Imperial College London

PROTEIN SEPARATION USING SURFACTANT PRECIPITATION

BY

FADZLIE WONG BIN FAIZAL WONG

A Thesis Submitted for the Degree of Doctor of Philosophy of Imperial College

DEPARTMENT OF CHEMICAL ENGINEERING

IMPERIAL COLLEGE

2015

ABSTRACT

Protein precipitation using a variety of surfactants has been shown to have considerable potential as a protein separation technique, and considerable work on using anionic surfactants has been carried out by previous researchers. However, anionic surfactants are only suitable for high pI proteins due to concerns about protein stability. Therefore, the first aim of this work was to develop a surfactant precipitation method for low pI proteins based on using cationic surfactants. The effect of important parameters such as the molar ratio of TOMAC to protein (R_p), and pH on the precipitation of bovine serum albumin, α-amylase, and trypsin inhibitor were examined. Recovery of the TOMAC-protein complex by solvent extraction and counter-ionic surfactant (AOT) was also studied. Varied results were obtained for the three proteins, and were correlated with protein properties, and it was found that the protein's hydrophobicity and molecular weight were the best predictors for precipitation efficiency and recovery.

The second aim of this research was to examine the feasibility of using a biocompatible surfactant – methyl ester sulphonate (MES) as a precipitating-ligand for target proteins in this surfactant precipitation technique. This work was a major breakthrough in the application of a new generation of 'green' surfactants for protein extraction. Lysozyme was used as a model protein in a single component system, and the influence of R_p, and pH were examined. Similarly, the recovery of the precipitate using solvent extraction and a counter-ionic surfactant, AOT, was studied. The performance of MES in precipitation was compared to a conventional surfactant, AOT, and it was found that their performance was comparable. This further highlighted its potential to be used as precipitant in protein purification.

The third aim of this work was to apply the surfactant precipitation method to the purification of a target protein from a real industrial sample. Bacteriocin produced by *Pediococcus acidilactici* Kp10 was chosen as a target protein for this purpose. With a concentration of 11.56 mM of AOT (pH 4), precipitate recovery by acetone (0.99 mM NaCl), and a final recovery phase of 20 mM PBS (pH 7), about 86.3% of overall activity recovery, and a purification factor of about 53.8 was obtained. Further, this separation technique was shown to be better than reverse micellar extraction, and aqueous two-phase extraction in terms of performance. Hence, the surfactant precipitation technique was proven to be an effective and a viable separation method.

DECLARATION OF ORIGINALITY

The work described in this thesis was carried out in the Department of Chemical Engineering, Imperial College London, United Kingdom between November 2011 and September 2014. 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.

Fadzlie Wong Bin Faizal Wong

Imperial College, August 2015

COPYRIGHT DECLARATION

ACKNOWLEDGEMENTS

Grateful to Allah Subhanahuwataala, who mercifully favoured me to accomplish this study. With his blessings, thanks to Him for this valuable experience.

I would like to thank my supervisor, Professor David C. Stuckey for his guidance and attention given to me during this study. The same goes to my other supervisory committee member from Malaysia, Prof. Dr. Arbakariya Ariff. It was through their critical comments and ideas that significantly helped me to complete the study. I would also like to extend my appreciation to Dr. Jerry Heng for his advice as my academic mentor at Imperial College.

I would also like to thank my beloved parents, wife and brothers for their support and motivation to get me through the study. They are always my biggest source of motivation and inspiration.

Furthermore, I would also like to thank my colleagues at the laboratory and officemates who created such a harmonious environment, providing help, comments, support and most importantly, friendship. They are Keeran, Nazri, Bonahis, Wen Qian, Beatriz, Yu Qiong, Jeong, Ana, Yati, Esteban, Somnuk, Rami and Juan.

Last but not least, I would also like to show my gratitude to the Ministry of Education, Malaysia who sponsored my study in this PhD programme.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 INTRODUCTION

1.1 DOWNSTREAM PROCESSING OF PROTEINS

Production of commercially important proteins such as industrial enzymes and therapeutic proteins is one of the most important areas in industrial biotechnology. Downstream processing of these products is the most challenging and overall cost-determining step immediately after the fermentation step (Sadama and Beelaram, 1995). There are various choices of unit operations available to be considered as a primary purification step of the target protein from the crude extract. To date most of the primary protein purification methods rely on chromatographic techniques which are based on the retention of a target protein in the stationary phase followed by a selective elution step. The method has been well established, and offers high resolution product recovery. However, chromatography is known to be high in capital and operational costs, occasionally complicated, and has low process throughput. Thus, there is a pressing need to develop alternative separation methods in the field of the bioseparation of proteins.

1.2 PRECIPITATION OF PROTEINS

Classical precipitation has the advantage of enabling purification in addition to concentration of the protein. A typical way of precipitation is by adding a compound to precipitate the target protein followed by centrifugation to obtain the protein pellet; the protein pellet is then re-dissolved in a new buffer that is compatible with the subsequent downstream processing. Hence, a soluble target protein relatively free from other unwanted impurities is eventually obtained. Additionally, this two stage separation method is very suitable for use with a large volume of sample (Hilbrig and Freitag, 2003). Theoretically, precipitation occurs as a result of the removal of 'bound' water molecules from the hydrophobic surfaces of a protein that causes protein association by hydrophobic interaction (Wenk and Fernandis, 2007); the protein then precipitates due to aggregation and an increase in relative density.

Several precipitants have been used previously for protein precipitation including salts, polyethylene glycol (PEG) and organic solvents (Hönig and Kula, 1976; Shih et al., 1992; Chertov et al., 2004). However, varying degrees of success have been achieved due to limitations such as low recovery, non-specificity, solubility problems and denaturing effects (Scopes, 1994; Vaidya et al., 2001). Nevertheless, despite these drawbacks precipitation remains a simple and cost effective method for protein separation.

Shin (2002) developed the novel method of using a surfactant, di-(2-ethylhexyl) sulfosuccinate (AOT), in protein precipitation, and this method was also investigated further by Cheng and Stuckey (2011), and this work supported its potential for protein separation. In fact, surfactants are deemed to be an interesting choice for such a purpose since they are widely used in laboratories, simple, and inexpensive. Price et al. (2003) even suggested that the reaction behaviour of surfactants at interfaces are expected to be more representative of biological reactions than reactions in dilute aqueous solutions. Precipitation only requires a short processing time as there is instant formation of an insoluble protein-surfactant complex upon contact, and can operate at room temperature or even below, minimizing protein denaturation (Shin et al., 2003). Compared with the reverse micelle technique, direct surfactant precipitation uses significantly less surfactant. Furthermore, it is also proven that the protein recovered maintains its original activity (Shin, 2002). The purity of the final product is also not compromised as the recovered protein is free of surfactant and other contaminating proteins (Shin et al., 2004).

The aim this work was to look more closely at the feasibility of using surfactants as a precipitant to improve protein precipitation and separation. Basically this work is the continuation of previous work (Shin et al., 2003; Cheng and Stuckey, 2011) in order to improve the understanding and application of this technique.

1.3 MOTIVATIONS AND AIMS

Previously, significant work had been carried out on the development of the surfactant precipitation technique, especially for high pI proteins using the anionic surfactant, AOT. Nevertheless, little emphasis has been put on the use of its cationic surfactant counterparts in this application. To this end, it was unclear whether cationic surfactants could perform a similar function, and if a similar mechanism is shared in the case that it does. Thus, with this in mind, the first aim was to develop a surfactant precipitation method for low pI proteins based on using cationic surfactants.

A new generation of ‘green’ surfactants has also emerged in the detergent manufacturing industry due to raised concerns about environmental issues. One example of this class of surfactant is methyl ester sulphonate (MES) which is a palm-oil derived surfactant with abundant environmentally friendly features, not to mention with improved performance in detergency. Hence, the second aim of this research was to examine the feasibility of using a biocompatible surfactant - MES as a precipitating-ligand for target proteins in this surfactant precipitation technique.

In order to establish the surfactant precipitation technique as a viable bioseparation tool, its ability to perform in a real complex fermentation broth must be proven. Is that possible for surfactants to selectively precipitate a target protein molecule in the presence of high concentrations of impurities

such as found in a fermentation broth? Hence, the third broad aim of this work was to apply the surfactant precipitation method to the purification of a target protein from a real industrial sample.

1.4 THESIS OUTLINE

Chapter 2 presents the literature review associated with the subject of the protein separation technique by surfactant: fundamentals of proteins and surfactants, including their properties and previous studies on this separation technique.

Chapter 3 describes the experimental procedures for the precipitation of low pI proteins with cationic surfactants, and their recovery from the precipitates. The influence of various experimental parameters such as; molar ratio of surfactant to protein, pH, type of recovery solvent and counter-ionic surfactant, on performance was also discussed. In addition, the results of the analytical assays used in this work were also reviewed such as: circular dichroism to determine the secondary structure of the protein, and surfactant concentration measurements by methylene blue/methyl orange, to support the research findings. The possibility of establishing a correlation between various protein properties on the performance parameters such as the percentage of precipitation and recovery was also presented.

Chapter 4 explored the feasibility of using a biocompatible surfactant, methyl ester sulphonate (MES) derived from palm oil, to be used as a precipitating-ligand in this separation technique. Optimisation of process parameters during the precipitation (molar ratio of surfactant to protein and pH) and recovery stage (type of solvent and ratio of counter-ionic surfactant to protein) was also detailed. The effect of these experimental conditions on protein stability was also examined, and a comparison made with the conventional surfactant, AOT, in terms of performance was also presented.

In Chapter 5, the viability of this separation technique in protein downstream processing was investigated, and it application to purify an anti-bacterial peptide, bacteriocin from a complex *Pediococcus* M17 broth was examined. The influence of system parameters, namely pH and surfactant (AOT) concentration was described, and in addition, a general comparative study with other purification techniques was presented.

Finally, a summary of the results and recommendations for future work are concluded in Chapter 6.

CHAPTER 2 LITERATURE REVIEW

2.1 PROTEIN STRUCTURE

The native conformation of a protein is key to its biological activity or functionality; however, the structures are dynamic with localised variations in their conformation (Darby and Creighton, 1993). Proteins have a repeating backbone from which 20 different possible kinds of side chains protrude. Their single backbone is termed a polypeptide which is made up of amino acid residues that are linked together by peptide bonds. The peptide bond has a resonance effect (delocalisation of electrons over several atoms) that causes the atoms involved with the bond to be coplanar, although the other two bonds, the C-N and C-C bonds which are not involved with the peptide bond can have free rotation within certain angles. Thus, a protein is a unique polymer with rotatable covalent bonds alternating with rigid planar ones (Petsko and Ringe, 2004); this forms the basis of protein structure and folding.

In addition, the stability of proteins is maintained by several non-covalent interactions, namely; electrostatic interactions, Van der Waals interactions, and hydrogen bonds. Formation of a hydrogen bond is energetically favourable, either between the polar groups themselves (if present in the interior of the protein), or with water molecules (if present on the protein surface). The tendency to form hydrogen bonds amongst protein polar groups leads to the formation of the secondary structure of a protein.

2.1.1 THE SECONDARY STRUCTURE OF A PROTEIN

β-turn: The simplest form of secondary structure is formed by the hydrogen bond between the carbonyl oxygen of one residue (n) and the amide, N-H, of residue n+3; the hydrogen bond formed makes the chain turn in the reverse direction. These elements are prevalent on protein surfaces which are in contact with an aqueous environment (Petsko and Ringe, 2004).

α-helix: The most common secondary structure in a protein is formed by local hydrogen bonding between the carbonyl oxygen atom of each residue (n) and the amide nitrogen of residue n+4. A resulting cylindrical structure is formed as all of the polar amide groups form hydrogen bonds with one another except for the amino and the carboxy acid terminal group (Petsko and Ringe, 2004).

β-sheet: A secondary structure that is formed by hydrogen bonding between polar amide groups of two or more separate strands which are arranged side by side. 2 types of β-sheet exist: parallel, if the chains are in the same direction and anti-parallel, if the chains are antiparallel to one another (Petsko and Ringe, 2004).

2.1.2 PROTEIN FOLDING IN WATER

Proteins are made up of polar and non-polar amino acid residues. Hence, in water the polar and charged groups of the protein will form a favourable hydrogen bond with water molecule. On the other hand, the non-polar, hydrophobic patches cannot do so and their presence on the protein surface will disrupt the hydrogen-bonded structure of water (Petsko and Ringe, 2004). Hence, these hydrophobic side chains tend to clump together, forming Van der Waals interactions, making the polypeptide compact in order to minimize its contact with water. As a consequence, protein folding occurs with polar charged groups occupying the protein surface area, while the hydrophobic patches are buried to form the hydrophobic core unless they are part of a specific binding site on the protein surface (Petsko and Ringe, 2004).

2.1.3 PROTEIN STRUCTURE DETERMINATION BY CIRCULAR DICHROISM SPECTROSCOPY

Circular dichroism (CD) is the difference in the absorption (ΔA) of left and right circularly polarised light of the chiral amide chromophore (light-absorbing groups) in a protein. It is measured by spectropolarimeters, and the CD spectrum is displayed in ellipticity (θ) as a function of wavelength (Kelly et al, 2005). When a sample is impinged upon by a linearly polarized light, an ellipse is drawn from the resulting electrical field vector. The unit of ellipticity is defined by the ratio of the minor to major axes of the ellipse, and can be calculated as ellipticity (θ) =32.98 ΔA (Kelly et al, 2005).

Proteins will assume a specific three-dimensional structure which is made up of packed α-helices and β-strands, β-sheets and reverse turns. Each of the α-helices, β-sheets and reverse turns gives a different spectrum in the far-UV region (180 nm-240 nm). Hence, by comparing the spectra with an existing database of protein spectra with a known structure, the average content of such secondary structures can be estimated (Darby and Creighton, 1993). Thus, CD can be very useful in detecting changes in a protein's native conformation.

2.2 PHYSICOCHEMICAL PROPERTIES OF PROTEINS

The two important physicochemical properties of a protein that are frequently exploited to purify it using adsorption are surface charge and hydrophobicity. In fact, these properties have paved the way for ion exchange chromatography and hydrophobic interaction chromatography (HIC) to be used extensively in protein purification. Hence, if these protein parameters are known, then predicting protein downstream processing performance can be carried out.

i. **Overall surface charge (q)**

   Quantitative measurement of protein surface charge is indispensable in order to assess the electrostatic interactions involved in protein adsorption. The surface charge of a protein was

previously determined by hydrogen ion titration and conductometry. Horn and Heuck (1983) also proposed the polyelectrolyte titration method to determine the surface charge (with known amino acid composition), which is performed by the incubation of a protein solution with excess amounts of an oppositely charged polyelectrolyte, followed by re-titration of the remaining polyelectrolyte. The difference between the initial and re-titrated amount of polyelectrolyte is equal to the number of charge residues per protein, assuming a stoichiometric binding between the protein and polyelectrolyte (Horn and Heuck, 1983).

ii. **Charge distribution on the protein surface (%S)**

Cheng and Stuckey (2011) in their study of protein precipitation have suggested the use of *%S*, as an indication of the symmetry/asymmetry of the charged group distributed on the protein surface. %S is defined as the percentage of random charge distributions with calculated dipole moments greater than the observed value (Barlow and Thornton, 1986).

iii. **Average surface hydrophobicity (φ)**

The average surface hydrophobicity (Φ) of a protein is determined in 2 steps: the visualization of the protein's three-dimensional structure, and; the determination of Φ based on a specific equation (Lienqueo, 2002). The protein's three-dimensional structure is determined by a program from a known amino acid sequence, to provide the accessible surface area of each amino acid residue. Next, Φ is calculated based on the area and hydrophobicity scale of each amino acid (Lienqueo, 2002). However, there are many hydrophobicity scales to choose from, for example Lienqueo et al. (2002) applied the Miyazawa–Jernigan and the Cowan–Whittaker scale in their calculation, while relating the theoretical hydrophobicity of the protein and its behaviour in hydrophobic interaction chromatography. In addition, Cheng and Stuckey (2011) used the Φ determined from the hydrophobicity scale of Yano et al (1994) for several proteins in a surfactant precipitation study.

iv. **Hydrophobic Imbalance**

In order to consider the homogeneity of protein surface hydrophobicity, the term hydrophobic imbalance (HI) is used. HI represents the displacement of the superficial geometric centre of the protein after taking into account the effect of the amino acids hydrophobicity (amino acid property vectors) (Salgado et al., 2006). The calculation is based on the expression below:

$$rHI = rH - rC$$

Where rH and rC represent the new and original superficial geometric centre of the protein, respectively, and each are calculated by a certain equation. A higher value of HI will correspond to a more evenly distributed surface hydrophobicity of the protein.

v. **Grand average of hydropathicity (GRAVY)**

GRAVY is the sum of hydropathy values of all amino acids in a protein sequence (where the calculation is based on Kyte-Doolittle scale) divided by the number of residues in the sequence. In general, increasing positive scores indicates greater hydrophobicity. With a known protein sequence (e.g. stored in Swiss-Prot, TrEMBL, or for a user entered protein sequence), calculation of this parameter can be carried out through the bioinformatics and resource portal, ExPASy server.

2.3 SURFACTANT (SURFACE ACTIVE AGENT)

Surfactants consist of a hydrophilic head group which determines their different classes: anionic, cationic, zwitterionic and non-ionic, and also a hydrophobic tail which is made of hydrocarbon chains. The hydrophobic tail is the largest part of the molecule, and hence contributes to the major cost of a surfactant (Porter, 1994). Generally surfactants are associated with the detergent/cleaning industry. The first synthetic surfactant made in Germany during World War I was for general application, to compensate for the shortages of animal and vegetable fats, and the predominant types used were the alkylaryl sulfonates, and alcohol sulphates.

After World War II, a new variant propylene tetramer benzenesulfonate was formulated and used until the early 1960s. However, due to environmental concerns, a more biodegradable alternative, linear alkylbenzene sulfonates (LABS) was developed and used in the detergent industry. Beside its application in the detergent industry, it was also being used in mining, and petroleum recovery, and as an additive in products such as paints, cosmetics, pharmaceuticals and foods (Myers, 1992).

2.3.1 TYPE OF SURFACTANT

2.3.1.1 ANIONIC

Anionic surfactants are made by the addition of anionic species onto many types of hydrophobic groups (Myers, 1992). Compared with other types of surfactant, they are most extensively used for detergent applications because of their ease and low cost of manufacturing. According to Myers (1992), usually the alkyl groups are in the C12-C18 range in order to maintain surface activity and solubility. On the other hand, the types of hydrophilic group can be: carboxylate ($RCOO^-$), sulphonate ($RSO3^-$), sulphate ($ROSO_3^-$) or phosphate ($ROPO(OH)O^-$).

2.3.1.2 CATIONIC

Cationic surfactants are uniquely used as antiseptic agents, fungicides and germicides, and for chemical applications (Myers, 1992). They are relatively less used compared to other surfactant types such as anionic and non-ionics. In addition, they mainly consist of positive charges, based on the positive charge-carrying nitrogen atom. Categories of cationic surfactants are the quaternary ammonium compounds, amine salts and amine oxides. The general formula for a quaternary ammonium salt is:

$$R'R''R'''R''''N^{+}X^{-}$$

where R', R'', R''' and R'''' are alkyl chains which can be straight chain or heterocyclic (pyridinium, morpholinium or imidazolinium); and X is the counterion which may be halide, sulphate, acetate, etc.

2.3.1.3 NON-IONIC

These are the second largest class of surfactants (Jonsson et al., 1999) after anionics. They are electrically neutral, and this fact has made them insensitive to electrolytes in the system and has lessened their effect on solution pH (Myers, 1992). Interestingly, they are compatible with other types of surfactants in which mixed micelles with other surfactants are known (Porter, 1994).The most important example is the polyoxyethylenes (POE)-based surfactants with a general formula of:

$$RX(CH_2CH_2O)_nH$$

where R is the hydrophobic group, X is O, N, etc. The number of oxyethylene units in the hydrophilic group, n, is usually greater than 5 or 6 in order to provide the solubility required.

2.3.1.4 AMPHOTERICS

They have two ionic groups with different charges, and form cations in acidic solution, or anions in alkaline solutions, and will become 'zwitterions' in the middle pH range (Porter, 1994). Among the categories of amphoteric surfactants are: N-Alkyl betaines, N-Alkyl glycinates, imidazoline based amphoterics and amine oxides. The most common anionic and cationic groups are the carboxyl group (COOH) and the amine group (NH), respectively (Jonsson, 1999). The pH at which these two groups have equal ionisation is known as the isoelectric point. At this pH, its physicochemical behaviour resembles that of a non-ionic surfactant, while at below and above the pH, it shifts to cationic and anionic properties, respectively (Jonsson, 1999).

Furthermore, they are suitable to be used in shampoos and personal care products since they show low eye and skin irritation (Jonsson, 1999). However, this class of surfactant is not compatible with the anionic surfactants.

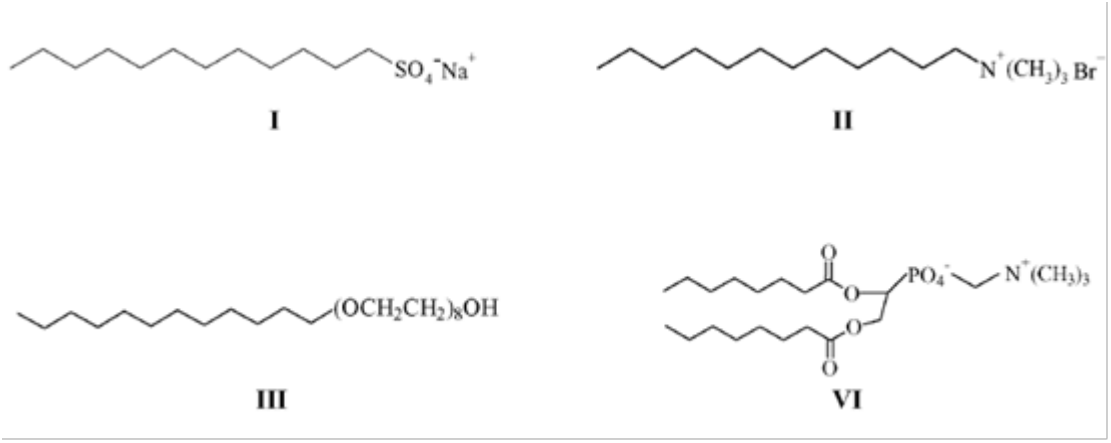


Figure 2.1 **Examples of surfactants: (I) anionic (Sodium dodecyl sulphate, SDS), (II) cationic (Cetyltrimethylammonium bromide, CTAB), (III) non-ionic (Tetraethylene glycol dodecyl ether, C12E4) and (VI) zwitterionic (Dioctanoyl phosphatidylcholine, C8-lecithin) (Rangel-Yagui et al., 2005).**

2.3.2 SURFACTANT PROPERTIES

2.3.2.1 SOLUBILITY

Generally, a surfactant refers to compounds that can alter interfacial interactions by enhancing the adsorption at interfaces. Surfactant molecules tend to concentrate at the interface as a result of their amphipathic structure. Such interfacial adsorption serves as the main mechanism to reduce the free energy of the system. However, when all of the available interfaces become saturated, the mechanism will shift to the thermodynamically favourable mode of crystallization/precipitation or aggregate (micelle) formation (Myers, 1992).

The solubility of ionic surfactants increased with increases in temperature, and there is a dramatic increase in the solubility at a certain temperature which is known as the Krafft temperature, T_K (Myers, 1992). Below T_K ionic surfactants follow the characteristics of a solid phase (hydrated crystal), while above T_K thermodynamically favourable micelles are formed (Porter, 1994). Conversely, the non-ionic counterpart has an inverse temperature/solubility relationship. Its solubility decreases dramatically above a certain temperature which is known as the “cloud point”.

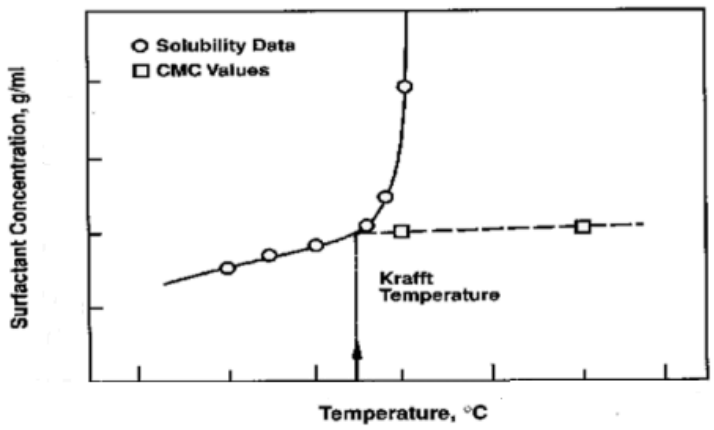


Figure 2.2 Temperature dependence of surfactant solubility (Jonsson, 1999).

