers to the lysozyme concentration in the initial aqueous solution before the addition of AOT, and Cp^e refers to the equilibrium lysozyme concentration remaining after the formation of an insoluble lysozyme-AOT complex. V_o and V_e indicate the volumes of the aqueous phase, initially and after the addition of AOT; $V_e = V_o + V_s$, where surfactant volume is denoted by V_s.

The activity of the lysozyme remaining in solution was another important parameter studied. According to the supplier of lysozyme used in this work (Sigma), one activity unit of lysozyme is defined as the amount of lysozyme that produces an initial linear decrease in absorbance of 0.001 per minute ($\Delta A_{450nm/min}$) in a 3.1 mL reaction mixture under the assay conditions described in the experimental procedure. This gives a reproducible relative measure of specific activity (units/mg) of lysozyme in the sample which can be calculated as:

$$\text{Specific activity} = \frac{\Delta A_{450nm/min}}{\text{mg lysozyme in the reaction mixture}} \left(\frac{1U}{0.001}\right) \qquad (3.2)$$

From the plot of absorbance versus time, it was observed that the initial velocity of the reaction was constant up to about 75 seconds from the start; after this the reaction began to slow down due to the substrate running out.

The percent activity lost as non-precipitated lysozyme was determined from the total activity of protein left in the reaction mixture.

$$\text{Total Activity}\,(\%) = \left(\frac{U_e / \text{mg} \times \text{Total protein in the reaction mixture}}{U_o / \text{mg} \times \text{Total protein in the initial aqueous solution}} \right) \times 100 \qquad (3.3)$$

where U_o and U_e are the protein activities before and after the precipitation step.

3.5.1 SURFACTANT PRECIPITATION OF LYSOZYME AS A FUNCTION OF R

The precipitation of lysozyme was performed using an initial aqueous solution of lysozyme at pH 6.2. The solution was adjusted to this pH because at pH 6.2 lysozyme produces a maximal activity for a wide range of ionic strengths (20mM to 100mM) (Davies *et al.*, 1969). The choice of surfactant for the experiment depends on the pI of the protein. For lysozyme, a protein with a relatively high pI, to remain within the stability range of its aqueous phase pH, an anionic surfactant, AOT, was used. A decrease in the CMC was observed in the phosphate buffer, which was due to the reduction of electrostatic repulsion between surfactant headgroups (Okada *et al.*, 2001). The increase in the CMC of AOT with lysozyme present indicates the formation of free micelles at higher concentrations as the protein reduces the free monomeric surfactant in bulk solution (Verdes *et al.*, 2008). To examine the effect of the molar ratio between AOT and lysozyme, R, varying concentrations of AOT (1.5 g/L – 11 g/L) were added to the lysozyme solutions of fixed protein concentration (1.0 g/L). For this analysis, the surfactant volume used (1 mL) was fixed for all the surfactant concentrations so that protein absorbance in the UV spectrophotometer and protein peaks shown in the chromatogram were free from the effect of the volume change in the protein solution when the surfactant was added, in contrast to the work of Shin *et al.* (2003c) which varied the volume of the AOT (5 g/L) added to the protein solution.

An aqueous surfactant solution containing from R=5 to R=35 of AOT was pipetted into the initial lysozyme solution to form an lysozyme-AOT complex. The reaction mixture was

vortexed and filtered during the precipitation process, and the protein concentration remaining in the aqueous phase was measured using UV absorption. Figure 3.1 shows the % precipitation increased with R, and was complete at R=16, and this was maintained at R=23. However, at larger AOT concentrations (R>23), there was a decrease in the percent removal of lysozyme, and this dropped to 32% when R approached 35. Where the lysozyme recovered from the aqueous phase was not 100%, it was assumed that lysozyme was not fully precipitated. An activity assay was performed to check that the activity lost in the aqueous solution accorded with the unrecovered mass of lysozyme observed (Figure 3.2). Increasing the molar ratio decreases the activity of lysozyme retained in the aqueous solution until a molar ratio of 16, at which no lysozyme activity was lost. At an R as high as 35, only a slight activity was detected in the aqueous solutions. The precipitating behaviour of lysozyme when it complexed with AOT demonstrated a similar trend for various AOT and lysozyme concentrations which produced a similar R as produced with 3.4 to 24.7 mM AOT. This agreed with the literature (Shin *et al.*, 2003b). Equilibrium pH before and after addition of AOT for all R (5 to 35) remained constant at pH 6.2 ± 0.02. Therefore, the AOT solution containing 3.4 to 24.7 mM surfactant used for different R had no significant effect on this method of precipitation, and the results of this study were solely a function of R.

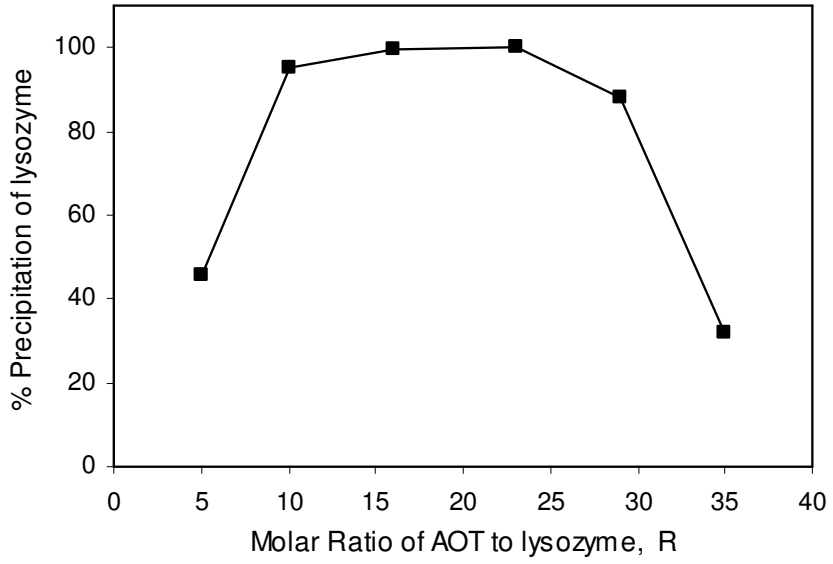

Figure 3.1 **Percent of lysozyme precipitated with AOT as a function of R: initial aqueous solution, 1.0 g/L lysozyme in phosphate buffer, pH 6.2 adjusted with NaOH.**

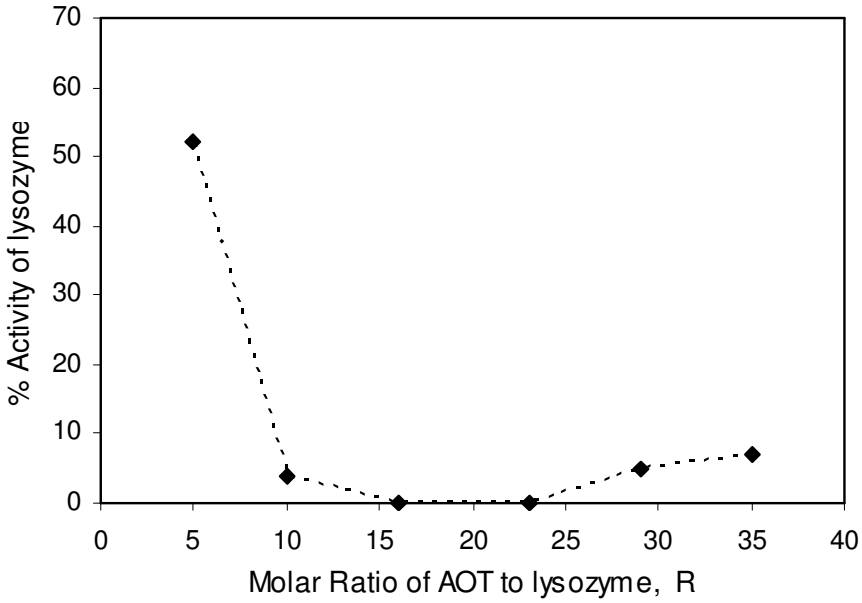

Figure 3.2 **Percent activity of lysozyme remaining in the aqueous phase after the addition of AOT: initial aqueous solution, 1.0 g/L lysozyme in phosphate buffer, pH 6.2 adjusted with NaOH.**

Overall, the results demonstrate a good correlation between mass and the activity of lysozyme in the aqueous phase at molar ratios of 5 to 23, with R=5 and 10 showing that the largest activity lost occurs from the non-precipitated lysozyme during extraction when there is insufficient AOT to form an insoluble complex with lysozyme. Measurement of the concentration and activity of lysozyme at R=16 and 23 showed that none of the original activity of the lysozyme remained in solution. This was due to 100% removal of lysozyme from the initial protein sample by precipitation with the surfactant. It appears that 1 mol of precipitated lysozyme was complexed with 16 moles of AOT. The amount of AOT forming an insoluble complex does not correspond to the net surface charge of lysozyme, which is about +9 at the pH of the solution used (Kuramitsu and Hamaguchi, 1980). It has been found in the literature that the concentration of buffer salt has a great effect on the R of precipitation; the R value shifted from 10 to 20 to obtain the same precipitation at 0.1 M and 0.3 M NaCl, with the increase in R due to anionic salt counterions decreasing the association of the AOT anion, and competing with AOT to bind with lysozyme (Shin *et al.*, 2003b).

At molar ratios of 29 and 35, however, the activities retained were much lower than expected, and the specific activity of lysozyme was reduced from that of the native lysozyme (Table 3.1). The lysozyme remaining in solution after precipitation at R=29 was approximately 5% active, while at R=35 it was 7% active. This discrepancy in activity with the amount of non-precipitated protein in solution suggests that lysozyme may have undergone some structural changes when large amounts of AOT were added to precipitate the lysozyme, e.g. denaturation. These findings seem to contradict the work of Shin *et al.* (2003b) who found complete removal of lysozyme with its original enzymatic activity, even at molar ratios greater than that required for protein removal. It would appear that the undetected protein denaturation from precipitation with surfactant by these researchers was due to analytical deficiencies; possibly because the absorbance of the denatured enzyme cannot be determined from HPLC at 210 nm because AOT also absorbs in this region (Ryu *et al.*, 2010). However, the absorbance of AOT at 280 nm was non existent in this study.

Table 3.1 Percent of lysozyme in solution measured after precipitation with AOT.

Non-precipitated lysozyme in aqueous solution		
Molar ratio AOT/Lysozyme	% Lysozyme*	% Activity (Specific Activity**)
R=5	52%	54% (50876 units/mg)
R=10	5%	4% (51632 units/mg)
R=16	0%	-
R=23	0%	-
R=29	12%	5% (22014 units/mg)
R=35	68%	7% (5439 units/mg)

* Experimentally determined with UV spectrophotometer.

** Specific activity of native lysozyme was 52833 units/mg.

3.5.1.1 Effect on Lysozyme Structure

The soluble lysozyme samples from the aqueous phase were further investigated using CD to analyse the conformational stabilities of lysozyme after interacting with different molar ratios of AOT ligands. CD is an effective method for studying the secondary structure of proteins (Greenfield, 1996). Probing the structural recognition process carried out with CD gave insights into conformational changes in the protein (Figure 3.3). Native lysozyme gives a negative band in the far-UV range of 200-240 nm, with a shoulder at about 222 nm, reaching its strongest intensity at 208 nm in the spectra (Yao and Gao, 2008). Figure 3.4 illustrates the original structure of lysozyme (Figure 3.4a), and its structure after precipitation at molar ratios of 5 to 35 (Figure 3.4b-d). For better structural recognition, and to enable comparability of the solution conformation, protein samples were diluted to about 0.1 g/L. Samples recovered below this concentration were analyzed at their original concentrations; exact protein concentrations used in the CD spectra are specified in the Figure 3.4 caption. The signal from CD is linear with protein concentration, and therefore the signal of the native protein can be scaled down based on the different concentrations of protein samples obtained.

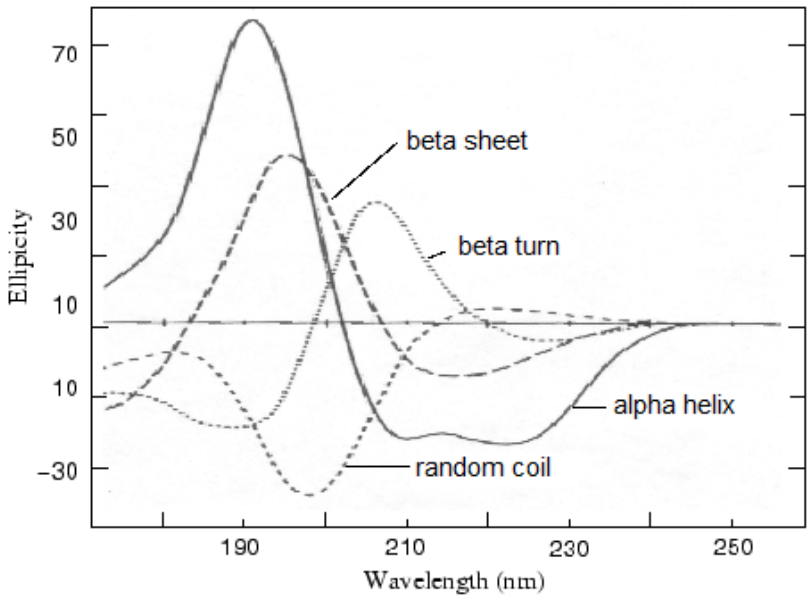

Figure 3.3 Far-UV CD spectra characteristics of alpha-helix, beta-sheet, beta-turn and random coil structures (Greenfield, 1996).

The secondary structure of lysozyme; α-helixes, β-turn fractions, and β-sheets, were observed in all the lysozyme samples. Duplicate scans of each sample and several degrees of protein

dilution provide confidence in these results. Lysozyme is an α/β globular protein (Raffaini and Ganazzoli, 2009); the native lysozyme spectrum yielded 32% α-helix, 11% β-sheet, 31% β-turn and 26% random coil. Figure 3.4b clearly shows that there was no significant conformational change in the recovered lysozyme at R=5. For R=10, although the sample was analysed at lower solution concentrations, the trace was seen to remain almost consistent throughout, with the peaks at 208 and 222nm recognizable without any significant shift despite the weaker signals (Figure 3.4c). The unaltered CD spectrum for R=5 and 10 indicates that lysozyme had retained its secondary structure in these molar ratio formulations. CD spectra were not provided for R=16 and 23 because measurement of lysozyme before and after precipitation revealed that none of the lysozyme remained in solution, nevertheless there is a strong indication that the samples do not undergo structural transformation based on fully functional active sites required for complete removal of the protein.

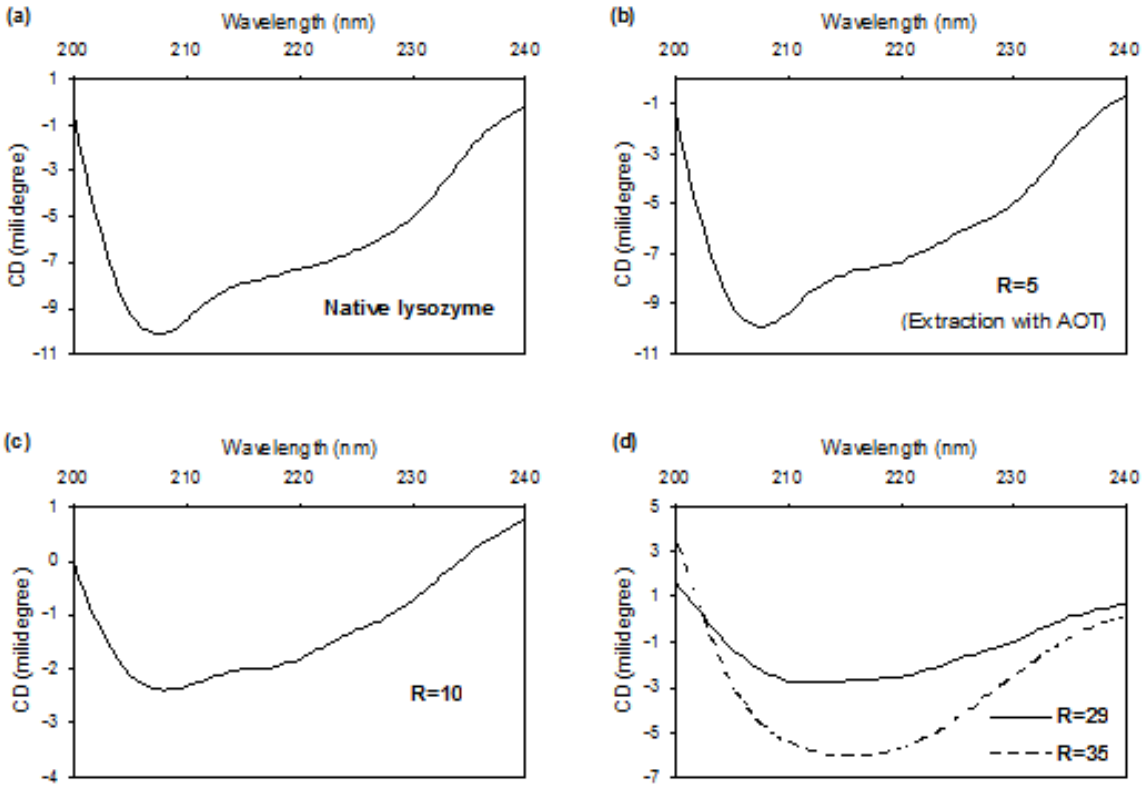

Figure 3.4 **Far-UV CD spectra of lysozyme in phosphate buffer (20mM), pH 6.2: (a) Initial lysozyme solution without AOT diluted to 0.1 g/L; Final aqueous phase left after precipitation of lysozyme at different AOT to lysozyme ratios (R) (b) R=5 at 0.1 g/L, (c) R=10 at 0.04 g/L, (d) R=29 at 0.1 g/L and R=35 at 0.09 g/L, respectively.**

The traces of the samples at R > 23, e.g. 29 and 35, also have negative bands in the range from 200 to 240 nm, however, they demonstrated inconsistency with the far-UV trace of the native protein. The α-helix fraction of lysozyme decreases rapidly from R=29 (29%) to R=35 (23%), conversely the β-sheet fraction increases from 12% to 15%, the β-turn content increases from 31% to 32% and the proportion of random coil increases from 28% to 30% in the media (statistically these results are significantly different at the 95% confidence interval). The CD data indicates that the overall conformation of the protein is strongly influenced by the surfactant concentration. The hydrophobic binding of large numbers of AOT ligands on the lysozyme surface caused the unfolding of lysozyme, so that some of the α-helixes changed into β-sheets (Chen *et al.*, 2009). The structural state of lysozyme in R=35 media changed appreciably from that of the native lysozyme; the helix structure is lost and β-structure is gained when there is a reduction of the disulfide linkages (Moriyama *et al.*, 2000) from the excessive AOT binding. The results obtained strengthen the belief of Hjelmeland and Conn (1986) that the structural features of the surfactant monomers do not determine denaturation, rather it is the protein structure.

3.5.1.2 HPLC Analysis of Lysozyme Samples

Through chromatography lysozyme was successfully retained and recovered by reversed phase HPLC (RP-HPLC) with a retention time of 5.8 min at 45% B gradient elution (Figure 3.5). Peaks that appeared at the start of the chromatogram, before protein is eluted, are the unretained substances from the sample solvent. Percent removal of lysozyme calculated by the areas under the eluted protein peak shows results similar to those obtained through UV spectrophotometer. Besides giving areas of the protein peak, plotted chromatograms can be used to compare the profile of lysozyme samples after interaction with AOT with the profile of the native lysozyme. These samples at AOT 1.5 g/L (R=5) (Figure 3.5b) and 3 g/L (R=10) (Figure 3.5c) have the same profile as the native lysozyme (Figure 3.5a), but with decreasing lysozyme peak areas and heights as the samples consist of remaining unbound lysozyme due to the lack of AOT molecules present. Complete removal of lysozyme is shown in Figure 3.5d-e where no protein peak was observed in the filtrate after precipitation with 5 g/L (R=16) and 7 g/L (R= 23) of AOT. When higher AOT concentrations, 9 g/L (R=29) and 11 g/L (R=35), were introduced to the aqueous protein solution, lysozyme was again detected in the filtrate. Samples analysed by chromatography confirm the results of the enzymatic assay

that suggest incomplete removal of lysozyme was achieved at these AOT concentrations because lysozyme becomes inactive from excess AOT in solution (Figure 3.5f).

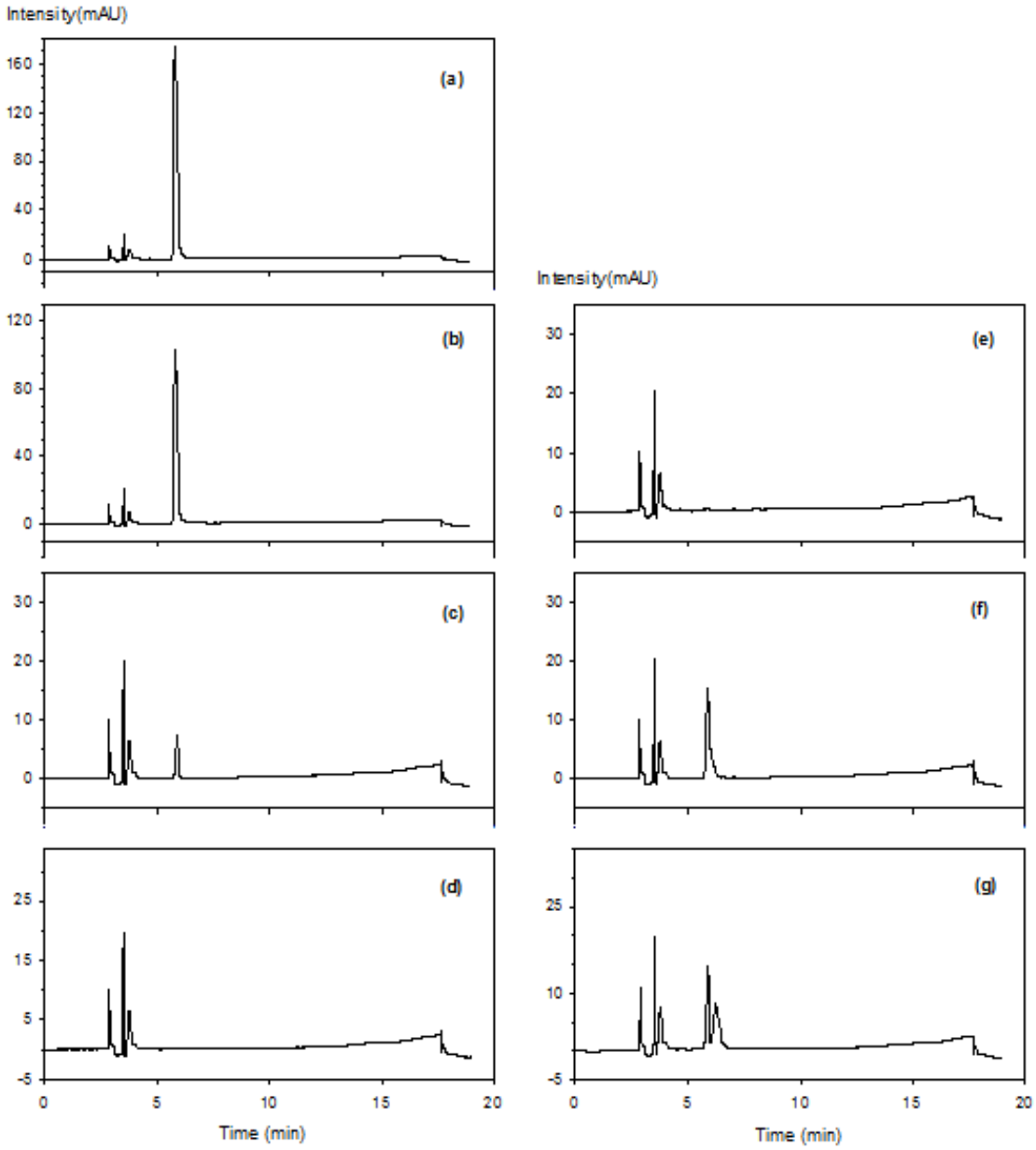

Figure 3.5 **Chromatogram of lysozyme samples: (a) Initial lysozyme solution without AOT (t_R = 5.8min) ; Soluble lysozyme in the aqueous solution after addition of AOT at (b) R=5, (c) R=10, (d) R=16, (e) R=23, (f) R=29, and (g) R=35.**

HPLC results also helped to interpret the results obtained from CD on the behaviour of lysozyme when surfactant was added beyond the concentration needed for complete formation of the lysozyme-AOT complex. CD spectra indicated that lysozyme at R=35 experienced hydrophobic binding with excess AOT ligands and was denatured. When the lysozyme sample at R=35 was subjected to chromatography, a sizeable peak at 6.2 min was produced near to the protein peak of the inactive lysozyme remaining in the filtrate (Figure 3.5g). The foreign peak, which appears as the protein peak decreases, was attributed to the unfolded lysozyme. The original lysozyme peak areas (t_R=5.8min) recovered in sample R=35 was calculated to be lower than that in sample R=29; presumably, inactive lysozyme was further denatured with increase in AOT concentration thus causing a rise in the peak area at t_R=6.2min.

Results showed that the shift of the lysozyme molecule to the denatured state was accompanied by an increase in the retention time. RP-HPLC relies on the strength of hydrophobic interactions between the protein and a non-polar support for separation (Sivasankar, 2005). The higher retention time of the unfolded protein observed during the chromatographic run can be explained by the exposure of hydrophobic residues initially buried in the interior of the native molecule, as a consequence of denaturation, and resulted in stronger binding to the column. This effect of protein denaturation on peak and retention times has been captured previously on HPLC (Bramanti *et al.*, 2003; Ingraham *et al.*, 1985). The trend of lysozyme denaturation in surfactant precipitation is shown in the HPLC to be a gradual alteration of protein from an inactive phase to a denatured phase. It is evident from this study that hydrophobic interactions can act as a parameter for protein stability during the formation of a protein-surfactant complex in surfactant precipitation.

Past research on surfactant precipitation detected excess AOT in the aqueous phase at molar ratios greater than that required for protein removal (Shin *et al.*, 2003b). However, rather than remain as free surfactant molecules, this work found that most of the excess AOT ligands had interacted with the lysozyme and contributed to the loss of lysozyme native structure and activity. This might have caused significantly less lysozyme being precipitated or the re-dissolution of the protein-surfactant precipitate due to an unstable precipitate forming at R=35. Figure 3.6 shows the binding of surfactant to protein increased continually after complete removal of lysozyme was attained. Results obtained here point to the likelihood that most of the AOT ligands bind to the hydrophilic outer surface of the lysozyme up to the molar ratios required for lysozyme removal (R=16). Electrostatic interactions with the AOT

ligands drives the neutralisation of the protein charges and the precipitation of proteins from the aqueous phase. With increasing concentration AOT molecules bind to the non-polar outer surface and enter the hydrophobic intracavity of lysozyme. This results in more attractive short-range hydrophobic interactions between the protein and surfactant at R > 23 after the molecules are brought together by the dominant intermolecular interaction (electrostatic forces). The secondary structure of lysozyme at R=29 seems less distorted compared to that at R=35; more unfolded lysozyme at higher surfactant concentrations produced more random coils. This is because non-covalent binding is normally a weak and non-specific interaction (Piekarska *et al.*, 1996), but a combination of more non-covalent hydrophobic bonds, such as that present at the highest AOT to lysozyme mole ratios in this study (R=35), may alter the conformation of the protein through its helical structure interacting with a surfactant ligand.

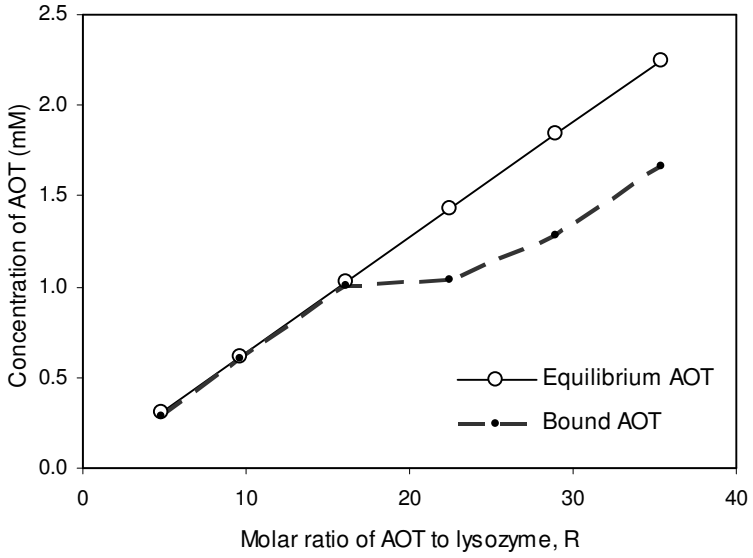

Figure 3.6 Equilibrium concentration of AOT in lysozyme solution and final concentration of AOT bound to lysozyme at pH 6.2 as a function of the molar ratio of AOT to lysozyme.

The severe activity loss reported for the mass of non-precipitated lysozyme at R=35 could have been caused by the hydrophobic binding of excess AOT ligands to lysozyme that inhibited its enzymatic activity, and affected the protein function. The structural transformation that occurs during protein folding is of great significance in the functioning of organisms (Hu and Xu, 1999), which in this case is lysozyme's ability to catalyze the hydrolysis of bacterial cell walls. It is also important to note that specific binding at the active site of a protein may result in enzyme inhibition before any appreciable conformational

change takes place (Jones, 1996). Higher mole ratios of surfactant monomers binding to a lysozyme molecule may have prevented the specific peptidoglycan component of *Micrococcus lysodeikticus* cell walls from entering the lysozyme cleft, thereby inhibiting the enzyme's hydrolysis ability, or/and the binding may have changed the protein conformation by producing overlapping enzyme active sites, thus the recovered lysozyme sample from the aqueous phase was only partially active.

3.5.2 EFFECT OF PH ON THE FORMATION OF A LYSOZYME-AOT COMPLEX

To examine the effect of pH on the surfactant precipitation of lysozyme, initial protein solutions containing lysozyme, at various pH values, were mixed with AOT solutions at a molar ratio (R) of 16. The pH of the initial lysozyme solution was adjusted with HCl or NaOH before AOT was added directly to form an insoluble complex. The pH before and after precipitation was kept fairly constant at its adjusted pH (standard deviation below ±0.30) in the 20mM phosphate buffer, rather than increasing with removal of lysozyme as demonstrated in a non pH adjusted and non salt aqueous system (Shin *et al.*, 2003b). For an adjusted pH range of 4 to 9, the percent precipitation after the formation of a protein-ligand complex was essentially 100%, as shown in Figure 3.7. A pH lower than 4 resulted in decreased precipitation removal, although nearly 98% lysozyme was still removed at pH 2; however, at pH 12 the percent precipitated dropped sharply.

The pH of the protein solution modifies the net charge distribution over the protein surface; at pHs below the pI the protein takes on a net positive charge, while at pHs above its pI it will have a net negative charge. When AOT was added at a pH set below the pI of lysozyme, favourable electrostatic attractions occurred. The positively charged lysozyme bound to the anionic surfactant to form an insoluble lysozyme-AOT complex. A pH higher than 11 had a negative effect on the formation of an insoluble complex, with only 39% precipitation achieved at pH 12. It is thought that lysozyme, at a pH considerably higher than the pI of the protein, finds it difficult to bind with the monomers of AOT because of the electrostatic repulsion between the protein and AOT headgroups, therefore, little lysozyme was precipitated. The system conditions in the initial aqueous phase, discussed in terms of pH in this work, affects the physiochemical state of protein and its interaction with the surfactant head groups in the formation of a protein–ligand complex.