2.3.2.2 CRITICAL MICELLE CONCENTRATION (CMC)

As highlighted previously, micellisation is an alternative mechanism to interface adsorption. It results from a balance between intermolecular forces of hydrophobic, steric, electrostatic, hydrogen bonding and Van der Waals interactions (Rangel-Yagui et al., 2005). Basically, the CMC is the most important characteristic of a surfactant, and refers to the highest monomeric surfactant concentration achievable in an aqueous solution of surfactant. The number of surfactant molecules in a micelle is known as the "aggregation number", and the CMC is influenced by several factors such as temperature, pressure, pH and salt concentration (Fresta et al., 2002). The CMC of an ionic surfactant is generally higher than that of a non-ionic; it is also higher for surfactants with higher hydrophilicity.

The physicochemical properties of a solution of a micelle-forming surfactant are dependent on its concentration **(Figure 2.3)**. Hence, CMC can be determined by the measurement of surface tension, solubilisation, self-diffusion, nuclear magnetic resonance (NMR) and fluorescence spectroscopy (Jonsson et al., 1998). Generally the CMCs for anionic surfactants are much lower than for their ionic counterparts, being of the order of 10^{-4} mol/L.

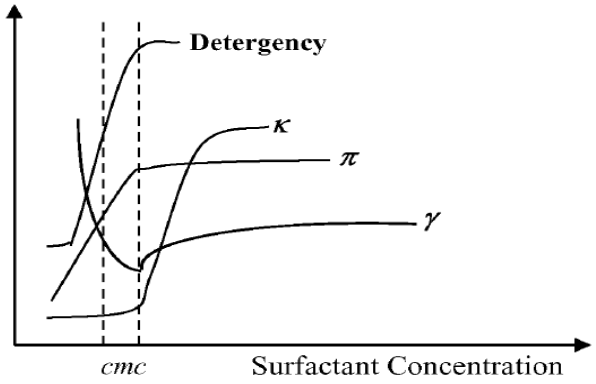


Figure 2.3 **Changes of physical properties: conductivity (κ), osmotic pressure (π) and surface tension (γ) of an aqueous solution of surfactant as a function of surfactant concentration (Rangel-Yagui et al., 2005).**

2.4 PROTEIN-SURFACTANT INTERACTION

Considerable research has been carried out studying protein-surfactant interactions through various approaches, for example: a phase diagram study (Morén and Khan, 1998; Stenstam et al., 2003); binding isotherm study (Jones, 1975); thermodynamics of adsorption (Randolph and Jones, 2002) and kinetics (Otzen, 2002 and 2002b). Moreover, the theoretical structures of surfactant-protein complexation have been proposed, namely rod-like (Reynolds and Tanford, 1970), decorated micelles (Jones, 1996) and pearl-and-necklace model (Turro et al., 1995), albeit this has not been conclusively proven.

The most established way to understand the interactions between proteins and surfactants is by scrutinising the binding isotherm of surfactants and proteins (specific binding-non-cooperative binding-cooperative binding) (Jones, 1975). A binding isotherm is displayed with the average number of surfactant molecules bound per protein molecule (ν) as a function of the logarithm of the free surfactant concentration. There are four characteristic regions in the binding isotherm: (I) specific binding, (II) non-cooperative binding, (III) cooperative binding, and (IV) saturation, as shown in **Figure 2.4**.

Specific binding: In general this is governed by electrostatic interactions between the head group of the surfactant and the oppositely charged binding site on the protein. Therefore, this binding is greatly influenced by pH as it mainly controls the net charge of a protein. As the pH is lowered, the anionic

binding isotherm is shifted to a lower surfactant concentration in contrast to the cationic binding isotherm (Valstar, 2000). What follows next is the non-cooperative binding stage: the binding causes the protein to expand somewhat and allows non-cooperative binding (Turro et al, 1995). Next is the cooperative binding stage, where subsequent binding of the added surfactant results in the formation of surfactant clusters on the protein (micelle-like structures at sub-CMC). This is a cooperative process (like micellisation), and the binding affinity increases as more surfactant is bound (Valstar, 2000). This is a critical stage as protein unfolding occurs at this stage. Otzen (2011) has described the fact that the clusters may be stabilised by the association of several protein molecules (extracted from Andersen, 2009), leading to small protein complexes driven by the formation of shared micelles (which are formed under a relatively small concentration window). As the concentration of surfactant increases (but still sub-CMC), each protein is able to form a cluster on its own. Finally, the interaction comes to a saturation stage, where further binding of the surfactant on the protein does not occur, and normal micelle formation occurs as excess surfactant is added (Turro et al, 1995).

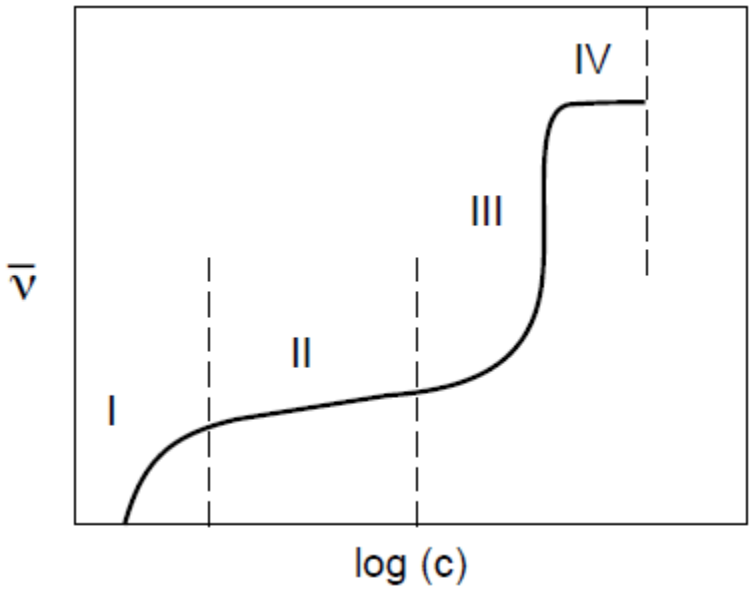


Figure 2.4 **Binding isotherm of surfactant and protein. The ordinate is the number of surfactants bound per protein molecule, $\bar{\upsilon}$, and the abscissa is the logarithm of free surfactant concentration, log (c) (Jones, 1975).**

2.5 PROTEIN SEPARATION BY SURFACTANT REVERSE MICELLES

Reverse micelles are formed when the added surfactant concentration in aqueous solution is higher than its CMC value in the presence of an organic solvent. For example, if we have 250 mM of an AOT micellar solution in isooctane. Due to the amphipathic property of the surfactant, aggregates (micelles) are formed which align themselves in a way such that the hydrophilic head groups are sequestered in the centre, while the hydrophobic tail groups are in contact with the surrounding solvent. Thus, many researchers have exploited the use of reverse micelles for liquid-liquid extraction of proteins (Aires-Barros and Cabral, 1991; Pires et al, 1996; Shin et al., 2003a). Basically, the steps involved consist of two stages: 1) forward extraction- in which the protein-containing buffered aqueous phase is mixed with the micellar solution; 2) back extraction- after phase separation, where the protein-loaded micellar solution is mixed with a fresh aqueous solution and addition of salt, solvent or counter-ionic surfactants enables us to recover the surfactant-free protein (Aires-Barros and Cabral, 1991; Shin et al., 2003a) back into the aqueous phase.

2.5.1 MECHANISM OF PROTEIN SEPARATION BY REVERSE MICELLES

As a result of electrostatic interactions between the charged protein groups and the surfactant head groups at the interface of the aqueous-solvent mixture, the proteins are solubilised inside the polar core of the micelles (entrapment), and stabilized by the surfactant shell layer that protects them from denaturation by the organic phase (Aires-Barros and Cabral, 1991; Pires et al, 1996). In addition, Shin et al. (2003a) proposed that there are 2 steps in the mechanism of reverse micelles extraction: 1) the formation of an insoluble lysozyme-AOT complex (precipitate); and, 2) the solubilisation of this complex in the reverse micellar phase, and this complex will remain as a precipitate if the micellar phase saturation limit has been reached. This is based on the findings of the formation of a white precipitate at the aqueous-organic interface when the lysozyme concentration exceeds the saturation limit, and the reduction of its concentration in the reverse micellar phase.

2.5.2 FACTORS INFLUENCING THE PERFORMANCE OF REVERSED MICELLE PROTEIN EXTRACTION

The effectiveness of reversed micellar extraction depends on the ease of protein extraction into the reverse micellar phase, its recovery from the micellar phase, and its capacity to maintain the protein biological function (Aires-Barros and Cabral, 1991). There are several factors that influence the performance of reverse micelle extraction which are highlighted below. Indeed these factors

determine the water content of the micellar solution (w_0), which is directly related to the size of the reverse micelles. The ease of protein transfer between phases is believed to be higher if the water pool radius ($r_{micelle}$) is larger than the protein size (Aires-Barros and Cabral, 1991; Shin et al., 2003a).

Factors that influence the performance of reversed micelle extraction (Pires et al, 1996) are:

i. **pH**

Electrostatic interaction is the main driving force in protein transfer, and since a change of pH will determine the protein net charge, pH has a strong influence on reverse micellar extraction. Another interaction involved are the hydrophobic interactions that predominate at pHs close to the pI, however, this effect is reduced as the temperature decreases (Pires et al, 1996).

ii. **Ionic strength**

Extraction efficiency is reduced when the ionic strength is increased because of the screening effect of the electrostatic interactions between the protein and surfactant, and the size exclusion effect as the water pool radius ($r_{micelle}$) approaches the protein radius (Pires et al, 1996).

iii. **Surfactant concentration**

The extraction efficiency can be increased with an increase in surfactant concentration; however, as a result the back extraction process can become more difficult.

iv. **Charge distribution**

The extraction efficiency of proteins with higher charge asymmetry into TOMAC reverse micelles is higher than its low charge symmetry counterpart.

v. **Type of electrolyte**

Different buffering systems and ionic species can influence the process efficiency.

vi. **Factors affecting reversed micelle structure**

The organic solvent used and temperature also affect the physicochemical properties of the reverse micelles, and thus influence the protein transfer efficiency in different phases.

vii. **Use of biospecific ligands**

Previous work by other researchers have also shown an improvement in extraction with the inclusion of affinity ligands or other co-surfactants.

2.5.3 DISADVANTAGES OF REVERSE MICELLAR PROTEIN EXTRACTION

Even though reverse micelle protein extraction has been proven to be effective; its main drawback is that there is normally a loss of protein by the formation of a precipitate at the interface which can eventually reduce the final yield (Jauregi and Varley, 1998; Lye et al., 1995; Shin et al., 2003a).

2.6 DIRECT PROTEIN PRECIPITATION BY SURFACTANT

2.6.1 MECHANISM OF DIRECT PRECIPITATION BY SURFACTANTS

The isoelectric point (pI) is the pH at which a protein will have a zero overall net charge (McMurry, 1992). The protein will have a net positive surface charge below this point, while it will possess a negative surface charge above this pH. Once the oppositely charged protein and surfactant make contact, the protein will form an ion pair with the surfactant head group resulting in the formation of an insoluble surfactant-protein complex (precipitate) (Shin et al., 2003b). Hence, the surfactant will act as a ligand to bind the target protein and later precipitating it. After precipitation the recovery of the target protein from the protein-surfactant complex (precipitate) is achieved by adding a solvent or counter-ionic surfactant. If a surfactant is used, due the attractive electrostatic interactions between the two oppositely charged surfactants, the target protein will be solubilised from the surfactant-protein complex. Furthermore, in comparison with reverse micelle extraction, the concentration of surfactant used for precipitation is lower than the CMC.

2.6.2 ADVANTAGES OF DIRECT PROTEIN PRECIPITATION

Among the highlighted advantages of direct precipitation from the literature are:

i. **Simple**;
ii. **Short processing time**;
iii. **High performance**; in previous work on lysozyme recovery, about 95% and 50% recovery efficiency were obtained for a lysozyme-albumin solution and hen egg white solution, respectively, by reverse micellar extraction (Chou and Chiang, 1998), and Shin et al. (2003b) showed that a similar yield can be achieved by direct precipitation;

iv. **More economic** in terms of the amount of surfactant used compared to surfactant mediated reverse micelle recovery (Cheng and Stuckey, 2011). The surfactant required to extract the same amount of enzyme using reverse micelles was orders of magnitude higher than the surfactant required for direct precipitation (Shin et al., 2003b);

v. The method of surfactant initiated precipitation has an advantage over micellar extraction in **its capability to recover protein without any unfolding**; it has less of a protein denaturing problem than is usually encountered in reverse micellar extraction while recovering protein that is electrostatically bound to surfactants. This point is confirmed during the recovery of trypsin from the precipitate (Cheng and Stuckey, 2011).

2.6.3 TYPE OF SURFACTANT USED FOR PROTEIN PRECIPITATION

2.6.3.1 ANIONIC

Basically, an anionic surfactant is usually used to precipitate a protein with a higher pI. As an example, Cheng and Stuckey (2011) used AOT for various proteins with a pI>8 with a pH under 6.2. The direct precipitation of lysozyme by the anionic ligand, di-(2-ethylhexyl) sodium sulfosuccinate (AOT) has been studied (Shin et al., 2003a; Cheng and Stuckey, 2011), and the effect of two important parameters: molar ratio (R) and pH, on lysozyme precipitation have been examined.

A molar ratio of AOT to lysozyme in the range 5-35 was tested for precipitation. Varying results for an optimum R were obtained by different researchers, even though the same buffer conditions were used: Shin et al (2002) achieved 100% removal at only R=10, while it was R=16 in the case of Cheng and Stuckey (2011). However, these findings were generally consistent given that there was only 5% of non-precipitated lysozyme at R=10 for the latter group. Notably, when a large amount of AOT was used (R>23), the lysozyme underwent structural changes or denaturation due to the hydrophobic binding of the excess AOT to lysozyme as determined by HPLC analysis (Cheng and Stuckey, 2011). Complete precipitation of lysozyme was obtained at pH 4-9, and the lysozyme recovered maintains its activity across this range. However, the amount of precipitate reduced markedly (60%) at pH 12 due to the electrostatic repulsion between the lysozyme and anionic surfactant; the lysozyme isoelectric point is 11. In addition, the percentage of precipitation drops at acidic pH 2 as probably most of the lysozyme becomes inactive and unable to interact with AOT, and there is ion exchange between the Na^+, the surfactant counter-ion, and the H^+, which reduce the solubility of AOT in the aqueous phase (Shin et al., 2002; Cheng and Stuckey, 2011).

Successful precipitation of other proteins by the anionic surfactant (AOT) has also has been achieved previously:

i. Ovalbumin

Complete precipitation of ovalbumin was attained at pH 4.0 in 20 mM phosphate buffer as the isoelectric point of ovalbumin is 4.9 (Shin et al., 2003b). It was found that 14 moles of AOT bind to 1 mole of albumin. Nonetheless, the ovalbumin was not recoverable from the precipitate even though various polar organic solvents such as acetone, methanol, isopropanol, pentanol, methyl acetate, methyl ethyl ketone and formaldehyde were tested. The researcher offered no explanation for the unsuccessful recovery of ovalbumin protein.

ii. Cytochrome c

At R=17 and pH 6.2, full precipitation of cytochrome c was achieved in 20 mM phosphate buffer (Cheng and Stuckey, 2011). From CD analysis of the remaining supernatant after the addition of AOT, the secondary structure of the protein only remains identical to the native one up to R=17. The β-sheet content increases while the helical structures are reduced; HPLC results also confirm the unfolding behaviour of the cytochrome c as another peak with a higher retention time was observed.

iii. Ribonuclease A

As for ribonuclease A, an optimum R of 22 for complete precipitation was obtained with the same buffer conditions (Cheng and Stuckey, 2011). Protein stability and conformation also show similar outcomes as cytochrome c with increasing R, as revealed by the CD and HPLC findings.

iv. Trypsin

In the interaction of AOT with trypsin, denaturation occurred with increasing AOT concentration. Initially, unbound trypsin maintained its native structure and activity at R=4. Then undesirable cooperative binding of the surfactant with the protein started at R=8, although no major conformational changes started to occur until R=14. However, denaturation begins to occur at R=20 and consequently trypsin lost its activity at R=31. Notably, the highest recovery achieved was only 43% at R=14. On the other hand, it was observed that the unfolded protein eluted earlier than the trypsin and this was different from previous proteins as they were normally being eluted later.

v. α-Chymotrypsin

Comparable to trypsin, complete precipitation was not achievable for α-chymotrypsin, and only 62% precipitation was reached at R=8. The protein remained structurally stable in the R range of 4 to 8. As the R was increased from 14 to 31, slight conformational changes occurred.

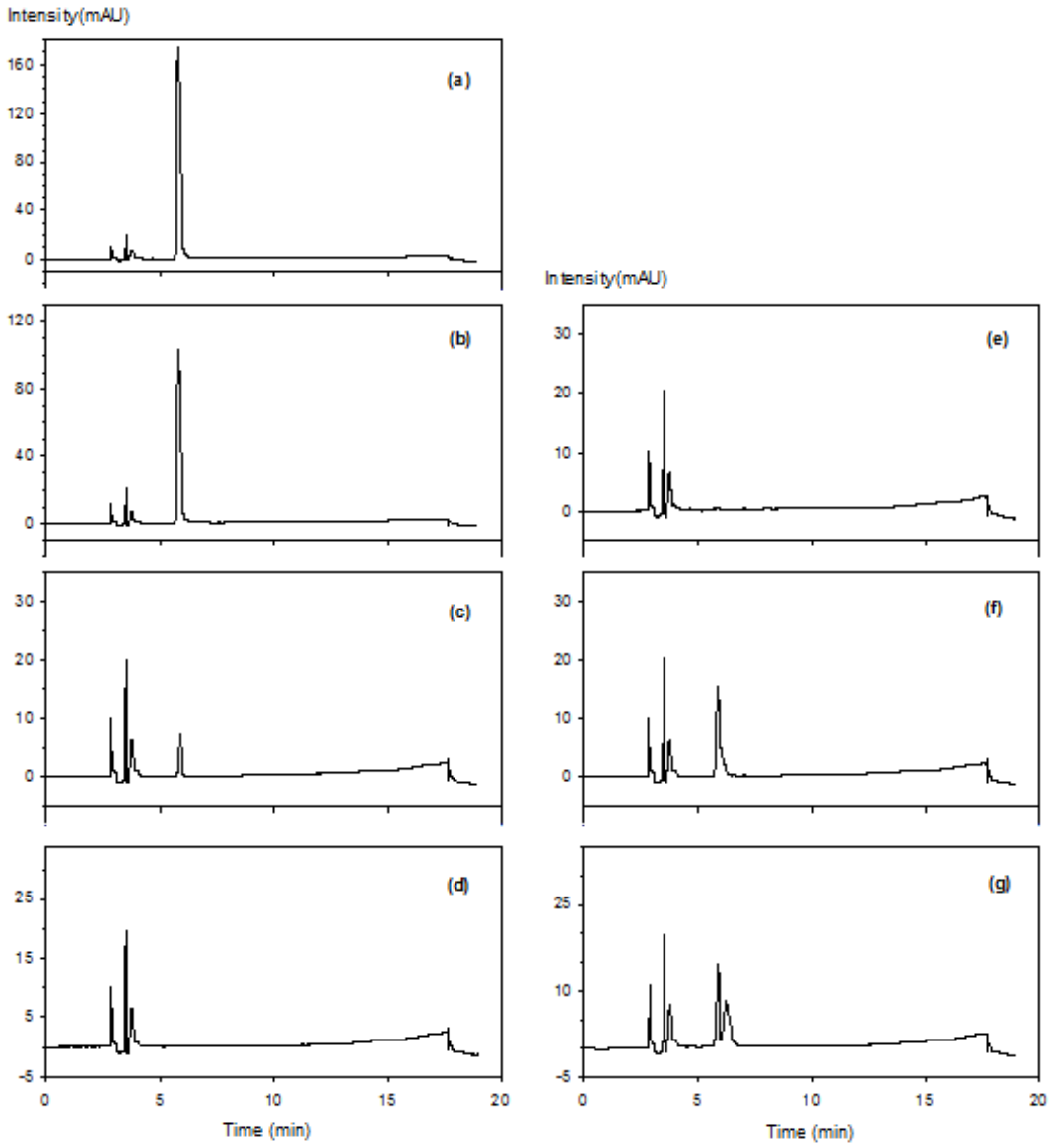


Figure 2.5 **Chromatogram of lysozyme samples: (a) Initial lysozyme solution without AOT (t_R = 5.8min); Soluble lysozyme in the aqueous solution after addition of AOT at (b) R=5, (c) R=10, (d) R=16, (e) R=23, (f) R=29, and (g) R=35 (Cheng and Stuckey, 2011).**

From the above results, it is clear that electrostatic interactions are the most important interactions involved in protein precipitation by surfactants. As in the case of hydrophilic proteins: lysozyme, cytochrome c and ribonuclease a, the level of precipitation increases until it reaches full precipitation with increments in the anionic surfactant, AOT. In order to gain insight into the precipitation process, more surfactant was added beyond the optimum R; it was found that the percentage removal for this group of proteins reduced slightly as the R exceeded this point. In addition, with counter-ionic surfactant (TOMAC) recovery, not only was complete recovery achieved, but these proteins also retained their conformation and activity (Cheng and Stuckey, 2011). Interestingly, there is also a

linear trend in the optimum R as a function of surface charge distribution (%S) **(Figure 2.5)**. It was also suggested that an increase in %S will require less R.

On the other hand, for the hydrophobic proteins, trypsin and α-chymotrypsin, different phenomena were observed. As the AOT concentration increased, the level of trypsin precipitation increased until it reached 80% at R=31 (maximum tested). However, the trypsin was strangely unrecoverable at this R. In fact, the maximum recovery of 43% was obtained at R=14 when the percentage precipitation was only 70%. For α-chymotrypsin, the precipitation trend was similar to the hydrophillic proteins, but with a significant reduction in the level of precipitation (from 70% to 30%) as the optimum R (8) was exceeded. Highest protein recovery (62%) was also achieved at this R.

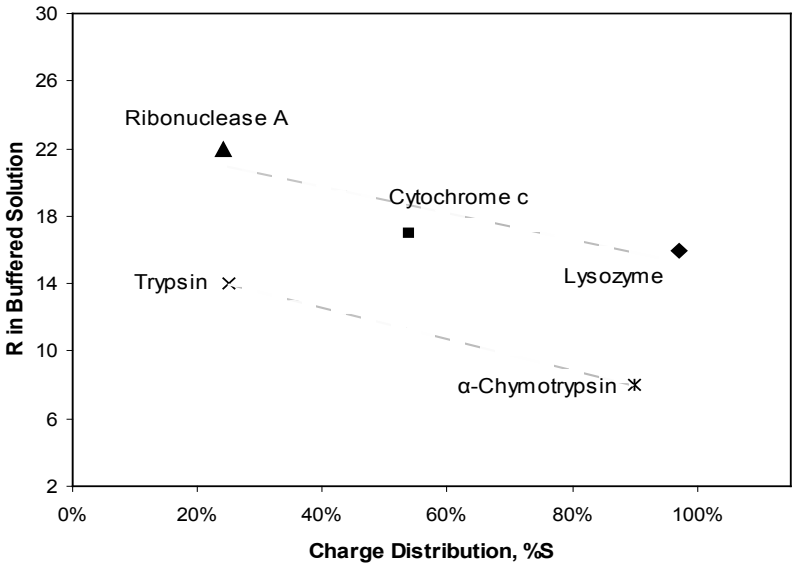


Figure 2.6 The molar ratio of AOT to proteins as a function of surface charge distribution (Cheng and Stuckey, 2011).

2.6.3.2 CATIONIC

In contrast, cationic surfactants were used to precipitate low pI proteins. For example, Ward and Stuckey (2011) employed cationic surfactants (DTAB, TOMAC and DODMAC) to precipitate bovine serum albumin (BSA- pI 4.7) at pH 6.2 and 9.0 from buffer. Intriguingly, in comparison with anionic surfactants, precipitation by cationic surfactants was achieved at high molar ratios above 40. In the case of DODMAC, BSA still maintains its conformation even at Rs as high as 100. The authors presumed that hydrophobic interactions were the main interactions after R=40, and as the R was increased, the precipitation increased mainly due to hydrophobic interactions. Nevertheless, even

though the highest precipitation was achieved at R=140 (56%), the conformation of BSA was affected, as shown by the HPLC and CD data.

Amongst the three surfactants tested, DODMAC was found to be the only one that could bind to BSA at pH 6.2. However, this finding was unforeseen considering that DODMAC was the lowest in the order of charge density, which is DODMAC<TOMAC<DTAB (Ward and Stuckey, 2011). By also taking into account the order of the hydrophobicity, DTAB<TOMAC<DODMAC, the authors claimed that only subtle electrostatic interactions were available to bring the BSA molecules close enough to support the hydrophobic interactions. As the pH was increased to 9.0, there was a marked increase in the precipitation level, and this was consistent with the postulate of charge density increment which promotes the electrostatic interactions between BSA molecules and the cationic surfactant. At this point, TOMAC reached a higher precipitation level than DODMAC (94% compared to 88%) since it had a higher charge density.

2.6.3.3 NON-IONIC

Protein extraction into non-ionic microemulsions, reverse micelles and aqueous two phase micellar systems have been successfully developed (Naoe et al, 1998; Nikas et al., 1992; Vasudevan and Wiencek, 1996). Non-ionic surfactants possess a relatively stable negative charge at pH 5-9, and are able to bind positively charged protein residues at these pHs (Vasudevan and Wiencek, 1996). In addition, non-ionic surfactants also have low affinity interactions and non-denaturing effects on proteins (Naoe et al, 1998; Nikas et al., 1992; Vasudevan and Wiencek, 1996). However, the application of non-ionic surfactants (Triton X-100, Tween 85 and Brij 30) for direct protein precipitation proved to be unsuccessful (Cheng and Stuckey, 2011). This result is also reported to be similar in mixed ionic/non-ionic systems. Precipitation or recovery of proteins by non-ionic surfactants failed to occur as the surfactant monomer binding does not occur. Hence, it was concluded that the non-ionic properties of these surfactants has hindered their use as a ligand for protein binding.

2.6.4 RECOVERY OF TARGET PROTEIN FROM THE PRECIPITATE

The precipitate is the insoluble surfactant/protein complex resulting from the addition of an ionic surfactant to the protein sample. Previously, Shin et al. (2004) reported on the use of acetone, a polar organic solvent, as a recovery solvent. Further electrolytes such as sodium chloride are required here to separate the protein from the surfactant in the acetone. The surfactant/protein complex solubilises once acetone is added, and later the desired protein precipitates out of solution after salt addition. Other solvents like ethanol, methanol and other solvent mixtures have also been tested for protein recovery. Varying results were achieved, and there was an undesired effect of protein unfolding/denaturation when the contact time with solvents was increased (Cheng and Stuckey, 2011;

Shin et al., 2004). In addition, Shin et al. (2004) found that the use of a large volume of a polar solvent such as acetone significantly decreased the recovered xylanase activity.

Rather than having the final target protein in an insoluble form, Cheng and Stuckey (2001) developed a recovery method based on counter-ionic surfactant addition that gave a free and soluble form of the protein, while simultaneously precipitating the surfactant. 4 types of cationic combination (TOMAC/ethanol, TOMAC/isooctane, DODMAC/ethanol and DTAB) were studied. DTAB is more hydrophilic, and therefore does not require a solvent to be dissolved into like the other surfactants used, in particular TOMAC, which is only soluble in organic solvents such as ethanol due to its high hydrophobicity attributed to its 3 alkyl chains (Ward and Stuckey, 2011). The effect of the recovery phase ionic strength and pH were also examined; the dissociation of the AOT-lysozyme complex was favoured at low ionic strength, and a high recovery was maintained over a wide range of pHs tested (4-7) for TOMAC/ethanol. From these results, TOMAC/ethanol in 20 mM potassium phosphate buffer at pH 6.2 was found to be the best recovery phase for lysozyme from the lysozyme-AOT complex in that it gives a higher yield (100% recovery at R=2 compared to only 81% recovery at R=2 for DODMAC/ethanol), is more miscible, more economic (only R=2 to achieve complete recovery as compare to R=2.5 for TOMAC/isooctane to achieve a similar result) and does not interfere with absorbance measurements at 280nm as with DTAB.

Nevertheless, DTAB was still being used to recover lysozyme after its precipitation by AOT, and it was found to follow a similar trend to TOMAC (Ward and Stuckey, 2011). As it is known that DTAB has a higher charge density than TOMAC, it was expected that DTAB would give a higher yield than TOMAC at low molar ratios (1.0), which were 83% and 77%, respectively. However, as the R increased to 3.0, the performance of the two surfactants was found to be equal in that 100% recovery was obtainable. DTAB has the advantage over TOMAC in the sense that it can be directly dissolved in water due to its high hydrophilicity, thus eliminating the use of an organic solvent to aid solubility that might denature the protein (Ward and Stuckey, 2011). However, the application of DTAB for counter-ionic recovery is complicated because of its large absorption at the far-UV CD spectra (Cheng and Stuckey, 2011).

2.6.5 SELECTIVITY IN FERMENTATION BROTH/BINARY PROTEIN SYSTEM

A separation selectivity study was conducted based on a binary protein system. It was determined that the surface hydrophobicity (average surface hydrophobicity & surface hydrophobicity distribution) of the proteins did not seem to control their extraction selectivity, even though better separation tends to

occur with proteins closer to each other on the surface hydrophobicity scale (low ΔΦ). However, the selectivity from a protein mixture was found to be a strong function of the surface charge distribution, and this correlation only applied to the hydrophilic proteins studied (lysozyme, ribonuclease A and cytochrome c)(Cheng and Stuckey, 2011).

Previously, Shin et al. (2003a) had reportedly failed to separate two proteins with similar pIs in a binary mixture, however, it was suspected that this might be due to the large difference in hydrophobicity of the two proteins (Cheng and Stuckey, 2011). This hypothesis was proven later as two proteins (lysozyme and ribonuclease A) with close pIs and hydrophobicity (low ΔΦ) in a binary mixture were selectively separated by surfactant precipitation. It is believed that the hydrophobicity effect was minimal here, while the surface charge distribution (%S) was controlling.

In binary system extraction from a broth, the protein with the lowest R (previously determined in buffer) was extracted first to prevent the denaturation problem. After the precipitate was removed, a second extraction step was performed on the remaining supernatant with the optimum R for the other protein, and this was later recovered similarly; the extraction was easier for the protein with a higher overall surface charge and distribution (Cheng and Stuckey, 2011). Selectivity was also achieved from a fermentation broth of *Pichia pastoris*, and these results were consistent with the results obtained in a buffer system **(Table 2.1).**

Table 2.1 Selectivity of surfactant extraction from buffered protein mixtures (Cheng and Stuckey, 2011b).

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

Surface charge distribution (%S) was found to be the most important factor influencing selectivity, as there was a good correlation between the relative difference in precipitation (Δ% precipitation) and the relative difference in surface charge distribution (Δ%S) **(Figure 2.7)** in which each was determined as:

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

where the x and y refers to the parameter for each of the protein tested.

A further test with least square estimates and analysis of variance (ANOVA) also confirmed the significance of %S in influencing the precipitation efficiency, and this was modelled in the equation below:

$\Delta P_m = 0.65 + 1.13\ \Delta S$

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

This finding is vital in the sense that it highlighted the effectiveness of surfactant precipitation since most of the proteins have a large difference in surface charge distribution, although often with small differences in overall surface charge (Barlow and Thornton, 1986).

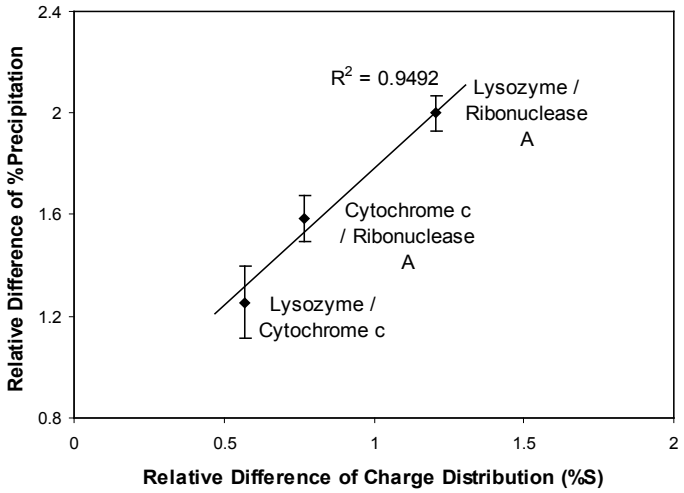


Figure 2.7 **Fitted straight line for selective precipitation of protein mixtures in broth according to the relative difference of surface charge distributions (Chen and Stuckey, 2011).**

2.6.6 SURFACTANT PRECIPITATION OF PROTEINS FROM COMPLEX SAMPLES

When dealing with mixtures, it has been reported that lower protein precipitation is obtained in contrast to using a single protein sample (Shin et al., 2004). Shin et al. (2004) successfully recovered 78% of xylanase from an industrial cellulase mixture, with 4 g/L of total protein. At pH 4.5, xylanase (pI = 9) will have a stronger electrostatic interaction with AOT compared to cellulose (pI about 6). This might be the reason why AOT can precipitate xylanase more selectively, and even the bound cellulose can still be removed from the AOT-xylanase complex by an additional washing step. This proves that selective recovery is achievable despite the proteins having similar charges at the same pH.

Nevertheless, the sample employed was not as complicated as a fermentation broth as there were only 2 types of proteins present in the mixture, and even with a different molecular weight range, each of the proteins had a distinct pI that could be easily manipulated. A close scrutiny of the SDS-PAGE results revealed that xylanase was the major protein in the sample compared with cellulases, even though it was claimed that there was only 15-20% xylanase.

Previous experiments also used spiked *Pichia pastoris* fermentation broth with proteins (lysozyme, cytochrome c or ribonuclease A) to evaluate selectivity. In buffer solution, the protein initially unfolded reversibly, but later unfolded irreversibly out of the solution with an increment in surfactant addition (increased molar ratio, R). However, proteins in the broth will directly undergo irreversible denaturation even at a concentration of AOT below that needed for efficient precipitation (Cheng and Stuckey, 2011). Most proteins were efficiently extracted when adequate AOT ligands were added both to the buffer or broth system. Even though complete precipitation was achieved at the same R in the buffer system, only about 85% of the protein was recovered. This was attributed to the formation of insoluble aggregates caused by hydrophobic compounds in the broth. Furthermore, it was observed that minor denaturation occurred even below the optimum R, and significantly increased above it, and this effect was also shown to be proportional to the increase in protein surface hydrophobicity.

2.6.7 MIXED SURFACTANT SYSTEM FOR PROTEIN PRECIPITATION

A combination of ionic/non-ionic surfactants for protein recovery has been proven to be quite successful in the reverse micelle technique (Fan et al., 2001; George and Stuckey, 2010; Goto et al., 1998; Shen, 2005). However, this is not the case for direct precipitation as demonstrated previously

with the combination of AOT (anionic)/Triton X-100 and DTAB (cationic)/Triton X-100 (Cheng and Stuckey, 2011). Non-ionic surfactants do not seem to improve the performance of ionic surfactants for direct precipitation due to the formation of monomers. The explanation for this is that non-ionic surfactants lack the charged head group, and even the amount that can be used is restricted by its low CMC. In addition, the previous study also showed that the sequence of adding it to a mixed surfactant experiment does not have any influence on its outcome.

2.7 SUMMARY

Previous work so far has elucidated the mechanisms behind the surfactant precipitation of proteins. This separation is initiated through electrostatic interactions that allow the surfactant monomer to interact with the protein molecules. Subsequently, following charge neutralisation, the surfactant and protein bind together by hydrophobic interactions, and eventually precipitate out of the solution (Cheng and Stuckey, 2011). This conclusion is supported by several experiments:

i. The precipitation study that used hydrophilic proteins (lysozyme, cytochrome c, ribonuclease a), found through circular dichroism and HPLC that the structure of proteins remained intact up to the optimum molar ratio (R), but began to unfold and denature as the R value increased above the optimum.
ii. The low level of precipitation and recovery of hydrophobic proteins (trypsin and α-chymotrypsin) where complete precipitation and recovery were not achievable.
iii. The inability of non-ionic surfactants to precipitate these proteins.
iv. In the protein precipitation study using cationic surfactants, DODMAC (even with the lowest charge density as compared to TOMAC and DTAB) was the only cationic surfactant that was able to bind to BSA at pH 6.2, while TOMAC and DTAB only started to interact with BSA at pH 9.0.

In addition, amongst the protein surface properties studied, surface charge distribution (%S) was found to be the main parameter that affected precipitation. There was a linear correlation between (%S) of these hydrophilic proteins, and the optimum molar ratio (R). The optimum R also reduced as %S increased.

When the method was also applied to protein mixtures in buffer and fermentation broth, similar extraction levels were obtained, and selectivity was achieved on the basis of the difference in surface charge distribution between proteins. However, the extent of protein unfolding in fermentation broth was greater due to the presence of hydrophobic constituents in the fermentation broth.

Nevertheless, the work to date on cationic surfactants for low pI protein separation was not as comprehensive as those using anionic surfactants. Basically, 3 proteins (BSA, lipase and trypsin inhibitor) have been used for the separation study by using cationic surfactants, and surprisingly only trypsin inhibitor was successfully precipitated and recovered (Cheng and Stuckey, 2011). The extraction of lipase failed, due to the difficulty in dissolving the lipase-TOMAC complex, and denaturation problems (Cheng and Stuckey, 2011). In the case of BSA, even though full precipitation was achieved, there was no attempt to recover the BSA from its precipitate by counter-ionic surfactants (Ward and Stuckey, 2011). Hence, more work on protein precipitation by cationic surfactants is necessary in order to gain a better understanding of surfactant precipitation. Moreover, it is currently still not clear if the protein precipitation mechanism and selectivity using cationic surfactants is similar to their anionic counterparts. Finally, provided that sufficient understanding has been gained of surfactant precipitation, it would be very interesting to try this technique on the separation of a high value protein.

2.8 OBJECTIVES

1) **Developing the surfactant precipitation method for low pI proteins based on using a cationic surfactant.**
   - 1.1 Examine if the precipitation mechanism is identical to anionic surfactant precipitation
   - 1.2 Evaluate the effect of experimental factors (molar ratio of surfactant to protein, R_p, and pH) on precipitation performance.
   - 1.3 Assess the performance of different organic solvents in recovering a target protein from its surfactant-protein complex
   - 1.4 Optimise the recovery of a target protein from its precipitate through the counter-ionic surfactant method.
   - 1.5 Investigate the influence of various protein properties (molecular weight, hydrophobicity, GRAVY, total charge and charge density, as well as adiabatic compressibility) on the overall performance of surfactant precipitation.
   - 1.6 Examine the structural integrity of a protein that has been subjected to surfactant precipitation.

2) **Feasibility of using a biocompatible surfactant - MES as a precipitating-ligand.**
   - 1.1. To study the feasibility of using a "biosurfactant" - MES as a precipitating ligand for lysozyme in the surfactant precipitation technique.
   - 1.2. To compare the performance of MES to a conventional surfactant (AOT) with lysozyme precipitation.

3) Application of the surfactant precipitation method to the purification of a target protein from a real complex sample.

1.1 To develop the method of separation based on the surfactant precipitation technique for bacteriocin from lactic acid bacteria, *Pediococcus acidilactici* Kp10 by using AOT.

1.2 To compare the performance of the surfactant precipitation method to another surfactant based separation technique, reverse micellar extraction.

CHAPTER 3 PRECIPITATION OF LOW PI PROTEINS WITH A CATIONIC SURFACTANT

3.1 INTRODUCTION

Since its development in 2003 by Shin et al., research on the protein separation technique using direct precipitation by surfactant has been mainly continued by Cheng and Stuckey (2011a, 2011b and 2012). Literature on the study of surfactant precipitation thus far has focused almost exclusively on the use of an anionic surfactant – AOT, and only a little weight was given to the use of cationic surfactant-based precipitation, for example some of the preliminary work done by Cheng and Stuckey (2011b) and Ward and Stuckey (2011). However, for separating low pI proteins, because of the importance of charge neutralisation during precipitation, and most importantly for this protein category to remain within its stability range, a cationic surfactant based precipitation should be examined and developed in more detail.

Protein unfolding/denaturation is known to be of great concern during surfactant precipitation. Ironically, it was suggested in the literature that cationic surfactants should cause fewer structural perturbations compared to anionic surfactants because they have less affinity for the protein during its interactions (Nozaki et al., 1974; Somasundaran, 2006). This was explained by the fact that cationic arginine and lysine side chains projected further from surface and contributed with more CH_2 groups for incorporation in a cluster than the anionic aspartate and glutamate side chains, and thus the magnitude of attraction to proteins was higher for anionic surfactants (Nozaki et al., 1974). Another reason may be the fact that the stronger steric hindrance exerted by the multiple hydrophobic tail groups of the cationic surfactant (e.g. quaternary ammonium compound) diminishes the ionic links between the protein charge residues and the surfactant head group (Blinkhorn and Jones, 1973; Somasundaran, 2006). Therefore, the application of a cationic surfactant as a precipitant-ligand in surfactant precipitation has strong theoretical reasons why it should be effective.

Thus, in this work we have extended the current precipitation technique by using cationic surfactants to complement the previous work with anionic surfactants, with the ultimate aim of establishing this technique as a viable purification method relevant to all proteins. It was also of interest to investigate whether cationic surfactants shared identical precipitation mechanisms with their anionic counter-parts in precipitating proteins. In this initial Chapter, the cationic surfactant trioctyl-methyl ammonium chloride (TOMAC) was used to precipitate 3 model proteins with low isoelectric points

(pI): bovine serum albumin (BSA), α-amylase and trypsin inhibitor. In-depth studies were conducted examining the effect of various experimental conditions during precipitation and the recovery stage. In addition, the relationship between performance (% protein removal) and various protein properties (molecular weight, hydrophobicity, GRAVY, total charge and charge density, as well as adiabatic compressibility) was also being investigated, to determine the best parameter that could predict performance. Last but not least, the structural integrity of the protein products subjected to surfactant precipitation was also examined.

3.2 MATERIALS AND METHODS

Materials: Lyophilised powders of the proteins: α-amylase from *Aspergillus oryzae* (MW≈51,86 kDa) , trypsin-chymotrypsin inhibitor from soybean (MW=7.9 kDa) and bovine serum albumin (MW=66.43 kDa) were purchased from Sigma Aldrich (UK). Commercial surfactants: Sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) (MW=444.56 g/mol) and trioctyl-methyl ammonium chloride (TOMAC)/aliquat® 336 (MW=404.16 g/mol) were obtained from Sigma Aldrich (UK). For surfactant concentration measurement, methylene blue and methyl orange were obtained from Sigma. For protein activity determination, Remazolbrilliant Blue (RBB)-starch, N-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) and trypsin from bovine pancreas were obtained from Sigma, UK. Buffer components: potassium phosphate monobasic (KH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic, disodium hydrogen orthophosphate ($Na_2HPO_4.7H_2O$) and citric acid were from Sigma, UK. Concentrated hydrochloric acid (HCl) (37%) and sodium hydroxide (NaOH) pellets were from AnalaR (VWR Ltd, UK). Solvents for high-performance liquid chromatography (HPLC): acetonitrile and 99.5% trifluoroacetic acid (TFA) were from Sigma, UK. For the protein recovery study, absolute ethanol was from AnalaR, 99% purity methanol, acetone and sodium chloride were from Sigma, UK. Distilled and deionised water was used throughout the experiment.

Instruments:

An analytical balance (AL204 from Mettler-Toledo AG, Greifensee, Switzerland) was used to weight all chemicals. A vortex mixer (VWR Ltd) was used for mixing. A Hanna Instruments benchtop microprocessor pH/mV/°C meter model pH 213 was used to monitor the pH (standard deviation = ±0.02). Disposable syringes (B.Braun Melsungen AG, Germany) and 0.2 μm Minisart syringe driven filter units (Sartorius Stedim Biotech, Germany) were used to remove any particulates before analyses. A centrifuge (Biofuge Stratos, Heraeus Instruments) was used for solid separation. A UV-VIS scanning spectrophotometer (UV-2101PC, Shimadzu, Japan) and 1-cm quartz cuvettes were used to measure the absorption of protein at 280nm and surfactant concentration. Chirascan CD spectroscopy (Applied Photophysics Ltd, Leatherhead, UK) and 1-mm quartz cell was used to perform circular dichroism spectral analysis.

3.2.1 PREPARATION OF PROTEIN SOLUTION

0.1 g of protein powder was weighed and dissolved in 10 mL of 20 mM potassium dihydrogen phosphate/sodium phosphate dibasic, pH 3.91-9.19 to be the stock solution. 1 mL of the stock solution was then diluted to the volume of 10 mL in a 15 mL tube to make up a 1 g/L solution that was used as the starting solution for the precipitation experiment.

3.2.2 PREPARATION OF TOMAC PHASE FOR PRECIPITATION

TOMAC was dissolved into ethanol at various concentration ranges, from 1.50 to 26.28 mM. Basically, TOMAC is water insoluble and thus ethanol was used as its solubiliser as it is miscible with water. The final concentration of TOMAC after being added to the protein solution was between 0.11 to 2.39 mM, and this falls well below its critical micelle concentration, CMC (in the range of 1-10 mM for ionic surfactants - Mackie and Wilde, 2005).

3.2.3 PREPARATION OF AOT PHASE FOR RECOVERY

The AOT solution was prepared by dissolving it in distilled water with a concentration range of 0.98 to 30.12 mM; at the moment of AOT addition to the protein sample, the final AOT concentration ranged from 0.09 to 2.74 mM. Therefore, AOT was present in the monomeric form at these concentrations as the CMC of AOT in water is 2.5 in 20 mM phosphate buffer; 4.1 mM in water at 25°C (Linfield, 1976), and increases to 6.7 mM with lysozyme prepared in buffer (between 0.03 to 0.2 mol%).

3.2.4 PRECIPITATION METHOD

10 mL (1g/L) of protein solution was contacted with 1 mL of surfactant solution (TOMAC) followed by 10 s of vortex mixing. Instant formation of an insoluble complex was observed right after the addition of the surfactant. The sample was then centrifuged at 7,500g for 10 min at 4°C to separate the insoluble solid from the solution. The supernatant was collected and analysed for the protein content and enzyme activity. The amount of protein left in solution indirectly indicated the amount of protein precipitated by difference. Meanwhile, the surfactant-protein precipitate formed was collected and underwent recovery with either solvent or counter-ionic surfactant. All experiments were performed in at least triplicate, at 25°C.

3.2.5 RECOVERY METHOD

In the recovery study, the precipitate from the optimum R for the respective protein was further recovered with either solvent or counter-ionic surfactant, TOMAC. All experiments were performed in at least triplicate, at 25°C.

3.2.5.1 Recovery by Organic Solvent

The precipitate was washed with 10 mL of phosphate buffer, followed by 10 s of vortexing and then centrifuged at 7,500g for 10 min. The supernatant (wash fraction) was collected and further analysed for protein concentration and activity. 10 mL of solvent was later added to the pellet, vortexed in 10 s and subjected to a second centrifugation. The supernatant (solvent) was then removed and the surfactant-free precipitates were then dissolved in a fresh phosphate buffer at pH 6.2, before being subjected to the analysis.

3.2.5.2 Recovery by Counter-Ionic Surfactant (AOT)

The anionic surfactant, AOT, was employed for the counter-ionic surfactant recovery of lysozyme from the insoluble complex of protein-TOMAC. After the addition of 10 mL of fresh phosphate buffer to the recovered precipitate, 1 mL of AOT solution with various concentrations (0.98 to 30.12 mM) was added to the final phase. The mixture was later vortexed for 10 s to promote the interaction between the cationic surfactant, TOMAC, and its anionic counterpart, AOT, to form a TOMAC-AOT dimer complex, while leaving lysozyme in solution. The sample was then subjected to centrifugation (7,500g, 10 min) to remove the complex, and the supernatant was then analysed for protein concentration and activity.

3.2.6 ANALYTICAL METHODS

All measurements were performed in triplicate.