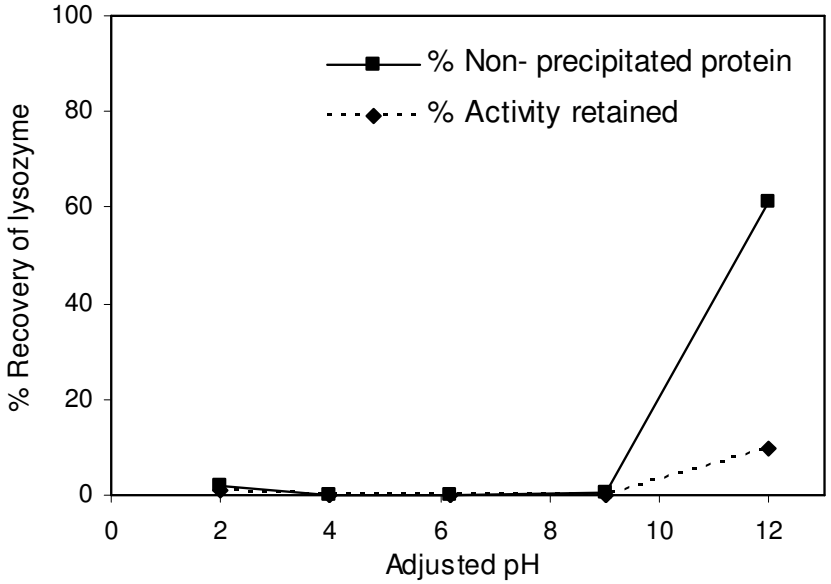

Figure 3.7 **Effect of pH on the precipitation of lysozyme with AOT: initial lysozyme concentration = 1.0 g/L, molar ratio between AOT and lysozyme = 16. Activity and amount of lysozyme left in solution after precipitation.**

An enzyme activity test carried out on the soluble lysozyme remaining after precipitation displayed matching results at pH 4 to 9, with protein being recovered without loss of enzyme activity to the aqueous phase. At pH 2 some activity was retained in the solution owing to the non-precipitated lysozyme with the surfactant (Figure 3.7). At an optimum ionic strength, determined as 20mM for this study, lysozyme is active over a broad range of pHs because it is possible that the enzyme conformation necessary for activity requires the presence of certain concentrations of small ions (Davies *et al.*, 1969). Lysozyme prepared in 20 mM phosphate buffer is an effective lytic agent from pH 4 to pH 9. A relatively acidic solution at a pH of 2 suggests a less active lysozyme interacting with AOT.

Lysozyme precipitated with the same method of extraction and a similar amount of AOT at pH 12 showed a severely reduced enzyme activity (~9%) in the aqueous phase in spite of more than 50% of the lysozyme not being precipitated. In the catalytic mechanism which operates during the enzymatic hydrolysis of a glycoside, one of the two carboxyl groups in the active conformation of the lysozyme is ionised, while the other is un-ionised and serves as a proton donor for the glycosidic cleavage of cell wall material (Vernon, 1967). The activity of the enzyme recovered is significantly reduced from its original activity as both the

carboxyl groups in the cleft are, presumably, fully ionised when lysozyme interacts at the extreme pH of 12 with surfactant ligands. The CD spectra of the lysozyme sample not precipitated at pH 12 in Figure 3.8 shows a noticeably altered protein conformation after precipitation with AOT (25% α-helix, 14% β-sheet, 31% β-turn and 30% random coil), and the methylene blue assay revealed highly bound AOT in the solution (>70%) (Figure 3.6). Pure lysozyme is not denatured at pH 12. With the lack of affinity for ionic binding, hydrophobic binding takes place when lysozyme interacts with surfactant molecules at a high pH, causing the protein to lose its original structure. This implies that unfolding of lysozyme by AOT was initiated by non-specific hydrophobic interactions.

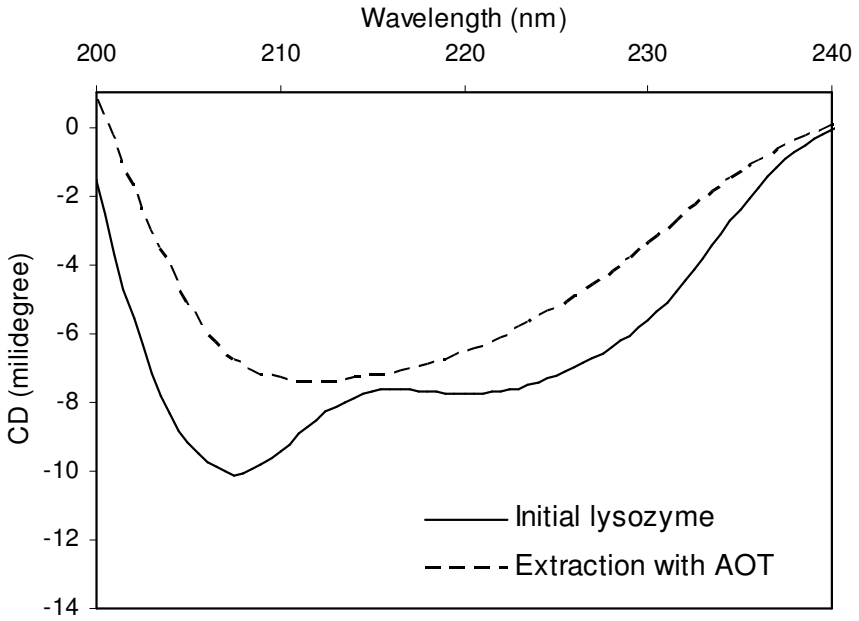

Figure 3.8 Far-UV CD spectra of lysozyme in phosphate buffer (20mM), pH 12.0, before and after precipitation with AOT (R=16). Samples are diluted to 0.1 g/L.

The solution containing the unfolded lysozyme at pH 12 was dialysed to about pH 6 and the protein content checked. Lysozyme in the sample was fully precipitated when the favourable charges on the amino acid side chains were regained upon dialysis, and no significant surfactant molecules were found in the solution using the methylene blue assay. Electrostatic interactions promoted the re-binding of an anionic surfactant from the non-polar sites to the polar sites of the protein, and hence dialysis was capable of restoring the original protein conformation. CD analyses were carried out on the initial lysozyme sample for other values of pH (2-9), and the CD profiles resembled that of the native lysozyme (CD spectra not

shown here). Lysozyme had a stable protein conformation within the range of pH values studied, thus maintained an original enzymatic activity when surfactant ligands were not introduced.

The results of the precipitation of lysozyme as a function of pH showed protein removal was dependent on the pH of the initial protein solution, proving electrostatic interactions are the dominant attractive forces between protein and surfactant molecules leading to precipitation. Results also show that pH could potentially be manipulated to enhance the separation of proteins with different pI's in a filtered buffer media when surfactant precipitation is applied. This separation technique should allow for a more specific precipitation of a target protein than other precipitation methods, eg. 'salting-out'.

3.6 CONCLUSIONS

The surfactant precipitation of lysozyme was successfully analysed using AOT in a solvent-free non-micellar system. The precipitation of lysozyme from aqueous solution was investigated as a function of the AOT and lysozyme molar ratio between 5 and 35, and a pH ranging from 2 to 12. An optimum stoichiometric molar ratio of 16:1 (AOT:lysozyme) achieved a complete (100%) removal efficiency of lysozyme at pH 6.2. The complete precipitation of lysozyme, and a potentially preserved secondary structure [i.e. biological activity] of the protein can be achieved when lysozyme is precipitated with an optimum amount of surfactant present in the form of monomers.

The original biological activity was maintained for lysozyme precipitated in an aqueous phase at a pH below the pI of the protein, and the effect of pH on protein removal indicated that electrostatic interactions between oppositely charged protein and surfactant molecules drives the extraction process. This ionic interaction induces the formation of an uncharged lysozyme-AOT complex which is not soluble and hence precipitates. The change of lysozyme structure in the aqueous phase after precipitation was measured using circular dichroism spectroscopy and liquid chromatography, and considerable insight has been gained into surfactant initiated protein precipitation. For this technique to become commercially viable, however, the protein removed from the aqueous phase has to be efficiently recovered. The recovery of protein from a surfactant precipitate using various extraction procedures is investigated in the next chapter.

CHAPTER 4 PROTEIN RECOVERY FROM SURFACTANT PRECIPITATION*

4.1 INTRODUCTION

Previous work on surfactant precipitation has focused on recovering the precipitated protein-surfactant complex by dissolving it in acetone (Shin *et al.*, 2003b), in which the insoluble complex was reported to resolubilise without loss of enzyme activity (Shin *et al.*, 2003c). One drawback of this method was that protein recovery was a strong function of the time the protein-surfactant precipitate was dissolved in the solvent to retrieve protein. The conformational stability of cytochrome c recovered within 10 minutes showed an original peak shape and 95% recovery; when left in acetone for more than 30 minutes, a severely distorted peak with HPLC and 0% recovery was measured (Shin *et al.*, 2004a). There remains a need to enhance and optimise this purification method to expand the use of surfactants as a precipitating ligand, and develop low cost methods for protein separation.

Organic solvents have a tendency to affect enzymatic stability, and the choice of an appropriate solvent cannot be decided arbitrarily. The aim of this work was to recover protein from the surfactant precipitate by examining commercially viable solvents besides acetone. Also, past work has shown that addition of counterionic surfactants can cause the backward transfer of proteins encapsulated in AOT reverse micelles (Jarudilokkul *et al.*, 1999; Juang and Mathew, 2005). Attempts were made to recover lysozyme precipitated by direct extraction with AOT by dissolving the protein-surfactant complex in a solution of trioctylmethylammonium chloride (TOMAC), dodecyltrimethylammonium bromide (DTAB) and dimethyldioctadecylammonium chloride (DODMAC). The effect of counterionic surfactant concentration and the ionic strength of the buffer solution on extraction efficiency and the secondary structure of the protein were also examined.

* This work presented in this chapter has been published as:
Cheng, S.I. and Stuckey, D.C. 2011. Protein recovery from surfactant precipitation. Biotechnology Progress 27: 1614-1622.

4.2 MATERIALS AND METHODS

Materials: Cationic surfactants, trioctylmethylammonium chloride (TOMAC), dodecyltrimethylammonium bromide (DTAB) and dimethyldioctadecylammonium chloride (DODMAC) were obtained from Sigma. Sodium chloride (NaCl) was obtained from Sigma. Reagent grade solvents used were ethanol at absolute 100% purity from AnalaR, and methanol, acetone, 2,2,4-trimethylpentane (isooctane) with minimum 99% purity from Sigma.

4.2.1 PREPARATION OF COUNTERIONIC SURFACTANT

The cationic surfactant solution used contained 11.3 mM to 39.4 mM of surfactant. The various TOMAC concentrations were prepared by dissolving it in ethanol and isooctane due to their low solubility in distilled water. Likewise, DODMAC was prepared in ethanol while DTAB was prepared in distilled water. The CMC of DTAB in water is 15 mM at 25ºC (Rosen, 1978). The CMC of ionic surfactants is often in the 10^{-3}–10^{-2} M (1-10 mM) range (Mackie and Wilde, 2005). Therefore, the surfactant concentration in the total aqueous mixture was kept between 1.03 and 3.58 mM, which is well below the CMC.

4.3 EXTRACTION PROCEDURES

4.3.1 PRECIPITATION OF LYSOZYME USING AOT AT R=16

Separation using surfactant precipitation is a two-step process; the precipitation and the recovery of protein. The precipitation procedure has been elaborated in the previous chapter. This part of the work will focus on the recovery of the protein from the precipitate using just one R value (R=16) for precipitation. To give a molar ratio of AOT to lysozyme of 16, 1 mL of 5 g/L (11.3 mM) AOT was added directly to 10 mL of the 1.0 g/L initial protein solution. At the moment of addition, the AOT concentration in the total aqueous mixture was 1.02 mM. The mixture was vortexed and centrifuged to separate the insoluble complex. The supernatant liquid was removed and analyzed for protein content. The precipitated lysozyme-AOT was collected for the protein recovery process.

4.3.2 RECOVERY OF LYSOZYME WITH SOLVENT

The recovery of lysozyme is the final step in the extraction procedure. After collecting the white surfactant precipitate by centrifugation, 10 mL of recovery solvent (ethanol, methanol, ethanol-acetone, or ethanol-water mixture) was added to the test tube containing the lysozyme complexed with AOT. It was subjected to 5 sec of vortex mixing for the insoluble complex to dissolve into the solvent phase. A small amount of 0.1 M NaCl solution of 10 μl was added to the solvent phase to neutralize the charges of the lysozyme dissociated from the AOT (Shin *et al.*, 2003c). The lysozyme then precipitated out of the solvent phase while AOT remained in solution. The addition of NaCl was followed by a second centrifugation to obtain the recovered lysozyme. Analyses of the final product were carried out by dissolving the recovered lysozyme into a fresh aqueous phase of phosphate buffer solution at pH 6.2.

4.3.3 RECOVERY OF LYSOZYME WITH COUNTERIONIC SURFACTANT

For recovery with TOMAC, 10 mL of fresh buffer solution (pH 6.2) was added to the surfactant precipitate. The precipitated lysozyme-AOT containing solution was mixed with 1 mL of various TOMAC concentrations in ethanol. In order to prevent the effect of volume change when the surfactant solution was added, the volume used was fixed for all the surfactant concentrations. 1 mL of ethanol by itself did not have any effect on protein recovery, nonetheless it was blanked off for accurate measurement of protein recovery with counterionic surfactant by measuring for soluble protein content in the precipitated lysozyme-AOT containing solutions for all samples in 1 mL of ethanol. The mixture was vortexed to encourage the interactions between TOMAC and AOT. Lysozyme dissociated from the AOT as the AOT complexed with TOMAC, and the surfactant dimer (TOMAC-AOT complex) precipitated out of the recovery phase while lysozyme remained in solution; the sample was analysed for lysozyme concentration and activity. The cationic surfactant, TOMAC, was prepared in isooctane to determine the effect of the solvent on recovery. DTAB and DODMAC were also prepared to determine the effect of the cationic surfactant properties on counterionic recovery. These recovery solutions were used in the same way as the TOMAC/ethanol.

4.4 ANALYTICAL TECHNIQUES

All samples containing the recovered lysozyme solution were filtered using 0.2 μm disposable syringe filters prior to analysis to ensure that any remaining non-dissolved solids were removed. Quantitative and qualitative analyses, as described in the last chapter, were carried out on the filtrate and are discussed below. The coefficient of variation (CV) of the extraction process for concentration and activity measurements was within ±5% for lysozyme recovery with counterionic surfactant, and ±7% for recovery with solvents. The reproducibility is indicated in the standard deviation of the measurements shown in the tables.

4.5 RESULTS AND DISCUSSION

4.5.1 OPTIMUM PRECIPITATION OF LYSOZYME

In these samples, AOT was added to pure lysozyme solution at pH 6.2 (pH < pI of lysozyme) for the positively charged lysozyme to bind to the anionic surfactant to form an insoluble lysozyme-AOT complex. The percent precipitation of lysozyme was calculated and the protein concentration remaining in the aqueous solution after precipitation with the AOT, which in this case was fixed at a molar ratio (R) of AOT to lysozyme of 16, was found to be optimum. According to the experimental results, when R was 16 100% lysozyme and its activity was removed from the initial protein sample by precipitation with the surfactant. Lysozyme did not “leak back” into the aqueous phase after the formation of a protein-surfactant complex; a proof of principle that separation by centrifugation managed to efficiently remove all insoluble complex from the precipitation phase. The surfactant precipitate consisting of a lysozyme-AOT complex produced from the precipitation process was then used to examine the effect of solvents and a counterionic surfactant on the recovery efficiency of lysozyme.

4.5.2 RECOVERY OF LYSOZYME

The effect of solvents on the recovery of lysozyme precipitated with AOT was quantified in terms of the concentration and activity of lysozyme recovered from the final aqueous phases. The percent of lysozyme recovered was calculated as:

$$\%\ \text{Protein recovered} = \left(\frac{C_{P^f} \cdot V_f}{C_{P^o} \cdot V_o} \right) \times 100 \qquad (4.1)$$

where Cp^o refers to the lysozyme concentration in the initial aqueous solution before the addition of AOT, and Cp^f is the recovered protein concentration. V_o and V_f are the volumes of the initial and final aqueous solutions used to solubilise the recovered lysozyme. The efficiency of the recovery process of lysozyme from the precipitated lysozyme-AOT complex was obtained from:

$$\text{Recovery Efficiency}\ (\%) = \left(\frac{C_{P^f} \cdot V_f}{C_{P^o} \cdot V_o - C_{P^e} \cdot V_e} \right) \times 100 \qquad (4.2)$$

Cp^e refers to the equilibrium lysozyme concentration remaining after the formation of an insoluble lysozyme-AOT complex and V_e indicates the volume of the aqueous phase after the addition of AOT; V_e=V_o+V_S, where surfactant volume is denoted by V_S.

Specific activity (units/mg) of lysozyme in the sample can be calculated from equation (3.2). Percent activity of lysozyme remaining in the final product was reported as:

$$\%\ \text{Activity recovery} = \left(\frac{U_f\ / \text{mg} \times \text{Total protein in the reaction mixture}}{U_o\ / \text{mg} \times \text{Total protein in the initial aqueous solution}} \right) \times 100 \qquad (4.3)$$

where U_o and U_f are the protein activities in the initial solution, and after the recovery step, respectively.

The standard deviation of the process is influenced by precipitate formation and the mass loss during the recovery process. The samples used were small, and therefore a small loss represents a significant change in the results. A larger deviation on the recovery with solvents was caused by the requirement for a second centrifugation step to separate solid lysozyme from the solvent phase. For recovery with counterionic surfactant, the final product was separated by filtration without any mass loss in the supernatant (data of protein content before and after filtration showed that adsorption of protein on the filter used was negligible).

4.5.3 EFFECT OF SOLVENT ON RECOVERY

Lysozyme recovery from the precipitate was carried out using four solvents; ethanol, methanol, ethanol-acetone (50% v/v) and ethanol-water (50% v/v). Solvent was added to the surfactant precipitate obtained by adding AOT to lysozyme at a molar ratio of 16. The white lysozyme-AOT precipitate dissolved in all the solvents evaluated, and lysozyme precipitated with the addition of NaCl as the AOT remained in the solvent phase; the recovery of lysozyme varied with the solvents being used (Table 4.1).

Table 4.1 Effect of solvent type on the percent recovery of lysozyme from a lysozyme-AOT precipitate.

Recovery Solvent	% Recovery of Lysozyme	% Recovery of Activity
Ethanol (100%)	78.8 ± 2.5	79.7 ± 3.1
Ethanol-Acetone (50% v/v)	-	75.0 ± 2.9
Ethanol-Water (50% v/v)	2.0 ± 0.1	2.0 ± 0.1
Methanol (100% v/v)	29.1 ± 1.2	27.9 ± 0.9

Initial protein solution 20 mM buffer, pH 6.2 (V_o=10 mL); AOT 11.3 mM (V_s=1mL); NaCl buffer (0.1 M) added to solvent (0.01mL); final aqueous phase 20 mM buffer (V_f=10mL). % Recoveries were of total concentrations of pure enzyme.

The recovery of lysozyme with ethanol in this study was about the same as that recovered with acetone, which was reported as 70 ± 18 % in the literature (Shin *et al.*, 2003b). Ethanol-acetone surprisingly gave a recovery that was above the absolute value. However, spectrum analysis showed that acetone alone absorbed strongly at 280 nm (Figure 4.1), and traces of acetone were still present in the assay after recovery by centrifugation and precipitated lysozyme was solubilised in fresh aqueous solution (Figure 4.2). Therefore, quantitative analysis of lysozyme was quite limited when acetone was used for recovery because acetone absorbed at the wavelength of the protein; however, ethanol had no influence on the absorbance profile, although the ethanol-water mixture gave the lowest recovery of lysozyme. It was inappropriate to use solvents with water as most of the lysozyme

precipitated out of the ethanol phase and dissolved in the water before it could be recovered from solution.

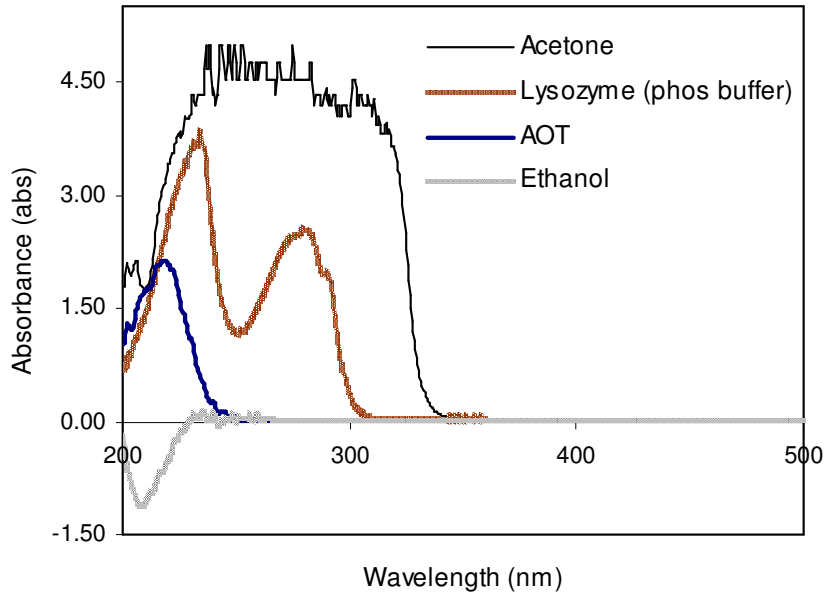

Figure 4.1 **Spectrum analysis of AOT surfactant, pure lysozyme solution, ethanol and acetone solvents: UV spectrophotometer 200-500 nm.**

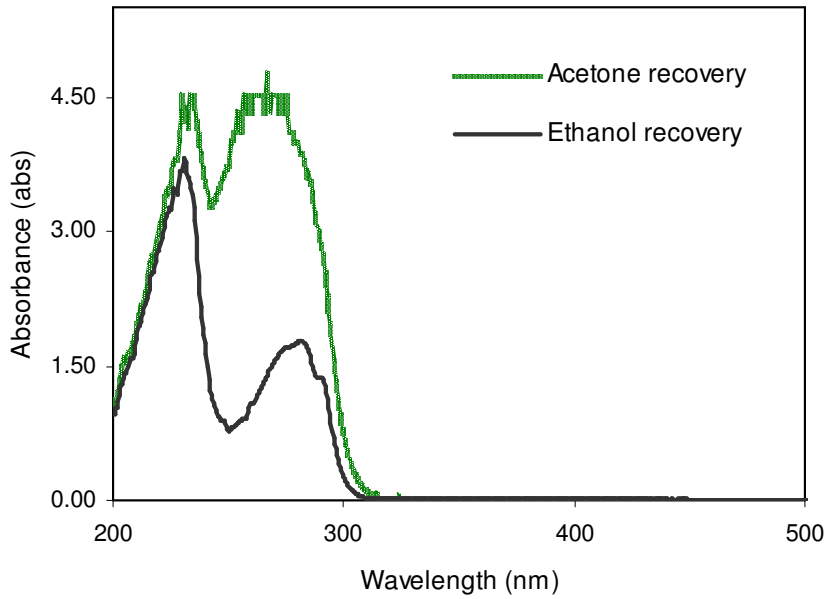

Figure 4.2 **Spectrum analysis of lysozyme sample in final aqueous solution recovered by ethanol and acetone solvent: UV spectrophotometer 200-500 nm.**

The recovery of lysozyme with methanol was low compared to that achieved with pure ethanol. The properties of the solvents were studied to determine the cause of the difference in recovery efficiencies. Ethanol and methanol, unlike a majority of solvents, have both polar and nonpolar characteristics due to a polar C-O-H group as well as nonpolar C-H bonds present in the molecules. For these solvents, a key concern affecting protein recovery is the protein's solubility in organic solvents. Dissolving lysozyme at pH 6 in solvents for 16 hours determined that the solubility of lysozyme was much higher in methanol (100% solubility) than in the more hydrophobic ethanol (4% solubility) (Bromberg and Klibanov, 1995). In our work, 70% of the lysozyme concentration was lost in methanol while only 20% was lost in ethanol. Despite the different recoveries, the methylene blue assay found no significant surfactant concentration (<4mg/L) in the final product of all samples; thus protein was recovered as AOT-free lysozyme. The activity of lysozyme in the final aqueous phase was retained, within experimental error, for all the solvents.

Besides looking at the effect of solvent type on protein recovery efficiencies, research also focused on protein stability. It was essential to determine whether lysozyme suffered any irreversible deterioration when recovered with either ethanol or methanol. After the addition of solvent and NaCl, lysozyme was left in the solvent phase for 60 minutes before being collected and redissolved in fresh buffer. Positive results ruling out protein denaturation were obtained for lysozyme samples tested with ethanol and methanol. Recovery of lysozyme in the final aqueous solution was maintained throughout the experiment, specific activity was the same as the native lysozyme, and CD spectra provided a sensitive measure of its conformational integrity.

Samples were free from the denaturation that had resulted from acetone recovery. Addition of a solvent that is miscible but less polar than water (ethanol, methanol, or acetone) enhances protein interaction by decreasing the dielectric constant, and disrupting the hydration layer around the protein so that the solubility of the hydrophilic protein is reduced. Large amounts of less polar solvent will also weaken the hydrophobic bonds in the interior of the protein (Li-Chan, 1996). Protein unshielded by water molecules can be unfolded from the extensive exposure of its hydrophobic groups to the surrounding solvent molecules. The reversibility of the process depends on the nature of the solvent, the extent of protein unfolding and the rate of solvent removal. Acetone, which is less polar than ethanol and methanol, promotes irreversible unfolding after prolonged exposure to the solvent (Shin *et al.*, 2004a).

It was observed that NaCl buffer was not necessary when recovering lysozyme from the ethanol and methanol phases. A probable explanation for this is that ethanol and methanol are protic solvents, which solvate negatively charged solutes strongly via hydrogen bonding (Lowery and Richardson, 1987). They are able to stabilise the charges of AOT when the anionic ligands are separated from the lysozyme-AOT complex and dissolved in the solvent. Once the lysozyme-associated water is stripped away by the solvent, the interactions between charged groups on the surface of lysozyme are stronger and the net electrostatic attraction may lead to protein aggregation. Acetone, on the other hand, is an aprotic solvent which can only solvate positively charged solutes (Lowery and Richardson, 1987). Therefore, the negatively charged surfactant monomers remained in the vicinity of the enzyme and inhibited the protein charge interactions: hence NaCl buffer had to be added to the solvent to neutralize the lysozyme charges to enable recovery.

Considering the low recovery with methanol, and the denaturing effect of acetone, ethanol seemed to be the solvent of choice in recovering lysozyme. However, the disadvantage with solvent recovery of the precipitate was that the AOT added to precipitate lysozyme reduced the removal of lysozyme into the solvent phase. Bromberg and Klibanov (1995) showed that surfactants below their CMC improve protein solubility in organic solvents through hydrophobic ion pairing with the oppositely charged protein.

4.5.4 EFFECT OF COUNTERIONIC SURFACTANT ON RECOVERY

Adding a cationic surfactant, TOMAC, dissolved in either ethanol or isooctane, to the insoluble lysozyme-AOT complex released lysozyme into solution, while the TOMAC-AOT complex precipitated. Jarudilokkul *et al.* (1999) showed that the electrostatic interactions between oppositely charged surfactant molecules leads to the collapse of reverse micelles, and it is clear in our work that the formation of nonpolar ion pairs resulted in the solubilisation of protein from the surfactant precipitate. Figure 4.3 shows the effect of TOMAC/ethanol concentrations on protein and activity recovery of lysozyme from a lysozyme-AOT complex; higher TOMAC concentrations increased the removal of lysozyme until complete recovery was achieved. Lysozyme was fully resolubilised at TOMAC concentrations of 22.5 mM or higher, and it can be seen that TOMAC complexes with AOT in the molar ratio of 2:1. As a surfactant solubiliser, both ethanol and isooctane were found to have no effect on the protein absorbance profile and protein stability in this work.

The trend of increasing lysozyme recovery using a counterionic surfactant was also found with TOMAC/isooctane (Figure 4.4). However, a greater concentration of TOMAC was required (28.1 mM -2.5:1 molar ratio TOMAC:AOT) to release all the complexed lysozyme. These results can be explained in terms of the miscibility of the solvents used to prepare TOMAC with water. Ethanol is miscible with the aqueous phase, while isooctane is immiscible. Solvent immiscibility in counterionic extraction of a surfactant precipitate is not beneficial, and could be expected to significantly affect the diffusion of TOMAC/isooctane in the aqueous solution interacting with AOT. Due to this effect, a higher concentration of TOMAC/isooctane was required compared to TOMAC/ethanol for the same recovery time. When complexation was complete at 2 moles of TOMAC/mole of AOT, excess TOMAC remained in the aqueous phase with ethanol. With isooctane, excess TOMAC remained in the solvent layer on top of the water.

Since isooctane is immiscible with water, the use of TOMAC/isooctane enabled a visual observation of the formation of the TOMAC-AOT complex. After addition of TOMAC/isooctane, the insoluble lysozyme-AOT complex immediately dissolved into the aqueous phase. Simultaneously, the TOMAC-AOT complex was seen to precipitate out of solution and accumulate between the top isooctane phase and the bottom aqueous solution. A probable explanation for this is that the TOMAC-AOT surfactant complex has a high hydrophobicity, and therefore is more soluble in the solvent phase. This complex is insoluble in water and can easily be removed by filtration using 0.2 μm disposable syringe filters, as was done in this experiment, or by adsorption with Montmorillonite (Jarudilokkul *et al.*, 1999).

The recovery of lysozyme from the lysozyme-AOT complex with TOMAC is possible because the electrostatic interactions between AOT and TOMAC are stronger than those between AOT and the positively charged groups on the lysozyme surface. Therefore, the protein and anionic surfactant complex can be broken by the addition of a cationic surfactant. It was also discovered that such a recovery concept, which evolved out of reverse micelle back-extraction, is well-adapted to surfactant precipitation because AOT surfactant molecules when present in the form of monomers, display a similarly strong interaction with an oppositely charged headgroup, TOMAC, as when in the form of reverse micelles (Jarudilokkul *et al.*, 1999).

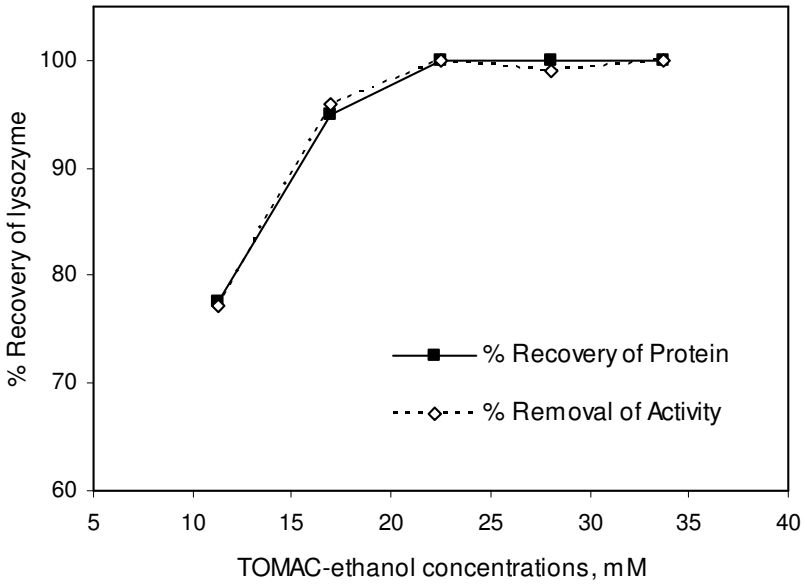

Figure 4.3 **Percent recovery of lysozyme from the precipitated lysozyme-AOT complex for an increasing TOMAC/ethanol concentration (11.3 – 33.7 mM).**

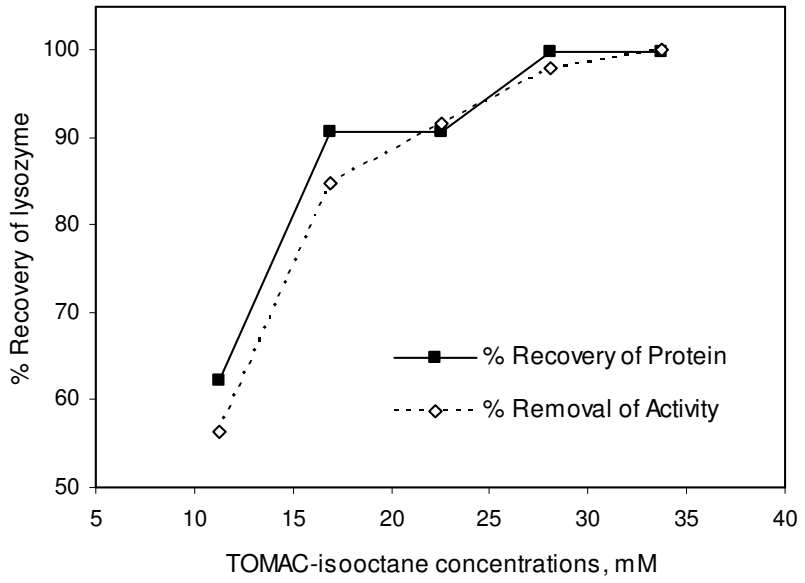

Figure 4.4 **Percent recovery of lysozyme from the precipitated lysozyme-AOT complex for an increasing TOMAC/isooctane concentration (11.3 – 33.7 mM).**

The lysozyme activity measured in solution after recovery with TOMAC was that of the original lysozyme activity (Figure 4.3 and Figure 4.4). No trace of surfactant was detected by the methylene blue assay after the removal of the TOMAC-AOT complex confirming that protein was released into the solution surfactant-free. The structural changes of lysozyme after interacting with the AOT ligands were characterized using CD since it is an effective method to study the secondary structure of proteins (Greenfield, 1996). CD analysis shows that there was no significant conformational change in the recovered lysozyme after counterionic extraction using TOMAC.

The activity and secondary structure of the recovered protein were analysed at 30-minute intervals for up to 3 hours to check protein stability against recovery time (data not shown), and no sign of denaturation was found. The favourable activity profile and the CD spectra obtained demonstrate that this approach to recovering a surfactant precipitate retains the protein's original activity and native structure.

4.5.5 PRECIPITATION OF LYSOZYME WITH TOMAC

For counterionic extraction it is inferred that lysozyme is recovered as a surfactant free protein in the final aqueous solution after the formation of TOMAC-AOT complex. We hypothesized that TOMAC added to the precipitated lysozyme-AOT binds to the AOT instead of the protein, and excess TOMAC remains in solution. To investigate this hypothesis, we studied the interaction between TOMAC and lysozyme in the absence of AOT. An initial protein solution was brought into contact with TOMAC by adding 1 mL of 22.5 mM of TOMAC/ethanol solution to 10 mL of lysozyme solution. The concentration of TOMAC which produced the highest amount of lysozyme from the lysozyme-AOT complex in the recovery experiment was chosen based on the assumption that the binding sites on the TOMAC surfactant for the AOT were the same as that for lysozyme. The percent precipitation of lysozyme was calculated with the equation 3.1.

These results are given in Table 4.2, and the percentage values fall within the analytical error of the method (standard deviation of the measurements ±3%). A "Student t-test" carried out for the removal of lysozyme and the recovery of activity showed that both were not significantly different (95% confidence interval), and hence indicate that TOMAC did not

precipitate lysozyme from solution. It is thought that lysozyme, at a pH considerably lower than its pI, has difficulty binding with the monomers of TOMAC because there are no strong ionic interactions between the positively charged protein and the cationic surfactant. CD spectra of the lysozyme solution after precipitation with TOMAC show original protein conformation; thus it is evident that TOMAC did not interfere with protein structure. This experiment confirms our predictions that the counterionic surfactant only binds with the oppositely charged surfactant to achieve full recovery of the protein, and excess counterionic surfactant does not interact with the protein released.

Table 4.2 Percent precipitation of lysozyme removed as a lysozyme-TOMAC complex at pH 6.2, with 22.5 mM TOMAC.

	Concentration (g/L)	**Total Activity (Units)**
Initial solution	1.0 ± 0.01	511000 ± 9000
After TOMAC addition	0.97 ± 0.03	491000 ± 12000
% Lysozyme removed	< 5%	< 5%

4.5.6 EFFECT OF BUFFER SALT SOLUTION ON THE RECOVERY OF LYSOZYME

Encouraged by the results of lysozyme recovery using a counterionic surfactant, we further examined the effect of ionic strength of the protein buffer solution on protein-surfactant interactions. These experiments were conducted by firstly adding AOT (AOT:lysozyme =16) to pure lysozyme prepared, respectively, in distilled water and in 20 to 100mM potassium phosphate buffer at pH 6.2. The various sets of protein solutions were analyzed by UV absorbance, enzyme activity assay and circular dichroism spectrum following the precipitation of lysozyme with surfactant. Next, the lysozyme was recovered by adding TOMAC/ethanol at a molar ratio of 2 TOMAC per AOT, and then all the final solutions were again analyzed after protein recovery. The TOMAC-AOT formed, and any lysozyme remaining as an insoluble complex in the final aqueous phase was filtered out of solution before the analyses.

Results of the protein concentration and activity remaining in solution after precipitation with AOT revealed a complete removal of lysozyme for all the different ionic strength buffers used (Figure 4.5). The amount of lysozyme complexed with AOT was consistent, and was not influenced by buffer solution strengths up to 100mM. At pH 6.2, lysozyme produces a maximal activity for a wide range of ionic strengths (20mM to 100mM) (Davies *et al.*, 1969). Dilute salt solutions stabilise charged groups on protein molecules and increase the solubility of proteins (salting-in) (Jakoby and William, 1971), and at the salt concentrations studied in this work, electrostatic interactions between proteins and surfactant molecules appeared stronger compared to protein-protein forces influenced by solvation of ions in an electrolytic solution. Beyond 100mM salt concentration, it was noticeably more difficult to dissolve lysozyme powder into the buffer solution, and increasingly less lysozyme was precipitated with AOT. Increasing buffer concentration after a point of maximum protein solubility allows less and less water molecules to interact with protein molecules, and with excess salt "salting-out" will occur (Jakoby and William, 1971).

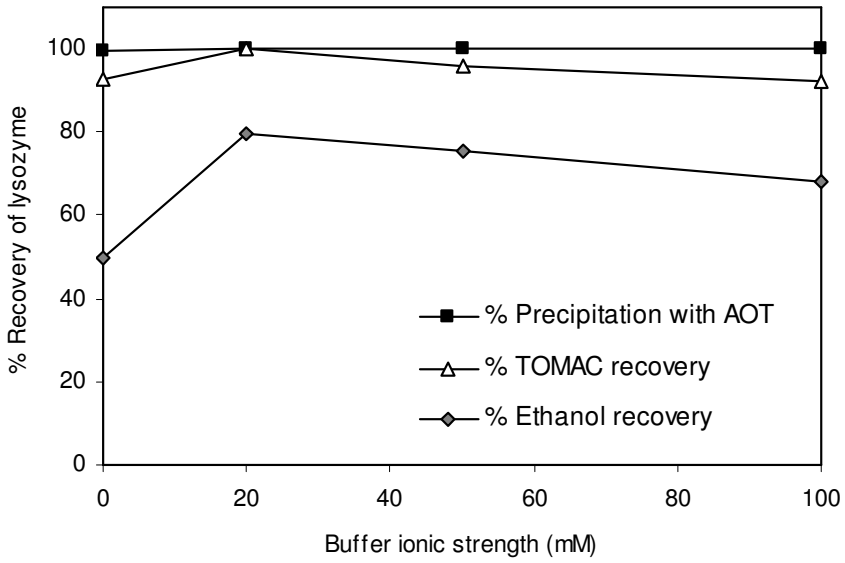

Figure 4.5 Effect of phosphate buffer concentration on the percent of lysozyme recovered with TOMAC, and ethanol: initial aqueous solution, 1.0 g/L lysozyme in phosphate buffer, pH=6.2 adjusted with NaOH.

Although in all cases (distilled water, 20mM, 50mM or 100mM buffer), the results of the % precipitation with AOT were similar (100%), lysozyme recovery with TOMAC/ethanol in the final solutions gave varying results (Figure 4.5). 100% recovery of lysozyme was found in 20 mM phosphate buffer compared to higher concentrations, and in distilled water. Total recovery of lysozyme with a small amount of buffer salt in the aqueous solution was an interesting observation. These results show that an optimum K_2PO_4 concentration (20 mM) can release lysozyme from the lysozyme-AOT complex completely, but higher K_2PO_4 concentrations slightly reduce the disassociation of the complex; protein recovery decreased to 92% at 100 mM (results of these samples are statistically different at the 95% confidence interval). The interactions of lysozyme are affected by the ionic strength of the media because it is possible that the enzyme conformation necessary for activity requires the presence of a certain concentration of small ions (Davies *et al.*, 1969). The equilibrium of the ionic species for the buffer system chosen for this experiment can be written as (Hainsworth, 1986):

$$H_2PO_4^- + H_2O \leftrightarrows H_3O^+ + HPO_4^{2-}$$

When sodium hydroxide was added to the buffer to adjust the pH to 6.2, the reaction equilibrium shifted to the right as the hydronium ions donated protons to the hydroxide ions. One explanation for the decrease in recovery is that an increase in potassium phosphate increased the concentration of hydrogen phosphate ions formed in solution when the buffer attempts to restore system equilibrium. As a result, more hydrogen phosphate ions compete with the AOT anion in the complex composed of lysozyme-AOT to bind with the cationic surfactant, thereby reducing complex formation between AOT and TOMAC. In distilled water, the recovery of lysozyme was 93%. Initial pH of the lysozyme solution without a buffer salt and pH adjustment depends on the lysozyme concentration (Shin *et al.*, 2003b). The concentration of lysozyme used in this experiment gave a pH of about 4.5 in distilled water. Lysozyme in distilled water in the absence of buffer salt ions (Chang and Carr, 1971) may suggest a fairly low enzyme activity, thus a lower concentration being recovered in distilled water. In comparison to lysozyme recovery with counterionic surfactant, the effect of buffer ionic strengths was also examined when recovering with ethanol (Figure 4.5). These results highlight the weakness of solvent recovery due to the protein solubility issue discussed above.

According to the activity assays and CD spectra in Figure 4.6, lysozyme recovered from phosphate buffer was not denatured at higher buffer salt concentrations, and all samples were

recovered with original lysozyme activities. The CD spectra of each set of lysozyme samples obtained before and after precipitation were the same, but only the post-recovery CD spectra are shown here for comparison. Despite the peaks at 208 and 222nm being clearly recognizable without any significant shift, a relatively larger negativity of the band is seen with the addition of phosphate buffer salts. The samples do not undergo structural transformation, but display only slight structural arrangement. The increase in helix structure content of lysozyme is more prominent in the 100mM phosphate buffer, and suggests that a high concentration buffer salt can weaken lysozyme solvation with water molecules, and reduces the self-association within the lysozyme chain. Consequently, lysozyme is dominated by the more compact secondary structure of an α-helix (Wang *et al.*, 2008).

Another parameter that we looked into was the relative specific activity (RSA), or the specific activity of each lysozyme sample relative to the specific activity of lysozyme in the control solution (20 mM phosphate buffer). Product detail specifies a specific activity of ~50,000 units/mg for the lyophilized lysozyme powder purchased from Sigma. The same batch of pure lysozyme should always exhibit, within a considerable experimental error, the same specific activity values. Specific activity is dependent on enzyme unit definitions stated by the supplier, and is of no relevance to the percent recovery of each lysozyme sample as far as the same assay condition is concerned. Table 4.3 shows that for each lysozyme sample, the specific activity is constant before and after the extraction regardless of the buffer concentration, whereas the RSA varies considerably between different samples. The specific activity of the enzyme recovered could be reduced because of the extremes in salt concentration used, and this leads to a decrease in the RSA with increasing ionic strength (Watanabe *et al.*, 1992).

The findings in this work that lysozyme is inactive in distilled water, is activated by low concentrations of salt and is inhibited by high concentrations of salt in surfactant precipitation agree well with those of Chang and Carr (1971). These workers used lysozyme in buffer solution without addition of surfactant where activation at low salt concentration was explained as closely correlated with a non-specific ionic strength effect, while inhibition at high salt concentrations was closely correlated with cationic concentration and charge. From these data it can be concluded that the reduction in specific activity of the lysozyme recovered can be attributed not to the extraction itself, but to contact with different buffer medium.

Table 4.3 **Specific activity (units/mg) of lysozyme in buffer solutions of different ionic strengths measured before and after surfactant precipitation (recovery with TOMAC, 22.5 mM).**

Specific activity (units/mg) of lysozyme solution			
Protein Buffer	**Initial solution**	**Final solution (recovery)**	**Relative Specific Activity (%)**
De-ionised water	43976 ± 958	44674 ± 1278	86%
Phosphate 20mM	52833 ± 874	51970 ± 1533	control solution
Phosphate 50mM	49520 ± 953	48338 ± 1526	93%
Phosphate 100mM	36365 ±1108	36183 ± 1156	70%

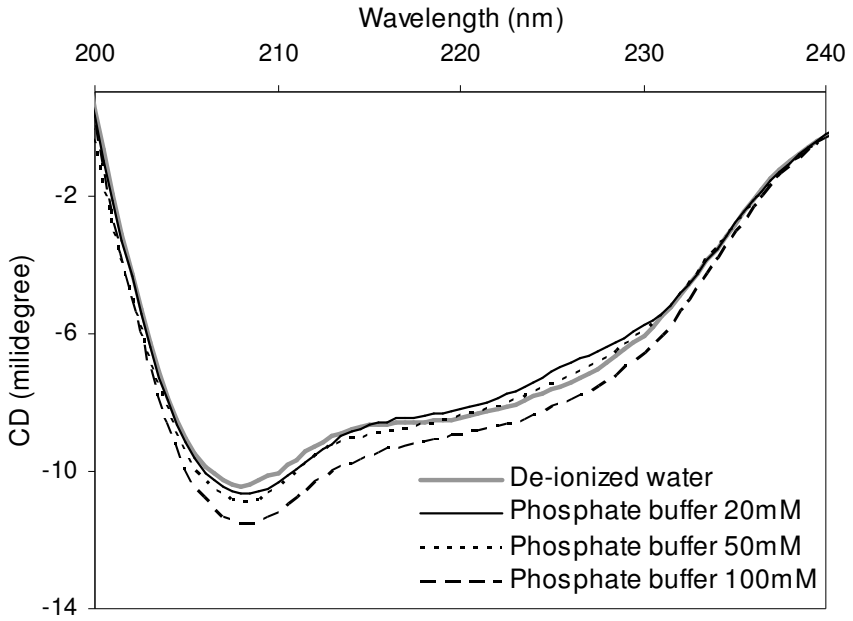

Figure 4.6 **CD spectra of the lysozyme-AOT complex in different buffer solutions recovered with TOMAC. [TOMAC]:[AOT]=2:1.**

4.5.7 EFFECT OF pH ON THE RECOVERY OF LYSOZYME

Comparison of the lysozyme recovery data in ethanol with those from TOMAC reveals some differences at carefully controlled buffer ionic strengths; 1 mL of TOMAC (22.5 mM, dissolved in ethanol) recovered 43% more lysozyme in non-buffered solution and 22±2% more lysozyme in buffered solution (20mM to 100mM), than a large volume (10 mL) of pure ethanol solvent (Figure 4.5). At the optimum buffer ionic strength, 20 mM, we could recover up to 1 g/L lysozyme in TOMAC, whereas only 0.8 g/L in ethanol. We ascribed these discrepancies to lysozyme's solubility in ethanol. To explore this hypothesis, we carried out lysozyme recovery experiments for aqueous solutions of four additional pH values than the pH 6.2 studied; pH 4, pH 5, pH 7 and pH 9, within the pH range (4 - 9) where complete formation of the protein-ligand complex (AOT:lysozyme =16) was determined to take place in the previous chapter. The resultant samples of the adjusted pH were each recovered with two recovery procedures, TOMAC and ethanol.

The data obtained, plotted with the results of recovery of lysozyme from hen egg white solution with acetone (Shin *et al.*, 2003c) in Figure 4.7, are quite remarkable. There is a striking similarity in the decreasing trend of protein recovery in alkaline solution. Specific ionic binding is significantly affected by changes in pH which changes the state of ionization of the amino acid side chains (Jones, 1996). As the pH is increased in alkaline solution, the glutamyl and aspartyl side chains will be fully ionized to interact repulsively towards an anionic surfactant, while cationic sites (lysyl, histidyl and arginyl) will partially lose their positive charge, so that the protein will progressively lose affinity for specific ionic binding with anionic surfactant molecules, and apolar binding occurs. In acid solution, however, Jones (1996) explained that the cationic sites will be fully protonated, while glutamyl and aspartyl residues will be partially protonated, so that favourable binding of an anionic surfactant can be expected. A favourable binding under acidic conditions can only be identified from TOMAC recovery and acetone recovery depicted in Figure 4.7.

Ethanol recovery showed contrary results, in particular unfavourable lysozyme recovery the more acidic the solution. Analysis of the ethanol phase found a large amount of soluble lysozyme, an indication that the cause of low recovery of protein was solubility in the system rather than poor release of protein from the insoluble complex. These results are supported by the literature (Chin *et al.*, 1994); the farther away the pH is from the isoelectric point, the greater the protein solubility in protic solvents. Recovery at pH values furtherest from

lysozyme's isoelectric point of 11, pH 5 and 4, was 50% and 11%, respectively. Although ethanol recovery carried out at a pH closest to the pI, pH 9, has the lowest protein solubility in the solvent, the downsides are the hydrophobic protein interactions and the non-maximal enzyme activity. The data clearly demonstrate that the pH of the protein aqueous solution prior to recovery defines the initial protein-surfactant interactions and their subsequent solubility in ethanol.

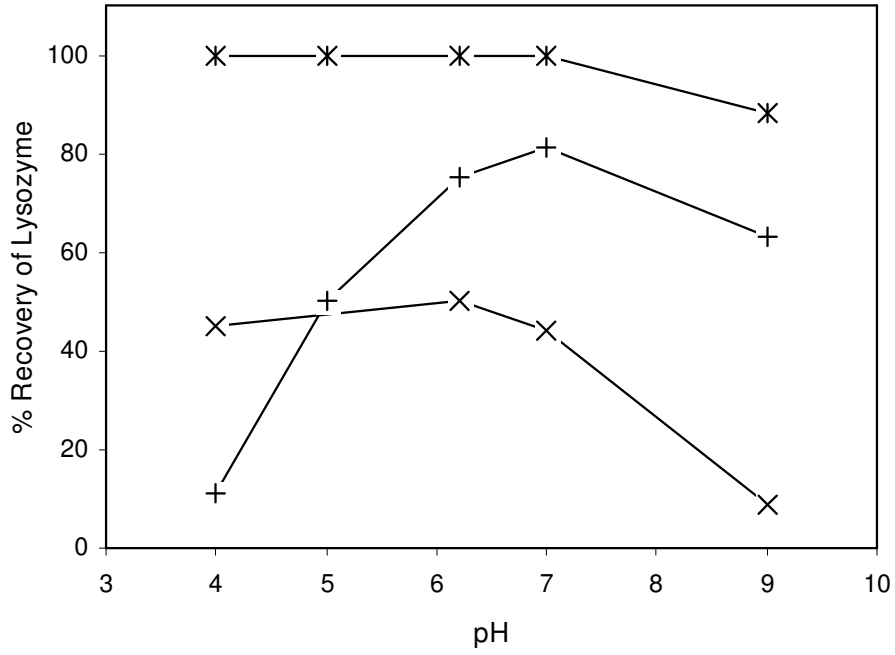

Figure 4.7 **Lysozyme recovery from a 20 mM buffer solution after complete precipitation with 11.3 mM AOT (standard deviation= ±5%): Counterionic surfactant recovery - TOMAC (✳); Solvent recovery - ethanol (+), and acetone (x) (Shin *et al.*, 2003c).**

4.5.8 EFFECT OF TYPE OF CATIONIC SURFACTANT ON THE COUNTERIONIC RECOVERY OF LYSOZYME

The use of cationic surfactant for the recovery of lysozyme was successful using TOMAC. The effect of the counterionic surfactant was investigated by considering the surfactant chain length, the size of the headgroup, and the nature of the counterion for the homologous series of surfactants. DODMAC and DTAB were the cationic surfactants chosen for comparison with TOMAC (in ethanol) recovery. TOMAC and DODMAC are quaternary ammonium chlorides, while DTAB is a quaternary ammonium bromide. These cationic surfactants are different in structure (Figure 4.8); TOMAC (mw=404.16) has three tails of an 8-carbon chain attached to the hydrophilic ionic head; DODMAC (mw=586.50) has two tails of an 18-carbon chain; DTAB (mw=308.34) has only one tail of a 12-carbon chain. DTAB with the least alkyl chain (more hydrophilic) was dissolved in the water phase, while the more alkyl chain surfactants, TOMAC and DODMAC could only be dissolved in the solvent phase (ethanol was used in this experiment) before being added to the recovery phase.

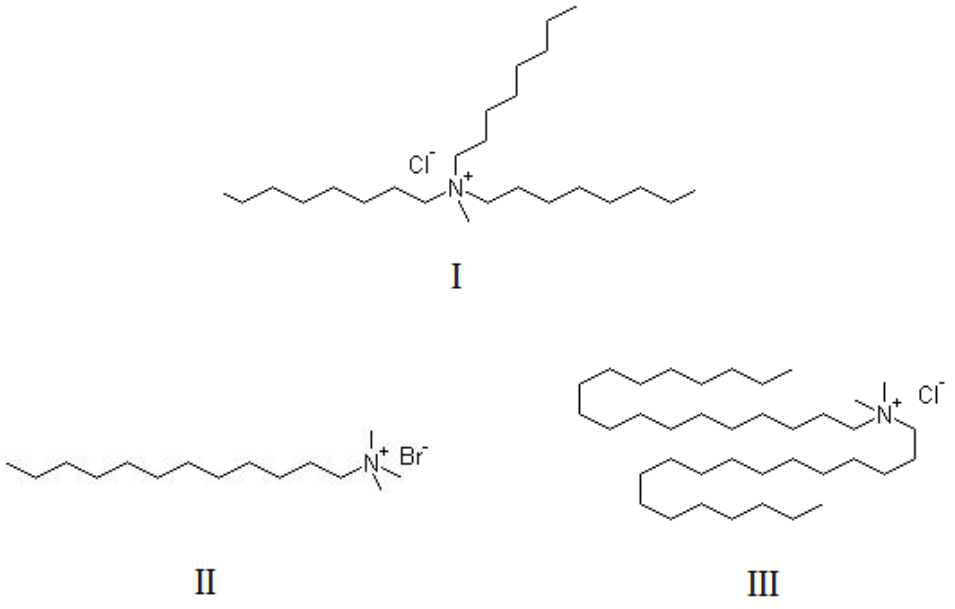

Figure 4.8 **Structures of the different type of cationic surfactants: (I) - trioctylmethylammonium chloride (TOMAC), (II) – dodecyltrimethylammonium bromide (DTAB) and (III) – dimethyldioctadecylammonium chloride (DODMAC)**

The recovery of lysozyme from the lysozyme-AOT complex with DTAB increased with DTAB concentrations, and the protein was fully solubilised at higher concentrations with its original activity (Figure 4.9). The longer the surfactant alkyl chain, the lower the charge density (Kötz *et al.*, 2001). Single-tailed DTAB, which is water soluble, has a higher charge density than TOMAC and was expected to improve the molar ratio of cationic surfactant to anionic surfactant for recovery, but this was not obvious from the results; DTAB complexes with AOT at the same molar ratio as TOMAC (Figure 4.11). The charge of the surfactant counterion (Br^- versus Cl^-) was not expected to influence the recovery because although chlorine is smaller, it still carries a charge of -1, the same as bromide.

Scrutinizing the plot (Figure 4.11), it can be seen that at a low molar ratio (1:1), DTAB (83%) gave a slightly higher recovery of lysozyme than TOMAC (77%). However, there was a subtle decrease in the complexation rate of DTAB-AOT at 1.5:1, where it was overtaken by TOMAC recovery before both cationic surfactants achieved complete complexation with 11.3 mM AOT at 2:1. It was likely that the higher charge density effect of DTAB was at work at lower DTAB concentrations. Literature shows that the addition of bromide surfactants provides a stronger affinity of the counterions to the cationic surfactant heads than chloride surfactants (Abuin *et al.*, 1993; Kim and Shah, 2003; Tofani *et al.*, 2004). We can then presume that the fast exchange of the bromide with the sulphate anion of AOT is prevented by a strong competition of the bromide counterion which is more prominent at the higher the R, thereby slowing the complexation of DTAB-AOT. One concern of DTAB recovery in surfactant precipitation could be that cationic surfactants with bromide counterions have shown an unacceptable large absorption in the far-UV spectrum in the analysis of protein and the complexed surfactant molecules, and hence chloride ion is preferable (Otzen, 2002). Excess DTAB in the recovered phase might pose a difficulty when analysing the far-UV CD spectra of recovered protein.

The lysozyme was also released from the lysozyme-AOT complex with DODMAC (Figure 4.10) through the formation of a DODMAC-AOT dimer. The molar ratio of DODMAC to AOT for the recovery to peak was 2:1. The DODMAC recovery plot did not resemble those of TOMAC and DTAB. Besides only reaching a maximum 81% recovery of lysozyme, a greater amount of DODMAC reduced the recovery of lysozyme significantly (54% at 39.4 mM). The original activity of lysozyme was recovered despite the lower recovery efficiencies, and lysozyme was found to have no interaction with DODMAC under the

experimental conditions used. Evidently, protein was neither precipitated nor denatured by DODMAC.

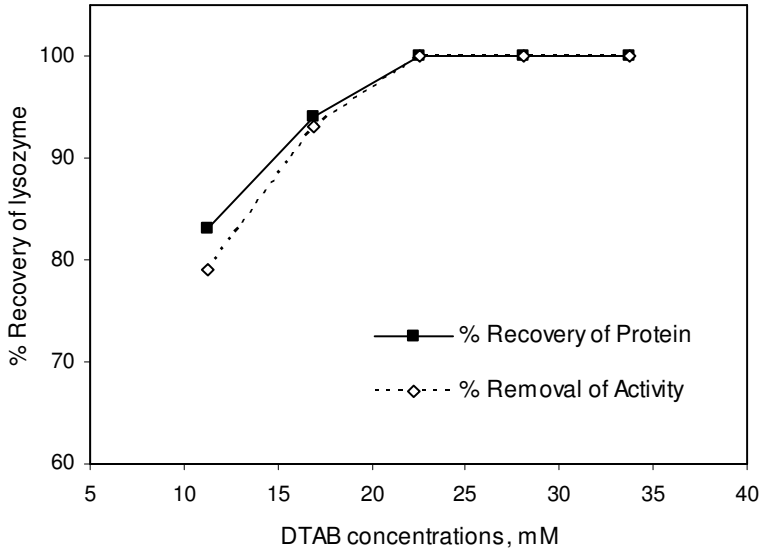

Figure 4.9 **Percent recovery of lysozyme from the precipitated lysozyme-AOT complex for an increasing DTAB concentrations (11.3 – 33.7 mM). (SD<±5%).**

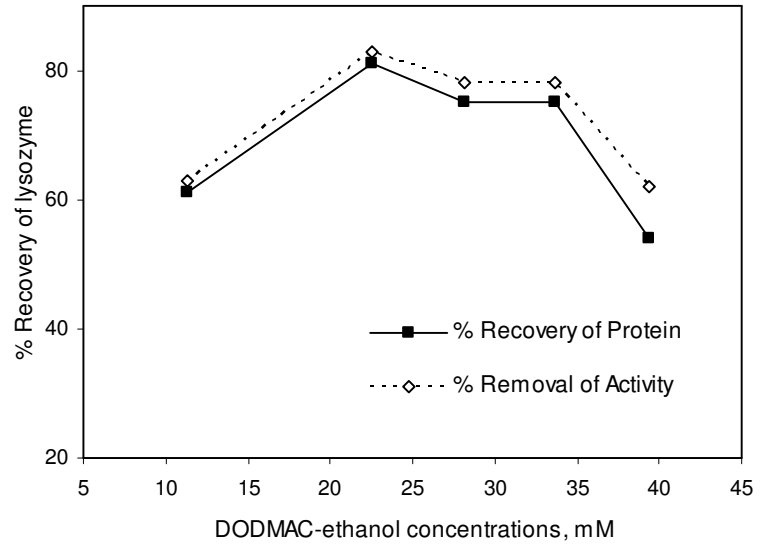

Figure 4.10 **Percent recovery of lysozyme from the precipitated lysozyme-AOT complex for an increasing DODMAC/ethanol concentrations (11.3 – 39.4 mM). (SD<±5%).**

The DODMAC molecule has the highest regions of low charge density (the nonpolar alkyl side chains), and it is more hydrophobic than the rest of the cationic surfactant studied (Tonova and Lazarova, 2008). The probable explanation for low DODMAC recovery was that the lower charge density of DODMAC hindered the complete formation of the DODMAC-AOT complex, while the increasing hydrophobicity of the long chain surfactant drove the formation of nonpolar structures with the protein-AOT complexes which inhibited the further release of lysozyme. Hence, the charge density and hydrophobic interactions of cationic surfactants played important roles in the efficiency of counterionic recovery in surfactant precipitation.