3.2.6.1 High Performance Liquid Chromatography (HPLC)

The YMC J'Sphere ODS-M80 column was first equilibrated with mobile phase A (0.1% TFA in water) at 25°C. 15 μL of sample volume was injected into the column and this was followed by an elution step with mobile phase B (0.1% TFA in acetonitrile). 15 min linear gradients were run from 0-90% B at a flowrate of 1 mL/min. A diode array detector was operated at 280nm for protein detection with an absorbance unit of mAU. Protein concentration was measured based on the integrated area of the peak obtained. For each of the proteins used in the study, its respective standard curve with a concentration range of 0 to 1.0 mg/mL was used to determine the concentration of protein in the sample. OriginPro 9.1 software was used to analyse and calculate the data obtained.

3.2.6.2 Determination of Circular Dichroism

The concentration of the protein sample in solution was diluted to 0.1 g/L in 20 mM potassium phosphate buffer, or immediately analysed if the original sample was below this concentration prior to the CD measurement. The sample was scanned over a far-UV wavelength range of 190 to 260 nm at 20°C in a thermostated cell holder. The scan speed was set at 10 nm/min with path length of 1 mm, step resolution of 0.5 nm and the bandwidth of 1 nm. The phosphate buffer used was set as the

baseline of the measurement to determine the spectra of the protein. The signal was presented as molar ellipticities [θ] based on a mean molecular mass per residue of 129 Da. The spectra were analyzed by using a deconvolution software, CDNN program version 2.1 (Böhm, 1997), and the software calculates the secondary structure of the protein by comparing it with a base set of 13 known protein structures.

3.2.6.3 Measurement of Alpha-Amylase Activity

The activity of α-amylase was determined based on the modified method of Rinderknecht et al. (1967). A 2% suspension of Remazolbrilliant Blue R (RBB)-starch (Sigma) was used as a substrate in 4.5 mL of 20 mM sodium phosphate buffer, with 50 mM sodium chloride at pH 7.0. 500 µL of the protein sample solution was later added to the suspension and incubated in a shaker at 37°C for 15 min. 2 mL of acetic acid was later added to the suspension that reduced the pH to 4, to terminate the enzymatic reaction. The mixture was then centrifuged at 10,000 g for 10 min, and the supernatant was assayed colorimetrically at 595 nm against a blank.

3.2.6.4 Measurement of Trypsin Inhibitor Activity

The activity of trypsin inhibitor was determined by a continuous rate spectrophotometric assay and expressed as the inhibition of BAEE units, in which one unit is defined as ΔA_{253} of 0.001 per min at pH 7.6 and 25°C with BAEE as a substrate. The total volume was brought up to 10 mL by adding 1 mM HCl. The mixture was allowed to stand at 25°C for 5-6 mins.

100 µL of the prepared above standards was pipetted into the cuvettes that were preloaded with a mixture of 3 mL of BAEE and 100 µL of 1 mM HCl (equilibrated to 25°C) to make up the final reaction volume to 3.2 mL. The reaction mixture was immediately mixed by inversion and the increase in A_{253nm} was recorded for approximately 5 mins. The ΔA_{253}/min was obtained by using the maximum linear rate. The trypsin activity was determined as:

$$\begin{matrix}\text{Trypsin Activity in} \\ \text{BAEE units/mL enzyme}\end{matrix} = \frac{(\Delta A_{253nm} \,/\, \text{min Sample} - \Delta A_{253nm}/\text{min Blank})(\text{df})(10)}{(0.001)(0.10)(0.5)}$$

df = Dilution factor

0.001 = The change in A_{253nm}/min per unit of trypsin at pH 7.6 at 25°C in a 3.2 mL reaction mixture

0.10 = Volume of standard/sample used in final reaction mixture (mL)

10 = Total volume of standard prepared (mL)

0.5 = Volume of trypsin (1 mg/mL) used in standard preparation (mL)

The activity was determined as the difference between the trypsin activity in the absence and in the presence of inhibitors (BAEE units/mg trypsin inhibitor).

3.2.6.5 Determination of TOMAC Concentration

The method for determining the concentration of the cationic surfactant, TOMAC, was taken from Wang and Langley (1975), but with a slight modification. The method was based on the use of an anionic dye, methyl orange. A specified volume of the sample was brought up to 5 ml with distilled water. 500 µL of disodium hydrogen orthophosphate/citric acid buffer (pH 3) was then added, followed by the addition of 50 µL of 0.1% methyl orange reagent in distilled water. The mixture was subsequently added to 5 mL of chloroform and vortexed for 30 s in order to reach equilibrium. The water and chloroform were allowed to separate by gravity for about 10 min. The chloroform layer was collected and analysed for TOMAC concentration by spectrophotometer, at 415 nm with reference Cell A of pure chloroform. The concentration of TOMAC in the sample was determined from a standard curve of known TOMAC concentrations ranging from 0.1 to 1 mM.

3.2.6.6 Determination of AOT Concentration

The assay for the determination of AOT concentration was performed by using a cationic dye, methylene blue that formed a complex with an anionic surfactant (Takagi et al., 1975; Fuda et al., 2004). 1 mL of 0.007% methylene blue that was prepared in 0.1% sodium sulphate solution was added to 5 mL of chloroform and this was followed by mixing. 100 µL of the sample was then added to the initial mixture and this was followed by mixing with a vortex mixer for 20 s. The bottom phase (chloroform) was separated from the water phase and analyzed for AOT concentration by spectrophotometer at the wavelength of 650 nm. The concentration of AOT was determined based on a linear standard curve with a concentration range of 0 to 1 mM.

3.3 RESULTS AND DISCUSSION

3.3.1 SURFACTANT PRECIPITATION OF BSA

3.3.1.1 Effect of Molar Ratio of TOMAC to BSA (R_p) on the Precipitation

Unlike AOT, the interference of TOMAC with the protein concentration assay at A280 nm in the UV spectrophotometer was not negligible. Thus, HPLC was used in this work for both quantitative and qualitative analysis of the protein samples. It was observed that a white insoluble complex formed

upon addition of the TOMAC solution into the sample, as described by previous researchers (Cheng and Stuckey, 2012). The percentage of protein precipitation was calculated by using the equation below:

$$Protein\ precipitated\ (\%) = \left(1 - \frac{C_e \cdot V_e}{C_i \cdot V_i}\right) \times 100\%$$

where C_i refers to the concentration of protein in the initial solution, while C_e is the equilibrium concentration of protein remaining after the formation of a surfactant-protein complex. The volumes of the initial and the recovered phase are denoted as V_i and V_e, respectively.

As shown in Figure 3.1, it is apparent that the percentage of protein precipitated increased with an increase in the molar ratio (R_p) of TOMAC to BSA up to R = 50. At pH 6.2, about 23% of the protein was precipitated at R_p = 10, followed by an increase up to 76% at R_p = 30, and eventually achieved almost complete precipitation (99%) at R_p = 50. This suggested that below the optimum R_p (50), the amount of TOMAC monomer was not sufficient to form insoluble complexes with BSA in the sample. In addition, it appeared that the binding stoichiometry was approximately 50 moles of TOMAC to 1 mole of BSA. This precipitation trend was similar to the findings of Shin et al. (2003) in the precipitation study of ovalbumin and lysozyme by AOT, in which the maximum precipitation level was maintained even after the optimum R_p was exceeded. Moreover, identical trends in precipitation increment as a function of R_p was observed at both pH 6.2 and 7.0 but not at pH 5.4 where it decreased above R=70. This can be explained by the theory of degree of ionization as a function of pH. At lower pHs, i.e., pH 5, the number of negatively charged groups is supposedly lower compared to those in higher pH range, i.e., pH 6.2 and 7.0. As a result, under the same concentrations of TOMAC employed, some of the TOMAC molecules may have interacted with BSA molecules through hydrophobic interactions after all the ionic groups on the protein surface have been occupied, therefore leaving the ionic groups of the surfactant free. Such circumstances resulted in a slight solubilisation of the BSA-TOMAC complex as a repulsion effect between the complexes is introduced. This phenomenon is consistent with the redissolution effect of a protein precipitate in solution by surfactant aggregates (micelles) as reported by previous researchers (Morén and Khan, 1998; Stenstam et al, 2001).

CD is an effective method to study the secondary structure of proteins (Greenfield, 1996), and a change in the CD spectra reflects a change in protein conformation (Chang et al., 1997). Furthermore, the computational analysis applied to calculate and estimate the changes in secondary structure in the protein proved to be useful.

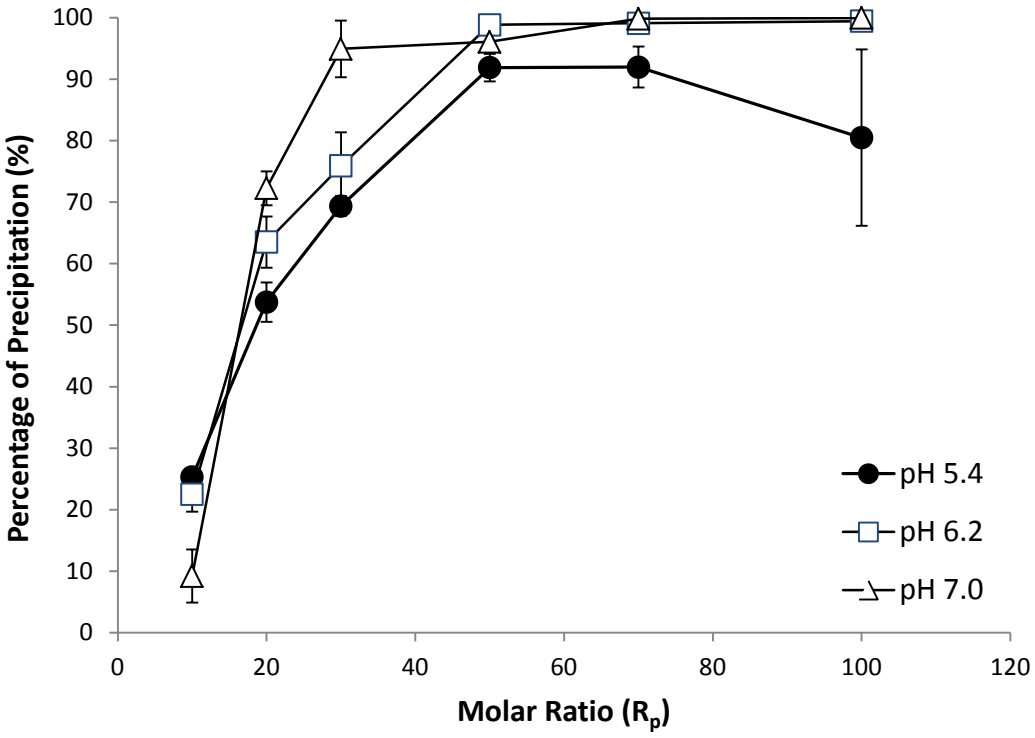


Figure 3.1 **Percentage of BSA precipitation as a function of molar ratio (R_p) between TOMAC and BSA at different pHs.**

In this work, CD was used to compare the physical characteristics of the remaining BSA in the supernatant after precipitation of the native BSA. The CD spectra of the sample obtained were essentially quite similar to the native structure, suggesting that no major structural change had taken place (Figure 3.2). In accordance with the work of Takeda et al. (1987), the spectrum of BSA exhibits a double-minimum, at 208 and 222 nm, indicative of an α-helix structure (Takeda et al., 1987; Pi et al., 2006). From the deconvolution of the CD data by CDNN software, it was also confirmed that the secondary structure was maintained as the R_p increased to 30 (R = 30: 63.4% α-helix, 5.6% β-sheet, 13.6% β-turn, 17.3% random coil; native: 63% α-helix, 5.7% β-sheet, 13.8% β-turn, 17.4% random coil; average difference to native state for the secondary structures = -1.1%).

The deconvolution obtained was also in agreement with the literature (alpha helix = 54-66%) (Putnam, 1975; Takeda et al., 1987). Basically these proportions depend on the reference spectra difference, and experimental conditions including protein concentration (Takeda et al., 1987). However, this finding is in contrast with a previous report (Takeda et al., 1987) on the changes in the secondary structure of BSA after the addition of surfactants which found a significant decrease in the α-helix fraction, a slight increase in the β-structure, and an increase in the random coil fraction as the surfactant concentration was increased in aqueous solution. Basically, this difference is likely due to the precipitation effect in conjunction with the additional centrifugation step employed right after the

formation of complex that removed the insoluble complex formed or possibly the partially unfolded BSA.

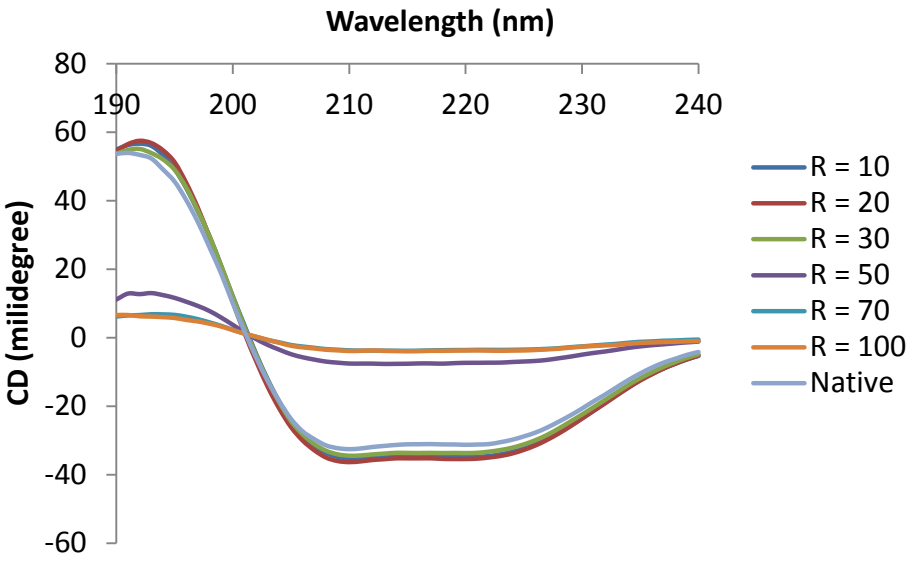


Figure 3.2 **CD spectra of the residual BSA in the supernatant solution after precipitation (from R_p = 10 to R_p = 100) and a scan of the native structure. The protein concentration was 0.1 mg/mL for the R_p = 10 to 30 separations and the native structure, while the samples at R_p = 70 and 100 were run in the CD as negative controls (no BSA).**

3.3.1.2 Precipitation as a Function of pH

The precipitation of BSA by TOMAC was then tested as a function of pH. The objective of this study was basically to investigate any effect of electrostatic interaction in the precipitation of BSA by TOMAC. Since the pI of BSA is 4.7, and its stable pHs are between 5 to 7, the pH experiment was performed for pHs of 5.4, 6.2 and 7.0. Thus, at these pHs it was expected that BSA had a negative overall net surface charge.

Theoretically, the overall net surface charge of a protein depends on the pH of the solution. At below the pI a protein assumed a net positive charge, while as the pH was increased above its pI the protein takes on a negative charge (McMurry, 1992). Thus, in the interaction of a protein with an ionic surfactant, as the pH is increased, the cationic binding isotherm was shifted to a lower surfactant concentration, while the anionic binding isotherm to a higher concentration (Valstar, 2000).

From Figure 3.1 it can be clearly seen that the precipitation level increased with an increment in the pH. For instance, at R_p = 30, 95% precipitation was obtained at pH 7, while only 69% was achieved at

pH 5.4. In addition, under the set of R_ps examined here, notably, almost complete precipitation was achieved at pH 6.2 and above, however, only a maximum of about 92% was reached at pH 5.4.

In this work, at pHs set higher than BSA's pI (~4.7), a favourable electrostatic interaction occurred between the negatively charged BSA and the positively charged head group of the TOMAC monomer to form an insoluble BSA-TOMAC complex, as evidenced by the development of turbidity in solution. Basically, the added TOMAC offsets the repulsive electrostatic interactions between the protein molecules that existed when surfactant was not introduced. By mixing the solution through the vortex, increased collisions between the TOMAC-BSA complex occurred, and it rapidly aggregates to form seed particles for growth of larger aggregates. At this point, the resulting increased hydrophobic attraction leads to agglomeration (Guzman et al., 1990). As the density of the aggregates formed is higher than the bulk solution, further centrifugation then lead to the precipitation.

Basically, this result was in accordance with the previous work of other researchers (Shin et al., 2003a, 2003b; Cheng and Stuckey, 2011) which highlighted the important role of electrostatic interactions in protein precipitation. Thus, due to the importance of electrostatic interactions, this work further highlights the specificity of this precipitation technique.

3.3.1.3 Recovery of BSA from the Precipitate

In the BSA recovery study, the precipitate from the R_p = 50 mixture was further recovered with organic solvent and the counter-ionic surfactant, AOT. The percentage of protein recovered was calculated from:

$$Protein\ Recovered\ (\%) = \frac{C_f \cdot V_f}{C_i \cdot V_i} \times 100\%$$

where C_f refers to the concentration of protein in the final buffer solution, while C_i is the concentration of protein in the initial solution. The volumes of the initial and the recovered phase are denoted as V_i and V_f.

3.3.1.3.1 Recovery by Organic Solvent

The use of a solvent (acetone) to recover the protein from its precipitate was first proposed by Shin et al. (2003), and lysozyme was fully recovered from a lysozyme-AOT precipitate with its original enzymatic activity. This method drew parallels with the conventional acetone precipitation technique typically employed in a protein purification step to concentrate the protein. The aim of this method was to resolubilise the surfactant precipitant in the added solvent, while leaving the protein in the precipitate form. Next, surfactant-free protein was obtained by resolubilising the protein precipitate in a new buffer.

In this work, however, the BSA precipitate was found not be recoverable by any means of solvent addition (neither with salt added). Several solvent types were indeed examined as shown in Table 3.1. Besides, the precipitates from an R_p below the optimum of 50 (i.e., 10, 20 and 30) were also treated with solvent to try and recover them, but to no avail.

3.3.1.3.2 Recovery by Counter-Ionic Surfactant (AOT)

Cheng and Stuckey (2011) previously proposed the use of a counter-ionic surfactant (TOMAC) to recover lysozyme precipitated by the anionic surfactant, AOT. Thus, following on from this concept, the use of AOT to recover protein from the protein-TOMAC complex was examined in this work.

In order to recover BSA from the BSA-TOMAC complex (precipitate), AOT was used. Essentially, AOT is an anionic surfactant and thus possesses a permanent negative charge which can form salts with cations over a wide pH range. This recovery method basically evolved out of reverse micelle back-extraction technique (Jarudilokkul et al., 1999; Matthew and Juang, 2005; Günther and Stuckey, 2011). It was postulated previously that the mechanism of back extraction by counter-ionic surfactant is caused by the electrostatic interaction between the two oppositely charged surfactant head groups that eventually lead to the collapse of reverse micelle (Jarudilokkul et al., 1999). Meanwhile, in surfactant precipitation, in principle the electrostatic interactions between AOT & TOMAC are stronger than those between the surfactant and the oppositely charged groups on the protein surface (Cheng and Stuckey, 2011). Hence, as the counter-ionic surfactant is added to the precipitate, protein will be released from the complex into solution while the two surfactants form a dimer complex that will be precipitated simultaneously.

Table 3.1 Summary of the various types of recovery phase and conditions employed during the BSA recovery study.

Recovery Phase	
Ethanol	50 – 100%
Acetone	100%
Acetone/Ethanol	50% (v/v)
Isopropanol	30%
Counter-ionic Surfactant, AOT	AOT to TOMAC molar ratio (R_r): 0.25 to 1

Nevertheless, the attempt to recover the precipitated BSA in this work by means of a counter-ionic surfactant (AOT) proved unsuccessful, even at an AOT to TOMAC molar ratio (R_r) of up to 1, far exceeding the complex ratio of 0.5, as suggested by Cheng and Stuckey (2011). Similarly with organic solvent recovery, all the precipitates from below the optimum R_p (50) were also treated with counter-ionic surfactant recovery and still it did not work. There was no clear explanation for this finding, although this observation will be discussed in more depth as this work progresses.

3.3.2 SURFACTANT PRECIPITATION OF α-AMYLASE

3.3.2.1 Effect of R_P on the Precipitation

After the addition of TOMAC to the protein solution, its final concentration fell between 0.18 to 1.78 mM, which makes up an R_p of between 10 and 100; after addition a white insoluble complex formed. A "negative control" was constructed by adding 100 µL of ethanol to 1 mL of α-amylase solution in 20 mM phosphate buffer at pH 6.0; no formation of the white insoluble complex was observed, and therefore no precipitation had taken place. Figure 3.3 shows the percentage of precipitation as a function of R_p; the error bars indicate the standard deviation of each point. The relationship between the degree of protein precipitation and increasing molar ratio was observed from the molar ratios of 10 to 25 (precipitation level of 57.7% and 99%, respectively), followed by a plateau. Similarly with the trend in BSA precipitation, complete precipitation was still achieved even beyond R_p = 25 (optimum), up to R_p = 100. At the optimum point (R_p = 25), the binding between surfactant and protein in forming the insoluble complex occurred at a stoichiometry molar ratio of about 25 to 1 (TOMAC: α-amylase).

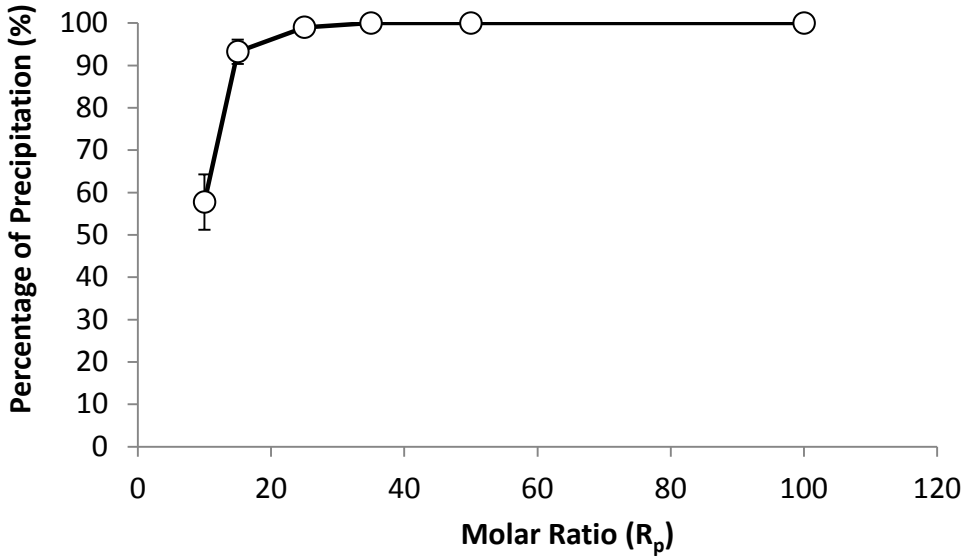


Figure 3.3 Percentage of α-amylase precipitation as a function of R_p.

In order to examine the conformational change of the residual α-amylase in solution after precipitation at R_p = 10 and 15, their CD spectra were recorded and computational analysis using CDNN software was applied to determine the type and fraction of secondary structure. Table 3.2 illustrates the calculated content of the secondary structure and its relative difference with the native structure. From the deconvolution of the CD data, a change of the secondary structure was observed as the R_p was increased from 10 up to 15. The general tendency of the change was a reduction in the α–helix and random coil, and an increment in the β-sheet content. It was found that the α-helix content reduced from 13.3 ± 0.2 (native) to 12.1 ± 0.5% (R= 15), with the estimated average difference of 9.4%. Although overall there was an increment in the β-sheet content with reference to the native structure at both R_ps (10 and 15), their calculated amounts were not significantly different to each other based on a t-test analysis. Hence, it can be concluded that the change in the secondary structure was not substantial with the increment in R_p at pH 6.0.

Table 3.2 The secondary structure of the residual α-amylase remaining in solution after precipitation, and its relative difference from the native form.

Sample	Percentage of Secondary Structure (%)			Average Difference (%)[c]
	α-helix	β-sheet	Coil	
Native[a]	13.3 ± 0.2	37.1 ± 0.3	34.4 ± 0.0	-
R_p = 10, pH 6.0	12.6 ± 0.1	39.6 ± 0.2	32.5 ± 0.2	
Difference (%)[b]	-5.6	+7.0	-5.4	6.0
R_p = 15, pH 6.0	12.1 ± 0.5	39.2 ± 0.4	33.7 ± 0.6	
Difference (%)	-9.4	+5.9	-2.0	5.8
R_p = 25, pH 7.6	13.0 ± 0.1	38.2 ± 0.4	33.5 ± 0.6	
Difference (%)	-2.6	+3.2	-2.5	2.8
R_p = 25, pH 9.19	12.5 ± 0.2	38.8 ± 0.7	33.4 ± 0.6	
Difference (%)	-6.0	+4.8	-2.8	4.5

[a]The native structure was based on the average of all pHs (5.5 to 9.19). [b]The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis (Chen et al., 1977). In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10, as suggested by Chang et al. (1997). [c]The average difference for the three kinds of secondary structure.