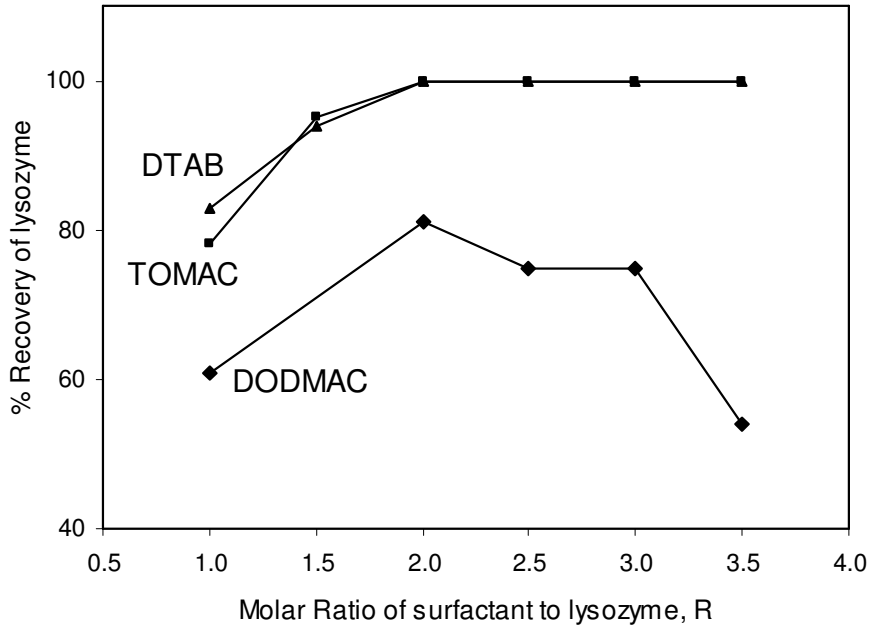

Figure 4.11 **Lysozyme recovery as a function of the molar ratio, R, of a counterionic surfactant: TOMAC recovery, DTAB recovery, and DODMAC recovery.**

4.6 CONCLUSIONS

The recovery of lysozyme from an aqueous solution containing precipitated lysozyme-AOT complexes was compared using different solvents. Ethanol, methanol and solvent mixtures dissolved the surfactant precipitate and recovered lysozyme as an insoluble protein precipitate. Recovery efficiency and protein stability varied with the type of solvent used. A new improved method of protein recovery in surfactant precipitation was proposed. The method uses the direct precipitation of lysozyme with AOT followed by the addition of a counterionic surfactant, TOMAC, to dissociate the lysozyme-AOT complex by binding to AOT, and lysozyme is released back into an aqueous solution. Strong electrostatic interactions between the oppositely charged AOT and TOMAC molecules were identified as the driver of the recovery process.

Experimental results demonstrated successful recovery of lysozyme with TOMAC/ethanol, TOMAC/isooctane, DTAB and DODMAC/ethanol. Under the conditions studied, all counterionic surfactant solutions gave full recovery efficiencies except DODMAC/ethanol, with TOMAC/isooctane requiring a higher concentration to achieve complete release of lysozyme. The lysozyme was recovered free of surfactant as well as retaining its original activity in the final aqueous phase and its native structure as observed in CD spectra. Specific activity studies showed that counterionic surfactant extraction does not alter the biological activity of the enzyme. It is postulated that 2:1 complexes of TOMAC and AOT were formed in the aqueous phase.

There is a marked dependence of protein recovery from surfactant precipitation on the type of recovery solution from which the protein-AOT complex was suspended. Protein solubility affects the recovery if a solvent phase is used, while the pH at which recovery took place and the type of cationic surfactant affect the recovery if a counterionic surfactant is employed. The addition of potassium phosphate buffer to the lysozyme solution improves recovery, however, the buffer salt concentration should be kept at the optimum concentration (20 mM) as increasing strengths reduce activity recovery.

The use of a counterionic surfactant has clear advantages over the use of solvents in recovery, and this study showed that the secondary structure of the protein was preserved over a long period of time in the presence of TOMAC without being denatured. Among the counterionic surfactants studied, TOMAC/ethanol recovery was preferred because the recovery phase was

more miscible, and the amount of counterionic surfactant required per mole of purified protein was lower than TOMAC/isooctane. The recovery of lysozyme was higher than DODMAC/ethanol, and it did not interfere with protein absorption in the far-UV spectrum as anticipated for DTAB. The high specificity of counterionic surfactant interactions towards AOT offers the possibility of using surfactant precipitation extraction for the rapid concentration of very dilute protein solutions.

Research has advanced compared to Shin's (2004a); solvent recovery was improved using ethanol and did not promote protein unfolding after prolonged exposure to the solvent, while a new counterionic surfactant recovery process was proved to be more efficient. In summary, we have developed a surfactant protein purification method (precipitation and recovery processes) which could have a substantial impact on bioprocessing because it has; potentially low overall costs, it is simple, achieves excellent product recovery and maintains virtually all the enzyme bioactivity, all of which are prerequisites for a feasible alternative to current bioseparation techniques. This novel method of protein separation has possible applications in the isolation of proteins from complex mixtures and industrial broth, as well as being incorporated with other downstream processes such as chromatography, membrane separation and affinity based separation. In further work in this thesis we will be investigating the effectiveness and biocompatibility of surfactants with proteins of varying characteristics (molecular weight, isoelectric point, hydrophilicity).

CHAPTER 5 MECHANISM OF PROTEIN EXTRACTION IN SURFACTANT PRECIPITATION*

5.1 INTRODUCTION

The protein-surfactant interactions in surfactant precipitation for separating proteins in an aqueous system were explored in Chapter 3. Proteins exhibited a high binding capacity for surfactant molecules and precipitated as a surfactant-protein complex; electrostatic as well as hydrophobic forces are involved in precipitation. Since the surfactant, which is an amphiphilic compound, mediates the purification, two properties of the protein surface, namely its charge and hydrophobicity, should play important roles in the separation of proteins. The surface of most native proteins have a significant number of accessible charged and non-polar residues, and this affects its interaction with the environment and its conformational changes. Many studies on a variety of protein complexes have demonstrated the importance of surface characteristics of proteins (Pettit *et al.*, 2007; Sael *et al.*, 2008) on the molecule's interactions (Berggren *et al.*, 2002; Goldenberg and Steinberg, 2010; Spelzini *et al.*, 2005; Xu *et al.*, 2005).

Protein surface charge is a key determinant of protein function (Goldenberg and Steinberg, 2010), yet protein structural stability is heavily dependent on hydrophobic interactions in surfactant extraction (Chapter 4). These two parameters were explored to find out which predicted the ability of proteins to complex with surfactants more accurately. Overall surface charge (q) is given based on the buffered pH in the experiment (Horn and Heuck, 1983; Kuramitsu and Hamaguchi, 1980). Average surface hydrophobicity (Φ) can be estimated from knowledge of the three dimensional structure of a protein, by taking into account the hydrophobic contributions of the amino acids (content of nonpolar residues) on the protein surface (Lienqueo *et al.*, 2002). The Φ of proteins is evaluated using hydrophobic interactive chromatography (HIC) (Hearn, 2002; Rao et al., 2006) and aqueous two-phase systems

* Parts of the work presented in this chapter have been submitted for publication;
Cheng, S.I. and Stuckey, D.C. 2011. Mechanism of protein extraction in surfactant precipitation systems. Biochemical Engineering.

(ATPS) (Franco *et al.*, 1996), which retain and partition proteins by differences in the hydrophobic character of their surfaces. A high retention time in HIC implies a big hydrophobic contact area and thus a big hydrophobic patch accessible to the hydrophobic matrix. The relative elution order for native proteins in HIC is cytochrome c, ribonuclease A and lysozyme (Kato *et al.*, 2002; Rao *et al.*, 2006); this indicates that the order of the surface hydrophobicity of the proteins is $\Phi_{lysozyme} > \Phi_{ribonucleaseA} > \Phi_{cytochromec}$. The surface hydrophobicity scale was derived by Yano *et al.* (1994) from log *K*, the partition coefficient of proteins, in ATPS; the smaller the value of Φ (even negative), the more hydrophobic the protein is.

Surface charge and hydrophobicity can be homogeneously or heterogeneously distributed on a protein surface, and hence it is reasonable to consider a surface charge and hydrophobicity distribution. Charge distribution on a protein surface, expressed as *%S*, was calculated from the electric moments of randomly distributed charged groups of a protein over a set of surface points (Barlow and Thornton, 1986). Hydrophobic imbalance (*HI*) correlated to the surface hydrophobicity distribution, and represented the displacement of the superficial geometric centre of the protein with the hydrophobic effect of each amino acid considered (Salgado *et al.*, 2006). The smaller the value of *HI* (even negative), the less distributed the surface hydrophobicity of the protein is.

Acknowledging the importance of protein surface analysis, researchers have explored surface properties associated with protein function, especially relating to protein-ligand and protein-protein interactions, (Pettit *et al.*, 2007; Rigden *et al.*, 2009; Via *et al.*, 2000). Protein surface-dependent systems have benefitted from the surface charge of proteins for solubilisation in reverse micellar extraction (Cassin *et al.*, 1994), and from surface hydrophobicity allowing partitioning selectivity into the micellar phase in cloud point extraction (Terstappen *et al.*, 1993).

We wanted to understand the influence these surface properties had on protein separation using surfactant precipitation. We analysed the effect of surfactant precipitation on cytochrome c and ribonuclease A individually, before examining the effect on the two proteins with lysozyme in relation to their surface properties. Lysozyme, cytochrome c and ribonuclease A are hydrophilic proteins (Kato *et al.*, 2002), and polar groups on the proteins are prevalent in protein interactions and likely to influence their binding with surfactant. As a comparison, trypsin and α-chymotrypsin, a hydrophobic group of proteins (Kato *et al.*, 2002),

were also subjected to this method of extraction where we mainly studied the hydrophobicity effect which dominated the weaker polar charge. Some of these proteins have been shown to precipitate by the direct addition of AOT in previous studies (Shin *et al.*, 2003b; Shin *et al.*, 2004a; Shin *et al.*, 2004b). Our other aim in this chapter was to explore protein structure and stability during protein-surfactant binding in surfactant extraction as we had done with lysozyme in Chapter 4.

5.2 MATERIALS AND METHODS

Materials: The experiments were carried out using lyophilized powders of cytochrome c from equine heart (Ferricytochrome c), ribonuclease A (EC 3.1.27.5, RNase A) from bovine pancreas, trypsin from porcine pancreas (EC 3.4.21.4, Peptidyl peptide hydrolase), and α-chymotrypsin from bovine pancreas (EC 3.4.21.1, Peptidyl peptide hydrolase); these powders were all purchased from Sigma (USA). For enzymatic measurements, yeast ribonucleic acid (RNA), β-nicotinamide adenine dinucleotide (β-NADH), *cytochrome c reductase (CCR)*, Na-benzyol-L-arginine ethyl ester (BAEE) and N-benzoyl-L-tyrosine ethyl ester (BTEE) were purchased from Sigma. Sodium acetate, potassium bicarbonate ($KHCO_3$), glycylglycine, sodium phosphate monobasic (NaH_2PO_4), tris(hydroxymethyl)aminomethane (trizma base) and calcium chloride ($CaCl_2$) from Sigma, USA, were used to make a buffer when preparing substrate solution. Reagent grade acetic acid and ammonium hydroxide (NH_4OH) were also purchased from Sigma.

5.2.1 SINGLE PROTEIN IN BUFFER SYSTEM

An initial aqueous solution containing 0.08 mM of cytochrome c and ribonuclease A were prepared in a 20 mM potassium phosphate buffer solution and the pH adjusted to 6.2. Preparation of trypsin and α-chymotrypsin were slightly different; self digestion of trypsin and α-chymotrypsin, if not inhibited, can cause inactivation and proteolytic degradation of the enzyme which is unfavourable to any purification. Furthermore, autolysis at room temperature was severe enough to inactivate trypsin appreciably in 2 hours, however, calcium ions are known to stabilize these proteins against autolytic attack (Vestling *et al.*, 1990). Hence, trypsin and α-chymotrypsin stock solution (1.2 mM) were first prepared in pH 3 buffer solution containing 1 mM calcium chloride. 1 mL of this enzyme stock solution was diluted to 0.08 mM with 20 mM phosphate buffer at pH 6.2 just before the experiment. Extra

care was taken not to exceed the $CaCl_2$ concentrations to prevent the formation of calcium phosphate (Simpson, 2004). The precipitation of protein was carried out like that of lysozyme, in the range of R within 1 mL of 1.5 to 11 g/L of AOT. After centrifugation, the supernatant was analyzed for protein concentration and stability when binding with the various molar ratios of surfactant, while the protein-AOT complex was collected and re-dissolved in fresh buffer for recovery with TOMAC (22.5 mM dissolved in ethanol). AOT free protein released into solution was analysed for both concentration and conformation.

5.3 ANALYTICAL TECHNIQUES

The CV of the extraction process for concentration and activity measurements of the proteins were within ±5%.

5.3.1 RIBONUCLEASE A ACTIVITY ASSAY

The enzymatic activity of ribonuclease A was determined by the method of Kunitz (1946), with 1.0 mg/ml of yeast ribonucleic acid (RNA) in 100 mM sodium acetate buffer at pH 5.0, 25˚C. The hydrolysis of RNA on digestion with ribonuclease A is accompanied by a decrease in absorbance at 300 nm. An RNA in acetate buffer volume of 1.5 mL was pipetted into two sample cuvettes. 1.3 mL of deionised water and 0.2 mL of ribonuclease A sample (diluted to 0.2-0.3 Kunitz units/mL) was added into the first sample cuvette then placed into the UV cell holder and the timer started. The decrease in the turbidity of the substrate was monitored at 1-minute intervals over a period of 10 minutes, and the A_{300nm} values were taken as the rate determination (E_t). The second sample cuvette was added with 1.5 mL of the same enzyme sample (diluted to 0.5-0.75 Kunitz units/mL) and incubated at 25˚C for 120 minutes. A_{300nm} was recorded until a constant reading and the final value was the total hydrolysis determination (E_f). A graph of ln A_{300nm} (E_t - E_f) as a function of time was plotted, and the rate of enzyme action ($\Delta A_{300nm/min}$) in the substrate reaction mixture was measured from the slope of the curve. The shift in the absorption spectrum produced by acid-soluble oligonucleotides from the RNA substrate in the reaction is directly proportional to the enzymatic activity of ribonuclease A. Specific activity values of ribonuclease A in the samples were calculated as Kunitz units/mg protein in the equation below. Kunitz is defined as a unit of activity for the amount of enzyme capable of causing a 100% per minute decrease in the A_{300nm} (E_t - E_f) of a solution at the specified assay conditions.

$$\text{Kunitz units/mg} = \frac{[\Delta \ln A_{300nm/min}(E_t - E_f)](3\text{ ml of assay})(df)}{\text{mg lysozyme in the reaction mixture}}\ (1U) \qquad (5.1)$$

5.3.2 CYTOCHROME C ACTIVITY ASSAY

This activity was assayed by a modification of the procedure of Mahler (1955) essentially used for enzymatic study of *cytochrome c reductase* (*CCR*). This enzyme belongs to the family of oxidoreductases, specifically those acting on diphenols and related substances as a donor, with a cytochrome c as an acceptor.

$$\text{Cytochrome c} + \beta\text{-NADH} \overset{CCR}{\leftrightarrows} \text{Cytochrome c (reduced)} + \beta\text{-NAD}$$

The assay was carried out at 25˚C by measuring the reduction of cytochrome c by *CCR* at 550 nm. A solution of 7.05 mM of β-NADH was prepared in a 300 mM glycylglycine buffer, and 0.05 units/ml of *CCR* enzyme solution was prepared in 20 mM potassium bicarbonate ($KHCO_3$) buffer. All the buffers were at pH 8.5. Reaction mixtures containing 0.1 mL of 0-1% (w/v) aqueous cytochrome c sample, 0.1 mL of β-NADH, 0.2 ml glycylglycine buffer and 2.5 ml of deionised water were pipetted into the reference and sample cuvettes. Immediately after adding 100 µl of *CCR* solution to the sample cuvette, both the cuvettes were placed into the UV cell holder and the increase in A_{550nm} for 5 minutes at 15-second intervals was recorded. *CCR* was omitted from the blank. A graph of absorbance (A_{550nm}) as a function of time was plotted, and the rate of enzyme action ($\Delta A_{550nm/min}$) in the reaction mixture was measured from the maximum linear portion of the curve. One activity unit is defined as the amount of enzyme that reduced 1 µmole of cytochrome c per minute ($\Delta A_{550nm/min}$), following a rate of 21.0 $m^{-1}M^{-1}$ as the difference in extinction coefficient (between cytochrome c and the reaction product, reduced cytochrome c) under the assay conditions described in the experimental procedure. This is the actual activity of *CCR,* and cytochrome c activity was quantified and expressed in terms of the enzyme reduction as an indirect measure of bioavailability of cytochrome c in the samples. A standard curve for *CCR* activity was prepared using different concentrations of cytochrome c. The specific activity (units/mg) can be calculated as:

$$\text{Units/mg} = \frac{(\Delta A_{550nm/min}\ \text{Sample} - \Delta A_{550nm/min}\ \text{Blank})(3\text{ ml of assay})(df)}{\text{mg } CCR \text{ in the reaction mixture}} \left(\frac{1U}{21.0}\right) \qquad (5.2)$$

5.3.3 TRYPSIN ACTIVITY ASSAY

Trypsin activity was assayed using a spectrophotometric assay at 25˚C (Bergmeyer *et al.*, 1974) which measures trypsin digestion of Na-benzyol-L-arginine ethyl ester (BAEE). A substrate solution of 0.25 mM BAEE was prepared in 67 mM sodium phosphate monobasic (NaH_2PO_4) buffer solution at pH 7.6. A BAEE volume of 3.0 mL was pipetted into the reference and sample cuvettes. Immediately after adding 200 µl of the trypsin sample solution to the sample cuvette, the absorbance was measured at 253 nm at 15-second intervals over 5 minutes against a blank containing the identical components except buffer in the place of trypsin. A graph of absorbance (A_{253nm}) as a function of time was plotted, and the rate of enzyme action ($\Delta A_{253nm/min}$) in the substrate reaction mixture was measured from the linear portion of the curve. Dilution of samples to give 350-700 BAEE units/mL of trypsin was necessary for the activity assay, and correction for dilution was made in the calculation of BAEE units of enzyme activity. One BAEE activity unit of trypsin produces an initial linear increase in absorbance of 0.001 per minute ($\Delta A_{450nm/min}$) in 3.2 mL of reaction mixture with BAEE as a substrate under the assay conditions described. Specific activity (units/mg) of trypsin in the sample is calculated as:

$$\text{BAEE units/mg} = \frac{(\Delta A_{253nm/min}\ \text{Sample} - \Delta A_{253nm/min}\ \text{Blank})(df)}{\text{mg trypsin in the reaction mixture}} \left(\frac{1U}{0.001}\right) \qquad (5.3)$$

5.3.4 α-CHYMOTRYPSIN ACTIVITY ASSAY

The α-chymotrypsin activity was assayed using a spectrophotometric assay at 25˚C (Wirnt, 1974) which measures the hydrolysis of N-benzoyl-L-tyrosine ethyl ester (BTEE). A substrate solution of 1.18 mM BTEE was prepared in 63% (v/v) methanol, and a 80 mM Tris-HCl buffer solution was prepared at pH 7.8. Reaction mixtures containing 1.40 mL of BTEE, 1.42 ml of Tris-HCl buffer and 0.08 ml of 2 M calcium chloride ($CaCl_2$) solution were pipetted into the reference and sample cuvettes. Immediately after adding 100 µl of α-chymotrypsin sample solution into the sample cuvette, absorbance at 256 nm was measured at 15-second intervals over 5 minutes against a blank containing the identical components except α-chymotrypsin. A graph of absorbance (A_{256nm}) as a function of time was plotted, and the rate of enzyme action ($\Delta A_{256nm/min}$) in the substrate reaction mixture was measured from

the linear portion of the curve. Dilution of samples to give 2-5 BTEE units/mL of α-chymotrypsin was necessary for the activity assay, and correction for dilution was made in the calculation of BTEE units of enzyme activity. One activity unit is defined as the amount of enzyme that hydrolyzes 1 μmole of BTEE per minute ($\Delta A_{256nm/min}$), following a rate of 0.964 $m^{-1}M^{-1}$ as the extinction coefficient of the reaction product, N-Benzoyl-L-Tyrosine at 256 nm, under the assay conditions described in the experimental procedure. Specific activity (units/mg) of α-chymotrypsin in the sample was calculated as:

$$\text{BTEE units/mg} = \frac{(\Delta A_{256nm/min}\ \text{Sample} - \Delta A_{256nm/min}\ \text{Blank})(3\ \text{ml of assay})(\text{df})}{\text{mg α-chymotrypsin in the reaction mixture}} \left(\frac{1U}{0.964}\right) \qquad (5.4)$$

5.4 RESULTS AND DISCUSSION

5.4.1 SEPARATION OF PROTEIN USING SURFACTANT

The research which examined protein-surfactant interactions versus protein stability with lysozyme was extended to look at cytochrome c, ribonuclease A, trypsin and α-chymotrypsin. These proteins (pI > 8) were buffered to pH 6.2. In these experiments, initially all the proteins were tested with the direct addition of TOMAC and there was no observable interaction between the molecules under the experimental conditions. Positively charged proteins were found to bind efficiently to the anionic surfactant, but not to the TOMAC used in its recovery. As used in the recovery of lysozyme, formation of nonpolar ion pairs between the oppositely charged surfactant molecules leads to the resolubilisation of the protein into solution, and the percent precipitation and recovery of final protein samples were calculated.

When two proteins with similar pIs are present in a mixture, it is not a straightforward process to determine the selectivity of extraction. In this work, we will discuss the effect of surface properties in terms of surface charge and surface hydrophobicity on protein separation from binary mixtures. The properties of the proteins studied are summarized in Table 5.1.

Table 5.1 Data on the properties of proteins used in surfactant precipitation.

Protein	MW[a] (kDa)	pI[a]	q	%S[e]	Φ[f] (kJ.mol^{-1})	HI[g]
Lysozyme	14.3	11.4	+9[b]	97	-40	-1.00
Cytochrome c	12.4	10.5	+10[c]	54	-99	-1.30
Ribonuclease A	13.7	9.6	+5[c]	24	-62	-1.25
Trypsin	23.8	10.8	+7[c]	25	-	-
α-Chymotrypsin	25.0	8.8	+4[c]	90	-	-
Trypsin inhibitor	27.0	4.1	+11[d]	29	-	-
*Haemoglobin	64.5	6.8	-	80	-210	-

[a]Sigma-Aldrich; [b](Kuramitsu and Hamaguchi, 1980); [c](Horn and Heuck, 1983); [d](Kopaciewicz *et al.*, 1983); [e](Barlow and Thornton, 1986); [f](Yano *et al.*, 1994); [g](Salgado *et al.*, 2006); *(Shin *et al.*, 2004a).

5.4.2 SURFACTANT PRECIPITATION OF CYTOCHROME C

Cytochrome c samples, analysed by HPLC after precipitation with AOT at 1.5 g/L to 11g/L, were retained and recovered with a retention time of 5.2 min at 40% B gradient elution (Figure 5.1). Gradient and retention time (t_R) for the protein is given in the figure captions. Peaks that appeared at the start of the chromatogram, before the protein was eluted, were the unretained substances from the sample solvent (e.g. buffer salts). Cytochrome c decreased in filtrate samples when it reacted with AOT at concentrations of 1.5 g/L (Figure 5.1b) to 6 g/L (Figure 5.1e). The precipitation of cytochrome c from the initial protein solution (Figure 5.1a) was; 32% at R=4, 66% at R=8, and 93% at R=14, while complete recovery was achieved at R=17. The originally added cytochrome c activity was recovered in the activity assay, and CD analysis showed that there was no significant conformational change in the protein after counterionic extraction using TOMAC (Figure 5.3). The secondary structure contents were 37% α-helix, 2% β-sheet, 21% β-turn and 40% random coil, identical to the native cytochrome c (±0.5%) in AOT up to R=17 (Figure 5.2).

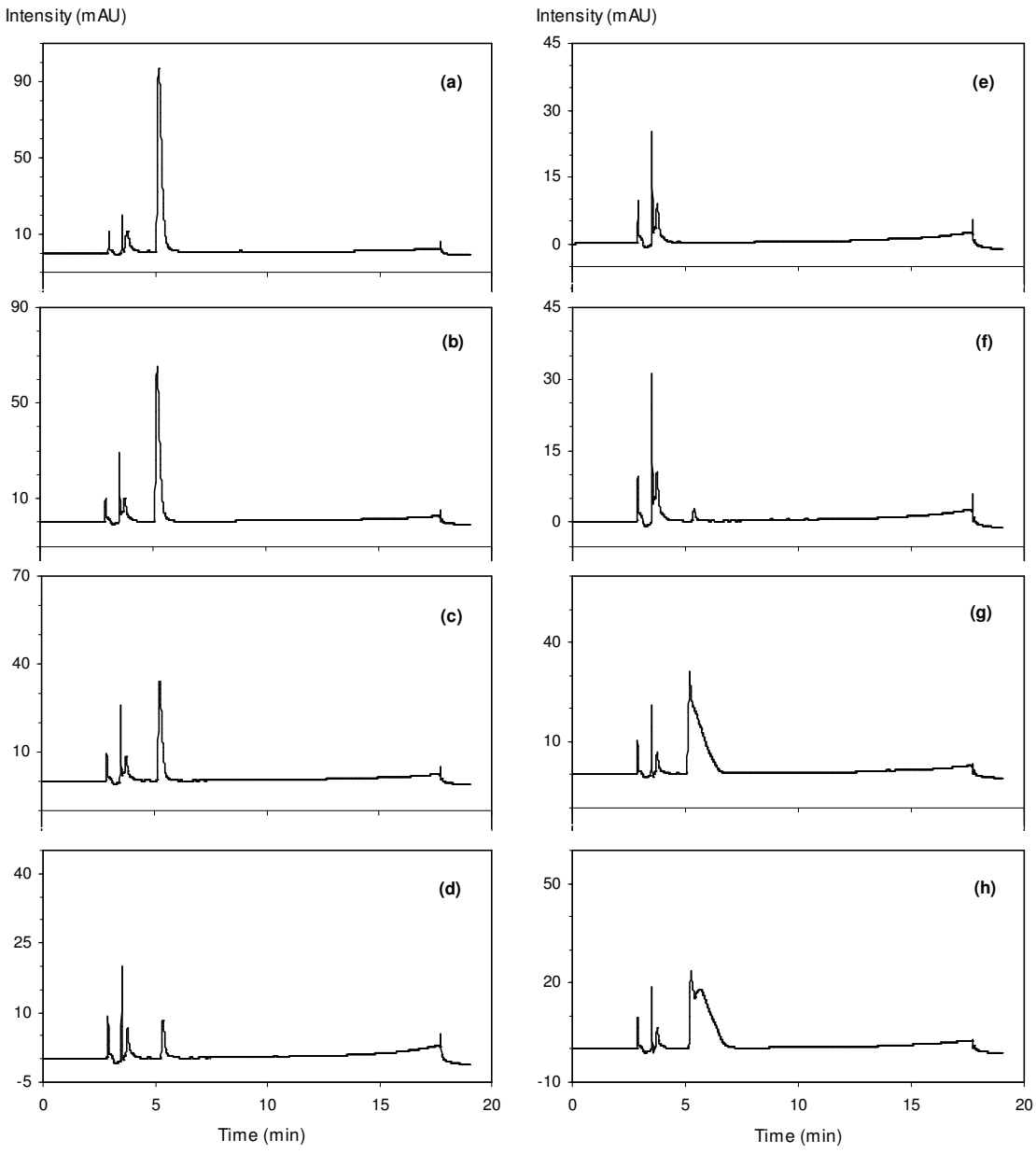

Figure 5.1 **Chromatogram of cytochrome c samples: (a) Initial cytochrome c solution without AOT (t_R = 5.2min); Soluble cytochrome c in the aqueous solution after addition of AOT at (b) R=4, (c) R=8, (d) R=14, (e) R=17, (f) R=19, (g) R=25, and (h) R=31. Gradients were run from 0-90% B in 15 min at a flowrate of 1 mL/min.**

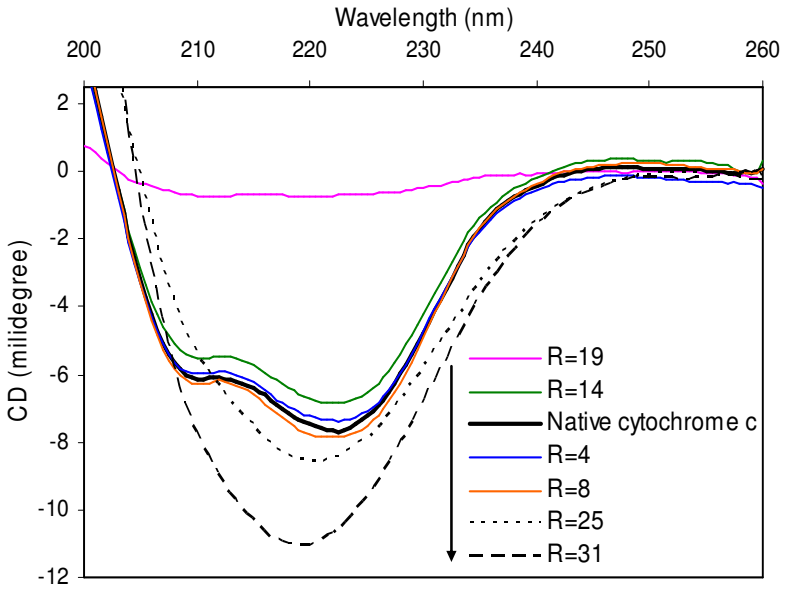

Figure 5.2 Far-UV CD spectra of cytochrome c in phosphate buffer (20mM), pH 6.2: (a) Initial cytochrome c solution without AOT diluted to 0.1 g/L; final aqueous phase left after precipitation of cytochrome c at different AOT to cytochrome c ratios (R=4 at 0.1 g/L, R=8 at 0.1 g/L, R=14 at 0.07 g/L, R=19 at 0.03 g/L, R=25 at 0.1 g/L and R=31 at 0.1 g/L).

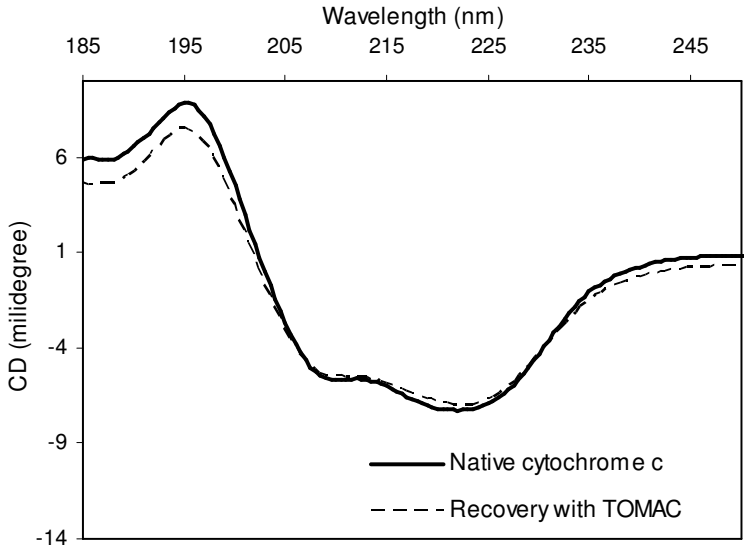

Figure 5.3 CD spectra of the cytochrome c sample (R=17) in phosphate buffer (20 mM), pH 6.2, recovered with TOMAC.

Increasing the AOT concentration to R=19 reduced precipitation efficiency by 3% (Figure 5.1f). A "Student t-test" showed the removal of cytochrome c was significantly different (95% confidence interval) at R=17 (99.5±0.6%) and R=19 (96.6±1.6%), and hence indicates that some supernatant cytochrome c was inactivated at R=19. The inactivated cytochrome c reported for the non-precipitated cytochrome c at R=19 could have been caused by binding with more surfactant thereby exceeding the saturation point of the protein binding sites, and this inhibited its enzymatic activity. However, there was no gross denaturation in its structure, and therefore the protein eluted as an original peak. In solutions of AOT at 9 g/L (Figure 5.1g) and 11 g/L (Figure 5.1h), cytochrome c was identified as well as another protein with a higher retention time of 5.6 min in the sample, and this appears to be the unfolded cytochrome c.

The cytochrome c peak recovered in sample R=31 was lower than those in sample R=25 because inactive protein was denatured and formed another peak with increases in AOT concentration added. The shape of the CD spectra revealed that denatured protein was dominated by β-sheet structures and lacked helical structures. This was reflected by the single negative peak at around 210-220 nm which depicting a β-rich protein (Sreerama *et al.*, 1999). The β-sheet increases at R=25 (7%) and R=31 (10%), the β-turn increases to 23%. The AOT unfolded cytochrome c shows a partial decrease in helix content (32%), indicating that a significant amount of helical structure remains folded in contrast to a complete loss of helical structure in GnCl denatured cytochrome c (Das *et al.*, 1998).

5.4.3 SURFACTANT PRECIPITATION OF RIBONUCLEASE A

Injecting ribonuclease A into the RP-HPLC column resulted in a retention time of 8.7 min at 28% B gradient elution (Figure 5.4). From the chromatogram, ribonuclease A decreased at AOT concentrations of 1.5 g/L (Figure 5.4b) to 7 g/L (Figure 5.4e). Precipitation of ribonuclease A from the initial protein solution (Figure 5.4a) was 11% at R=5, 25% at R=9, 88% at R=15, and full precipitation at R=22. Ribonuclease A is an α/β protein with more sheet than helical residues; 21% α-helix, 38% β-sheet, 13% β-turn and 28% random coil. CD spectra indicated that ribonuclease A retained its secondary structure (±0.5%) with TOMAC recovery (Figure 5.6). The recovered activity corresponded to that expected from the amount of protein recovered.

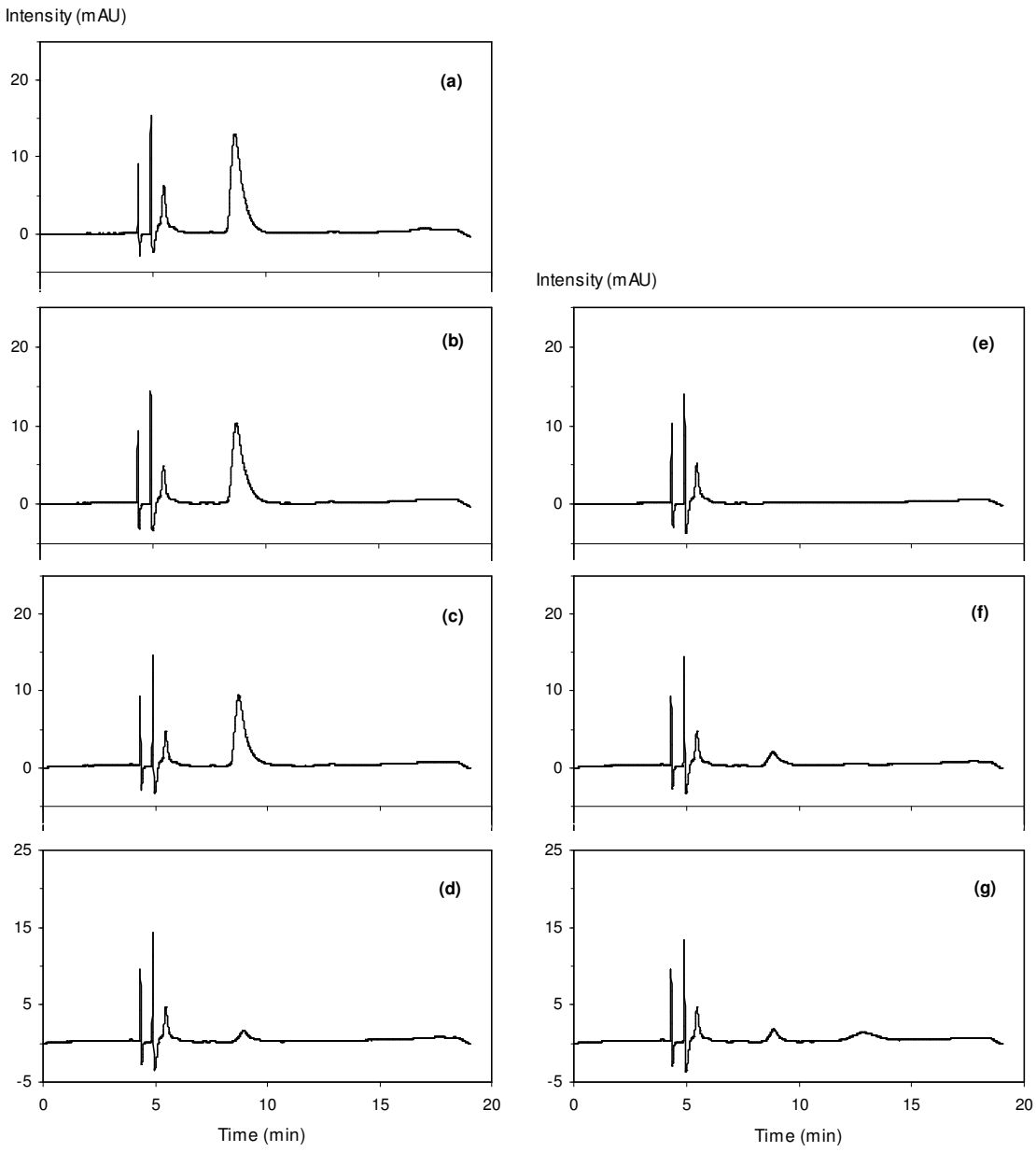

Figure 5.4 **Chromatogram of ribonuclease A samples: (a) Initial ribonuclease A solution without AOT (t_R = 8.7min); Soluble ribonuclease A in the aqueous solution after addition of AOT at (b) R=5, (c) R=9, (d) R=15, (e) R=22, (f) R=28, and (g) R=34. Gradients were 0-35% B in 15 min at 0.65 mL/min.**

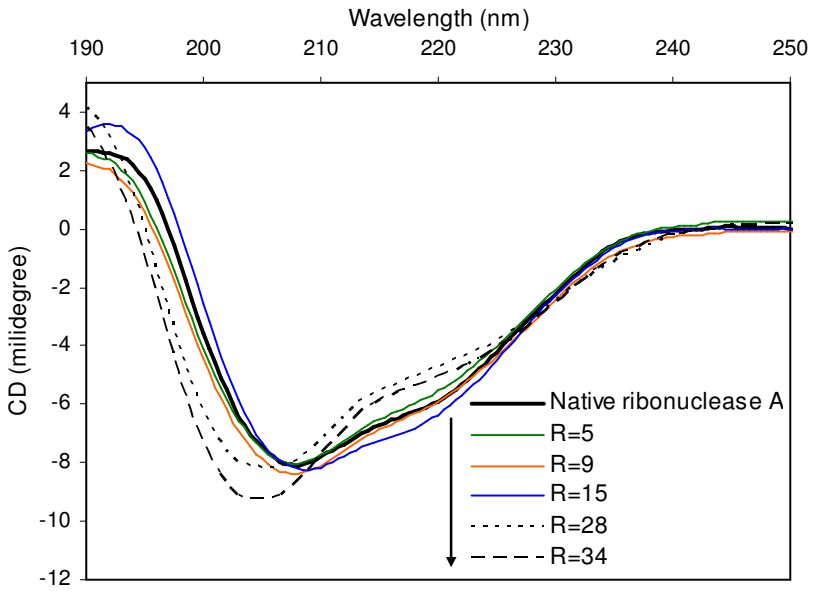

Figure 5.5 **Far-UV CD spectra of ribonuclease A in phosphate buffer (20mM), pH 6.2: (a) Initial ribonuclease A solution without AOT; final aqueous phase left after precipitation of ribonuclease A at different AOT to ribonuclease A ratios (R=5, R=9, R=15, R=28 and R=34). All samples diluted to 0.1 g/L.**

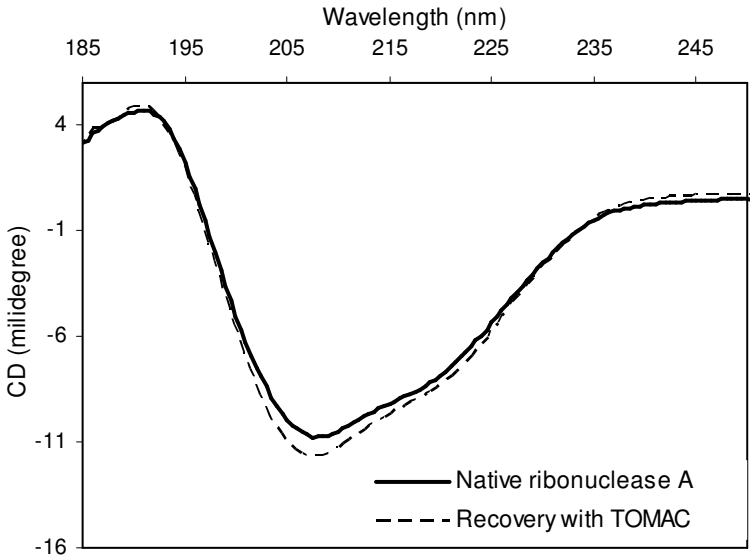

Figure 5.6 **CD spectra of the ribonuclease A sample (R=22), in phosphate buffer (20 mM), pH 6.2, recovered with TOMAC.**

Following optimum recovery, the sample with R=28 produced 5% inactive ribonuclease A in the filtrate, while R=34 resulted in two protein components; 3% inactive ribonuclease A, and its denatured component being retained at a retention time of 12.8 min. The longer retention time of the unfolded protein during chromatography can be explained in the same way as lysozyme. CD spectra of these two samples showed skewed regions due to denaturation compared to samples at lower AOT concentrations (Figure 5.5). The α-helix fraction decreases from R=28 (19%) to R=34 (15%), and the proportion of random coil increases from 30% to 33% (these results are statistically different at the 95% confidence interval). It seemed that at higher AOT concentrations, the main transition in the secondary structure was the unwinding of helices as the far-UV spectra changes to a shape more characteristic of a random coil structure. The structure has some similarity to ribonuclease A in thermal unfolding (Stelea *et al.*, 2001), and in this paper more information regarding the unravelling of helices resulting in such spectra are shown.

5.4.4 SURFACTANT PRECIPITATION OF TRYPSIN

Trypsin was recovered by HPLC column at a retention time of 6.3 min at 47% B gradient elution (Figure 5.7). Trypsin concentrations in the filtrate samples decreased with increasing concentrations of AOT, but protein was not completely removed in all the samples; 83% at 1.5 g/L (R=4), to 20% at 11 g/L (R=31). The amount of trypsin released after TOMAC recovery, and the protein left in the supernatant after precipitation with AOT did not add up to 100% (Table 5.2). Highest recovery of trypsin was achieved at R=14 (43%), and recovered protein from all samples was at an original activity. From the chromatogram, a protein component with a retention time of 5.6 min was seen to be increasing in peak areas and heights alongside the decreasing peak of trypsin in the sample solutions, starting from a concentration of AOT as low as 1.5 g/L (Figure 5.7b-g). This was likely to be due to the unfolding of trypsin, which could be a derivative being produced containing less enzymatic activity and a faster eluting peak than native trypsin (Hopkins and Spikes, 1973), or the intermediate state of the partially unfolded protein (Bramanti *et al.*, 2003). The soluble denatured components and, if any, insoluble denatured components would account for the amount of trypsin that was unsuccessfully recovered in this experiment.

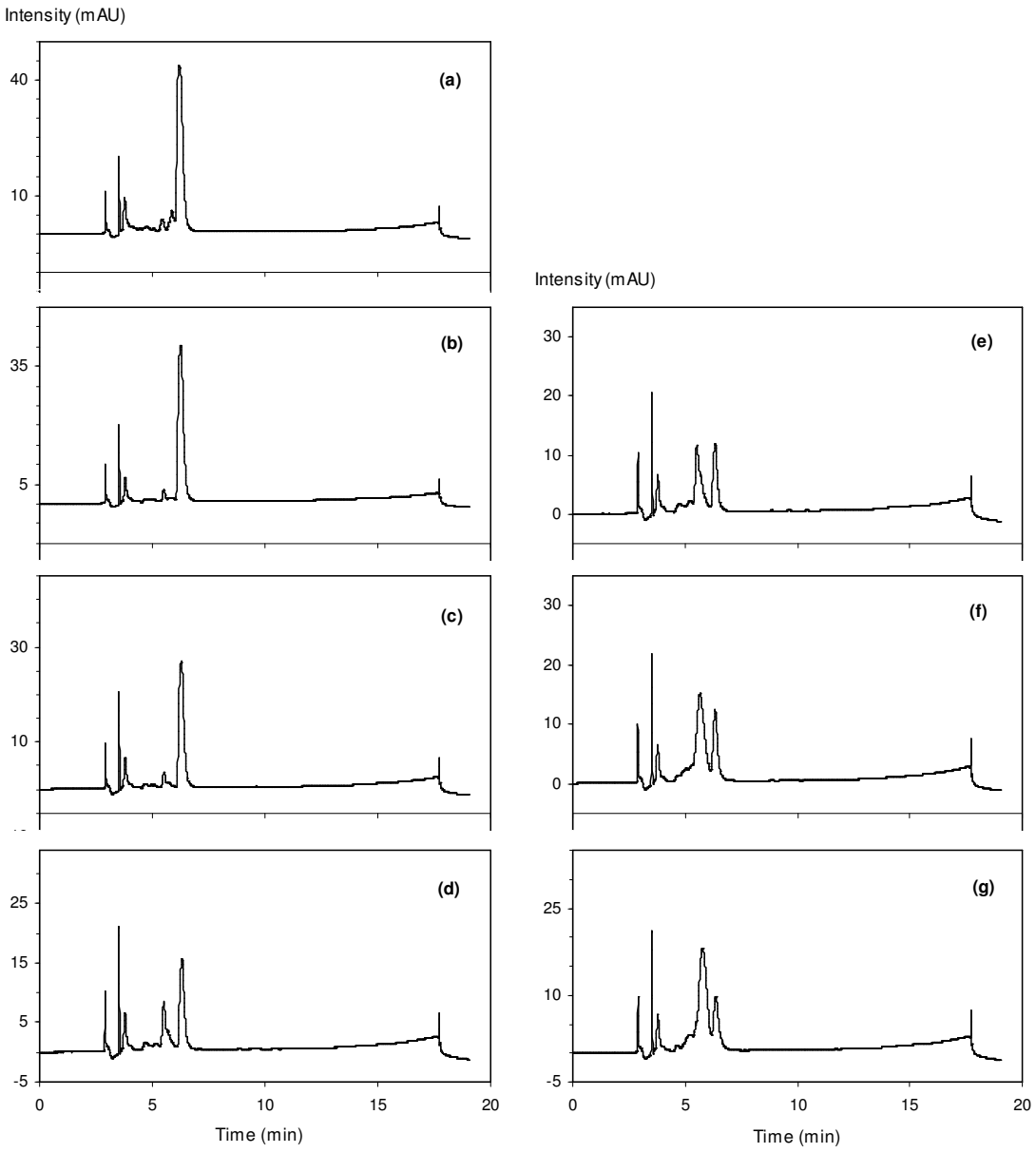

Figure 5.7 **Chromatogram of trypsin samples: (a) Initial trypsin solution without AOT (t_R = 6.3min); Soluble trypsin in the aqueous solution after addition of AOT at (b) R=4, (c) R=8, (d) R=14, (e) R=20, (f) R=25, and (g) R=31.**

Table 5.2 **Percent concentration of trypsin in the aqueous phase after addition of AOT, and in final solution after recovery of the trypsin-AOT complex with TOMAC, determined with HPLC (t_R = 6.3 min).**

Concentration (%) of trypsin in solution		
	Supernatant solution	**Recovery solution**
Initial trypsin concentration	100%	-
Recovered with AOT at:		
R=4	83%	9%
R=8	53%	32%
R=14	30%	43%
R=20	21%	43%
R=25	23%	5%
R=31	20%	0%

The extent of unfolding in the sample solutions was analysed with far-UV CD spectra (Figure 5.8). The spectrum for pure trypsin (11% α-helix, 32% β-sheet, 19% β-turn and 38% random coil) differs from the spectra for trypsin-AOT complexes. Within its range of negative bands, the samples shows a shift of a minimum from 210 nm to about 205 nm, thereby favouring the possibility of an intermediate state of the partially unfolded structure of trypsin with AOT over the formation of a new derivative from denaturation. At low AOT concentrations, the surfactant molecules bind specifically through ionic and perhaps some hydrophobic interactions to the protein, which causes the protein to expand and allows cooperative binding when AOT concentrations increase (below the CMC) (Turro *et al.*, 1995); sample R=4 resulted in no change in the secondary structure content; R=8 (9% α-helix, 33% β-sheet, 20% β-turn) and R=14 (5% α-helix, 36% β-sheet, 20% β-turn) experienced a decrease in α-helix with an increase in β structures.

AOT continues to change the profile exhibited by trypsin complexes; a prominent shoulder at 220 nm at increasing concentrations of AOT in solution indicates less random coil with the

increase in α-helix of the trypsin molecules to which AOT binds. Also, based on the calculations of secondary structure contents using CDNN deconvolution software; R=20 (12% α-helix, 34% β-sheet, 19% β-turn, 35% random coil), R=25 (26% α-helix, 30% β-sheet, 16% β-turn, 28% random coil) and R=31 (59% α-helix, 19% β-sheet, 8% β-turn and 14% random coil). The secondary structures of these unfolded trypsin molecules have some similarities to the results reported in a trypsin-SDS system where the α-helix preference of trypsin molecules is related to the formation of trypsin-surfactant clusters during unfolding of trypsin in the cooperative binding region (Ghosh and Banerjee, 2002). In addition, it has similarities to another beta-barrel protein, bovine beta-lactoglobulin, which also cooperatively transits to non-native alpha-helical intermediates in its unfolding pathway (Ragona *et al.*, 1999). The similarity is most probably in the protein structure.

Overall, we found three modes of interaction between trypsin and increasing AOT concentrations; (1) the majority of AOT molecules were associated with specific binding sites on the native trypsin at R=4, and unbound trypsin was in its native conformation and activity; (2) increasing numbers of AOT molecules began cooperatively associating with trypsin at R=8 and R=14 without major conformational changes, and unbound trypsin still retained its activity; (3) large numbers of AOT were cooperatively associating with trypsin at R=20, R=25 and R=31 causing gross denaturation, and unbound trypsin lost more than half of its activity as zero trypsin was recovered at R=31.

An important conformational structure of trypsin is that the strands of the polypeptide chains are held together by six disulfide bridges (Stroud *et al.*, 1974). The change in conformational properties of trypsin takes place when its long and flexible polypeptide chains interact with surfactant molecule clusters hydrophobically (Ghosh and Banerjee, 2002). In this region, unfolded trypsin-AOT aggregates can be formed through surfactant clusters possibly nucleating at hydrophobic sites along the protein chains (Turro *et al.*, 1995). The electrostatic repulsion between the charged surfactant headgroups along the trypsin chain results in coil expansion and protein unfolding. Our method of surfactant initiated precipitation has an advantage over micellar extraction in its capability to recover protein which is electrostatically bound to surfactants without any unfolding; trypsin recovered from the precipitate retained its secondary structure and activity (Figure 5.9).

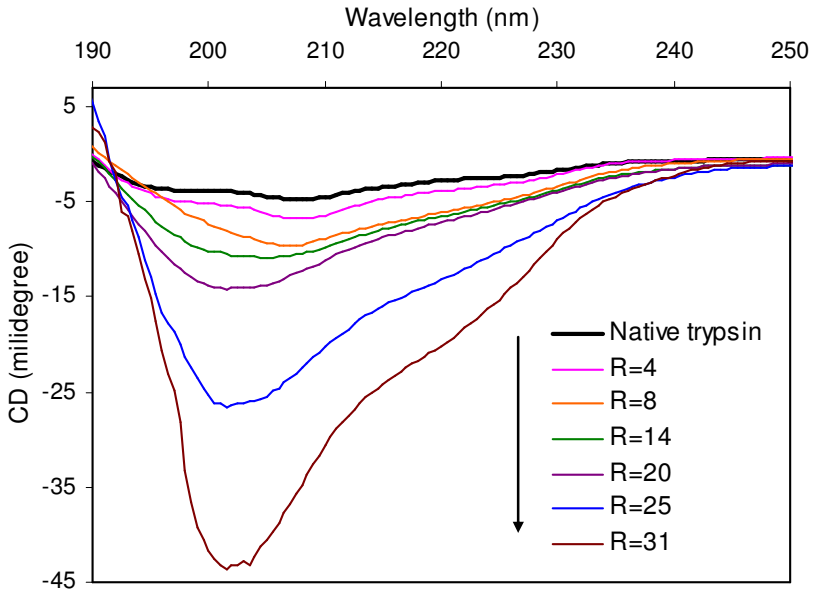

Figure 5.8 **Far-UV CD spectra of trypsin in phosphate buffer (20mM), pH 6.2: Initial trypsin solution without AOT; final aqueous phase left after precipitation of trypsin at different AOT to trypsin ratios (R=4, R=8, R=14, R=20, R=25 and R=31). All samples diluted to 0.1 g/L.**

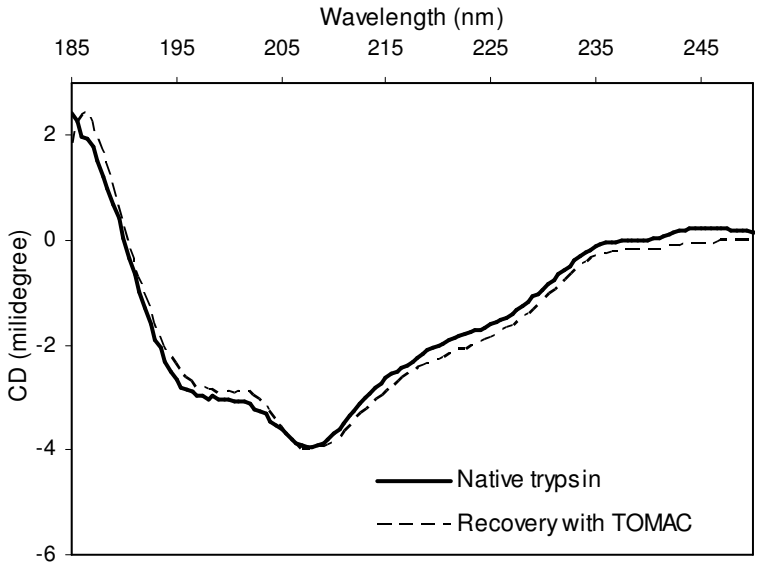

Figure 5.9 **CD spectra of the trypsin sample (R=14) in phosphate buffer (20 mM), pH 6.2, recovered with TOMAC.**

5.4.5 SURFACTANT PRECIPITATION OF α-CHYMOTRYPSIN

Chromatograms of the samples after α-chymotrypsin was precipitated with AOT showed mobile phase B eluted α-chymotrypsin from the column at a retention time of 6.6 min at 49% B gradient elution (Figure 5.10). α-Chymotrypsin concentrations measured in the filtrate samples decreased to a minimum of 30% at 3 g/L AOT (R=8), and 62% of the total protein at this R was recovered with TOMAC (Table 5.3) at its original activity and protein conformation (Figure 5.12). Increasing concentrations of AOT (5 - 11 g/L) in the precipitate solution recovered noticeably lower amounts of α-chymotrypsin (~20%). A small peak, other than α-chymotrypsin's was retained by the column at the retention time of 5.2 min in sample R=4. As the R increased to 20, the peak was resolved into a doublet which had stronger resolution by R=31. The probability that the doublet was due to contamination of the peptides was ruled out with careful purification and analysis of the samples by frequent column cleaning. Formation of a peak with lower retention time and a doublet on the peak due to multiple conformational states of the peptides is in agreement with literature data on the denaturation in α-chymotrypsin induced by urea (Ke *et al.*, 2009).

Table 5.3 Percent concentration of α-chymotrypsin in the aqueous phase after addition of AOT, and in the final solution after recovery of α-chymotrypsin-AOT complex with TOMAC, determined with HPLC (t_R = 6.6 min).

Concentration (%) of α-chymotrypsin in solution		
	Supernatant solution	Recovery solution
Initial α-chymotrypsin concentration	100%	-
Recovered with AOT at:		
R=4	85%	10%
R=8	30%	62%
R=14	70%	22%
R=20	61%	20%
R=25	61%	19%
R=31	55%	20%

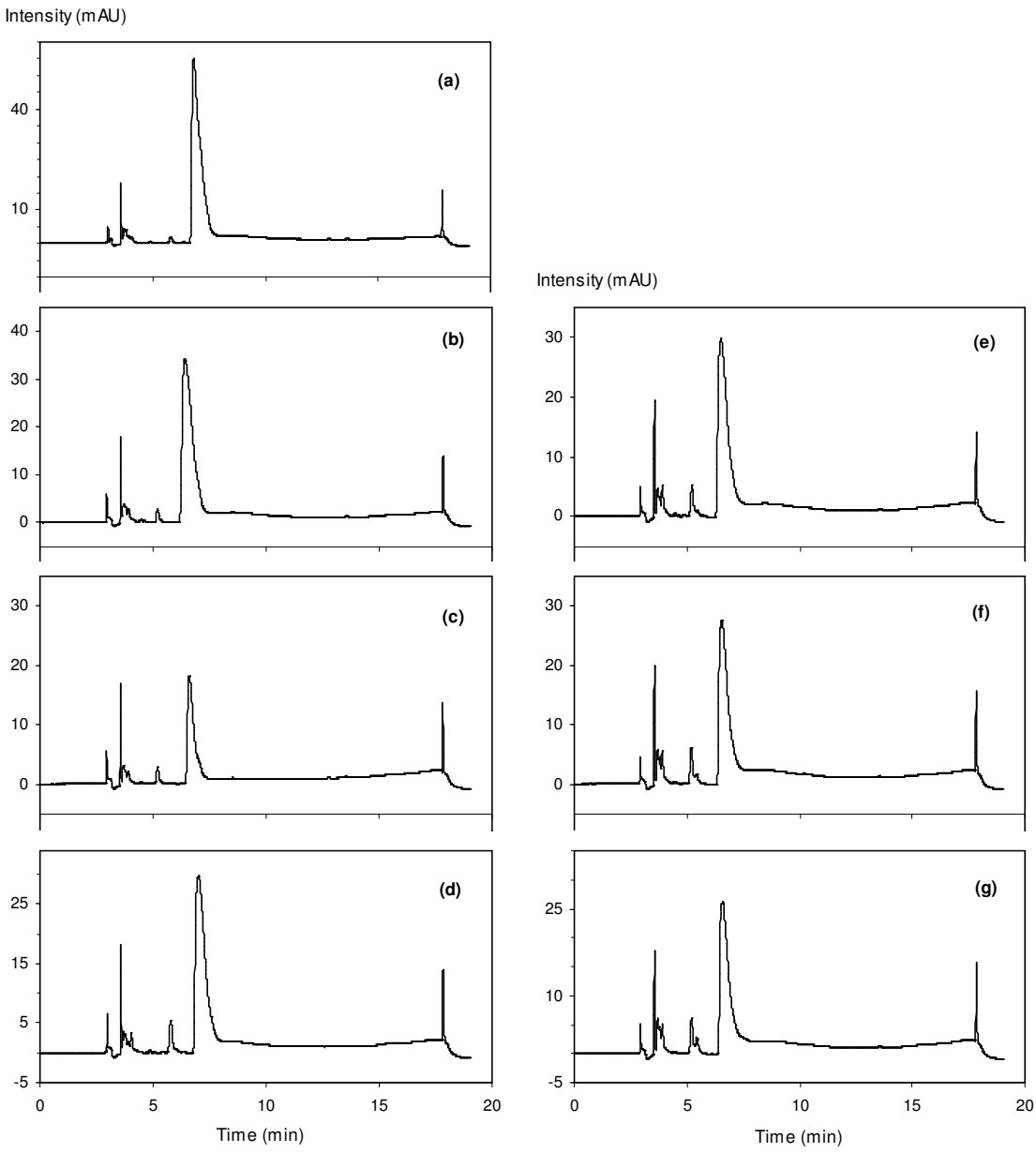

Figure 5.10 Chromatogram of α-chymotrypsin samples: (a) Initial α-chymotrypsin solution without AOT (t_R = 6.6min); Soluble α-chymotrypsin in the aqueous solution after addition of AOT at (b) R=4, (c) R=8, (d) R=14, (e) R=20, (f) R=25, and (g) R=31.

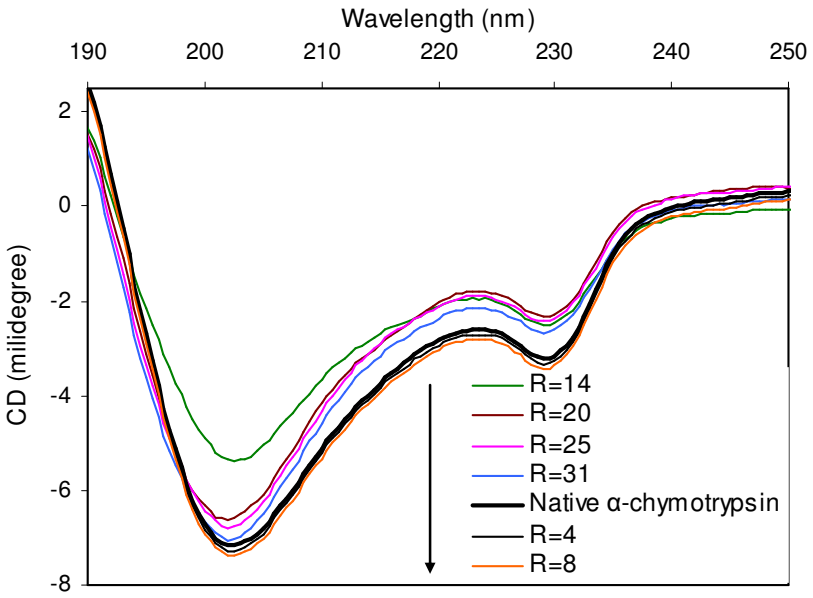

Figure 5.11 **Far-UV CD spectra of α-chymotrypsin in phosphate buffer (20mM), pH 6.2: Initial α-chymotrypsin solution without AOT; final aqueous phase left after precipitation of α-chymotrypsin at different AOT to α-chymotrypsin ratios (R=4, R=8, R=14, R=20, R=25 and R=31). All samples diluted to 0.1 g/L.**

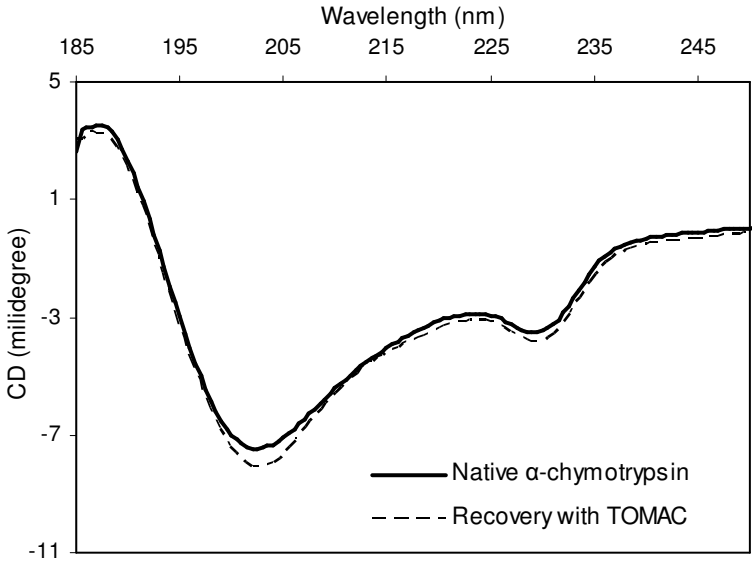

Figure 5.12 **CD spectra of the α-chymotrypsin sample (R=8), in phosphate buffer (20 mM), pH 6.2, recovered with TOMAC.**

The native α-chymotrypsin far-UV CD spectrum was characterized by a minimum of 205 nm and a negative band in the 230 nm region, which yielded 9% α-helix, 32% β-sheet, 25% β-turn and 34% random coil. Deconvoluting the CD spectra of the supernatant samples gave no change in the secondary structure content at R=4 to 8, and only a slightly distorted soluble α-chymotrypsin structure at R=14 to 31 (6% α-helix, 36% β-sheet, 24% β-turn) (Figure 5.11). CD spectra did not show a major conformational change to the samples in contrast to the development of a new doublet peak in the HPLC results, and the substantial reduction in α-chymotrypsin recovery at R=14. An explanation for this is that the CD conformation was measured on the considerably large inactive soluble α-chymotrypsin in the sample (≥55% at R≥14) that AOT did not denature, but rather inhibited. The decrease in intensity of the negative peak at 230 nm that is closely related to the catalytic active conformation of α-chymotrypsin (Celej *et al.*, 2004) was with concomitant loss of enzyme activity. At an R of 31, only 34% activity was detected in the 55% α-chymotrypsin. Analysis of structural changes in the protein that forms intermediates with surfactants is complicated by the likelihood of the partitioning of protein intermediates with surfactants (Viseu *et al.*, 2007), and therefore the conformational change might not be represented by the bulk protein solution.