3.3.2.2 Precipitation as a Function of pH

Almost complete precipitation was achieved at a pH of 6.0 (R= 25), thus it was expected that a similar outcome could be attained at a higher pH at a similar R_p. However, interestingly, the results showed that percentage of precipitation dropped with an increase in pH from 6.0 to 9.19 (Figure 3.4). It was also noteworthy to highlight that complete precipitation was obtained at just pH 5.5 (knowing that the pI of α-amylase is about 5.4). On the other hand, at pH 7.6 and 9.19, α-amylase was detected in the solution after precipitation, at 11.3 ± 1.77% and 7.7 ± 0.9%, respectively.

CD analysis performed on these remaining protein (Table 3.2) samples, indicated that the structural integrity was mainly retained. This was indicative of a lesser hydrophobic interaction between the protein and surfactant since any cooperative binding (hydrophobic interaction) might have resulted in a significant change in the secondary structure. It is well understood that hydrophobic interactions are mostly linked with protein unfolding or denaturation (Cheng and Stuckey, 2012; Pi et al., 2006; Valstar, 2000). Hence, at these pHs presumably an electrostatic interaction seems to be the predominant force that governs the protein-surfactant interaction, and possibly the precipitation.

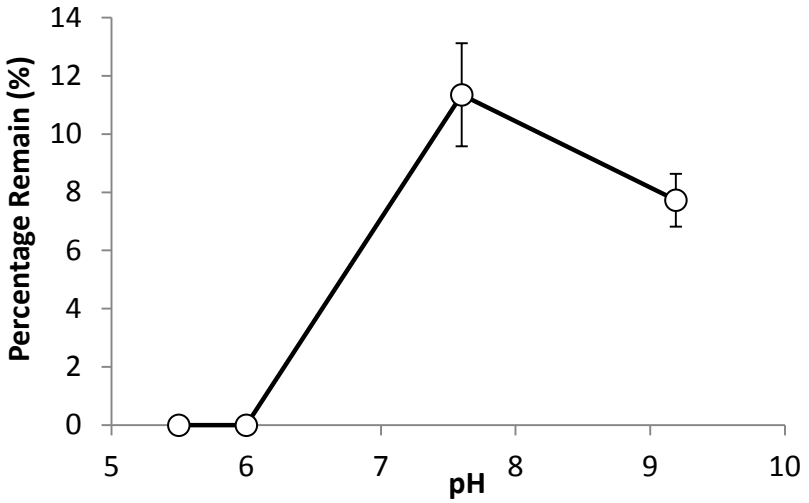


Figure 3.4 Percentage of non-precipitated α-amylase in solution after precipitation as a function of pH.

The influence of pH on precipitation can be explained in the context of a binding isotherm as a function of pH. In an earlier study on cationic surfactants (CTAB, DTAB and CPB) binding with lysozyme, Subramanian et al. (1986) highlighted that as the pH was increased from 5.0 to 9.0, the binding isotherm shifted to a higher surfactant concentration. It was claimed that the binding ratio between the surfactant and protein increased because the number of binding site increased. Therefore, if the above finding can be used to explain our data, the reduction in precipitation at higher pHs might

be the result of a lack of TOMAC monomers present to accommodate the additional number of anionic binding sites which need to be neutralised for precipitation to occur. Unfortunately, measuring the residual TOMAC concentration in solution after precipitation was not done, and further work on this will be needed to shed light on this hypothesis. The next question raised was why the above phenomenon was not encountered with BSA. Presumably, at their optimum R_p for precipitation (i.e. 50), excess TOMAC was present in solution and they can bind with the additional binding sites exposed with the increase in pH; as a result, complete precipitation was retained. Again, this hypothesis was not supported by the determination of the bound TOMAC concentration.

3.3.2.3 Recovery

3.3.2.3.1 Recovery by Organic Solvent

Previous researchers (Shin et al., 2003) had highlighted the need for NaCl addition (less than 0.5 mM) in lysozyme recovery during the recovery step with acetone after AOT precipitation to neutralize the protein charges. In contrast, Cheng and Stuckey (2011) found that NaCl was not required during lysozyme recovery by methanol and ethanol because of the protic nature of these solvents, which solvate the negatively charged AOT via hydrogen bonding that consequently caused the released lysozyme to become attracted to each other and precipitate.

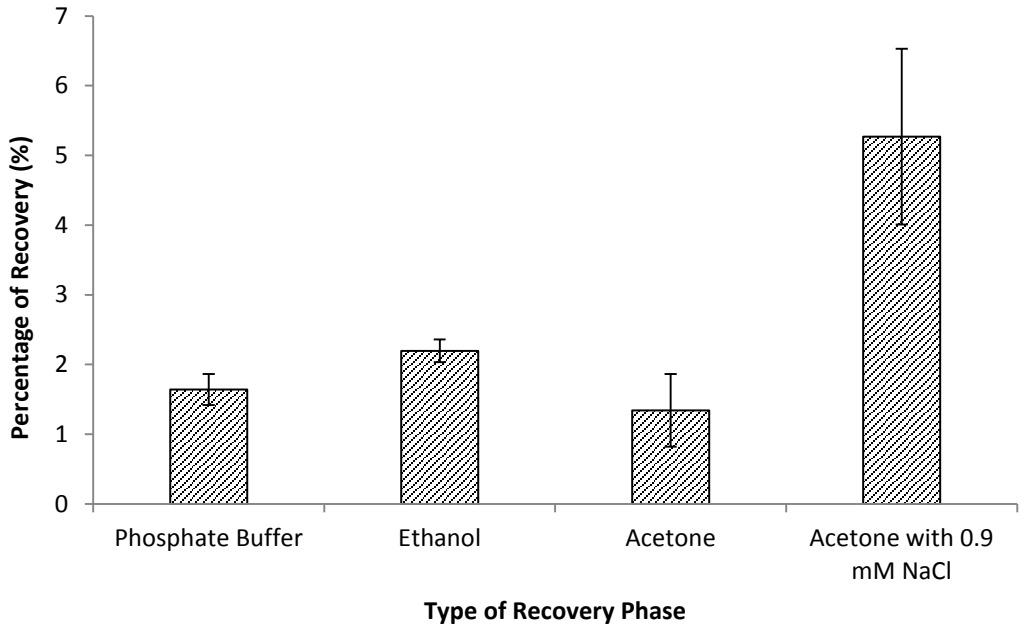


Figure 3.5 The effect of solvent on the recovery of α-amylase.

Moreover, Crowell et al. (2013) demonstrated that a synergistic relationship exists between salt and a solvent that controls the efficiency of acetone precipitation. Through their work it was shown that the recovery of BSA remains poor (below 15%) at low NaCl concentrations. Nevertheless, as the

concentration exceeded 0.01 mM, maximal recovery of about 95% can be reached. A model of ion-pairing was proposed which exists between Na^+ and Cl^-, and charges on the surface of proteins that shield the electrostatic repulsive forces which are responsible for protein solubility.

In this work, however, since the improvement using acetone recovery with salt addition proved insignificant, coupled with the poor recovery by the various solvents employed (Figure 3.5), it can therefore be concluded that the poor overall recovery is not merely because of solubility effects alone, but also related to denaturation effects caused by the precipitant- TOMAC.

3.3.2.3.2 RECOVERY BY COUNTER-IONIC SURFACTANT (AOT)

In the α–amylase recovery study, the precipitate from R_p = 25 was recovered with the counter-ionic surfactant, AOT; the concentration of AOT used ranged from 0.98 (R_r= 0.2) to 4.9 mM (R_r = 1.0).

Figure 3.6 shows the recovery of α-amylase as a function of the molar ratio of AOT to TOMAC (R_r); the standard deviation is indicated by the error bars shown in the graph. It is noteworthy to mention that the pattern of recovery resembled the recovery profile of DODMAC on a lysozyme-AOT precipitate as performed by previous researchers (Cheng and Stuckey, 2011), who found that the recovery increased up to 81% (R= 2) before it dropped to only 54% at an R_r= 3.5.

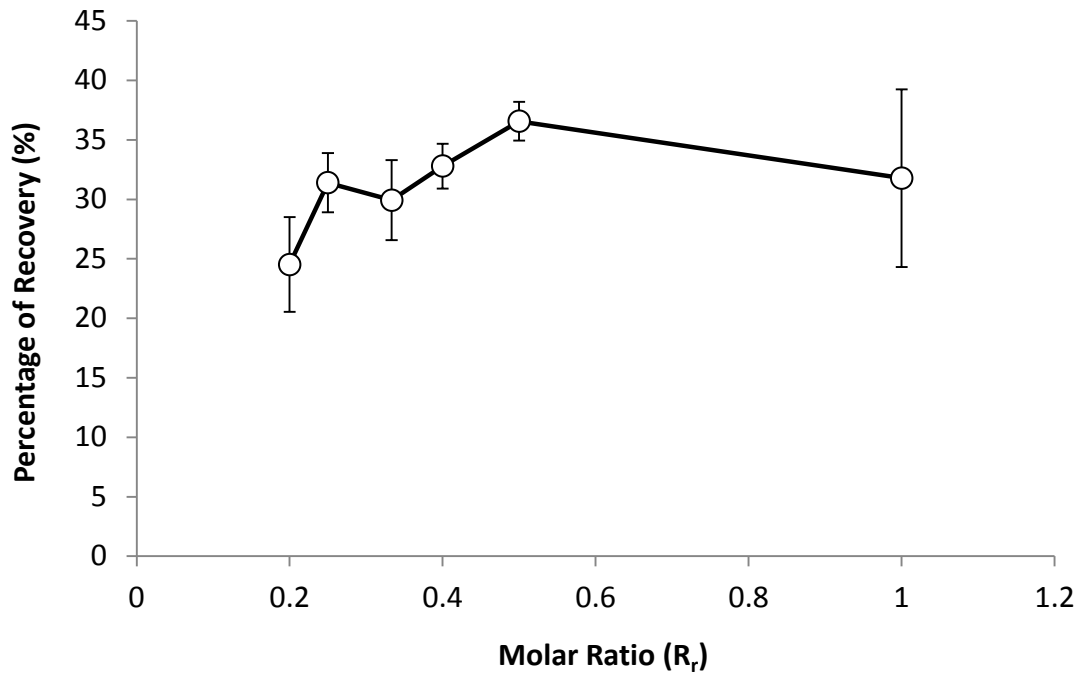


Figure 3.6 Percentage of α-amylase recovery as a function R_r (AOT to TOMAC).

In this work, the percentage recovery generally increased as the R_r was raised from R_r= 0.2 to 0.5, where a maximum of 36.6% was reached. Based on the calculated SD value, there was no significant

difference in yield as R_r increased further to 1.0. Basically, α-amylase became solubilised and released from the TOMAC-α-amylase complex as the counter-ionic surfactant concentration was raised. The counter-ionic surfactant, AOT, was capable of recovering α-amylase from the precipitate (even though the extent was low at just 37%), in contrast to the use of the solvents acetone and ethanol. However, the addition of sodium chloride to the organic solvent only slightly increased recovery by about 5.3%.

3.3.2.3.2.1 Measurement of surfactant concentration after recovery (carry-over of surfactant)

In general, the yield and purity of the final product after the downstream processing of proteins is of great importance. With surfactant precipitation it was found that there was a small carry-over of surfactant into the final protein phase after the recovery step performed here (Figure 3.7). The results show that there was a carry-over of AOT even at an R_r= 0.2 of about 0.02 mM. Nevertheless, no attempt was made to remove the residual AOT at this point, but further discussion of this topic will occur in the following Chapter 4. In contrast, TOMAC, which was used as the precipitant in this work, was not detected.

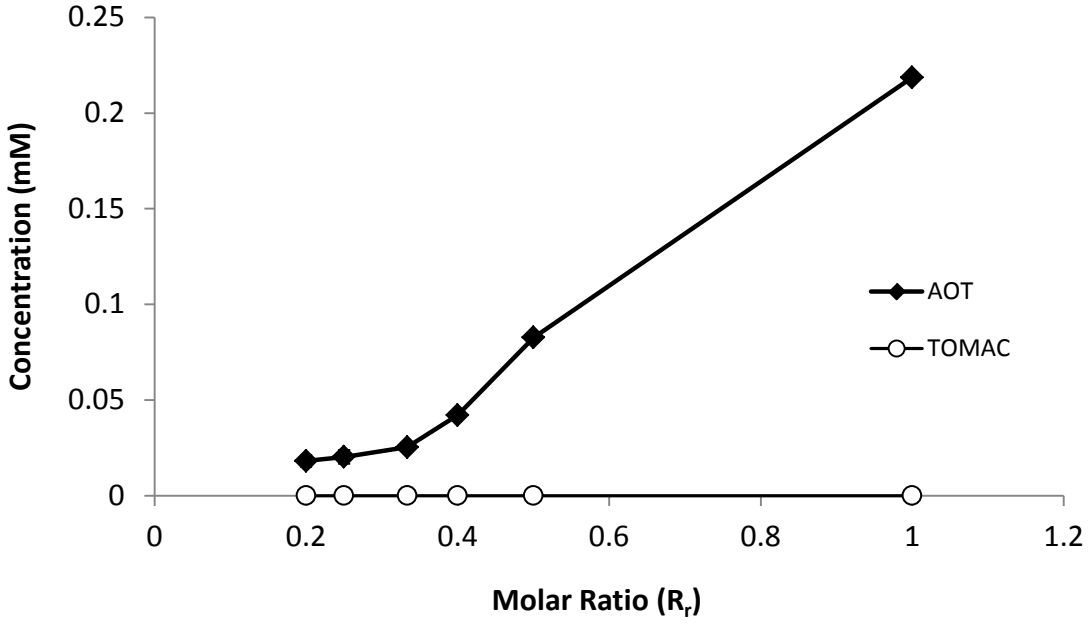


Figure 3.7 Concentration of residual surfactants (TOMAC and AOT) in the recovered phase of α- amylase as a function of R_r.

3.3.2.3.3 Structural integrity and activity of the recovered α-amylase

The main conformational structure of the recovered α-amylase was preserved after recovery, as determined from the CD spectra (Figure 3.8) and deconvolution analysis (Table 3.3), and only a very minimal change in the secondary structure (average difference ≤ 3.6%) was detected. Furthermore, the calculated values of the secondary structure content for the various R_rs (AOT to TOMAC) tested were also consistent, indicating that the slight changes detected were not being influenced by R_r.

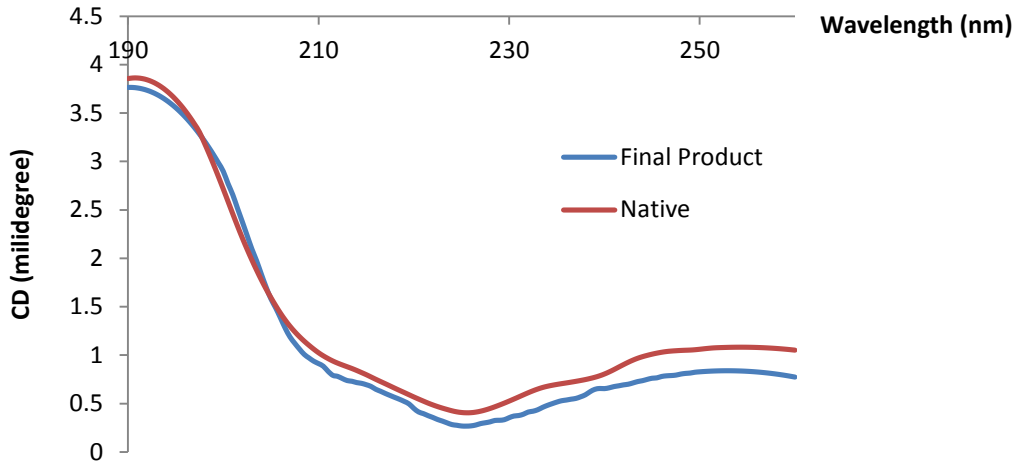


Figure 3.8 **CD spectra of the recovered α-amylase relative to its native structure at pH 6.0. The protein concentration of the recovered α-amylase and native form were about 0.25 g/L. For simplification, only the final product recovered at R_r = 0.5 (AOT to TOMAC) was shown, and the detail results of the deconvolution for all final products is presented in Table 3.3.**

Table 3.4 highlights the specific activity of recovered α-amylase as determined by the RBB-starch analysis. This method is based on the colorimetric determination of Remazolbrilliant Blue R (RBB) at 595 nm after it is released from RBB-starch as a result of α-amylase digestion. All of the enzymes recovered at the various R_rs are shown to be active, and the calculated specific activities are surprisingly higher (and statistically significant at the 95% confidence level) than the crude protein sample, averaging 39.5 U/mg (% SD= 4) compared to about 33.6 ± 0.0 U/mg for the crude control.

It was suspected that the presence of AOT (as detected by the methylene blue assay in the solution after the counter-ionic recovery) might have contributed to this increment in enzyme activity, rather than as the result of the initial interaction with TOMAC (which was not detected in the recovered solution). This result is similar to the findings of previous researchers (Muga et al., 1993; Lamb and Stuckey, 1999) who reported an increase in activity of the enzymes α-amylase and β-galactosidase in

the presence of anionic surfactants such as SDS. The theory of a protein structure's "loosening'' was proposed by these authors to explain the phenomenon of enzyme activation. However, further work is still needed to confirm the effect of the AOT alone on the α-amylase activity in aqueous solution. In addition to that, physical filtration (sieving) could possibly be carried out to remove any carry-over of the surfactant, and the resulting surfactant-free protein solution can be analysed in terms of activity/CD profile, to verify if the unbound AOT may have contributed to this observation.

Table 3.3 **The secondary structure content of the recovered α-amylase by counter-ionic surfactant, AOT at various R_rs (ranging from 0.20 to 1.00) and its relative difference from the native form. The result was based on the average of at least 3 samples.**

Sample/R_r	Percentage of Secondary Structure (%)			Average Difference (%)[b]
	α-helix	β-sheet	Coil	
Native	13.3 ± 0.2	37.1 ± 0.3	34.4 ± 0.0	-
0.20	12.8 ± 0.1	38.7 ± 0.2	33.4 ± 0.4	
Difference (%)[a]	-3.8	+4.0	-2.9	3.6
0.25	12.9 ± 0.1	38.6 ± 0.1	33.3 ± 0.1	
Difference (%)	-3.0	+4.0	-3.2	3.4
0.33	12.9 ± 0.1	38.7 ± 0.3	33.2 ± 0.1	
Difference (%)	-3.0	+4.0	-3.5	3.5
0.40	12.9 ± 0.1	38.6 ± 0.1	33.2 ± 0.1	
Difference (%)	-3.0	+4.0	-3.5	3.5
0.50	12.9 ± 0.1	38.6 ± 0.6	33.3 ± 0.5	
Difference (%)	-3.0	+4.0	-3.2	3.4
1.00	12.8 ± 0.2	38.3 ± 0.3	33.5 ± 0.6	
Difference (%)	-3.8	+3.2	-2.6	3.2

[a]The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis (Chen et al., 1977). In general, it is accepted that there is a major deviation from native state when the difference (%) is larger than 10, as suggested by Chang et al. (1997). [b]The average difference for the three kinds of secondary structure.

Table 3.4 **Measured activity of the recovered α-amylase at various R_rs (AOT to TOMAC) employed, and in its crude form.**

Sample/R_r	Specific Activity (U/mg)
Crude*	33.6 ± 0.0
0.2	38.1 ± 2.0
0.3	41.8 ± 1.9
0.4	38.8 ± 5.3
1.0	39.4 ± 0.0

*The crude activity was determined in 20 mM phosphate buffer, pH 6.0, in accordance with the condition employed in the precipitation and recovery study.

3.3.3 SURFACTANT PRECIPITATION OF TRYPSIN INHIBITOR

3.3.3.1 Effect of R_P on the Precipitation

The effect of R_p on the percentage of precipitation was investigated from R_p = 2.3 (2.94 mM) to 20.8 (26.28 mM) at pH 7.6. Typically, the onset of turbidity was observed with the addition of TOMAC into the trypsin inhibitor solution to indicate the formation of a protein-surfactant complex. A comparable trend in the increase of precipitation as a function of R_p was observed for trypsin inhibitor as with α-amylase (Figure 3.9). Optimum R_p for the precipitation was determined to be 7.7 where about 95% precipitation was reached. The conformational change of the residual protein in solution after precipitation was also examined (Table 3.5). In contrast to α-amylase, there was no significant change detected in the non-precipitated protein at R_p = 2.3 and 4.0.

3.3.3.2 PRECIPITATION AS A FUNCTION OF PH

By taking into account the stable pH range (1-10) and pI (4.0-4.3) of the trypsin inhibitor, an examination of the pH effect on precipitation was performed from pH 3.91 to 9.19, under the optimum R_p determined earlier (R_p = 7.7). The plot of trypsin inhibitor precipitation as a function of pH (Figure 3.10) concurred with the charge mechanism concept in this surfactant mediated precipitation. At pH 3.9 (lower than its pI point, 4.0 – 4.3), only about 20 ± 2.9% of the protein precipitated. Notably, once a sufficient degree of ionization was reached, complete precipitation was obtained as observed at pH 6, and contrary to α-amylase, this level was maintained up to pH 9.2. Presumably, due to the smaller size of trypsin inhibitor (7.9 kDa), as compared to α-amylase (51.9 kDa), the charge density for trypsin inhibitor was more consistent as the pH increased; the effective

ionic interaction between the surface ionic groups of trypsin inhibitor and the cationic group of TOMAC was retained, and therefore the neutralisation for precipitation was possible at higher pHs.

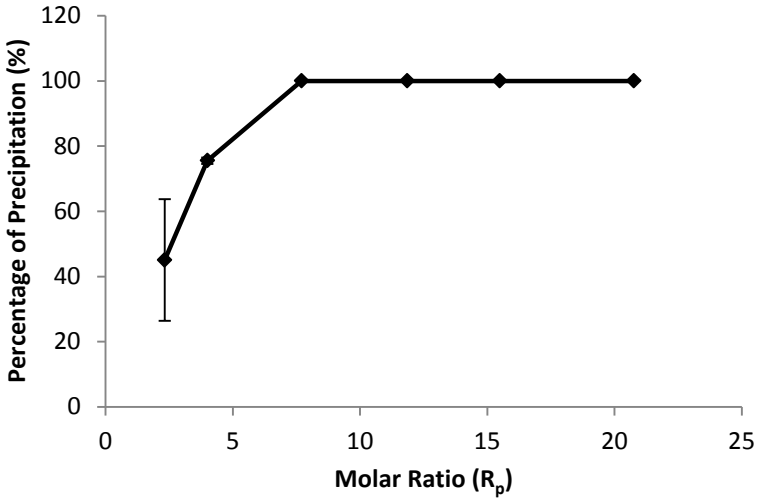


Figure 3.9 Percentage of trypsin inhibitor precipitating as a function of R_p.

Table 3.5 The secondary structure of the residual trypsin inhibitor remaining in solution after precipitation, and its relative difference from its native form.

Sample/ R_p	Percentage of Secondary Structure (%)			Average Difference (%)[b]
	α-helix	β-sheet	Coil	
Native, pH 7.6	13.3 ± 0.1	42.5 ± 0.3	28.3 ± 0.4	-
R_p = 2.3, pH 7.6	13.4 ± 0.0	43.4 ± 0.5	27.1 ± 0.5	
Difference (%)[a]	+0.8	+2.1	-4.2	2.4
R_p = 4.0, pH 7.6	13.2 ± 0.1	43.6 ± 0.1	27.1 ± 0.1	
Difference (%)	-0.8	+2.6	-4.2	2.5
Native, pH 3.91	12.6 ± 0.0	42.3 ± 0.0	29.5 ± 0.0	-
R_p = 7.7, pH 3.91	12.8 ± 0.1	41.2 ± 0.2	30.5 ± 0.1	
Difference (%)	+1.6	-2.5	+3.3	2.5

[a]The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis (Chen et al., 1977). In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10, as suggested by Chang et al. (1997). [b]The average difference for the three kinds of secondary structure.