The structure of α-chymotrypsin has some resemblance to trypsin since they share 41% (101) identical sequence positions of the amino acid residues, including four disulfide bridges (Stroud *et al.*, 1974), and therefore the general pattern of folding might be complementary. It would seem that for trypsin and α-chymotrypsin in the buffer used in this work, the binding isotherm for electrostatic interactions between the protein and surfactant has shifted to higher free surfactant concentrations, while the binding isotherm for hydrophobic interactions is strengthened and shifted to a lower free surfactant concentration. Therefore, protein was hydrophobically bound to the surfactant before reaching complete recovery through charge interactions. The effect of AOT was existent in both proteins, although the different stages of binding was not as prominent as trypsin with AOT because of considerably less formation of intermediate state components.

5.4.6 PROTEIN EXTRACTION BEHAVIOUR

The instant formation of an insoluble complex was observed in cytochrome c, ribonuclease A, trypsin and α-chymotrypsin solutions when adding AOT, and the results obtained were within the CV of ±5%. Positively charged proteins bound electrostatically to the anionic surfactant, with cytochrome c, ribonuclease A, trypsin, α-chymotrypsin, and lysozyme (analysed in Chapter 3 and 4) each showing different binding behaviour with increasing AOT concentrations (Figure 5.13). The results show that the highest recovery of protein was obtained for a molar ratio (R) of 16 for lysozyme, 22 for ribonuclease A, 17 for cytochrome c, 14 for trypsin and 8 for α-chymotrypsin. At these R's protein in the samples were precipitated with AOT and no significant concentration of surfactant was found in the solution using the methylene blue assay. Even though the molecular weights of trypsin and α-chymotrypsin are almost twice of that of cytochrome c, ribonuclease A and lysozyme, the number of moles of AOT required to precipitate a mole of trypsin and α-chymotrypsin were lower than that required to precipitate a mole of cytochrome c, ribonuclease A and lysozyme, indicating that the formation of a protein-AOT complex is not a simple function of protein size.

Previous work carried out for lysozyme, cytochrome c, ribonuclease A and α-chymotrypsin by Shin (2002), with no pH and salt adjustment to the aqueous phase, also found protein molecular weight had no effect on the optimum R, but this work gave differing Rs for the precipitation of these proteins. An experiment with trypsin in a non-buffered solution (distilled water) being precipitated with AOT was carried out in our work, and these results were included in the Figures below. Comparisons were made on the R's in buffered and non-buffered solutions as a function of q (Figure 5.14) and $\%S$ (Figure 5.15). In non-buffered solution, q predicts the exact moles of AOT required to form 1 mole of a protein-surfactant complex (Figure 5.14b); the R's required to neutralize the charge on the protein were equivalent to the surface charge present at the natural pH of the protein. Non-buffered solution seemed to be an ideal environment for the precipitation of single pure proteins with AOT because the R's for complete removal of proteins were lower than those in buffered solution. Moreover, trypsin and α-chymotrypsin (Shin *et al.*, 2004b) were totally precipitated and unfolding only started to occur at R=25 for trypsin, and R=14 for α-chymotrypsin. Despite the advantage and accuracy of the surface charge model in non-buffered solutions, when experiments were performed in 20 mM buffered solutions at pH 6.2 (Figure 5.14a), there was a lack of consistency in the plots of q.

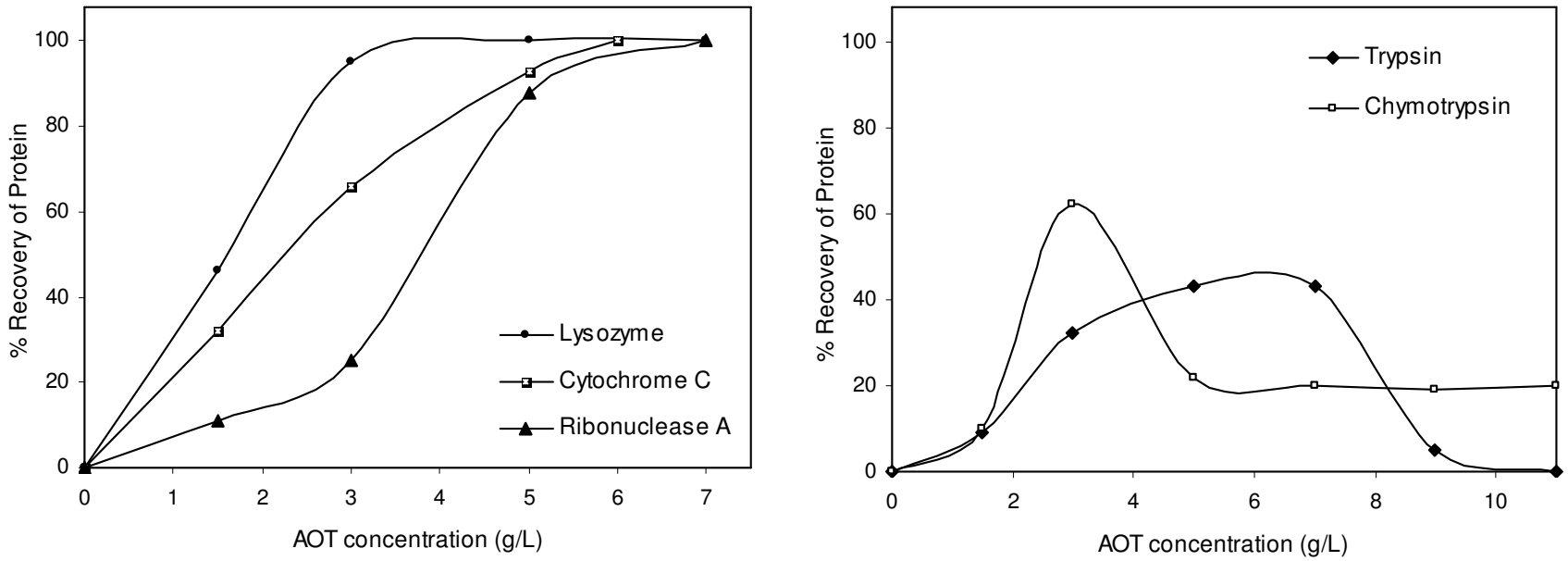

Figure 5.13 Recovery curves for lysozyme, cytochrome c, ribonuclease A, trypsin and α-chymotrypsin as a function of AOT concentration (g/L) added to 0.08 mM of the protein solutions in phosphate buffer, pH 6.2.

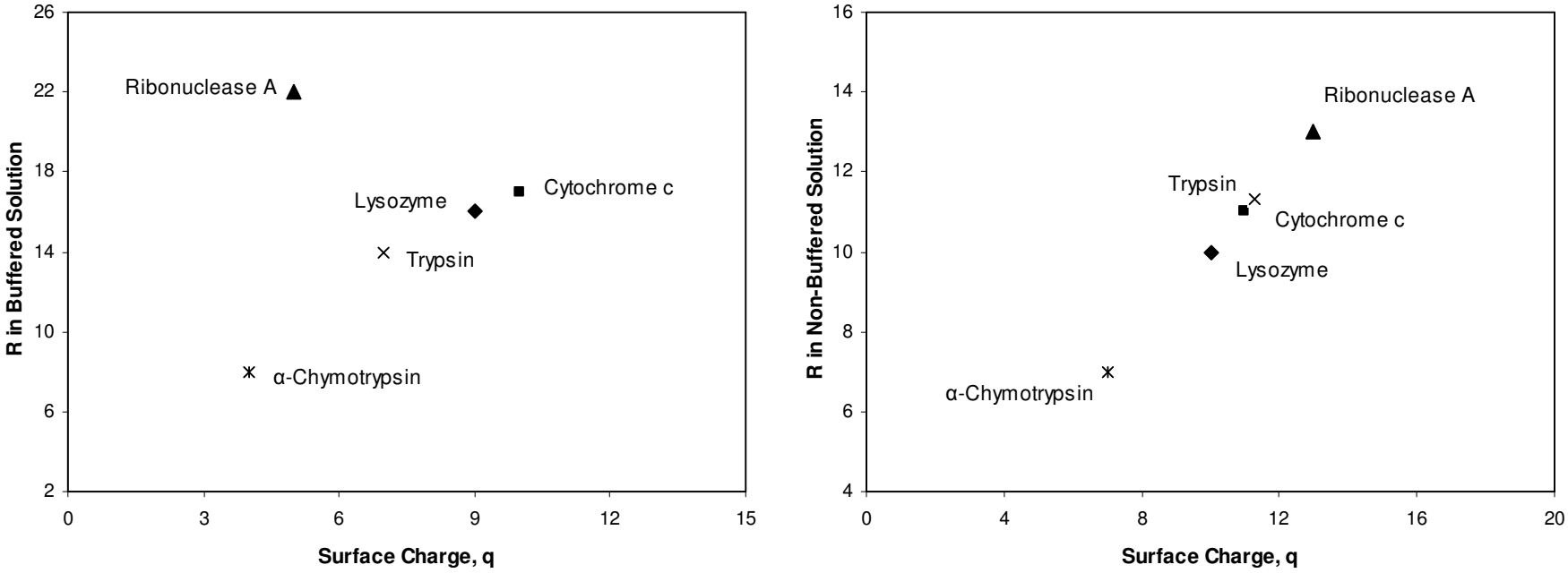

Figure 5.14 Effect of surface charge, *q*, on the molar ratio of AOT to protein, R, required for total recovery of protein in buffered and non-buffered solutions.

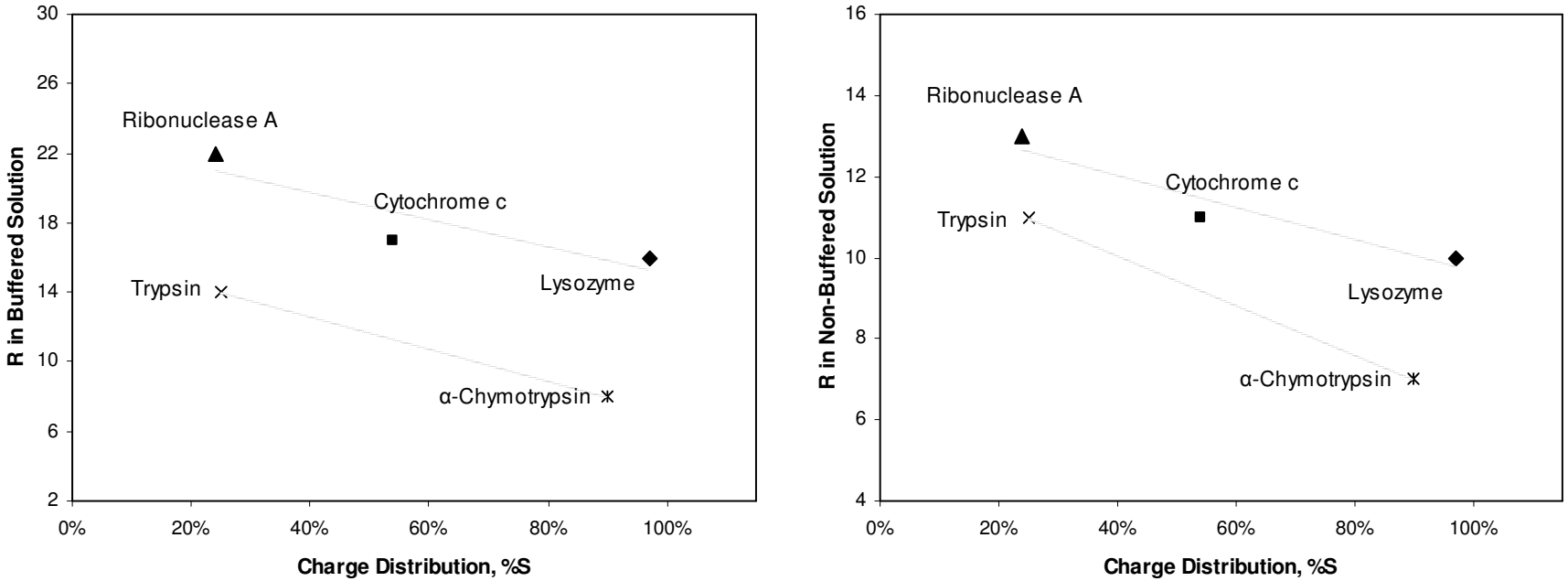

Figure 5.15 **Effect of surface charge distribution, *%S*, on the molar ratio of AOT to protein, R, required for total recovery of protein in buffered and non-buffered solutions. Lines included for guidance to indicate two different groups of protein based on the ability to achieve complete recovery in surfactant precipitation.**

In a separate model involving surface charge distribution, the R's of precipitation of the set of proteins are shown to be consistent in the plot of buffered systems (Figure 5.15a) and non-buffered systems (Figure 5.15b); R decreases with increase in *%S*. This means that *%S* is more reliable in representing the extraction behaviour of each protein in surfactant precipitation without being influenced by the system composition. When *%S* is high, the charges of the protein are distributed evenly over its surface. This might account for more specific electrostatic interactions between protein and surfactant molecules, thus reducing the moles of AOT required for complete binding with protein. However, an irregular charge distribution on the protein surface, evident from the *%S* of each protein, requires more AOT molecules to neutralize the respective surface charge of the proteins. Surface charge distribution has been shown to have an effect on the electrostatic interactions at the binding sites of proteins (Wen *et al.*, 2010). Thus, an irregular charge distribution is likely to be more influential than net surface charge in the binding specificity and the affinity of a protein for ligands. *Φ* and *HI* were only found in the literature for lysozyme, cytochrome c, ribonuclease A; nonetheless they showed no distinguishable correlation with the R's of the proteins.

In our research we have chosen to focus on improving the interactions of protein and surfactant in a buffered system, to enhance the bioprocess significance of this purification technique. It is more realistic to have a downstream process that is efficient in a buffered system because protein extraction from fermentation culture and industrial broth might involve complex mixtures containing proteins with different natural pH's and various salt contents. Without buffers, the pH of the initial solution easily changes with the addition of surfactant and protein, and this influences the R for the protein to achieve the desired precipitation efficiency (Shin *et al.*, 2003c). Furthermore, the enzyme conformation necessary for activity requires a certain concentration of ions, and they could be supplemented by buffer salts (Chang and Carr, 1971; Davies *et al.*, 1969).

It was observed that the surfactant precipitation behaviour of trypsin and α-chymotrypsin differed from those of lysozyme, ribonuclease A, and cytochrome c. Formation of a partially unfolded intermediate component appeared as faster eluting peaks for trypsin and α-chymotrypsin, while formation of a denatured form of the peptide appeared as slower eluting peaks for lysozyme, ribonuclease A, and cytochrome c. In buffered solution, the unfolding of trypsin and α-chymotrypsin occurred throughout the concentration range of AOT without achieving complete precipitation. Contrary to interactions with AOT, the unfolding of trypsin

and α-chymotrypsin was not observed when tested with TOMAC (22.5 mM - an adequate amount of counterionic surfactant to bind with all the AOT in solution). This can be explained by the lack of affinity for monomer surfactant binding, which is a prerequisite for protein unfolding (Otzen, 2011) in TOMAC due to charge repulsion.

Examination of Figure 5.15 also found that analysing trypsin and α-chymotrypsin as a separate group of proteins would more coherently express the trend of the plots (lines were drawn on the two plots to show the correlation of the two groups of proteins). Literature has classified trypsin and α-chymotrypsin as hydrophobic proteins, while the other three proteins studied in this work were hydrophilic (Kato *et al.*, 2002). Trypsin and α-chymotrypsin are more hydrophobic in nature, and have more tendencies to interact with the alkyl chain of AOT molecules instead of the hydrophilic head of the surfactant in the presence of buffer salt counterions; this provided a probable explanation for the expedited formation of an unfolded component in a buffered system. Limitations to the current work involved the inability to determine the amount, if any, of insoluble denatured protein-surfactant product being formed aside from the TOMAC-AOT complex produced, and the method to separate them if both were present simultaneously.

Unlike trypsin and α-chymotrypsin, the results of the hydrophilic proteins recovered from the surfactant precipitate corresponded closely to the mass balance calculation of protein remaining in the supernatant up to the amount of AOT required for complete removal of protein. Thereafter, for an AOT concentration higher than 7 g/L for lysozoyme, 7 g/L for ribonuclease A, and 6 g/L for cytochrome c, a large deviation (>±20%) from total recovery with TOMAC occurred, probably due to an unstable precipitate forming, and mass loss during the recovery process. Unfolded and denatured protein detected in the supernatant at these Rs might be the cause of interference in the recovery of the protein-AOT complex. The formation of nonpolar ion pairs between AOT and TOMAC would not assist in the recovery of protein in this case because hydrophobic bonding is involved in protein-AOT interactions. However, no protein recovery pattern was obtained in the absence of an accurate quantitative measure when increasing hydrophobic interactions between proteins and surfactants took place.

Cytochrome c, ribonuclease A, and lysozyme showed comparable results on their chromatogram profiles when precipitated with AOT. From HPLC analysis it was concluded that the proteins studied exhibited a similar trend of denaturation with surfactant

precipitation; the process was shown to be a slow, gradual alteration of the protein from an inactive phase to a denatured phase. The results show that unfolded protein was found in the supernatant phase at a molar ratio of 35 for lysozyme, 34 for ribonuclease A, and 25 for cytochrome c. Lysozyme enabled the highest binding of the excess AOT (19 moles AOT/protein) upon total protein recovery before it was denatured, followed by ribonuclease A (12 moles AOT/protein), then cytochrome c (8 moles AOT/protein). Hydrophilic proteins with more non-polar groups on their surface ($\Phi_{lysozyme}$) allowed more hydrophobic interactions with surfactant ligands before the hydrophobic residues in their interior were exposed thereby denaturing the protein; this principle of protein hydrophobicity is exploited in a number of chromatographic systems.

From HPLC analysis, the appearance of peaks during the course of a run were related to the simultaneous existence of various unfolded states (Ingraham *et al.*, 1985). Based on these findings, the equilibrium amongst the various unfolded states has been investigated by measuring the changes in the retentions and peak characteristics. Further work can be done on these samples by isolating the fractions to investigate each feature in a particular state. Such work will provide additional information for understanding the folding mechanism of a target protein in surfactant precipitation.

Overall, the recovery of protein with surfactant precipitation is interesting to pursue because the amount of AOT required was markedly less than in other surfactant mediated purification, e.g. the R's required to extract protein into AOT reverse micelles were about 100 moles AOT/mole lysozyme (Lye *et al.*, 1995; Shin *et al.*, 2003b), 260 moles AOT/mole cytochrome c (Ichikawa *et al.*, 1992), 55 moles AOT/moles ribonuclease A (Lye *et al.*, 1995), and 57 moles AOT/mole α-chymotrypsin (Paradkar and Dordick, 1994), and recovery efficiencies were sufficiently high for a one-step recovery, even for proteins that did not precipitate with AOT completely such as α-chymotrypsin; other downstream processes reported 45% recovery of α-chymotrypsin in a three-step chromatographic procedure (Al-Ajlan and Bailey, 2000), and 37±18% α-chymotrypsin was recovered by contacting α-chymotrypsin-AOT with acetone (Shin *et al.*, 2004b).

5.5 CONCLUSIONS

The separation of globular proteins cytochrome c, ribonuclease A, trypsin and α-chymotrypsin from buffer solution using surfactant precipitation (AOT), and their recovery with counterionic surfactant, TOMAC, was investigated. The proteins formed an insoluble complex with AOT and precipitated from solution: the molar ratio (AOT:protein) required for optimum removal was 17 for cytochrome c (100% recovered), 22 for ribonuclease A (100% recovered), 14 for trypsin (43% recovered) and 8 for α-chymotrypsin (62% recovered). It was found that amongst the factors controlling the extraction of these proteins that the surface charge distribution was the most important in being able to predict surfactant precipitation.

Hydrophilic and hydrophobic proteins exhibited different behaviour when subjected to the same precipitation procedure with AOT. Our work in 20 mM phosphate buffer at pH 6.2 concluded that all hydrophilic proteins achieved complete precipitation with AOT at different Rs, while hydrophobic proteins had difficulty interacting with the hydrophilic group of the surfactant, and tended to interact with AOT hydrophobically, and hence are more prone to unfolding in surfactant precipitation. Hence, the potential of incorporating downstream processes such as hydrophobic interaction chromatography (Kato *et al.*, 2002), affinity ultrafiltration (Luong *et al.*, 1988), or ammonium sulphate fractionation (Ee *et al.*, 2008) to separate hydrophobic proteins before sequential purification with surfactant precipitation may be a promising approach to separating mixtures of hydrophilic/hydrophobic proteins.

CHAPTER 6 PROTEIN SELECTIVITY AND SEPARATION FROM A FERMENTATION BROTH*

6.1 INTRODUCTION

The effect of the protein surface properties (charge and hydrophobicity) have not been studied thoroughly in relation to separating proteins from mixtures using surfactant precipitation. Only surface charge has been studied indirectly by manipulating the isoelectric values (pIs) of the proteins to give a desirable overall protein charge for reaction with the charged head group of a surfactant. With this approach, proteins with different pIs were selectively precipitated with AOT by controlling the pH of the initial protein mixture (Shin *et al.*, 2003c). However, when separating proteins with similar pI values, the surfactant precipitation method failed (Shin *et al.*, 2004b). When analysing Shin's experiments, we suspected that the different hydrophobicities of the proteins could have complicated the results of the study, and we proposed carrying out a series of experiments which excluded the influence of this parameter in order to study protein selectivity based on protein surface properties only.

We chose to investigate the selective extraction of three hydrophilic proteins with a monomeric structure, lysozyme, cytochrome c and ribonuclease A, from sets of protein mixtures within a small range of molecular weights (12,400Da – 14,300Da), and isoelectric points within two pH units (9.6 – 11.4). It was also advantageous that the proteins chosen had convenient assay methods, were well characterised, and were inexpensive. Although having some similar physical properties, the proteins chosen provided an excellent model for evaluating selective surfactant precipitation as they had a diverse range of surface properties; an overall surface charge of +5 to +10, a surface charge distribution of 24% to 97%, a surface hydrophobicity of -40 kJ.mol^{-1} to -99 kJ.mol^{-1}, and a surface hydrophobicity distribution of -1.0 to -1.3. To understand the influence these surface properties had on protein selectivity

* Parts of the work presented in this chapter have been submitted for publication;
Cheng, S.I. and Stuckey, D.C. 2011. Mechanism of protein extraction in surfactant precipitation systems. Biochemical Engineering.

using surfactant precipitation, we examined all three proteins in several mixtures prepared in buffer solution.

The recovery and purification of proteins from complex fermentation broths is usually a major obstacle to their commercialisation due to their cost of separation (Desai and Banat, 1997; Isa *et al.*, 2007). Although surfactant precipitation of lysozyme, cytochrome c and ribonuclease A is reported to be effective in buffer solutions, the mechanisms of separation has to be investigated in fermentation broth for the technique to be successful and potentially commercialisable. Therefore, surfactant precipitation of the same group of proteins was evaluated in fermentation broth by surface properties measurements; we conducted protein separation studies of the three proteins first in individual protein solutions, then in sets of protein mixtures.

6.2 MATERIALS AND METHODS

Materials: Fermentation broth was kindly provided by B. Edwards-Jones of the Department of Life Sciences, Imperial College, London, UK.

6.2.1 FERMENTATION BROTH

The fermentation broth used was taken from a yeast (*Pichia pastoris)* continuous culture of recombinant trypsinogen which was grown aerobically in a 1.5 L fermentor. The culture was supplied with 400 ml/min filtered air; pH was maintained at 5.0 by addition of 25% w/v potassium hydroxide; foaming was controlled with 0.01% v/v Acepol-83E. Continuous methanol-fed culture medium (Tredwell *et al.*, 2011) contained per litre; 0.2 g $CaCl_2.2H_2O$, 9 ml phosphoric acid (85%), 7.5 g KOH, 6 g K_2SO_4, 4.67 g $MgSO_4.7H_2O$, 5 g $(NH_4)_2SO_4$, 3 ml PTM4, and 30 ml 1% w/v histidine. Metabolites were present in the intracellular and extracellular locations of the *P. pastoris* culture. The fermentation broth was centrifuged at 4000 rpm for 10 min to separate the supernatant broth from the cell pellet and dead cells. Increasing lysed cells with cell death caused contamination of the supernatant with host cell protein. The host cell protein concentration at the end of the fermentation was approximately 0.5 g/L (Hohenblum *et al.*, 2003).

6.3 EXTRACTION PROCEDURES

6.3.1 SINGLE PROTEIN IN A FERMENTATION BROTH

0.08 mM of protein (lysozyme, cytochrome c, and ribonuclease A) was dissolved in a fermentation broth at the same concentration as in the buffer system. pH of the solution was then adjusted to the required pH, 6.2. The centrifuged broth, without being spiked with protein, was contacted with the AOT phase, and the solution remaining after equilibrium was used as a blank.

6.3.2 PROTEIN MIXTURES IN THE BUFFER SYSTEM

Equimolar mixtures of proteins (0.08 mM) were prepared in phosphate buffer (20 mM, pH 6.2) for three sets of binary mixtures; **A** - lysozyme and cytochrome c, **B** – lysozyme and ribonuclease A, and **C** – cytochrome c and ribonuclease A. These mixtures comprised all the possible combinations for the binary proteins. Protein extraction was performed using the same methods as for the single protein solutions above; precipitation with AOT and recovery with TOMAC. Quantitative and qualitative analyses were carried out on the final product and are discussed below.

6.3.3 PROTEIN MIXTURES IN THE FERMENTATION BROTH

Protein binary mixtures consisting of equimolar concentration, 0.08 mM, as in the buffer system, (binary mixture **A**, **B**, or **C**) were prepared in fermentation broth. Protein extraction in broth was performed using the same method as for the protein solutions in buffer; precipitation with AOT and recovery with TOMAC.

6.4 RESULTS AND DISCUSSION

6.4.1 SINGLE PROTEIN EXTRACTION IN BROTH

Purification of a single protein from fermentation media was carried out in our experiments. Centrifuged fermentation broth without additional protein turned turbid when contacted directly with 1 mL of AOT at 3.4 mM to 24.7 mM. AOT below the CMC of the surfactant was tested to analyze the effect of surfactant monomers on the broth. A semi-transparent precipitate was obtained after centrifugation, but it could be easily redissolved in deionised water. Additions of AOT to the supernatant broth upon removal of the precipitate reproduced turbidity. Concentration of the pre-expressed host cell protein, recombinant trypsinogen, in the fermentation broth did not change after AOT was added to the solution. These results pointed out that the surfactant might have precipitated out of the fermentation broth free from interactions with the host cell protein. Hydrophobic compounds likely to be found in the broth, such as hydrophobic amino acids, and phospholipids seem to reduce the repulsive forces between surfactant headgroups (Krei and Hustedt, 1992), thus resulting in the precipitation of AOT.

The broth spiked with lysozyme, cytochrome c, and ribonuclease A produced clear solutions, but the instant formation of an insoluble complex was observed with the addition of AOT. Proteins showed similar binding behaviour with increases in AOT as demonstrated in buffer solutions (Figure 5.13). Complete precipitation in broth was obtained for these proteins at the molar ratios identified in buffer; 16 for lysozyme, 22 for ribonuclease A, and 17 for cytochrome c, which will be known simply as R_{Total} in the following discussions. Proteins were completely removed from broth at $>R_{Total}$. The protein-AOT complex was recovered with TOMAC; Figure 6.1 shows the extraction yield of the three proteins recovered from broth into fresh buffer solutions. Proteins recovered were at their original activities.

The amount of protein recovered from the surfactant precipitate did not correspond to the mass balance calculations of proteins precipitated with AOT. The three proteins, in separate solutions, were recovered up to about 85±7% at R_{Total}, and then recoveries began to drop at $>R_{Total}$. Protein was lost as an insoluble aggregate, presumably denatured due to the hydrophobic compounds in the broth. The amount of insoluble aggregate obtained during the range of Rs studied is plotted in Figure 6.2. A trend was observed in the denaturation profile;

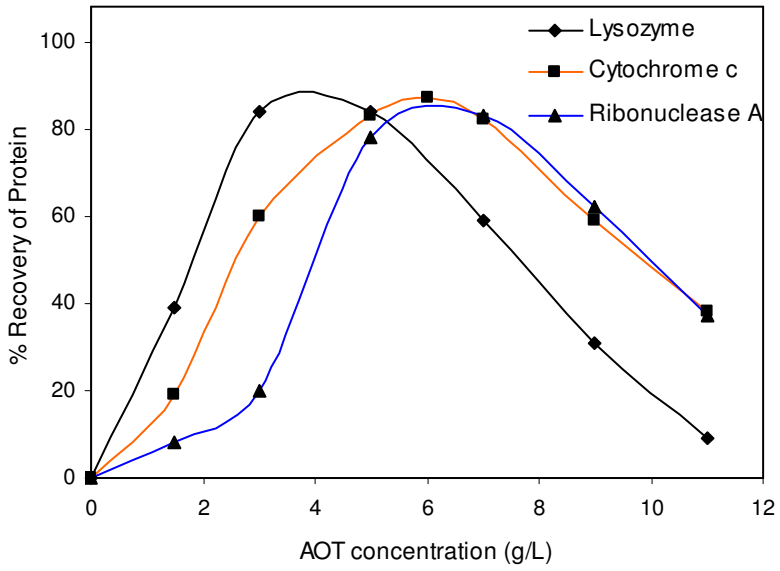

Figure 6.1 **Protein extraction from fermentation broth: 1 g/L lysozyme, 1 g/L cytochrome c, and 1 g/L ribonuclease A with various AOT concentrations: 3.4 mM to 24.7 mM, recovered with TOMAC 22.5 mM. The recovered proteins were fully active.**

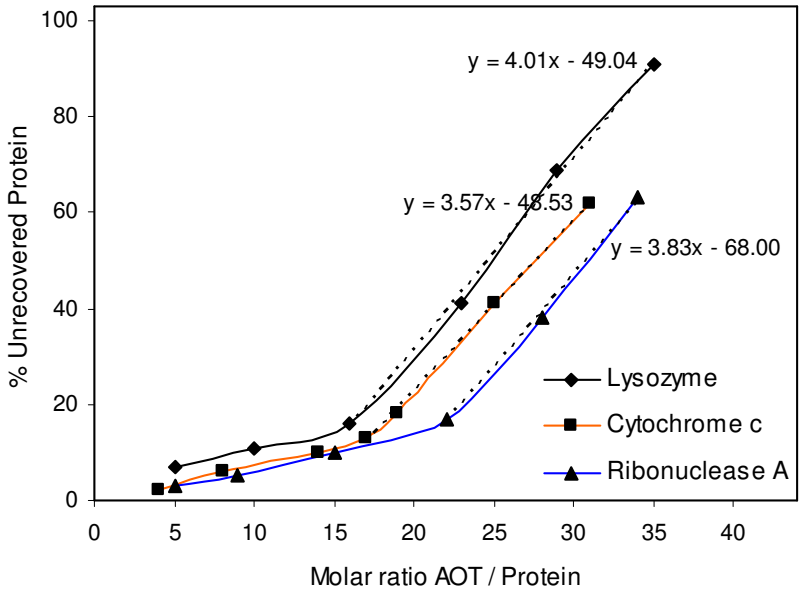

Figure 6.2 **Percent of lysozyme, cytochrome c and ribonuclease A lost as insoluble aggregate from precipitation with AOT in fermentation broth as a function of R.**

protein undergoes slight denaturation until R_{Total}, followed by a substantial increase in denaturation. The slope of the initial denaturation was approximately ~0.8 for all the proteins. However, the slope of the subsequent denaturation was shown to increase from cytochrome c, ribonuclease A to lysozyme indicating that the broth constituents were more influential with proteins with higher surface hydrophobicity. The reason for the change in the curves may be related to the type of protein-surfactant interactions dominating the protein precipitation procedure; electrostatic interactions drive the complexation of protein and AOT, upon neutralisation of the protein charges proteins experienced hydrophobic binding with excess AOT ligands and are denatured significantly.