In addition, the structural integrity of the non-precipitated trypsin inhibitor at pH 3.91 was examined using CD analysis; with regards to its native structure at pH 3.91, there was no significant change detected. This result indicated that presumably no hydrophobic interaction has taken place, and hence this is why no structural perturbations occurred at a pH<pI (where supposedly no ionic attraction existed). This observation was in contrast to the previous finding by Cheng and Stuckey (2012), where it was found that although the ionic attraction was theoretically not prevailing (pH>pI), structure-altering hydrophobic interactions were instead happening during the lysozyme-AOT (anionic surfactant) precipitation study. However, their result was not supported in terms of the amount of TOMAC in solution (as no measurement of surfactant was made), and additionally, the specific activity of the residual protein was not determined.

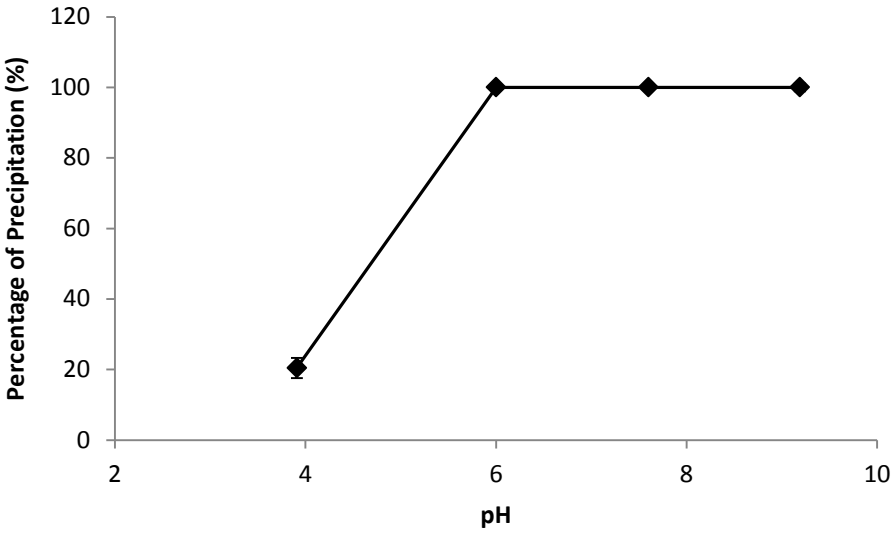


Figure 3.10 Percentage of trypsin inhibitor precipitated as a function of pH. Concentration of the TOMAC solution was 9.8 mM (R_p = 7.7).

3.3.3.3 RECOVERY OF TRYPSIN INHIBITOR FROM PRECIPITATE

3.3.3.3.1 Recovery by organic solvent

Figure 3.11 illustrates the percentage of trypsin inhibitor recovered in the final aqueous phase, and its corresponding fraction in the organic phase (wash-out) as detected by HPLC. Recovery by acetone was shown to be slightly better as compared to ethanol (8.8 ± 0.7% as compared to 6.9 ± 0.3%), however, overall it can be concluded that organic solvent was not effective to recover trypsin inhibitor from its precipitate. Apparently, the problem of trypsin inhibitor solubility was identified as the major cause of its poor recovery by ethanol. This result was in agreement with Frattali (1969) who claimed

that trypsin inhibitor from soybean is alcohol-soluble, however, the effect of salt on the recovery by organic solvent was not investigated further in this section.

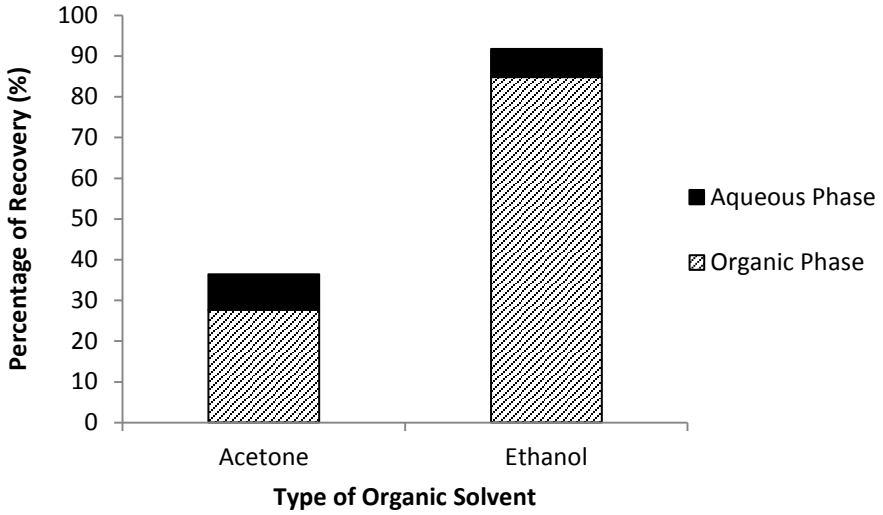


Figure 3.11 The effect of acetone and ethanol on the recovery of trypsin inhibitor.

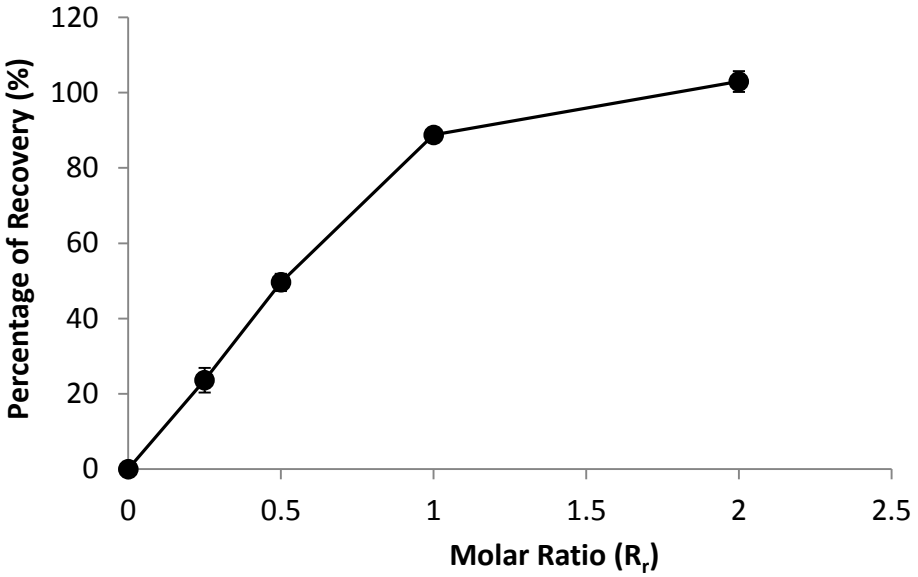


Figure 3.12 Plot of percentage of trypsin inhibitor recovery (%) as a function of R_r.

3.3.3.3.2 Recovery by counter-ionic surfactant (AOT)

Figure 3.12 shows the percentage of trypsin inhibitor recovery as a function of R_r from $R_r = 0.25$ (2.45 mM) to 2.0 (19.6 mM). The trend of the recovery curve was typical where an approximately

proportional relationship between percentage recovery and R_r was observed. Notably, about half (49.6%) of the protein was recovered at an R_r of 0.5, and this further increased to 89% as the R_r was doubled. Contrary to the case of BSA and α-amylase, quantitative recovery (100%) was obtained for trypsin inhibitor at an R_r = 2. As shown in Figure 3.13, there was carry-over of AOT in the final product; at an R_r of 2 the exact stoichiometric molar ratio of binding was actually 1.4 to 1 (AOT: TOMAC), and the carry-over was about 29% relative to the total AOT added. This implied that the manual mixing process performed, i.e. 10 s of vortex mixing, was not efficient. Therefore, in future work it is suggested that the mixing step be optimised, or that automated batch reactors be employed (Boychyn et al., 2000) to improve this contacting.

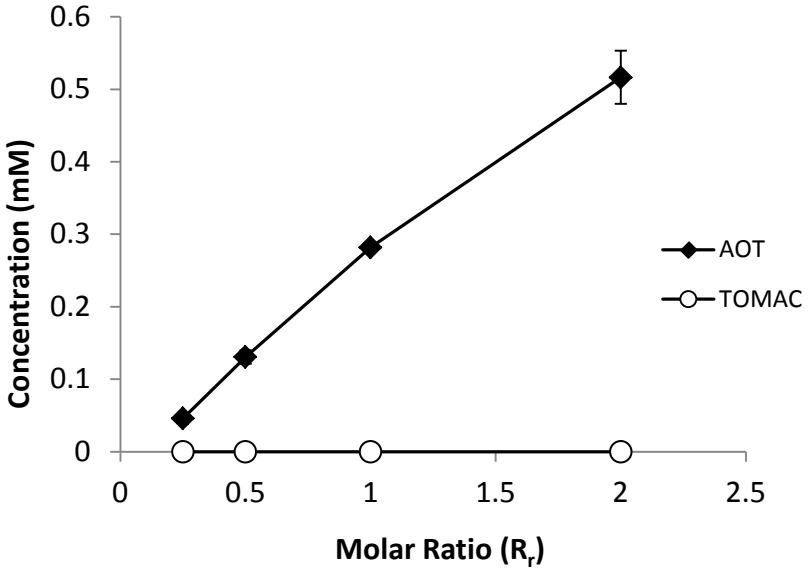


Figure 3.13 Concentration of residual surfactants (TOMAC and AOT) in the recovered phase of trypsin inhibitor as a function of R_r.

3.3.3.3.3 Structural Integrity and Activity of the Recovered Trypsin Inhibitor

The CD spectra of the trypsin inhibitor recovered at the optimum point by counter-ionic surfactant (R_r = 2; 100% recovery) is shown in Figure 3.14, and this is followed by the detail results of the CD deconvolution for all of the final products (R_r from 0.25 to 2.0) in Table 3.6. Apparently, there was no obvious change in the secondary structure content of recovered trypsin inhibitor compared to its native form. Moreover, in terms of activity, they were also fairly potent toward trypsin, comparable to their native form (Table 3.7).

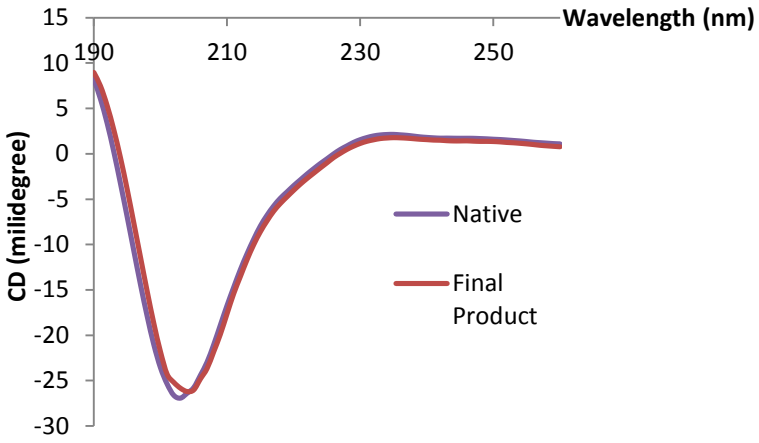


Figure 3.14 **CD spectra of the recovered trypsin inhibitor relative to its native form at pH 7.6. The protein concentration of the recovered trypsin inhibitor and native form were about 0.4 g/L. For simplification, only the final product recovered at R_r = 2.0 (AOT to TOMAC) is shown, and the detail results of the deconvolution for all the final products is presented in Table 3.6.**

Table 3.6 **The secondary structure content of the recovered trypsin inhibitor by counter-ionic surfactant, AOT, at various R_rs (ranging from 0.25 to 2.00), and their relative difference from the native structure.**

Sample/ R_r	Percentage of Secondary Structure (%)			Average Difference (%)[b]
	α-helix	β-sheet	Coil	
Native	13.3 ± 0.1	42.5 ± 0.3	28.3 ± 0.4	-
R = 0.25	13.0 ± 0.4	41.8 ± 0.5	29.5 ± 1.0	
Difference (%)[a]	-2.3	-1.6	+4.2	2.7
R = 0.50	12.9 ± 0.1	42.2 ± 0.5	29.2 ± 0.7	
Difference (%)	-3	-0.7	-3.2	2.3
R = 1.0	12.8 ± 0.1	41.7 ± 0.1	30.6 ± 0.1	
Difference (%)	-3.8	-1.9	+8.1	4.6
R = 2.0	12.9 ± 0.1	41.9 ± 0.2	30.6 ± 0.1	
Difference (%)	-3	-1.4	+8.1	4.2

[a]The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis (Chen et al., 1977). In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10, as suggested by Chang et al. (1997). [b]The average difference for the three kinds of secondary structure.

Table 3.7 **Specific activity of the recovered trypsin inhibitor recovered by the counter-ionic surfactant, AOT, across all the R_rs tested.**

R_r (AOT to TOMAC)	BAEE units/mg
0.25	2740.4 ± 193.5
0.50	2704.1 ± 268.9
1.00	2504.0 ± 429.5
2.00	2833.3 ± 394.2
Crude	2680.0 ± 226.3

3.3.4 CORRELATION BETWEEN PROTEIN PROPERTIES AND EXPERIMENTAL RESULTS

Table 3.8 summarizes the experimental results obtained with respect to several proteins' parameters which may influence the performance of the surfactant precipitation technique. Cationic surfactant – TOMAC precipitation as performed in this work exhibited similar behaviour to its anionic counterpart, AOT, in the sense that the precipitation was mainly governed by electrostatic interactions, while hydrophobic interactions seem to be the limiting factor. With that, the experimental data shown are also taken from the work of previous researchers, as well as the results for anionic surfactant, AOT, precipitation for high pI proteins in order to establish a correlation between the results obtained and protein properties. Another point which deserves to be highlighted is that all of the recovered protein is active compared to its native form.

3.3.4.1 RELATIONSHIP BETWEEN PRECIPITATION AND PROTEIN PROPERTIES

In terms of precipitation, because of the importance of the charge neutralisation mechanism as highlighted in this finding, it is intended to correlate the percentage precipitation, or optimum R_p of precipitation, with the protein parameters outlined earlier. There seems to be no correlation between the optimum R_p and proteins' charge during the precipitation. Nozaki et al. (1974) highlighted that the number of bound detergent ions is much larger than can be accounted for on the basis of one detergent per charged side chain because of significant hydrophobic attraction (surfactant clustering) between surfactant ions and protein. However, from Figure 3.15(c and d), it seems that a proportional relationship between precipitation level and protein charge/charge density at their optimum pH can still be observed by excluding BSA and trypsin inhibitor (27 kDa), as both seem to exhibit excessive hydrophobic binding during their precipitation. This was indicated by the R_p values of both proteins

(50 and 61, respectively) as they were high compared to other proteins. Based on this result, the role of charge - electrostatic interactions during the precipitation of proteins was shown.

Basically, this finding is in agreement with the previous work of Cheng and Stuckey (2011b) who suggested that surface charge distribution rather than total charge was the most accurate parameter to predict the efficiency of precipitation. However, their conclusion was only based on just 3 model proteins (lysozyme, ribonuclease A and cytochrome c) which were categorised as hydrophilic proteins by Kato et al. (2002), and that class of protein must be separated from the hydrophobic proteins so that the correlation can be established. In addition, the surface charge distribution parameter is only available in the literature from the work of Barlow and Thornton (1986). The protein database provided by these authors was also very limited, and the determination of the parameter for a new protein was deemed very difficult to reproduce due its complex calculation and modelling using the Hammer projection for instance.

In essence, this observation draws parallels with the concept of protein retention on strong ion-exchange chromatography as proposed by Kopaciewicz et al. (1983). It was reported that protein net charge alone does not satisfactorily explain the interaction of the protein with the ionic matrix, but must rather take into account the protein's intramolecular charge distribution. Furthermore, according to Kopaciewicz et al. (1983), protein retention on an ionic surface is the result of protein charge, surface charge (i.e. charge layers of ions extending from protein's surface into the external medium) and the charge characteristics of the surrounding medium. Therefore, such complex circumstances might explain the difficulty in precisely predicting precipitation using the protein's charge parameters.

3.3.4.2 RELATIONSHIP BETWEEN RECOVERY AND PROTEIN PROPERTIES

From the results obtained (Figure 3.16a), it can be argued that the percentage recovery for a specific protein was strongly correlated to its hydrophobicity (R^2 = 0.8772). Hydrophilic proteins such as lysozyme, cytochrome c, ribonuclease A (Kato et al., 2002) were effectively precipitated and recovered using the anionic surfactant, AOT (Cheng and Stuckey, 2012). Meanwhile, for hydrophobic proteins such as lipase, trypsin, α-chymotrypsin, α-amylase, BSA and ovalbumin (Kato et al., 1984 and 2002; Guo et al., 1986; Nozaki and Tanford, 1971; Bigelow and Channon, 1976), less satisfactory results were obtained, as detailed in the table above. Amongst the currently examined hydrophobicity parameters from the literature (great emphasis was given to these parameters because of the significant role of hydrophobic interactions in protein denaturation), only the one that was calculated based on the retention coefficients of amino acid residues by HPLC (Guo et al., 1986; Mant et al., 1989) provided the best estimate for recovery percentage, as compared to other hydrophobicity parameters reported in the literature such as surface hydrophobicity, which was generally derived from the binding of SDS molecule on the protein (Kato et al., 2002), determination based on amino

acid solubility in solvent (Nozaki and Tanford, 1971), or GRAVY (i.e. sum of hydropathy values of all amino acids in a protein sequence divided by the number of residues in the sequence) (Kyte and Doolittle, 1982). For example, ovalbumin, somehow regarded as a hydrophilic protein by Kato et al. (1984 and 2002), but contrastingly by Guo et al. (1986) achieved full precipitation by AOT, yet it was not recoverable from a precipitate (Shin et al., 2003b). Indeed, a similar outcome was obtained for BSA which was assigned a relatively high hydrophobicity value of 7.82 by Guo et al. (1986) compared to lysozyme (about 4.8 factors lower in hydrophobicity index) which was precipitated and recovered with ease.

Furthermore, an attempt was also made to perform denaturing purification on the BSA-TOMAC precipitate according to the method of Langenhof et al. (2005). A similar approach was also taken previously by Guzman et al. (1990) in the recovery of avidin by ligand-modified surfactants, and avidin was successfully resolubilized and refolded. However, in this work it was found that the BSA aggregate even failed to be solubilized during the solubilisation/denaturation step (50 mM Tris-HCl, 3 mM EDTA, 8 mM urea, 0.1 M DTT, pH 8.5 for 3 - 24 hours), and this indicated that BSA had undergone irreversible denaturation after being precipitated by TOMAC.

Table 3.8 **Summary of Experimental Results in Relation to Protein Parameters**

Protein	Protein Parameters									Experimental Result		
	Molecular Weight (mol/g)	Hydrophobicity				Charge	N	Charge Density	Adiabatic Compressibility[e]	Optimum R_p	Precipitation (%)	Recovery (%)
		I	II	III	IV							
BSA	66 430[a]	990	2729	7.82	-0.429	-0.3	607	0.05	10.5	50	100	0
α-amylase	51 860[b]	-	-	4.63	-0.208	-16.4	433	3.79	*5.12 (45.5 kDa)*	25	100	37
Trypsin inhibitor	7900[a]	-	-	1.16	-0.159	-16.7	110	15.18	-	7.7	100	100
Trypsin inhibitor[d]	27000[a]	*1031 (21.5 kDa)*	59	2.03		-8.0	210	3.81	*0.17 (21.5 kDa)*	61	58	58
Ovalbumin[c]	44300[c]	992	15	*6.5 (45 kDa)*	-0.001	28.7*	386	7.44	9.18	14	100	0
Lysozyme[d]	14300[a]	893	25	1.62	-0.15	9.7	147	6.60	4.67	16	100	100
Cytochrome c[d]	12400[a]	1049	-	*1 (11.7 kDa)*		11.2	105	10.67	0.066	17	100	100
Ribonuclease a[d]	13700[a]	777	30	1.1	-0.855	8.5	119	7.14	1.12	22	100	100
Trypsin[d]	23800[a]	884	94	2.53	-0.111	2.8	231	1.21	0.92	14	70	43
α-chymotrypsin[d]	25000[a]	908	-	2.81	0.051	4.5	241	1.87	4.15	8	70	62

I Gekko and Hasegawa (1986); Nozaki and Tanford (1971); Bigelow and Channon (1976). II Kato et al. (1984). III Mant et al. (1989); Guo et al. (1986). [a]Sigma Aldrich. [b]Stein et al. (1960). [c]Based on the work of Shin et al. (2003b). [d]Based on the work of Cheng and Stuckey (2011b). [e]Gekko and Hasegawa (1986), units: × 10-12 cm^2 dyn^{-1}. Since the protein property data is not available for every protein investigated, reference proteins were used (a similar protein which may not derived from same source). Denoted with an italic font and their respective molecular weight are indicated in parenthesis.

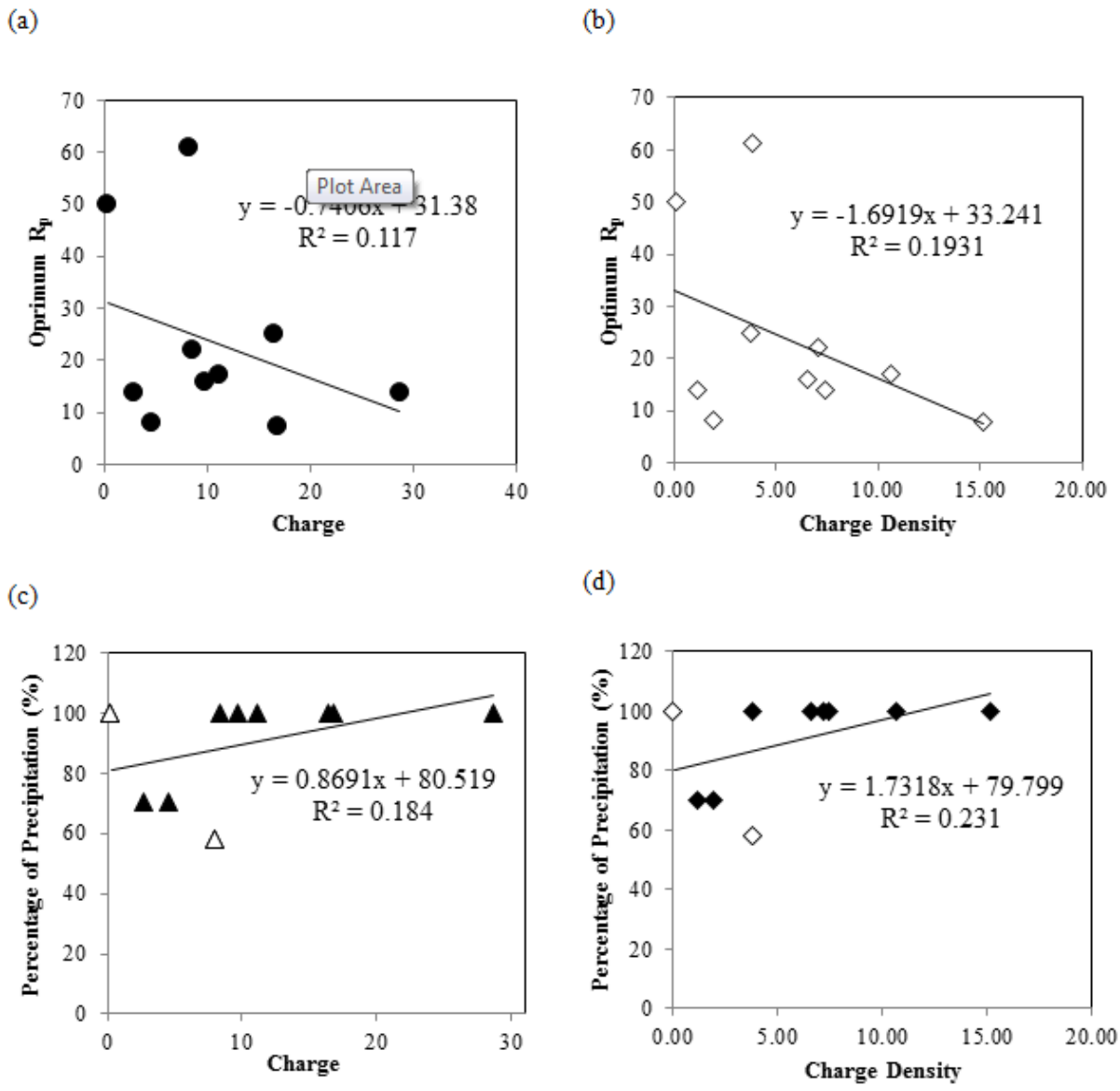


Figure 3.15 **Plots of optimum R_p (a and b) and percentage of precipitation (c and d) against a protein's hypothetical charge and charge density. The non-filled symbols in (c) and (d) indicate BSA and trypsin inhibitor (27 kDa), respectively. The least-squares linear regression was represented by the solid line, and the regression equation including the correlation coefficient, r^2 are shown on the respective graphs.**

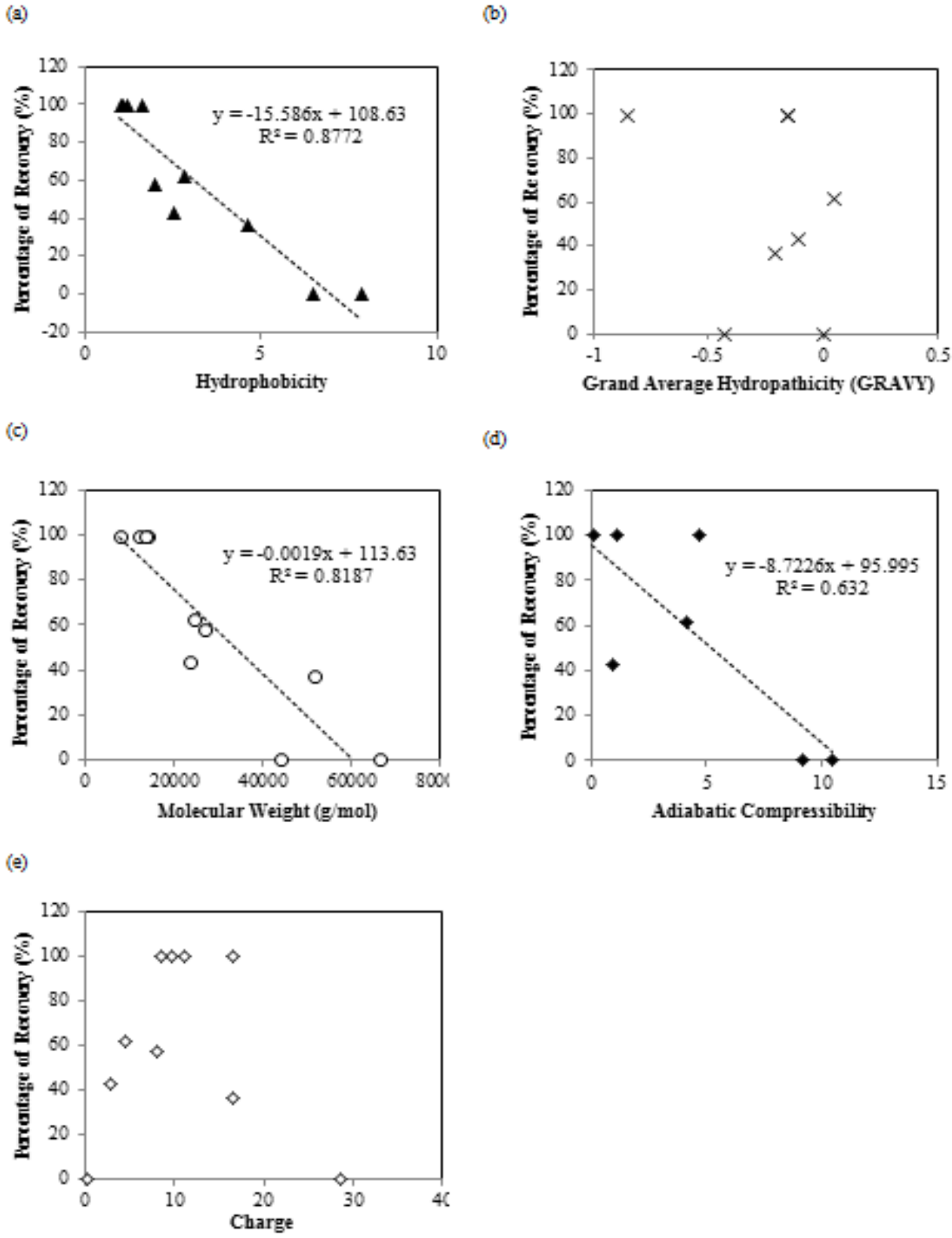


Figure 3.16 **Plots of percentage protein recovery against various protein's parameters: (a) hydrophobicity, calculated based on, (b) grand average hydropathicity, GRAVY, (c) adiabatic compressibility, (d) molecular weight. The least-squares linear regression was represented by the solid line, and the regression equation including the correlation coefficient, r^2 are shown on the respective graphs.**

Next, without neglecting the effect of charge and hydrophobicity, with the protein data set studied to date, a general trend in surfactant precipitation can also be observed based on protein size (Figure 3.16c). Additionally, it seems that size (molecular weight) of the protein does affect the efficacy of protein recovery from its precipitate. The experimental data obtained to date with regard to molecular weight (Table 3.9) demonstrated that:

1. For low molecular weight proteins (trypsin inhibitor, lysozyme, cytochrome c, ribonuclease A, with a molecular weight range of 7.9 to 14.3 kDa), for this protein set good performance (100% yields) was achieved both in precipitation and in the recovery stage.
2. For intermediate size proteins (trypsin, trypsin inhibitor and α-chymotrypsin, with a molecular weight range of 23.8 to 27 kDa), modest performances were obtained in the precipitation stage, while their recoveries were quite high, reaching about 61 – 100% recovery efficiency from the precipitated protein.
3. For large proteins (BSA, α-amylase and ovalbumin, with a molecular weight range of 44.3 – 66.43), even though complete precipitation can be achieved, their recoveries were poor. Presumably the extent of hydrophobic interactions and aggregation for large and multi-domain proteins was high, eventually causing denaturation after interacting with the surfactant. Likewise, in an attempt to extract BSA by using AOT reverse micelles, Shiomori et al. (1998) has found that BSA underwent denaturation and precipitation by a strong electrostatic interaction with AOT molecules at a pH below its pI value. However, for α-amylase, another large protein with a size of 51.9 kDa, about 37% recovery was obtained, and this might be due to its lower degree of flexibility (another parameter which will be discussed next) in relative to BSA and ovalbumin.

In addition, considering the amphiphilic nature of surfactants, the denaturing-causing hydrophobic interactions between the surfactant's tail and hydrophobic groups in the protein may not be avoided, especially with a protein that has a significant amount of the hydrophobic moiety on its surface, and one with a high degree of flexibility. During the binding of protein with surfactant, it was elucidated that the hydrophobic core of the enzyme is being attracted to the hydrophobic surface as it attempts to achieve a lower energy state (entropy). Such a tendency can lead to a conformational change and flattening of the enzyme (Barlow and Thornton, 1986; Lamb and Stuckey, 1999).

Finally, the influence of another parameter of interest - adiabatic compressibility that was introduced by Gekko and Hasegawa (1986) indicating the degree of protein flexibility, and therefore the degree of conformational change, was also scrutinized. The higher the value, the higher the degree of exposure of the protein's hydrophobic core, and eventually increased overall protein-surfactant hydrophobic interaction (Lamb and Stuckey, 1999). This parameter may account for the results obtained for BSA and ovalbumin, as both have a high adiabatic compressibility value (10.5 and 9.18

cm^2/dyn, respectively). This may shed some light on why full precipitation of BSA and ovalbumin was achieved, while the resulting precipitate (protein-TOMAC complex) was not recoverable; a high degree of hydrophobic interaction may result in irreversible unfolding. On the other hand, in the case of cytochrome c, ribonuclease A and lysozyme, all were hydrophilic proteins with low adiabatic compressibility values (0.066, 1.12 and 4.67 cm^2/dyn, respectively), and precipitation and recovery was complete. Meanwhile, for the hydrophobic proteins, trypsin and α-chymotrypsin, although they have low adiabatic compressibility values (0.92 and 4.15 cm^2/dyn, respectively), a lower yield in both the precipitation and recovery stages was still obtained.

3.4 CONCLUSIONS

The precipitation of three low pI proteins (BSA, α-amylase and trypsin inhibitor) by TOMAC was investigated as a function of R_p and pH. For all three proteins, the level of precipitation increased with an increase in R_p, until complete precipitation was eventually achieved. Moreover, for the proteins studied, it was found that the complete precipitation region extended well beyond their respective optimum R_ps. Meanwhile, the percentage precipitation was determined to be a strong function of pH, although overall the results were in agreement with the proposed theory of net charge and protein-anionic surfactant interaction by earlier researchers (Shin et al., 2003; Cheng and Stuckey, 2012). Especially for BSA and trypsin inhibitor, the level of precipitation increased as the pH was increased further away from their pI point (estimated to be around 4.7 and 4.0-4.3, respectively). Therefore, it was confirmed that the electrostatic attraction between the protein and cationic surfactant was the driving force for the precipitation. For α-amylase, non-consistency with this trend was observed presumably due to a lack of TOMAC monomer available to neutralise the additional ionic binding sites at higher pH, rather than as a consequence of hydrophobic interaction. The optimum conditions in which complete precipitation was achieved for BSA, α-amylase and trypsin inhibitor were $R_p = 50$ (pH 6.2), $R_p = 25$ (pH 6.0) and $R_p = 24$ (pH 7.6), respectively.

In terms of recovery of these proteins from the precipitate, mixed results were obtained. BSA was not recoverable with either organic solvents or a counter-ionic surfactant, AOT. For α-amylase, the recovery was also poor, either by solvent (less than 10%) or counter-ionic surfactant (37%, at $R_r = 0.5$). Due to its high solubility in organic solvent (acetone and ethanol), most of the precipitated trypsin inhibitor was lost in the organic phase, and therefore the overall recovery was low. However, trypsin inhibitor was efficiently recovered through counter-ionic surfactant (AOT) treatment, where complete recovery was achieved.

In light of the experimental results obtained, we can hereby conclude that the mechanism of cationic surfactant – TOMAC precipitation was similar to its anionic counterpart – AOT, as reported in some

depth previously, which was based on the charge neutralisation of the protein molecules followed by aggregation of the neutral hydrophobic moieties.

Among the protein parameters examined to be able to predict protein precipitation, the hydrophobicity index based on the method of Guo et al. (1986) and Mant et al. (1989), in addition to size (molecular weight) were highly correlated with overall protein recovery, and consequently the overall efficacy of this precipitation technique. Certainly it can be seen from the experimental results obtained that considerable difficulties were faced when dealing with large multi-domain and hydrophobic proteins, but this precipitation technique could still be applied for this class of protein by removing the impurities initially and leaving the target protein in solution, thus indirectly purifying it.

CHAPTER 4 A BIOCOMPATIBLE SURFACTANT, METHYL ESTER SULPHONATE (MES) AS A PRECIPITATING LIGAND

4.1 INTRODUCTION

To date the ground work on the surfactant precipitation method based on the use of conventional surfactants (including the work described in Chapter 3) has been substantial (Shin et al., 2003a, 2003b; Cheng and Stuckey, 2011; Ward and Stuckey, 2011). The most important finding of this body of work was that the charge of the ionic surfactant's head group is central to the mechanism of this precipitation technique, and this explains why conventional ionic classes of surfactant: anionic (AOT) and their cationic counterparts (TOMAC, DODMAC, DTAB and CTAB) have been successfully tested.

However, advances in the detergent production industry are focussing on the production of a biobased surfactant due to raised concerns about environmental sustainability (Hayes, 2009). One of the examples of a biobased detergent is methyl ester sulphonate (MES), a palm oil-derived surfactant which has attracted a great deal of interest in commercial application (LION Eco Chemicals, (n.d.); Chemithon Corp., 2008). Basically, the two main features of MES that are appealing to the detergent industry are its higher detergency, and environmentally friendly features. In fact, the overall cost of detergent production is reduced significantly as MES has shown higher detergency with a low dosage in comparison with linear alkyl benzene sulphonate (Satsuki et al., 1992). From an environmental point of view, MES is a 'greener' alternative than conventional surfactants because it is carbon neutral, results in lower organic substance discharge due to its lower dosage during washing, and also exhibits a higher biodegradability as a result of its faster and easier decomposition in domestic waste water (Masuda et al., 1993). In addition, it was reported that MES has shown good compatibility when coexisting with protease, and has no adverse effect on enzyme activity (Satsuki, 1992). Hence, coupled with its anionic properties, it appears that MES might be very useful to apply to protein purification through the surfactant precipitation technique.

In line with the development of this new generation of surfactants, the first aim in this chapter was to examine the feasibility of using MES as a precipitant in surfactant precipitation. Secondly, the performance of this biosurfactant was also compared to the conventional surfactant, AOT. In the

study conducted, lysozyme was used as a model protein in a buffer system, and its performance in both the precipitation and recovery stages was examined. Furthermore, the influence of various parameters such as: the molar ratio of surfactant to protein (R), pH, and type of organic solvent on the process, as well as the possibility of using a counter-ionic surfactant to recover the lysozyme were examined. Above all, the structural integrity of the target protein that was subjected to this technique was also analysed.

4.2 MATERIALS AND METHODS

Materials: Lysozyme from chicken egg white (EC 3.2.1.17, Mucopeptide N-acetylmuramylhydrolase, pI=11.0, MW=14.3 kDa) was from Sigma (Missouri, USA). Anionic surfactants: sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) (MW=444.56 g/mol) was obtained from Sigma (UK), and, methyl ester sulfonates (MES), MIZULAN FL-80 (MW=372-400 g/mol) was obtained from the LION group (Malaysia). *Micrococcus lysodeikticus* (ATCC No. 4698) for determination of lysozyme activity was purchased from Sigma.

4.2.1 PREPARATION OF LYSOZYME SOLUTION

0.1 g of lysozyme powder was weighed and dissolved in 10 mL of 20 mM potassium dihydrogen phosphate/sodium phosphate dibasic, pH 6.23 to act as the stock solution. 1 mL of the stock solution was then diluted to a volume of 10 mL in a 15 mL tube to make up a 1 g/L solution that was used as a starting solution for the precipitation experiments.

4.2.2 PREPARATION OF ANIONIC SURFACTANT, AOT PHASE

The AOT solution was prepared by dissolving it in distilled water with a concentration range of 1.7 to 26.6 mM. At the moment of AOT addition to the protein sample, the final AOT concentration ranged from 0.15 to 2.42 mM, and therefore AOT was present in its monomer form at these concentrations as the CMC of AOT in water is 2.5 in 20 mM phosphate buffer; 4.1 mM in water at 25°C (Linfield, 1976), and increases to 6.7 mM with lysozyme prepared in buffer (between 0.03 to 0.2 mol%).

4.2.3 PREPARATION OF ANIONIC SURFACTANT, MES PHASE

MES (marketed under the name MIZULAN FL-80 by LION) flake was dissolved in distilled water to make up the same concentration range as AOT (1.7 to 26.6 mM) in order to facilitate the performance comparison study of the both surfactants.

4.2.4 PREPARATION OF COUNTER-IONIC SURFACTANT, TOMAC PHASE

TOMAC was dissolved into ethanol (due to its low solubility in distilled water) at various concentration ranges, from 11.18 to 33.54 mM, to make up the different molar ratios of TOMAC to MES used in the recovery (1 to 3). Its final concentration in solution ranged from 1.02 to 3.05 mM.

4.2.5 DETERMINATION OF MES CRITICAL MICELLE CONCENTRATION

The critical micelle concentration (CMC) of MES was determined by using the conductivity method. Gradual addition of the MES solution into a 20 mM phosphate buffer was made, and the measurement of conductivity was performed simultaneously. A plot of conductivity as a function of MES concentration at equilibrium was constructed. In theory, below the CMC an increase in conductivity is observed as the concentration of monomer (charge carriers) increases with MES addition. Above the CMC, with further addition of surfactant the concentration of micelles increase while the concentration of surfactant monomer remains unchanged. Thus, the CMC was determined as the concentration where the rate of conductivity increment started to become considerably slower.

4.2.6 PRECIPITATION PROCEDURES

4.2.6.1 PRECIPITATION OF LYSOZYME WITH AOT/MES

10 mL (1g/L) of lysozyme solution was contacted with 1 mL of surfactant solution (AOT or MES), followed by 10 s of vortex mixing; the instant formation of an insoluble complex was observed right after the addition of surfactant. The sample was then centrifuged at 7,500g for 10 min at 4°C to separate the insoluble solid from solution, and the supernatant collected and analysed for protein content and enzyme activity. The amount of protein left in solution indirectly indicated the amount of protein precipitated. Meanwhile, the surfactant-protein precipitate formed was collected and underwent the following recovery study with either solvent or counter-ionic surfactant.

4.2.7 RECOVERY PROCEDURES:

4.2.7.1 Recovery of Lysozyme From Lysozyme-MES Complex By Solvent

The precipitate was washed with 10 mL of phosphate buffer, followed by 10 s of vortex mixing, and eventually centrifuged at 7,500 g for 10 min. The supernatant (wash fraction) was collected and further analysed for protein concentration and activity measurement. 10 mL of solvent was later added to the pellet, vortexed for 10 s, and then subjected to a second centrifugation. The supernatant (solvent) was then removed and the surfactant-free precipitates were then dissolved in a fresh phosphate buffer at pH 6.2 before being subjected to analysis.

4.2.7.2 Recovery of Lysozyme from a Lysozyme-MES Complex by Counter-Ionic Surfactant

The cationic surfactant, TOMAC, was employed for counter-ionic surfactant recovery of lysozyme from the insoluble complex of lysozyme-MES. After the addition of 10 mL of fresh phosphate buffer to the recovered precipitate, 1 mL of TOMAC-containing ethanol with various concentrations (11.18 - 33.54 mM) was added to the final phase; TOMAC was pre-dissolved in ethanol as it was not soluble in water. The mixture was later vortexed for 10 s to promote the interaction between the anionic surfactant, MES, and its cationic counterpart, TOMAC, to form a MES-TOMAC dimer complex, while leaving lysozyme in solution. The sample was then subjected to centrifugation (7,500 ×g, 10 min) to remove the complex, and the supernatant was analysed for protein concentration and activity.

4.2.8 ANALYTICAL TECHNIQUES

4.2.8.1 Protein Assay

The protein concentration in the initial lysozyme solution, aqueous phase after the precipitation, wash fraction of the insoluble complex, and the final phase after recovery were determined by measuring its absorbance at 280nm. The spectrophotometer was calibrated to zero absorbance with 20 mM phosphate buffer (pH 6.23) that was used in the precipitation experiment. The concentration of the protein was determined from a standard curve of lysozyme, with concentrations ranging from 0 to 1.0 g/L (R^2= 0.99). The lysozyme standards were also prepared in the same phosphate buffer.

4.2.8.2 Lysozyme Activity Assay

The activity assay of lysozyme was determined based on the enzymatic lysis rate of a *Micrococcus lysodeikticus* cell suspension by lysozyme (Shugar, 1952). The cell suspension was prepared in 20 mM phosphate buffer (pH 6.23) with an absorbance at 450 nm of between 0.6 to 0.7 versus a buffer blank. 2. 5 mL of the substrate was loaded into the two reference Cells A and B. 100 µL of buffer and lysozyme-containing sample was added to the substrate in the cell A and B, respectively. After immediate mixing by inversion, the decrease in the substrate turbidity at 450nm was recorded for 5 minutes. The rate (A450nm/min) was determined from the linear curve of the graph of absorbance (A450nm) as a function of time. The linear portion chosen was at least over a one minute interval.

The specific activity was calculated as:

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

4.2.8.3 Determination of the MES Concentration

The assay for the determination of MES concentration was performed by using a cationic dye, methylene blue, which formed a complex with any anionic surfactant (Takagi et al., 1975; Fuda et al.,

2004). 1 mL of 0.007% of a methylene blue solution that was prepared in 0.1% sodium sulphate solution was added to 5 mL of chloroform and mixed. 100 μL of sample was then added to the initial mixture and vortex mixed for 20 s. The bottom phase (chloroform) was separated from the water phase and analysed for MES concentration by spectrophotometer at a wavelength of 650 nm. The concentration of MES was determined based on a linear standard curve with concentrations ranging from 0 to 1 mM.

4.3 RESULTS AND DISCUSSION

4.3.1 EFFECT OF R_P ON THE PRECIPITATION LEVEL

Precipitation of protein by the biosurfactant MES, and a commercial surfactant AOT, was examined, and their performances compared. Lysozyme was chosen as the model protein as it is very well-studied, relatively cheap, and was reportedly used by previous researchers (Cheng and Stuckey, 2012; Shin et al., 2003) in studies on surfactant precipitation. Most importantly, the high pI of lysozyme (about 11), and its pH stability range (6 to 9), has made it very suitable for studying surfactant precipitation by these anionic surfactants. One advantage of using anionic surfactants such as AOT and MES in protein precipitation is their lack of interference in the spectroscopic measurement of proteins at A280 nm, i.e. an absorbance of about 0.0157 at 27 mM MES, while only about 0.0010 at 2.7 mM. Hence, at the maximum concentration employed in this work, 2.42 mM, to make up a molar ratio of 38 (at the moment of MES addition), the interference can be neglected. Hence, the use of an easy method like spectrophotometry for protein concentration determination is possible, rather than the more complicated method of HPLC. Furthermore, MES and AOT did not interfere with the activity determination of lysozyme also.

Both surfactants were prepared in the concentration range of 1.7 to 26.6 mM, which made an R_p of between 2.38 and 38 possible. Lysozyme was prepared in 20 mM phosphate buffer (pH 6.23) because it was reported that maximal activity was produced at pH 6 - 9, with varying ionic strengths of 20 to 100 mM (Davies et al., 1969). In this work, the salt concentration used in the phosphate buffer was kept at 20 mM as a minimum possible to prevent the salt ions from competing with MES and lysozyme in ionic interaction.

Figure 4.1 shows the remaining fraction of protein, and activity in solution after precipitation as a function of the molar ratio between MES and lysozyme (R_p). As the R_p was increased from 2.38 to 16, the percentage of precipitation increased as indicated by the drop in the fraction of protein and activity (74.5% to 0%). The fraction of activity also reduced accordingly from 71% (R_p = 2.38) to 0% (R_p = 16), at which point full precipitation of lysozyme was achieved. At the initial stage (R_p <16), there was insufficient amounts of MES to form a complex with lysozyme, and subsequently as more MES was added, the level of precipitation increased and finally approaching 100% at R_p of 16. This

complete level of precipitation for MES was found to be maintained up to an R_p of 38. It was also clear (Figure 4.1) that the amount of activity was in accordance with the remaining mass in solution after precipitation over the range of all the R_p s. In addition, the optimum R_p for complete precipitation of lysozyme by MES was also found to be similar to the case of AOT (Figure 4.2), suggesting that 16 moles of MES might have sufficiently interacted with 1 mole of lysozyme to form an insoluble complex.

On the other hand, in the case of AOT precipitation (Figure 4.2), the ascending trend of precipitation as a function of R_p was only observed until its optimum R_p (16), before the level of precipitation reduced to about 87.5% (R_p = 29) and eventually to 33.4% (R_p = 38). The activity of lysozyme in the remaining solution was also found not to correspond to the mass of protein: 2.8% (R_p = 29) and 16.4% (R_p = 38), for respective protein fractions of 2.8% and 66.6%. This finding was in agreement with the work of Cheng and Stuckey (2011).