6.4.2 PROTEIN FOLDING IN BUFFER AND BROTH SYSTEMS

Our work on the stability of the protein remaining in buffer after precipitation with AOT has found that soluble lysozyme, cytochrome c and ribonuclease A were present in an inactive and/or denatured state at above R_{Total}. Disordered structure and soluble aggregates of protein were formed via hydrophobic interactions (Jin *et al.*, 2009) with AOT. Protein was also found to be unfolded as a non-dissolvable precipitate ($>>R_{Total}$; 35 for lysozyme, 34 for ribonuclease A, and 25 for cytochrome c) with solvent recovery; this was not observed visually in TOMAC recovery because of the formation of an insoluble TOMAC-AOT complex. Insoluble aggregates may be attributed to an increase in non-specific hydrophobic interactions between the unfolded proteins (Tanford, 1970). The amount of protein lost as a soluble, or insoluble component cannot be determined accurately from an aqueous system due to the lack of information concerning denatured protein-AOT complexes. Interestingly, when extraction was performed on broth no soluble protein was detected at any R of the respective proteins. However, even at molar ratios below R_{Total}, protein was denatured and precipitated at increasing concentrations with increases in AOT concentration. The hydrophobic amino acids in the broth seem to enhance interactions between the solvent (broth and AOT) and the nonpolar groups of the protein, thus favouring the denatured state (Gopal and Ahluwalia, 1994).

It is apparent that the buffer and broth systems were different in terms of the extent of protein unfolding in the recovery solution at the same molar ratio of AOT (composition of the unfolded components and the Rs were presented in Figure 3.5, Figure 5.1, Figure 5.4 - buffer system, and Figure 6.2 – broth system). In order to obtain more details on protein folding

behaviour in these systems, soluble protein samples having lesser activity and suspected to be unfolded were tested for recovery with TOMAC (22.5 mM), while the insoluble aggregate (TOMAC-AOT and non-dissolved precipitate) were collected, solubilised in fresh buffer then subjected to the same recovery. Any remaining non-dissolved particle thereafter was removed and the solution was analysed for protein concentration, activity and conformation. Unfolded soluble protein regained its original activity and conformation, but the insoluble aggregate remained denatured. According to Tuszynski (2008), if a protein remains water-soluble when denatured, it can return to its native conformation when placed back into a 'normal' environment. The addition of TOMAC to the soluble aggregate contributed to the refolding/renaturation of the protein by removing the excess AOT from the unfolded protein. On the basis of these observations, it is suggested that protein samples in buffer were gradually unfolded reversibly in the solution to irreversibly out of the solution, whereas samples in broth went through a direct irreversible denaturation.

6.4.3 SELECTIVE SEPARATION OF PROTEIN MIXTURES IN BUFFER SYSTEM

The selectivity for specific proteins in mixtures was investigated with the surfactant precipitation technique by keeping the aqueous phase pH (6.2), buffer ionic strength (20 mM) and type of surfactant constant (AOT). Table 6.1 shows that the selectivity of proteins extracted into a protein-surfactant complex and then recovered with a counter-ionic surfactant ranged from 0 to 100%. Apparently, proteins having a higher overall surface charge with a symmetrical charge distribution, e.g. lysozyme, are more easily extracted, while proteins such as ribonuclease A, which has a lower overall surface charge with an asymmetric charge distribution are less well extracted, or not at all. Therefore, binary mixture B consisting of lysozyme and ribonuclease A has the highest selectivity amongst the protein mixtures studied.

The AOT needed for optimum selectivity in mixtures varied according to the proteins extracted, and is approximately the R of the protein with the highest degree of extraction; A and B - 16, C – 17. A two-step extraction of the binary mixtures can fully extract all the proteins from the aqueous phase; each step consists of a precipitation and a recovery procedure. In mixture A for example, an R of 16 selectively extracted 98% lysozyme with 33% cytochrome c after the surfactant precipitate of the first extraction was removed, and

then an R=17 extracted 60% of the remaining cytochrome c from the original solution. In experiment B, the technique achieved a good separation of the mixture into two single protein phases; the first step extracted 99% of lysozyme, while the second step 94% of ribonuclease A. The final supernatant was found to contain no protein, and the standard deviation of the measurement was attributed to a small mass loss during the recovery process. One insight into surfactant precipitation of proteins from mixtures was that it is crucial that the first protein precipitated from solution is removed before AOT exceeds its R for complete protein-surfactant binding. The removal of the first protein before the next protein was precipitated with surfactant prevented denaturation of the first protein from hydrophobic interactions with excess surfactant molecules.

Table 6.1 Selectivity of surfactant extraction from buffered protein mixtures. Each step consisted of a precipitation and a recovery procedure.

Equimolar Protein Solutions			
Binary Mixtures		% Recovery	
		Step (1)	Step (2)
(A)	Lysozyme	98	0
	Cytochrome c	33	60
(B)	Lysozyme	99	0
	Ribonuclease A	0	94
(C)	Cytochrome c	98	0
	Ribonuclease A	13	82

The effect of a protein's surface properties on separation efficiencies in binary mixtures was analysed by the relative difference in precipitation of the relevant proteins and their surface properties, which are documented in Table 5.1. The relative difference (Δ) of a certain parameter belonging to two proteins was calculated as:

$$\text{Relative difference} = \frac{|x-y|}{\left(\frac{|x+y|}{2}\right)} \qquad (6.1)$$

From Figure 6.3 it can be seen that no relationship could be established between Δ% precipitation with Δ*q* and Δ*HI* for the protein tested. Although it was discussed that *q* together with *%S* determines the selectivity in mixtures, selectivity was not significantly affected by *q* alone. However, *q* is likely the determining factor if protein charges are distributed homogeneously over the surface of all proteins in the mixture. Variation in the *HI* data between proteins was quite small, within just 0.3 units; therefore the relative importance of this parameter within the group of proteins may not be well represented.

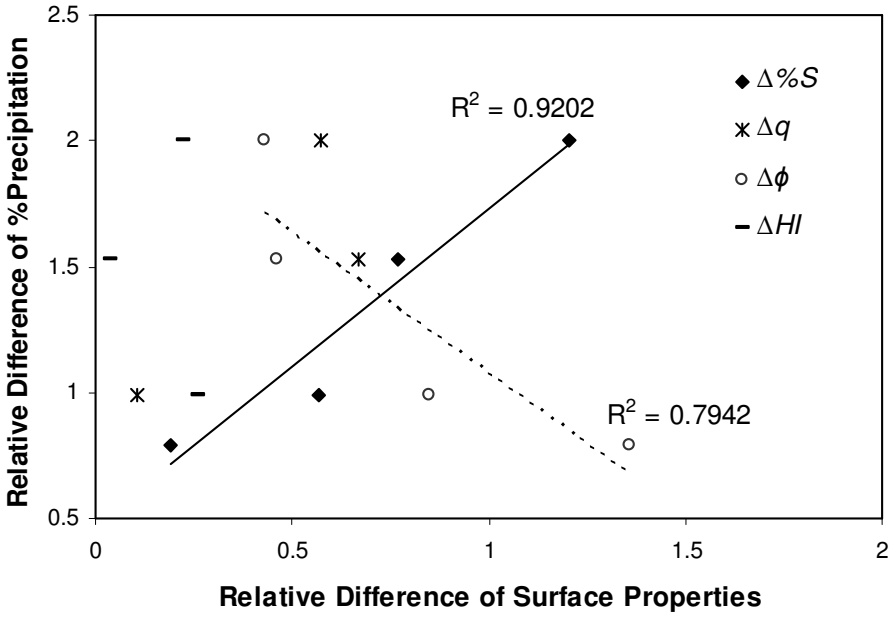

Figure 6.3 Relative difference of precipitation as a function of relative difference of surface properties; surface charge distribution (*%S*), overall surface charge (*q*), average surface hydrophobicity (*Φ*), surface hydrophobicity distribution (*HI*).

A good correlation was obtained by plotting Δ%precipitation against Δ*%S* (the error bars indicating the standard deviation of the measurements based on four replicates are seen in Figure 6.4). A linear fit was tested by least square estimates and an analysis of variance (ANOVA) approach. The parameters of the regression analysis are shown in Figure 6.4 and Table 6.2. The coefficient of determination (R^2) was 0.9185, which means that *%S* can represent the precipitation efficiencies of proteins in the mixtures well. At a significance level of 0.05, the best fit of the linear model was obtained due to the high R^2 value and an

insignificant lack of fit ($P_{LOF} \geq 0.01$). This correlation was tested with the results of protein selectivity using a similar method of extraction by other researchers to see how compatible it was with other protein mixtures not studied here. Shin reported a selectivity of 38±3% for haemoglobin and 88±10% for cytochrome c (Shin *et al.*, 2004a). This data fits well when the mixture consisting of haemoglobin, which has a higher molecular weight and lower pI than the group of proteins analysed in this paper, was incorporated into the correlation (R^2=0.9202). This supported our findings that *%S* is a strong determining factor for protein separation in surfactant precipitation.

When we related $\Delta\Phi$ to Δ%precipitation, it correlated reasonably well (R^2=0.7942). Better separation tends to occur with proteins closer to each other on the surface hydrophobicity scale (low $\Delta\Phi$) where the hydrophobicity effect was reduced, allowing other parameters e.g. *%S* to control protein selectivity. The effect of Φ on the selectivity of hydrophilic proteins is not fully understood, however, a comparative study using hydrophobic proteins is suggested to enhance understanding.

The results of protein selectivity in mixtures successfully yielded information about the degree of extraction of proteins based on their surface properties. Results showed that protein precipitation with surfactant is not only driven by ionic interactions between biomolecules and surfactant molecules (Shin *et al.*, 2003b), but also by the differences in surface charge distribution between biomolecules. Among the surface properties studied, surface charge distribution of biomolecules is probably the most important factor affecting separation performance. This suggests that a protein is most likely to precipitate in extraction when it has the most accessible charged residues on its surface.

Proteins with pI values differing by less than two pH units, and the same molecular weight (lysozyme and ribonuclease A) have been selectively separated using this method. Generally proteins tend to have small variations in their overall surface charge, but can have widely differing surface charge distributions (Barlow and Thornton, 1986), and therefore surfactant precipitation is a good technique to separate many proteins. Moreover, this technique can selectively separate a large number of proteins when carried out with modifications of the surface properties of the target proteins by protein surface engineering (Ono and Goto, 1998). The relationship obtained between Δ%precipitation and %S can be used to predict the separation efficiencies of other protein in mixtures.

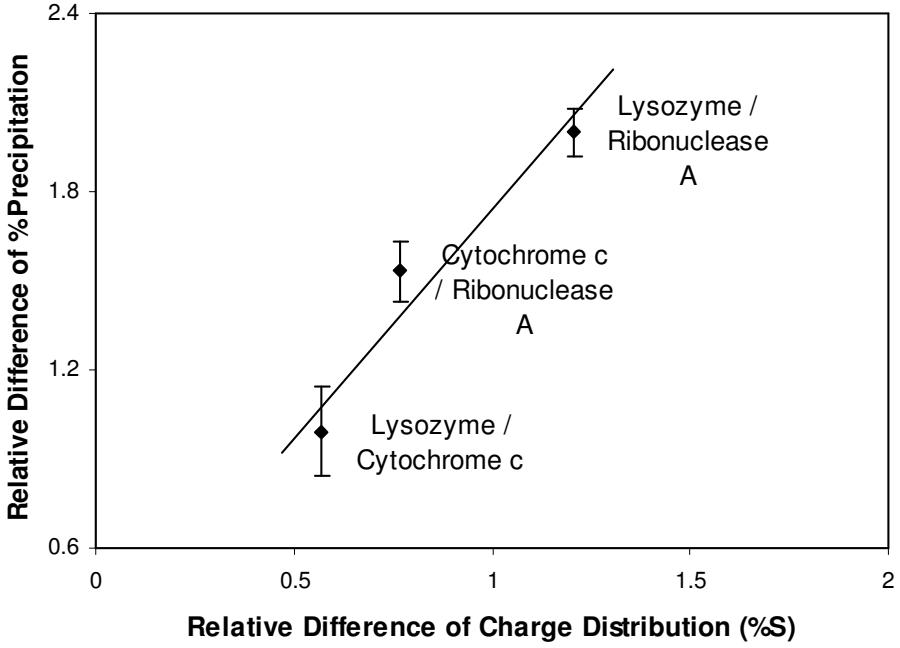

Figure 6.4 **Linear regression for the effect of surface charge distribution on the selectivity of three sets of binary mixtures. The calibration curve is given by equation: $\Delta P_m = 0.24 + 1.50\ \Delta S$, where ΔP_m (%) is the relative difference of precipitation in binary mixtures and ΔS (%) is the relative difference in surface charge distribution. Standard error of the intercept at the origin S_a (1.364), Standard error of the slope S_b (0.446), residual standard error of the regression S_r (0.291), correlation coefficient r (0.9584), and determination coefficient R^2 (0.9185).**

Table 6.2 ANOVA for the lack-of-fit test of the protein separation in buffer system based on variation in surface charge distribution, S.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio	P Value (Prob > F)
Model	1	0.953	0.953		
Charge Distribution	1	0.953	0.953	45.086	0.003
Residual	4	0.085	0.021		
Lack of Fit	1	0.064	0.064	9.044	0.057
Pure Error	3	0.021	0.007		
Total	5	1.038			

6.4.4 SELECTIVITY IN BROTH SYSTEM

A linear regression model is used to explain the selectivity characteristics in fermentation broth based on four replicates for each binary protein mixture. The calibration curve, as well as the standard deviations of the measurement are plotted in Figure 6.5. Equation 6.2 was obtained using least squares estimates, which minimises the sum of the squares of the errors (differences between the observed and predicted values for the dependent variable), and an analysis of variance (ANOVA) approach to test the significance of regression. Results of the statistical analysis of protein mixtures separation are shown in Table 6.3.

$$\Delta P_m = 0.65 + 1.13\ \Delta S \qquad (6.2)$$

where ΔP_m (%) is the relative difference of precipitation in binary mixtures, and ΔS (%) is the relative difference in surface charge distribution.

The parameters of the regression analysis were determined as follows; standard error of the intercept at the origin S_a (1.364), of the slope S_b (0.262), residual standard error of the regression S_r (0.171), and correlation coefficient r (0.9743). The high determination coefficient ($R^2 = 0.9492$), and an insignificant lack of fit ($P_{LOF} \geq 0.01$) indicates that the data was fitted well by the model. Therefore, at a significance level of 0.05, it can be concluded that the relationship between ΔS and ΔP_m is linear.

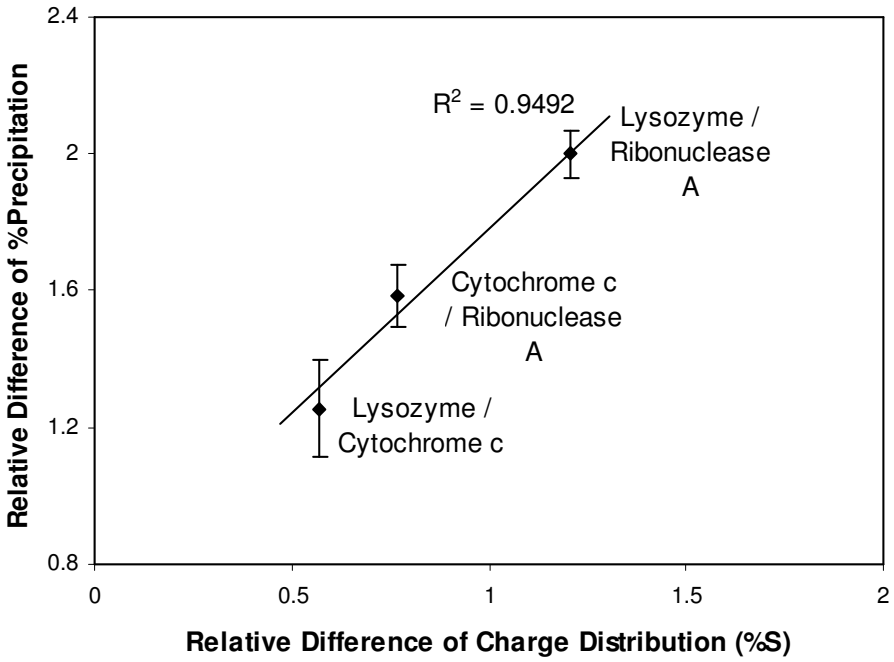

Figure 6.5 **Calibrated straight line for selective precipitation of protein mixtures in broth according to the relative difference of surface charge distributions.**

Table 6.3 **ANOVA for the lack-of-fit test of the protein separation in fermentation broth based on variation in surface charge distribution, S.**

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Squares	F Ratio	P Value (Prob > F)
Model	1	0.546	0.546		
Charge Distribution	1	0.546	0.546	74.741	0.001
Residual	4	0.029	0.007		
Lack of Fit	1	0.011	0.011	1.935	0.258
Pure Error	3	0.018	0.006		
Total	5	0.576			

Protein selectivity of mixtures in buffer provides a close representation of the selectivity achieved in fermentation broth. The advantage of such a system is that the development of surfactant precipitation carried out extensively with buffered aqueous phases can easily be adapted to fermentation broths from various upstream processes. Using the surfactant precipitation technique for the separation of proteins could prevent some problems encountered by specific purification techniques with fermentation broth, for example low product purity, long production period, and formation of emulsions in membrane separation, ion exchange, big aperture resin adsorption (Liu and Wang, 1994; Yan and Pang, 1991), and reverse micellar (Jarudilokkul *et al.*, 2000b) methods.

6.5 CONCLUSIONS

Protein mixtures consisting of cytochrome c, ribonuclease A and lysozyme were used to determine the factors controlling selectivity of protein separation in a buffer solution and fermentation broth. Selectivity was found to be a strong function of the surface charge distribution of each protein, indicating that specific charge interactions between the surfactant and surface groups of different proteins was the driving force for the separation of proteins in mixtures using surfactant precipitation. It was shown that protein mixtures can be resolved by surfactant precipitation on the basis of this difference; lysozyme and ribonuclease A have significantly different surface charge distributions with respect to their surface properties, and thus can be separated using surfactant precipitation.

The surface charge of a protein, manipulated by the control of media pH, was a crucial factor in the choice of an ionic surfactant for successful extraction. Nevertheless, the exact value of the surface charge did not influence protein selectivity. In addition, our results also showed that surface hydrophobicities (average surface hydrophobicity and surface hydrophobicity distribution) did not control selectivity during extraction. This explained why the proteins extracted from the mixture were in their native conformation because hydrophobic interactions had been shown in our previous work to weaken the structural stability of the protein if present.

The similarities of protein separation from buffer and broth systems were that most proteins were sufficiently extracted when adequate AOT ligands were present for neutralisation of the

protein charges, and the selectivity from a protein mixture was a strong function of the surface charge distribution of the proteins present. However, separation of proteins is more complicated in fermentation broth because it is based on an interplay between the hydrophobic compounds in the broth, the surface hydrophobicity of the protein, and the prevalent distribution of surface exposed polar amino acid residues. Nonetheless, protein separation from fermentation broth with surfactant precipitation was able to achieve high extraction efficiencies, with a high purity level in the proteins recovered, and preserve protein stability.

CHAPTER 7 CATIONIC/NON-IONIC SURFACTANT PRECIPITATION

7.1 INTRODUCTION

Anionic surfactants have been known to form surfactant clusters which begin to unfold typically water soluble proteins by association between protein molecules after the saturation point of protein binding sites has been reached, despite being below the CMC (Andersen *et al.*, 2009). This denaturing effect was observed with protein precipitation with AOT conducted in Chapters 3 and 5 where the distorted secondary structure of proteins (lysozyme, cytochrome c, ribonuclease A) was analysed beyond the molar ratio of AOT to protein required for complete precipitation. Furthermore, the unstable aggregate formed at this stage interfered with counterionic recovery causing a large deviation in the recovery of protein. The unfolding of proteins by surfactant clusters was initiated by monomeric binding of AOT through its electrostatic interactions with protein, and then the preceding hydrophobic forces.

In this Chapter we aim to explore the use of nonionic surfactants in surfactant precipitation because a nonionic surfactant solution does not form clusters, which is the precursor to sub-CMC protein unfolding, and nonionic surfactants would contribute to reducing the affinity in the protein-surfactant system. A weak electrostatic interaction and non-denaturing character of nonionic surfactants determines the underlying mechanisms of protein extraction into nonionic microemulsions, reverse micelles and aqueous two-phase micellar systems (Naoe *et al.*, 1998; Nikas *et al.*, 1992; Vasudevan and Wiencek, 1996). This system was adapted to protein extraction in surfactant precipitation in an attempt to reduce, if not to prevent, protein unfolding. Addition of nonionic surfactants is also suggested for the application of this technique because it is pH and temperature stable as well as water soluble (Andersen *et al.*, 1986). Nonionic surfactants also result in minimal interference with the UV absorbance spectrum for the assaying of protein concentrations to give an accurate experimental characterisation of the technique (Shin, 2002).

Tween 85 and several nonionic surfactants are reported to possess a small net negative charge at neutral pH and between the pH range of 5-9. Unfortunately, results are limited to the existence and the sign of the electrostatic charge, and the actual number of charges associated

with these non-ionic surfactants are not available (Vasudevan and Wiencek, 1996). Therefore, we seek to take advantage of the negative charge of a non-ionic surfactant as a possible precipitating ligand for positively charged proteins. The research evaluated three non-ionic surfactants, Triton X-100, Tween 85 and Brij 30, for lysozyme precipitation and non-ionic surfactant recovery before investigating a mixed surfactant approach to surfactant precipitation and recovery.

Recent studies investigating mixed surfactants showed that non-ionic surfactants introduced into ionic systems gave rise to higher efficiencies of protein recovery, protein stability and enzyme activity (Chiang, 1999; George and Stuckey, 2010; Lalonde *et al.*, 1995; Rong *et al.*, 1999; Russell and Britton, 2002). This was attributed to the ionic/non-ionic surfactant synergy leading to a more thermodynamically favourable surfactant-surfactant interaction (Stoner *et al.*, 2006), and a decrease in cooperative and high affinity binding of the ionic surfactant to a protein (Jones *et al.*, 1992). Since protein precipitation is a result of a combination of noncovalent interactions, a two-phase aqueous mixed (ionic/non-ionic) surfactant precipitation system generated in aqueous solutions aims to fine tune surfactant composition so that it attracts a desired protein of interest, when it is hydrophilic or hydrophobic.

Studying a surfactant mixture to enhance performance of surfactant precipitation has not been done in any literature reviewed. Our objectives included identifying the effect of anionic/non-ionic protein precipitation, and the effect of cationic/non-ionic protein recovery with the surfactant precipitation technique. Other alternative techniques carried out in this research were using cationic surfactants as a precipitating ligand, and using anionic surfactants as a recovery solution specifically to recover protein with a low pI which is not suitable for AOT precipitation.

7.2 MATERIALS AND METHODS

Materials: Non-ionic surfactants, Triton X-100, Tween 85 and Brij 30 were obtained from Sigma. The experiments were carried out using lipase protein powder from *Aspergillus niger* (EC 3.1.1.3, Triacylglycerol lipase) and trypsin inhibitor from chicken egg white purchased from Sigma (USA).

7.2.1 PREPARATION OF NONIONIC SURFACTANT

The nonionic surfactant solution used contained different ranges of concentration. The various Triton X-100 (up to 2.4 mM), Tween 85 (up to 0.08 mM) and Brij 30 (up to 0.26 mM) initial concentrations were prepared in distilled water. Surfactants with a non-ionic headgroup have less repulsive interactions than ionic surfactants. As a result, lower concentrations are required to force them into close proximity at the interface or to form micelles, thus the CMC of non-ionic surfactants is much lower than ionic surfactants and is in the 10^{-6}–10^{-5} M range (Mackie and Wilde, 2005). The CMC of Triton X-100 is between 0.22 to 0.24 mM at 25ºC (Tiller *et al.*, 1984), Tween 85 is about 0.01 mM and Brij 30 is 0.02 mM (Hinze and Pramauro, 1993). The surfactant concentration in the total aqueous mixture was kept within the CMC of the non-ionic surfactants.

7.2.2 PREPARATION OF MIXED SURFACTANT

Mixed AOT/Triton X-100 (90:10 mol%) surfactant solution was prepared for the precipitation of lysozyme. Triton X-100 solution (0.44 - 2.88 mM) was added to the AOT solutions (4.0 mM to 27.2 mM) that give the molar ratio of AOT to lysozyme (R=5 to 35) in the earlier AOT precipitation studies to allow comparison between the results. Another mixed surfactant, DTAB/Triton X-100 (95:5 mol%), was prepared for the recovery of lysozyme from precipitation using AOT (R=16). Triton X-100 solution (0.57 – 1.92 mM) was added to the DTAB solutions (11.3 mM to 33.7 mM) that give the molar ratio of DTAB to AOT (R=1.0 to 3.0) in the earlier DTAB recovery studies. These mixed surfactants were produced with final concentrations of each surfactant well below its individual CMC.

7.3 EXTRACTION PROCEDURES

7.3.1 PRECIPITATION OF LYSOZYME WITH NON-IONIC SURFACTANT AND MIXED (AOT/NON-IONIC) SURFACTANT

The precipitation of lysozyme with non-ionic (Triton X-100, Tween 85 and Brij 30) surfactant and mixed (AOT/Triton X-100) surfactant experiments were performed in the same way as the precipitation with AOT in Chapter 3, although the aqueous protein solution

was contacted directly with these surfactants, instead of AOT. The surfactant solution in each of the concentrations prepared was added to 10 mL of the lysozyme aqueous solution (1.0 g/L). Lysozyme remaining in the supernatant phase after centrifugation was taken to represent the efficiency of the non-ionic precipitation.

7.3.2 RECOVERY OF LYSOZYME WITH NON-IONIC SURFACTANT AND MIXED (DTAB/NON-IONIC) SURFACTANT

In a separate experiment to the one above, this recovery work began with the precipitation of lysozyme using AOT at an R value of 16 (refer to Chapter 4). The precipitated lysozyme-AOT was collected, followed by the protein recovery process with non-ionic (Triton X-100) surfactant and mixed (DTAB/Triton X-100) surfactant, respectively. The recovery solutions were used in the same way as the TOMAC, DTAB and DODMAC. The precipitated lysozyme-AOT solubilised in 10 mL of fresh buffer solution (pH 6.2) was added with the various surfactant concentrations.

7.3.3 PURIFICATION OF PROTEIN WITH CATIONIC SURFACTANT

An initial aqueous solution containing 0.04 mM of protein; lipase (mw=45 kDa) and trypsin inhibitor (mw=27 kDa), was prepared in a 20 mM potassium phosphate buffer solution and the pH adjusted to 6.2. A volume of 1 mL TOMAC solution dissolved in ethanol (11.3 to 28.1 mM) was added to 10 mL of the protein aqueous solution. The rest of the experimental method for precipitation of lipase and trypsin inhibitor with TOMAC was carried out as detailed in the surfactant precipitation technique. After centrifugation, the supernatant was analyzed for protein concentration and stability when binding with the various molar ratios of cationic surfactant, while the precipitated complex was collected and re-dissolved in fresh buffer for recovery with 1 mL of AOT (11.3 mM). Protein released into solution was analysed for both concentration and conformation.

7.4 RESULTS AND DISCUSSION

7.4.1 NON-IONIC SURFACTANT IN SURFACTANT PRECIPITATION

With the non-ionic surfactant concentrations used, Triton X-100 made up to a highest molar ratio of R=3.0 with lysozyme, whereas Tween 85 made up to merely R=0.1 and Brij 30 up to R=0.3. Upon the mixing of Triton X-100, Tween 85 and Brij 30 with lysozyme at pH 6.2, no precipitate was observed. From the UV assay and the enzymatic assay of the protein samples, results showed the original lysozyme concentration and activity in the supernatant phase. CD spectrum analysis of the samples showed no changes in the secondary structure of lysozyme. The possible reason for these observations could be the low concentrations used for direct surfactant precipitation was unable to exploit the weak negative charge of the non-ionic surfactant as a precipitating ligand.

Precipitation with non-ionic surfactants was repeated for pH values lower than 6.2 (pH 4 and 2), to enhance the overall surface charge; q of lysozyme increased from pH 6.2 (+9) to pH 4 (+12) and pH 2 (+16) (Kuramitsu and Hamaguchi, 1980). Even with a higher q to promote electrostatic interactions between the lysozyme and non-ionic surfactant, the results remained very similar. Below the CMC, lysozyme underwent a series of conformational changes as it bound to an increasing number of ionic surfactant molecules; in contrast, there were undetectable interactions with non-ionic surfactants in this concentration range. This observation confirmed the fact that non-ionic surfactants did not support direct precipitation of proteins using surfactant.

In the recovery of lysozyme from lysozyme-AOT with Triton X-100, Triton X-100 made up to R=0.2 with AOT in the sub-CMC concentrations of the non-ionic surfactant. Triton X-100 was used for both the non-ionic recovery and the mixed surfactant because other non-ionic surfactants (Brij 30 and Tween 85) have too low a CMC to be suitable to analyse the surfactant effect. Non-dissolved precipitate in the recovery phase was removed after addition of the surfactant. The Triton X-100 recovery process did not have any effect on the recovery phase; no solubilisation of protein from the lysozyme-AOT complex was found, and the final sample solutions were free of lysozyme. Non-ionic surfactants lack the charged head groups for electrostatic attraction to AOT, and were not involved in competitive binding for AOT with lysozyme to form a non-ionic-AOT dimer.

7.4.2 MIXED SURFACTANT IN SURFACTANT PRECIPITATION

The sequence of the addition of surfactant, whether preparing a mixture of ionic/non-ionic solutions separately before adding them to the protein, or adding an ionic surfactant prior to non-ionic surfactant to the protein solution, or vice versa did not seem to matter. The precipitation of lysozyme with AOT/Triton X-100 was not significantly different when compared to the precipitation with the values of AOT concentrations without Triton X-100 (Figure 3.1, Figure 3.2, Figure 3.4, Figure 3.5). The recovery with DTAB/Triton X-100 produced a plot of recovered lysozyme identical to the recovery with only the DTAB (Figure 4.9). DTAB was chosen to study the mixed cationic/non-ionic surfactants because of its solubility in water. The mixed ionic/non-ionic micelles and reverse micelles are able to improve on protein extraction in the literature. But in our work mixed ionic/non-ionic monomers did not seem useful with surfactant precipitation, most probably because in the form of monomers, ionic and non-ionic surfactants have no interactions. Therefore, the synergy of the mixed surfactant system and the charged surfactant affinity binding to protein were not exhibited.

7.4.3 SURFACTANT PRECIPITATION OF LOW pI PROTEIN

This part of the work examined the precipitation of proteins with low pIs (<5), and hence are inappropriate for surfactant precipitation with anionic surfactants. Lipase and trypsin inhibitor were used because they fulfilled the pI requirement of the test; pI = 4.5 and 4.1 (Stadelman and Owen, 1995). For proteins with relatively low pIs to remain within the stability range of their aqueous phase pH, a cationic surfactant was used in the precipitation process. In these samples, TOMAC was added to the protein solution at pH 6.2 (pH > pI) for the negatively charged protein to bound to the cationic surfactant.

7.4.3.1 Cationic Surfactant as a Precipitating Ligand

The instant formation of an insoluble complex was observed in lipase and trypsin inhibitor solutions when adding TOMAC, and a protein-TOMAC complex precipitated out of solution. Centrifuging the lipase samples at 5000 rpm for 1 min did not manage to separate the lipase-TOMAC precipitate from solution completely. Increasing the centrifugation to 7500 rpm for

30 minutes and resorting to filtration of the supernatant using 0.2 μm disposable syringe filters still resulted in some interference in the UV assay (~0.1 to 0.3 A_{280nm}) of the precipitate. Lipase samples were not analysed with HPLC to prevent blockage of the guard column due to the fine particles.

Running the far-UV CD spectrum surprisingly gave weaker traces of the undiluted lipase samples than the original lipase solution diluted to 0.25 g/L (Figure 7.1). The samples at TOMAC to lipase ratios of R=31, R=46, R=61 and R=76 were quite certain to have no soluble lipase due to the absence of the highly absorbing protein and its most prominent structure at 200-240 nm. The signal to noise ratio, which was particularly large despite the very weak signal, could be caused by some protein complex unsuccessfully removed from the supernatant. There are reasons to believe that all the lipase in the solution was denatured by TOMAC. Lipase being an extremely hydrophobic protein (Jürgens and Huser, 1981; Karadzic *et al.*, 2006), was most likely aggregated rapidly through a strong hydrophobic interaction after being attracted to the TOMAC electrostatically.

For precipitation of trypsin inhibitor with TOMAC, there was a good separation between the precipitate and supernatant using the current method. The soluble trypsin inhibitor in the samples gave the cationic precipitation efficiency, and the results obtained were within the CV of ±5%. The protein content in the samples decreased in the order of R=31 (63%), R=46 (50%), R=61 (42%) and R=76 (42%). The secondary structures of trypsin inhibitor in all the samples were that of the native protein shown in Figure 7.2 (only R=61 was drawn to simplify the graph). Trypsin inhibitor bound with 61 moles of TOMAC to produce the highest precipitate at pH 6.2, 20 mM buffer condition. Considering the trypsin inhibitor net charge of +10±2 (Kopaciewicz *et al.*, 1983), the mole ratio of TOMAC was much higher than the mole ratio of AOT needed (R=16) to precipitate lysozyme with a near net charge (Table 5.1). However, trypsin inhibitor is almost twice the size of lysozyme, and therefore has a lower charge density, and has more than a three-fold lower surface charge distribution %S (29%) (Barlow and Thornton, 1986) which might justify why more surfactant molecules were required to neutralize the protein charges. There is a possibility to improve the precipitation of trypsin inhibitor measured here since the optimal pH for trypsin inhibitor binding is known to be 8.0 (Laskowski and Laskowski, 1954).

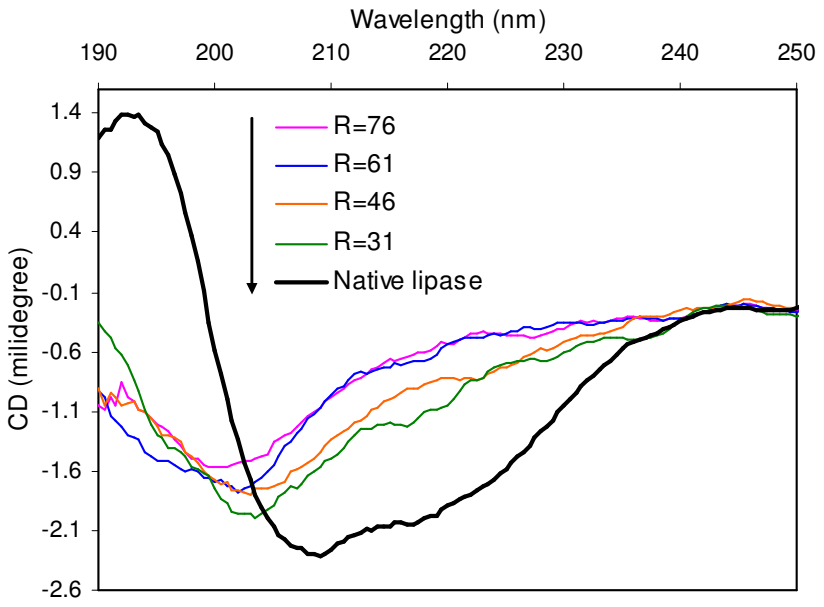

Figure 7.1 **Far-UV CD spectra of lipase in phosphate buffer (20mM), pH 6.2: Initial lipase solution without TOMAC diluted to 0.25 g/L; final aqueous phase left after precipitation of lipase at different TOMAC to lipase ratios (R=31, R=46, R=61 and R=76).**

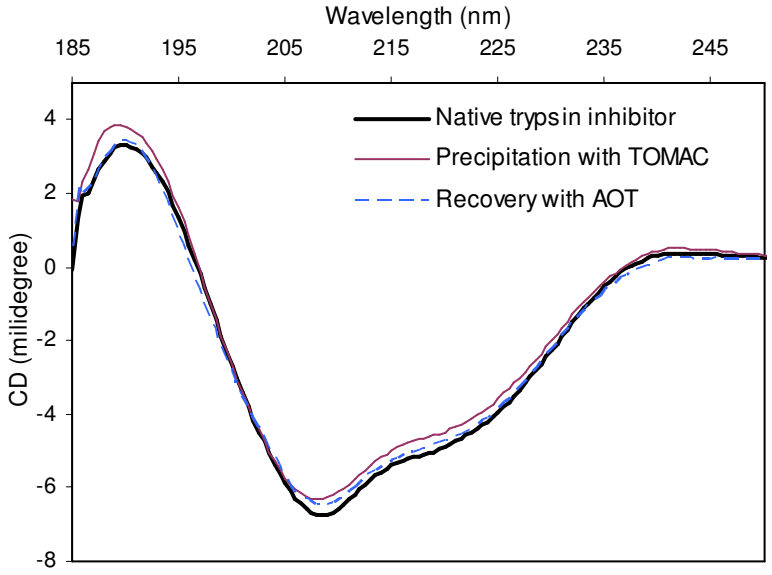

Figure 7.2 **CD spectra of the trypsin inhibitor sample, in phosphate buffer (20 mM), pH 6.2, precipitated with TOMAC 22.5 mM and recovered with AOT 11.3 mM. All samples diluted to 0.1 g/L.**

7.4.3.2 Anionic Surfactant in Counterionic Recovery

The sample with the highest trypsin inhibitor-TOMAC precipitate, R=61 at 22.5 mM TOMAC concentration, which was successfully collected was submitted to counterionic recovery using AOT. The concentration of AOT which produced two moles of TOMAC per mole of AOT as determined in the cationic recovery experiment (Chapter 4) was chosen to bind to the total TOMAC in solution to release trypsin inhibitor back into solution. A complete recovery of protein was achieved (58±4%) and the protein conformation was retained (Figure 7.2) to prove that TOMAC as a precipitating ligand did not denature trypsin inhibitor.

7.4.3.3 Precipitation of Low pI Protein with AOT

The purification of lipase and trypsin inhibitor has not been tested with the surfactant precipitation technique yet, but literature relating protein net charge and electrostatic interaction with surfactants (Andrews *et al.*, 1994; Shin *et al.*, 2003b) allow us to hypothesize that there is no formation of a protein-AOT complex when the two proteins are contacted with AOT at the pH of counterionic recovery. Hence, we experimented with the possibilities of any interaction, i.e. hydrophobic, between lipase or trypsin inhibitor and AOT which would lead to protein aggregation in the absence of TOMAC; experiments were as the one performed on lysozyme with AOT just that in this case proteins having a low pI were used.

Results showed that it is true that there was no removal of protein through lipase-AOT or trypsin inhibitor-AOT formation at pH 6.2 because of the electrostatic repulsion between the protein and AOT headgroups. Also, there was no aggregation of lipase and trypsin inhibitor from hydrophobic forces as was suspected for lipase and TOMAC in the above studies. AOT did not interfere with the lipase and trypsin inhibitor structure; a CD spectra of the protein after being contacted with R=9, 18, 30, 42, 55 and 67 of AOT showed their original conformations in Figure 7.1 and Figure 7.2. Therefore, it is safe to say that AOT added to the precipitated protein-TOMAC binds only to the TOMAC to achieve full recovery of the protein, and any excess AOT remains in solution without interacting with the protein. This experiment confirms that the lack of attractive forces prevent favourable monomer binding from being present to initiate sub-micellar protein unfolding (Nielsen *et al.*, 2007).

7.5 CONCLUSIONS

From the investigation, non-ionic surfactants proved incapable of surfactant monomer binding and hence cannot be used in either precipitation, or the recovery of protein. The advantages of non-ionic surfactant low affinity interactions and non-denaturing effects exhibited in reverse micelles and aqueous two-phase micellar extraction using mixed ionic/non-ionic system were not apparent in surfactant precipitation. Protein was virtually unaffected by the presence of non-ionic surfactants. Cationic surfactant proved otherwise, and varying outcomes were obtained for the precipitation of lipase and trypsin inhibitor with TOMAC. Lipase was fully precipitated but encountered complications in the recovery process due to its aggregated form. Trypsin inhibitor at a stoichiometric molar ratio of 61:1 (TOMAC:protein) at pH 6.2 achieved 58% precipitation efficiency, and 100% recovery efficiency with AOT. The low %S of trypsin inhibitor contributed to the large molar ratio of binding to TOMAC. The hydrophobicity of lipase was blamed for protein aggregation, while the unoptimised solution conditions for trypsin inhibitor binding was blamed for incomplete precipitation.

It is essential to note that the discussion on cationic precipitation with surfactant was based solely on an assumption that the mechanism of surfactant precipitation remained unchanged, with the cationic surfactant as a precipitating ligand in the place of an anionic surfactant. It was also assumed that surface charge distribution determines protein precipitation behaviour, and hydrophobicity determines protein unfolding. There is a possibility that the binding of protein with TOMAC follows different charge interactions and hydrophobicity effects. This type of surfactant precipitation is left to further research by studying a greater variety of cationic surfactants and proteins within the low pI category, armed with the knowledge from this research. One drawback of cationic precipitation is the analytical techniques employable are limited by the choice of the cationic surfactant; a majority of anionic surfactants such as alkyl sulfates are spectroscopically silent making them compatible with basically all spectroscopic techniques, whereas a selective group of cationics (i.e. chloride counterion) are preferable over those (i.e. bromide counterion) with strong absorption in the protein UV range.

Lastly, this work is no less important in discovering the workability and potential of an alternative methodology for surfactant precipitation. This research has provided insights into various surfactant initiated protein precipitation systems.

CHAPTER 8 CONCLUSIONS AND FUTURE WORK

8.1 INTRODUCTION

In this chapter a summary of the research findings is presented in order to provide a clear understanding of the insights gained into a new but not yet commercialised protein separation technique using surfactant precipitation. This summary will include; relationships between protein stability and some key parameters, improvements in protein recovery techniques, selective separation of a target protein, and applications of surfactant precipitation. The results obtained and their significance to the fundamental and practical understanding of the subject as detailed in the objectives is assessed. This is followed by a brief discussion on the limitations and future direction of research needed based on the present findings.

8.2 SUMMARY OF THE RESULTS

8.2.1 PROTEIN STABILITY

The precipitation of protein from an aqueous phase by addition of surfactant monomer is controlled by many system parameters in both the aqueous and surfactant solutions. All the previous work studied manipulated the key system parameters, e.g. ionic strength, molar ratio of surfactant to protein, and pH to optimise precipitation. However, the effect of these parameters on the stability of the protein have not been investigated when in contact with the monomeric structure of a surfactant. Furthermore, there have been questions in past research about which structural feature of the surfactants determine their denaturing properties. Arguments were arbitrary and experimental evidence was missing to allow researchers to rule out surfactant monomers being the cause of denaturation in these type of systems. **Therefore, the first objective of this study was to examine the unfolding behaviour and secondary structure of native lysozyme in a solution of AOT monomers.**

The study of the effect of the molar ratio of surfactant to protein showed that even monomer binding at relatively low surfactant concentrations has an effect on reducing protein stability

beyond a certain surfactant:protein ratio. A group of hydrophilic proteins with a monomeric structure, lysozyme, cytochrome c and ribonuclease A was tested with the anionic surfactant (AOT), and the protein samples began to unfold after achieving complete precipitation with AOT at a surfactant:protein ratio of 16, 17 and 22, respectively. The trend of denaturation of these proteins in surfactant precipitation was found to be a gradual alteration of the protein from an inactive phase to a denatured phase. The overall conformation of the protein was strongly influenced by the surfactant concentration. For this group comprised of α/β and α-rich globular proteins, denatured protein was dominated by β-sheet structures, and the more unfolded the protein was at higher surfactant concentrations the more it produced random coils. This work enabled us to state conclusively that surfactant monomers do not determine denaturation, rather it is the protein structure. Therefore, another group comprising basically β-rich proteins, trypsin and α-chymotrypsin, were analysed. Trypsin and α-chymotrypsin only achieved up to 43% and 62% precipitation at surfactant:protein ratios of 14 and 8, respectively. Formation of a partially unfolded intermediate component appeared for both proteins, and trypsin cooperatively transits to non-native alpha-helical structures in its unfolding pathway.

The study of the effect of phosphate buffer salt on protein stability showed that the enzyme conformation necessary for activity might require the presence of a certain concentration of ions. Previous work gave 63% protein activity recovery from 25 mM sodium acetate at pH 4.5. In this work, lysozyme prepared in 20 mM phosphate buffer was determined to be an effective lytic agent from pH 4 to pH 9, where the protein remains fully active. The use of a potassium phosphate salt has a similar effect as sodium chloride in the sense that the amount of lysozyme complexed with AOT was consistent (100%), and was not influenced by buffer solution strengths up to a high concentration of salt; 0.3 M for NaCl, and 0.1 M for K_2PO_4, before reduced solubility and precipitation. The lysozyme sample in phosphate buffer from 20 mM does not undergo structural transformation. However, a slight structural rearrangement in the 100mM sample was due to the high concentration of buffer salt weakening the lysozyme solvation with water molecules, reducing the self-association within the lysozyme chain so that lysozyme had a more compact α-helix structure. These findings resembled those without addition of surfactant, and therefore the effect of ionic strength was not attributed to the interactions between lysozyme and AOT.

System conditions affect the physiochemical state of the protein and its interaction with the surfactant head groups in the formation of a protein–ligand complex. **Thus in line with the**

first objective, our next objective was to understand the interactions involved during various protein-surfactant complex formation conditions.

From the above study, it was found that the binding of surfactant to protein increased continually after the complete removal of lysozyme, cytochrome c or ribonuclease A was attained. Results obtained point to the likelihood that most of the AOT ligands bind to the hydrophilic outer surface of the protein up to the molar ratios required for removal, while increasing concentration of AOT molecules beyond this point bind to the non-polar outer surface and enter the hydrophobic intracavity of protein. The more attractive short-range hydrophobic interactions between the protein and surfactant after the molecules are brought together by the dominant intermolecular interaction (electrostatic forces) caused the unfolding of lysozyme, and contributed to the change in secondary structure described above. With trypsin, the non-native secondary structure of the protein was in accordance to three modes of interactions it encounters in its unfolding pathway; (1) AOT molecules were associated with specific binding sites on the native protein, (2) AOT molecules began cooperatively associating with protein without major conformational changes, and (3) large numbers of AOT were cooperatively associating with trypsin through the formation of trypsin-surfactant clusters causing gross denaturation. The different stages of binding were less prominent for α-chymotrypsin with AOT because of considerably less formation of intermediate state components.

The study of the effect of pH on protein-surfactant complex formation indicated that electrostatic interactions between oppositely charged protein and surfactant molecules drive the precipitation process. Only 39% precipitation was achieved at pH 12 because lysozyme at a pH>pI finds it difficult to bind with the monomers of AOT due to the electrostatic repulsion between the protein and surfactant headgroups. This weak affinity for ionic binding initiated hydrophobic binding of lysozyme with surfactant molecules causing the protein to lose its original structure.

It is evident from this study that the unfolding of a protein is closely related to the hydrophobic interactions between the protein and surfactant molecules, and it can act as a parameter for protein stability during the formation of a protein-surfactant complex in surfactant precipitation. The knowledge of protein-surfactant interactions involved, and the protein conformational stabilities with respective precipitation parameters improves our capacity to preserve the function and structure of a protein during this method of extraction.

8.2.2 PROTEIN RECOVERY

In surfactant precipitation, most of the previous studies use acetone to dissolve the surfactant precipitate, and recovered lysozyme as a precipitate, but protein denaturation was a strong function of the time protein was solubilised in the solvent. A method of recovering protein from a protein-surfactant complex that does not promote denaturation is required in order to enhance the process of surfactant precipitation. **The objective of this part of the research was to optimise the solvent recovery method by examining commercially viable solvents (ethanol, methanol, ethanol/acetone and ethanol/water) besides pure acetone.** The recovery of lysozyme with ethanol in this study was about the same as that recovered with acetone (80%) in past work, however, the activity and conformation of the protein was retained when ethanol was used as a recovery phase. Furthermore, pure ethanol was not absorbed at the wavelength of the protein as it was with the 50% v/v ethanol/acetone mixture, and the lysozyme precipitated out of the ethanol phase was not redissolved in the water as it was with the 50% v/v ethanol/water mixture. In the solvent recovery study, a large amount of lysozyme was lost in methanol (70%), while only some was lost in ethanol (20%). The key concerns affecting protein recovery with solvent were found to be the solvent's polarity and protein solubility in the organic solvent. This work enabled us to evaluate commercially viable solvents for protein recovery based on their ability to maintain the conformational stability of the protein.

A new and improved method of recovery was proposed using a surfactant counterionic to the AOT used for protein precipitation in order to maximise the extraction yield and activity, as well as to avoid the concerns of solvent recovery. **The next objective was to evaluate the use of different cationic surfactants to develop the method of counterionic surfactant recovery.** The use of a cationic surfactant (TOMAC, DTAB and DODMAC) was evaluated to form a nonpolar ion pair with the negatively charged AOT molecules which could lead to the resolubilisation of the protein into solution. Complexes of the cationic surfactant and AOT were formed at a 2:1 molar ratio, and the length of the alkyl surfactant increases according to DTAB<TOMAC<DODMAC. All counterionic surfactant solutions gave full recovery efficiencies, except DODMAC (81%) where its lower charge density hindered the complete formation of a DODMAC-AOT complex; furthermore, the hydrophobicity of its long chain also inhibited further release of the lysozyme. The effect of the surfactant counterion (Br^- versus Cl^-) was seen at higher cationic surfactant concentrations where the fast exchange of the bromide with the sulphate anion of AOT was prevented thereby slowing

down the complexation of DTAB-AOT. Hence, the charge density, hydrophobic interactions and counterion effect of cationic surfactants are the key parameters in the efficiency of counterionic recovery in surfactant precipitation. Among the counterionic surfactants studied for protein recovery, TOMAC was the preferred cationic surfactant due to its high product recovery.

An efficient protein recovery procedure should retain protein's bioavailability in various solution conditions. **The next objective was to compare the efficiency between solvent and counterionic recovery based on the effect of the phosphate buffer conditions (ionic strength and pH) and activity recovery.** The study of buffer ionic strength on protein recovery showed that lysozyme was released from the lysozyme-AOT complex completely at an optimum phosphate concentration (20 mM), but higher buffer salt concentration reduces the dissociation of the complex. Counterionic recovery decreased slightly to 92% at 100 mM phosphate buffer, however, the weakness of solvent recovery due to the protein solubility issue was still more important in recovery. From the study on the effect of specific ionic binding with changes in pH it was determined that complete formation of the protein-ligand complex (AOT:lysozyme=16) was possible between pH 4 and 9. Nevertheless, the state of ionization of the protein amino acid side chains in alkaline solution (pH 9) was unfavourable for the recovery of lysozyme, and recovery was reduced to 88% with TOMAC surfactant and 63% with ethanol solvent. For the other pHs within the range studied, including an acidic solution (pH 4), TOMAC recovered the original concentration and activity of the lysozyme. Ethanol recovery at pH 4, on the other hand, showed a particularly low lysozyme recovery (11%) because greater protein solubility in ethanol was involved as it is farthest away from the pI. Therefore, in this study the pH of the protein aqueous phase also defined the protein's subsequent solubility in solvent during the recovery process. Hence, the use of a counterionic surfactant has clear advantages over the use of solvents in recovery, and this is one of the major contributions of this work to the field.

This research successfully demonstrated an improved protein recovery process for surfactant precipitation. The study showed that the secondary structure of the protein was preserved in the presence of TOMAC and ethanol without being denatured over a short period of time.

8.2.3 MECHANISM

This is the first piece of work within those in the same area, where protein separation from surfactant precipitation has been discussed as a function of the surface properties of the protein, which plays an important role in protein-surfactant interactions and protein stability. **This objective was to examine the influence surface charge and hydrophobicity have on extraction of a single protein in solution.** The study found that **surface charge distribution** is the main factor controlling protein extraction in surfactant precipitation. Lysozyme, cytochrome c, ribonuclease A, trypsin and α-chymotrypsin extracted from a phosphate buffer solution and a non-buffered solution gave differing optimum surfactant:protein ratios for the precipitation of these proteins, but the extraction behaviour were well represented by surface charge distributions in different system conditions. The trend of the plots (surface charge distribution versus surfactant:protein ratio) showed that the hydrophobic proteins, trypsin and α-chymotrypsin, should be analysed in a separate group from the three hydrophilic proteins. Hydrophobic proteins have hydrophobicity effects dominate the weaker polar charges on the protein surface, and are more prone to denaturation. Hydrophilic proteins with more non-polar groups on their surface (cytochrome c<ribonuclease A<lysozyme) allowed a higher excess of surfactant interactions (8 moles AOT/cytochrome c, 12 moles AOT/ribonuclease A, 19 moles AOT/lysozyme) after saturation of the protein binding sites have been reached for a stable unfolded component to be formed. Therefore, this study also found surface charge and hydrophobicity as influential protein characteristics determining the unfolding behaviour of a native protein in a solution of AOT monomers.

The selectivity of the surfactant precipitation method has not been previously reported in the literature when trying to separate proteins with similar pI values. **Hence, the next objective was to investigate the selective separation of sets of protein mixtures with the same pI and same range of molecular weights as a function of surface properties.** The study showed that proteins having a higher overall surface charge with a symmetrical charge distribution are most likely to precipitate in extraction. Proteins with different selectivities in mixtures can be extracted using surfactant precipitation by following a series of sequential precipitation and recovery steps. Based on these findings, lysozyme and ribonuclease A were selectively separated from a binary mixture. The study correlated accessible charged residues distributed on a protein surface with the degree of extraction in three separate binary mixtures (R^2=0.9185). The results showed that the differences in surface charge distribution between biomolecules is probably the most important factor affecting separation performance after the

ionic interactions between biomolecules and surfactant molecules. This novel method of protein separation has possible applications in the isolation of proteins from complex mixtures and industrial broth.

8.2.4 APPLICATION

Extraction of a protein from buffer solution has been used as a model in all of the previous studies. To be realistic, the extraction should be performed with fermentation broth. **Thus, the influence of fermentation broth on selectivity of extraction and protein folding was the next objective.** In this study, lysozyme, cytochrome c and ribonuclease A were dissolved in fermentation broth, then extracted individually and in mixtures. Each protein showed similar precipitation behaviour with increases in AOT as demonstrated in buffer solution, and they were recovered at their original activities despite lower yields (85±7%). On reaching the surfactant:protein ratio for highest recovery, significant denaturation of protein increased from cytochrome c, ribonuclease A to lysozyme. This trend in the denaturation profile was attributed to protein-surfactant interactions dominating the precipitation procedure. The study indicated that broth constituents were more influential with proteins with higher surface hydrophobicity. In the broth proteins were unfolded irreversibly as a non-dissolvable precipitate, contrary to their behaviour in buffer where renaturation of the unfolded soluble component was found to occur when the excess AOT was removed from solution. Protein selectivity of mixtures in buffer was found to be a close representation of the selectivity in fermentation broth, and the same strong correlation between the surface charge distribution of proteins and separation was achieved (R^2=0.9492). The ability to use surfactant precipitation with a real system, such as fermentation broth, is a key determinant for this technique to become commercially interesting.

The use of other types of surfactants besides anionic ones was investigated with surfactant precipitation in this work to ensure that protein purification could be applied to proteins where AOT was not suitable, e.g. proteins with a low pI. **The next objective was to develop a surfactant precipitation technique with TOMAC for trypsin inhibitor and lipase.** Surfactant precipitation was tested with using a cationic surfactant (TOMAC) as a precipitating ligand, and anionic surfactant (AOT) as a recovery solution, and proteins were extracted in 20 mM buffer at pH 6.2. Trypsin inhibitor at a TOMAC:protein ratio of 61 achieved 58% precipitation efficiency, 100% recovery efficiency, and the protein

conformation was retained. The large molar ratio of binding to TOMAC was likely to be due to the low surface charge distribution and charge density of trypsin inhibitor. Lipase, which is an extremely hydrophobic protein, was found to be fully precipitated but unsuccessfully recovered due to its aggregated form. Assuming that the mechanism of surfactant precipitation remained unchanged with TOMAC as a precipitating ligand in the place of AOT, surface charge distribution would define the protein precipitation behaviour and hydrophobicity would define the protein unfolding. There is potential for a more efficient separation system using cationic surfactants, e.g. the precipitation of trypsin inhibitor can be further improved by optimizing the key parameters of the process determined earlier in the study, such as pH and ionic strength.

Anionic surfactants offer great advantages for the purification of proteins in surfactant precipitation. The only concern is that at a certain sub-CMC concentration in the cooperative binding region, ionic surfactants form surfactant clusters which contribute to protein unfolding by association between protein molecules through hydrophobic forces. Non-ionic surfactants, however, do not form surfactant clusters, and benefit from their weak electrostatic interactions and non-denaturing character in protein extraction in non-ionic microemulsions, reverse micelles and aqueous two-phase micellar systems. In addition, a mixture of ionic and non-ionic surfactants is known to lead to a surfactant synergy which decreases the cooperative and high affinity binding of ionic surfactant. **To examine such possibilities in surfactant precipitation, the final objective of the research was to employ alternative surfactants (Triton X-100, Tween 85, Brij 30, AOT/Triton X-100 and DTAB/Triton X-100) for precipitation and recovery.** The range of non-ionic surfactant concentrations used was very small because of their much lower CMCs compared to ionic surfactants. The study showed that surfactant precipitation was unable to exploit the weak negative charge of the non-ionic surfactant for binding with AOT, or to form a non-ionic-AOT dimer for the recovery of protein. With mixed ionic/non-ionic monomers, the synergy of the mixed surfactant system and the charged surfactant affinity binding to protein were not exhibited because ionic and non-ionic surfactants in the form of monomers have no interactions. Therefore, non-ionic surfactants proved incapable of surfactant monomer binding and hence cannot be used in either precipitation, or the recovery of protein, or in a mixed ionic/non-ionic system.

In summary, we have developed a surfactant protein purification method (precipitation and recovery processes) which could have a substantial impact on bioprocessing because it has;

potentially low overall costs, it is simple, achieves excellent product recovery and maintains virtually all the enzyme bioactivity, all of which are prerequisites for a feasible alternative to current bioseparation techniques.

8.3 FUTURE WORK

8.3.1 PROTEIN STABILITY

- The unfolding of protein by surfactant monomers occurred in various unfolded states. The fractions of unfolded protein can be isolated to investigate each feature in a particular state to provide more information for protein folding in surfactant precipitation as an effect of the characteristics of a target protein.
- Cationic surfactant precipitation has been found to successfully recover full activity and conformation of trypsin inhibitor at the conditions that we fixed. The research provided high confidence in the workability and potential of the system, and further work should be carried out on parameters such as the surfactant:protein ratio, pH and ionic strength to improve the extraction efficiency, and at the same time to determine the protein folding behaviour and the interactions involved.

8.3.2 PROTEIN RECOVERY

- Limitations to the current work involved the inability to determine the amount, if any, of insoluble denatured protein-surfactant product being formed aside from the TOMAC-AOT complex produced. Therefore, a method to separate the precipitates if both were present simultaneously is necessary for accurate quantification of the unfolded component.
- The protein recovery study found that the bromide counterion of cationic surfactants provided competition for the exchange with anion of AOT. In order to understand the effect of bromide ion in counterionic surfactant recovery, different molecular weight and alkyl chain length bromide surfactants such as cetyltrimethylammonium bromide (CTAB) (mw=364.45) and dimethyldioctadecylammonium bromide (DODMAB) (mw=630.95) should be investigated.

- The "retrograde dissociation" process from literature which introduces a more hydrophobic surfactant (SDS) to dissociate surfactant from the complex can be examined with AOT. The effect of phosphate buffer conditions (ionic strength and pH) and activity recovery should be studied with "retrograde dissociation", and the efficiency then compared to that of counterionic recovery.

8.3.3 MECHANISM

- The key factors controlling separation in the cationic surfactant precipitation system should be investigated with various low pI proteins of different characteristics, in particular surface charge and hydrophobicity, in order to be able to state conclusively if the system behaviour is identical to that of anionic surfactant precipitation.

8.3.4 APPLICATION

- Research into the effect of broth constituents on surfactant precipitation extraction highlighted some interesting findings: known broth constituents and other fermentation broth should be investigated in more detail.
- Cationic surfactants with bulky head groups such as the trimethylammonium group is known to not denature proteins significantly due to the lack of strong ionic interactions with the negatively charged groups on the protein surface. The application of cationic surfactant precipitation should be tested with surfactants such as DTAB and CTAB, and in fermentation broth.
- The possibilities of incorporating surfactant precipitation with other downstream processes should be examined. For example, the addition of a non-ionic water soluble ligand such as polyethylene glycol (PEG) to couple to the hydrocarbon chain of anionic surfactant to prevent protein unfolding from hydrophobic forces, producing a anionic/polyelectrolyte system to improve the selectivity of hydrophilic protein extraction that is based on the accessibility of charged residues distributed on the protein surface, and using dead end filtration or microfiltration to separate precipitates from supernatants where centrifugation has encountered complications.

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