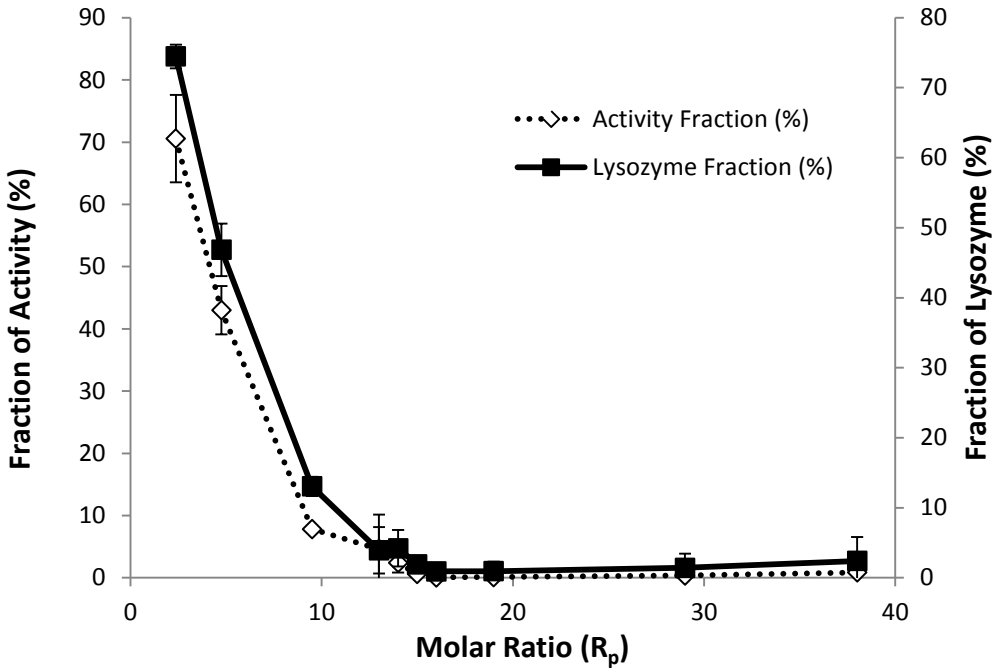


Figure 4.1 **Amount of the residual lysozyme and its activity in solution after precipitation with MES. The error bars highlight the standard deviation for each point.**

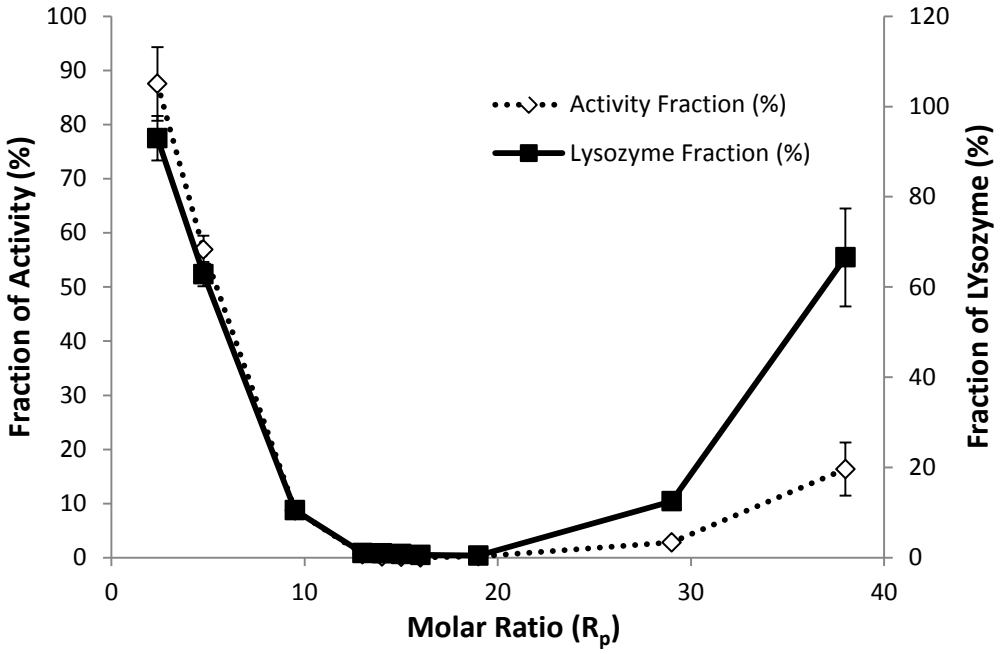
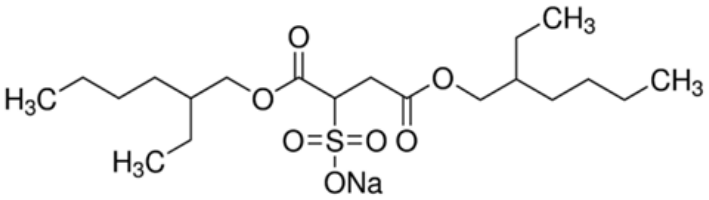


Figure 4.2 **Amount of the residual lysozyme and its activity in solution after precipitation with AOT. The error bars highlight the standard deviation for each point.**

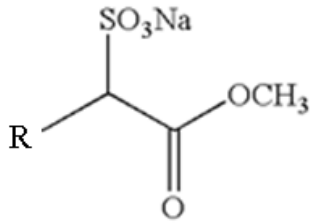
Previous research has highlighted the fact that surfactant aggregates (micelles) were responsible for the redissolution of the protein precipitate in solution (Morén and Khan, 1998; Stenstam et al, 2001). During protein-surfactant interactions, as the concentration of surfactant increases, surfactant molecules nucleate on the protein chain in a 'pearl neklace' structure (Turro et al., 1995). Normally, redissolution occurs when a critical concentration of surfactant molecules that aggregates on the complex is reached (Stenstam et al., 2001). Thus, in a homologous surfactant, redissolution at higher surfactant concentration (R) is more efficient with a more strongly associating surfactant (Morén and Khan, 1998; Stenstam et al, 2001). As the surfactant aggregates (micelles) are mainly responsible for the solubilisation of the neutral surfactant-protein complex, a surfactant with a lower CMC is thought to have a better solubilizing effect than one with a higher CMC value.

MES has a straight hydrophobic chain, rather than a branched one for AOT (Figure 4.3). Moreover, AOT has a hydrophilic group (sulphate group) positioned at the centre of the hydrophobic chain, rather than at the end of it like MES. Thus, AOT has two hydrophobic tails, each of about 6 carbon chain lengths, and an ethyl group that is branched at the second position. In contrast, in MES a single linear hydrophobic tail of about 12 to 14 carbon chain lengths exists. Two tail surfactants generally have a higher CMC in aqueous solution compared to an analogous single tail surfactant since the linear non-branched and flexible hydrophobic tail is more efficient in forming micelles than a two tail surfactant. In addition, the branched hydrophobic chain of AOT sterically inhibits micellization which results in a high CMC value (Ryoo et al., 2003). Hence, theoretically, as far as redissolution of the

precipitate is concerned, MES is expected to exhibit a better effect than AOT. However, the result obtained in this work is not consistent with the above theory, in the sense that although MES has a lower CMC (determined to be around 0.2 mM), the phenomenon of precipitate redissolution which occurred in AOT precipitation was not encountered for MES; this point will be discussed in more depth as the work progresses.



i) Sodium bis-(2-ethylhexyl) sulphosuccinate (AOT) (MW=444.56 g/mol)



R = 14 ~ 16

ii) Methyl ester sulphonates (MES) (MW=372-400 g/mol)

Figure 4.3 Chemical structure of AOT and MES. Position of linear alkyl chain: in AOT, the hydrophilic group is flanked by a double hexyl chain with an ethyl group branched at position 2. In contrast, in MES the anionic head group is tailed by a single linear alkyl chain, with a 14 to 16 carbon chain length.

Figures 4.4 and 4.5 show the binding isotherm at equilibrium for both MES and AOT. It can be observed that beyond the point of optimum binding for complete precipitation (R_p = 16), rather than remain as free surfactant, both MES and AOT anions continue to bind with protein-surfactant complex. However, in this work it was observed that the excess binding of MES (R_p > 16) to the lysozyme-MES complex was more pronounced in comparison with AOT. In fact, beyond the optimum R_p (equilibrium concentration above the CMC), and in the presence of lysozyme-MES complex in the aqueous phase, MES tended to bind with the complex rather than forming micelles among themselves to achieve thermodynamic equilibrium. The long linear hydrophobic chain in MES explained the greater adsorption of MES through cooperative binding on the neutral protein-surfactant complex through hydrophobic interactions. However, the "charging" of the complex with negative

charges due to excessive MES binding was somehow insufficient to provide a repulsive electrostatic force to overcome the hydrophobic attraction that kept the complex together.

In contrast, the concentration of AOT anions detected in the supernatant were significant after exceeding the optimum R_p (Figure 4.5) indicating that there was an unfavourable interaction between AOT and the neutral complex beyond the optimum R_p (16). Nevertheless, interestingly, the redissolution effect was pronounced in AOT precipitation, specifically at $R_p = 28$ onwards, although its binding ratio on the complex was significantly lower. Thus, the findings of the current study did not support previous research on the redissolution effect at higher R_p s. It is difficult to explain this result, but it might be related to the conformational integrity of lysozyme in the presence of surfactants at a particular concentration.

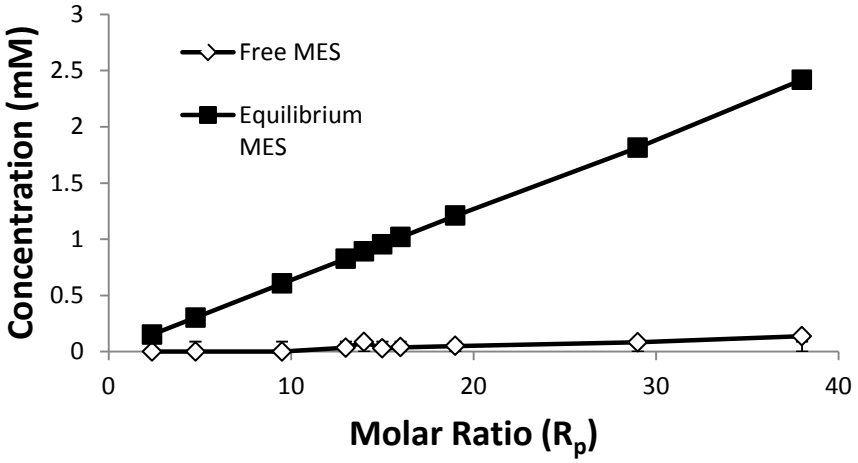


Figure 4.4 **Equilibrium concentration of MES, and concentration of free MES in lysozyme solution at pH 6.2 as a function of R_p.**

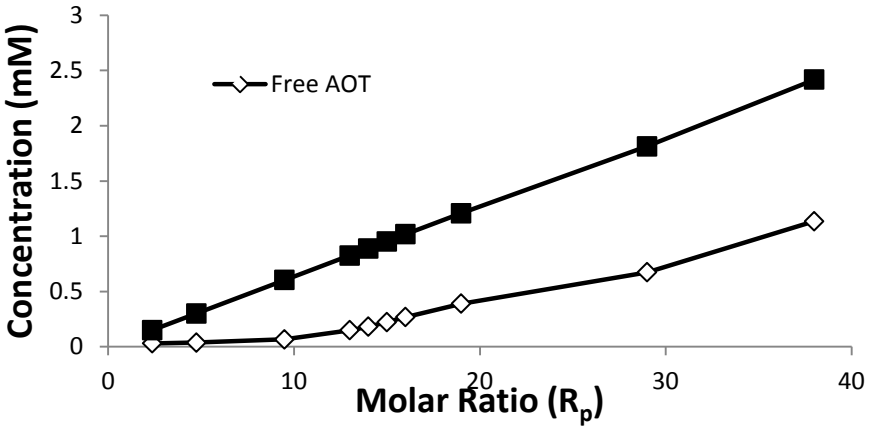


Figure 4.5 **Equilibrium concentration of AOT, and concentration of free AOT in lysozyme solution at pH 6.2 as a function of R_p.**

In this work it seems that the excess binding of MES (with a higher amount bound than AOT at similar R_ps) did not instigate the loss of native structure which was responsible for the drop in precipitation encountered in the AOT-lysozyme system (Cheng and Stuckey, 2011), and this extended in the precipitation region even up to R_p = 39. Cheng and Stuckey (2011) previously postulated that the structural integrity of lysozyme was a prerequisite for effective precipitation through the charge neutralisation mechanism driven by favourable electrostatic interactions between the AOT anionic group and ionic groups on the lysozyme surface. In fact, once the excessive binding of AOT on lysozyme occurs (R_p >23), its native structure and activity is lost as AOT started to bind with the protein's non-polar surface groups and enter its hydrophobic intracavity, eventually resulting in less lysozyme being precipitated. Furthermore, taking into account that the highest concentration of AOT employed in the system (2.4 mM at R_p = 38) was far below its CMC, it is logical to presume that the redissolution of the neutral precipitate was a result of structural changes in lysozyme after interacting with the bulkier hydrophobic structure of branched AOT, instead of the charging effect of the complex by excessive binding.

4.3.2 EFFECT OF R_P ON THE STRUCTURE OF LYSOZYME

CD was used to monitor any structural changes of the residual lysozyme in the supernatant after precipitation. Figure 4.6 shows the CD spectra of the lysozyme at R_p =2.4 to R_p =9.5 in conjunction with the native form in the far-UV range of 190-260. In general, lysozyme has a negative band in the far-UV range of 200-240 nm, with a shoulder at about 222 nm, and the strongest intensity at 208 (Yao and Gao, 2008). 30.9 ± 0.4% α-helix, 24.0 ± 0.1% β-sheet, 18.5 ± 0.1% β-turn and 26.7 ± 0.4% random coil were calculated to be present in native lysozyme through the CDNN deconvolution software. It was apparent that there were no significant changes in the secondary structure of the soluble lysozyme as the R_p was increased from 2.4 to 9.5 (Figure 4.6). As a control, the CD spectra (Figure 4.6e) including the deconvolution result (Table 4.1) of the residual lysozyme in the supernatant after precipitation with AOT at R_p = 39 was also presented, which merely indicated the significant structural changes that occurred. These results are consistent with the findings of previous researchers (Cheng and Stuckey, 2012); the unfolding pattern observed was the loss of the α-helical structure and a gain in β-sheet and random coil fractions.

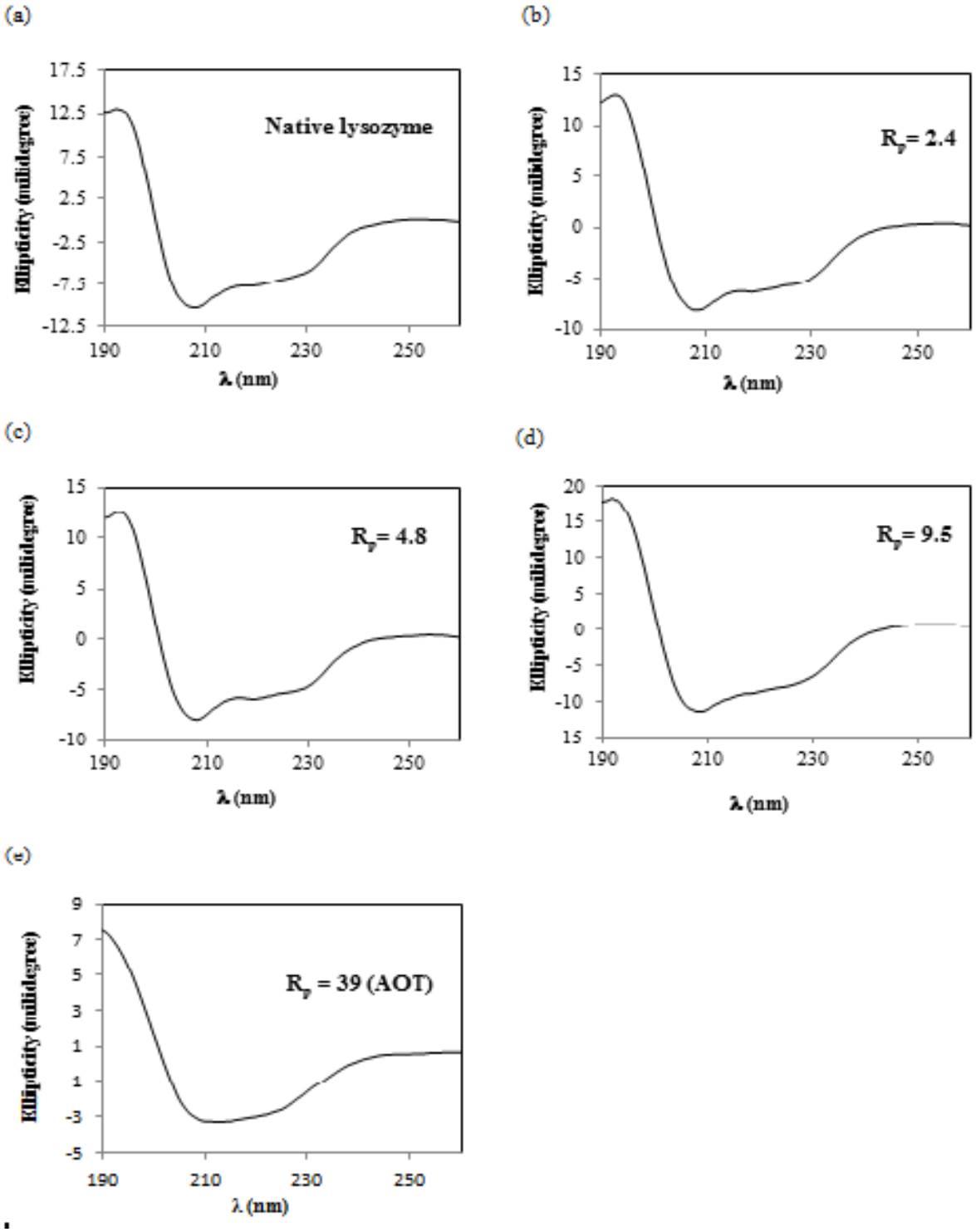


Figure 4.6 **Far-UV CD spectra of native lysozyme and the residual lysozyme in the supernatant from R_p =2.4 to 9.5 (MES) and R_p =39 (AOT), in 20 mM phosphate buffer, pH 6.23: (a) Native lysozyme, (b) R_p =2.4, (c) R_p =4.8, (d) R_p =9.5, (e) R_p =39 for AOT.**

Table 4.1 **Secondary structure content of the non-precipitated lysozyme at R_p =39 with AOT, and its relative difference (%) with regards to the native lysozyme structure.**

Secondary Structure (%)	Sample/R_p		
	Native	R_p = 39 (AOT)	Difference (%)[a]
α-helix	30.9 ± 0.4	19.5 ± 1.7	-36.9
β-sheet	24.0 ± 0.1	30.0 ± 2.1	+25.0
β-turn	18.5 ± 0.1	16.6 ± 0.2	-10.3
Coil	26.7 ± 0.4	34.0 ± 0.2	+27.3
Average Difference (%)[b]			24.9

[a]The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis (Chen et al., 1977). In general, it is accepted that there is a major deviation from native state when the difference (%) is larger than 10, as suggested by Chang et al. (1997). [b]The average difference for all of the secondary structure.

4.3.3 EFFECT OF PH ON THE PRECIPITATION OF PROTEIN

In order to study the effect of pH on the precipitation of lysozyme by MES, lysozyme was initially dissolved (1 g/L) in a pH-specified 20 mM phosphate buffer ranging from pH 6 to 12. Pure lysozyme solution at all of the pHs was used as the negative control for each of the pHs examined. 1 mL of MES solution was later added to 10 mL of these single-protein solutions, to make up an R_p of 16. The pH study was performed beyond the active pH range of lysozyme (at 6 to 9, as specified by product manufacturer, Sigma) up to 12, so as to evaluate the effect of electrostatic interactions between MES and the lysozyme molecule, which has a pI of about 11.

In general, the overall net charge of a protein depends on the pH of the solution; protein will assume an overall positive charge at a pH below its pI, a zero charge at its pI, and a negative charge at a pH above this point (McMurry, 1992); it is reported that lysozyme has 18 cationic amino acid residues (6 lysyl including 1 N-terminal, 11 arginyl and 1 histidyl) (Canfield and Liu, 1965). In surfactant precipitation, specifically in the case of MES and lysozyme at a pH<pI, it is hypothesized that the anionic head group of the MES monomer will form an electrostatic interaction with the positively charged groups on lysozyme molecules to produce neutral insoluble MES-lysozyme complex that will eventually precipitate (Shin et al., 2003; Cheng and Stuckey, 2011). At a pH>pI there will be an

electrostatic repulsion between the two similar negatively charged molecules of lysozyme and MES, thus preventing the formation of the neutrally charged complex.

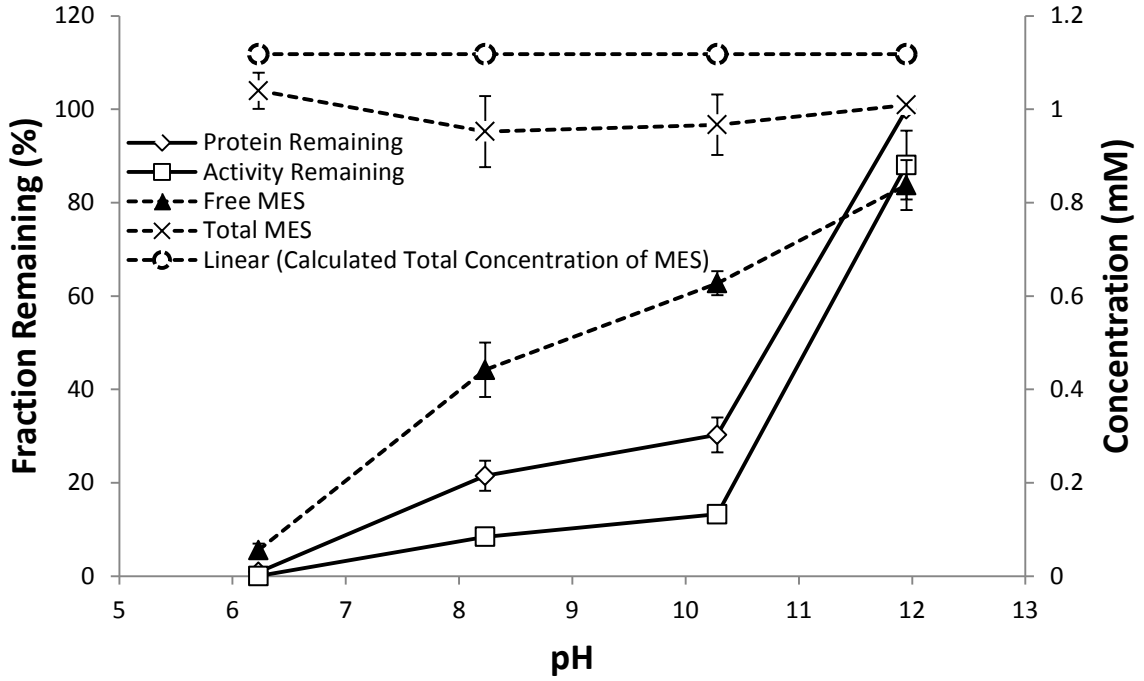


Figure 4.7 **Fraction of protein, and activity remaining, total concentration of MES, and final concentration of free MES in the after-precipitation solution.**

Figure 4.7 shows the fraction of the mass and lysozyme's activity in the remaining solution after precipitation, as a function of the pH of the solution. Full precipitation was obtained at pH 6.23 and the level of precipitation decreased with increasing pH up to 12, as indicated by the increase in the remaining fraction, from 21% (pH 8.2) to almost 100% (pH 12). This behaviour was due to the explanation discussed previously, i.e. that the further away the pH is away from the pI of lysozyme (i.e. 11), the more likely is the formation of an ion pair between the anionic MES head group and the positively charged protein surface charge, and thus the higher the amount of precipitate obtained.

An intriguing observation was that most of the non-precipitated lysozyme's activity remained at pH 12 (88%), contrary to the AOT precipitation study reported by Cheng and Stuckey (2011). They previously reported that despite more than 50% of the lysozyme not precipitating, only 9% of its activity was detected at an R_p of 16 and pH 12. It was claimed that hydrophobic interactions between AOT and the lysozyme took place at this pH (>70% bound AOT) which consequently caused a significant drop in its activity. However, it was apparent that for MES, even with mild (manual end-

over-end mixing) or rigorous (≈10 - 20s vortex) mixing were performed, no significant binding was observed at pH 12 as indicated by the non-formation of a white insoluble complex upon MES addition. Furthermore, in this study determination of the equilibrium MES concentration in the supernatant after precipitation was also being carried out to gain a better understanding of the interaction (Figure 4.7).

From Figure 4.7 it can be seen that there was an increase in the concentration of free MES in solution with increasing pH, and this result was in accordance with the amount of precipitated lysozyme.. Less MES was interacting with lysozyme as the pH increased, due to the reduced electrostatic interactions between MES and lysozyme as a consequence of the reduced net positive charge of lysozyme. Moreover, at higher pH (above the pI of about 11) a considerable amount of free MES (about 85%) can still be detected. This implied that the degree of hydrophobic interaction between lysozyme and MES at this point was not very significant compared to that encountered in AOT precipitation (Cheng and Stuckey, 2012), which explained why the activity of the non-precipitating lysozyme remained i.e. 88%, for the case of MES precipitation.

CD and deconvolution analysis of the non-precipitated lysozyme over the pH range examined was also performed to check on their structural integrity (Figure 4.8 and Table 4.2). Only a slightly altered CD profile of the lysozyme at pH 12 was observed, and this is understood to be a result of the binding (15% of bound MES). However, in comparison to the AOT results where major loss in conformational structure was observed (Cheng and Stuckey, 2012), the degree of change caused by MES was relatively minor, and in fact most of its activity was retained. It is believed that with AOT, the dominant effect of the hydrophobic interactions at higher pH (>pI) might be due to the larger steric effect of its bulky branched double hydrophobic tail.

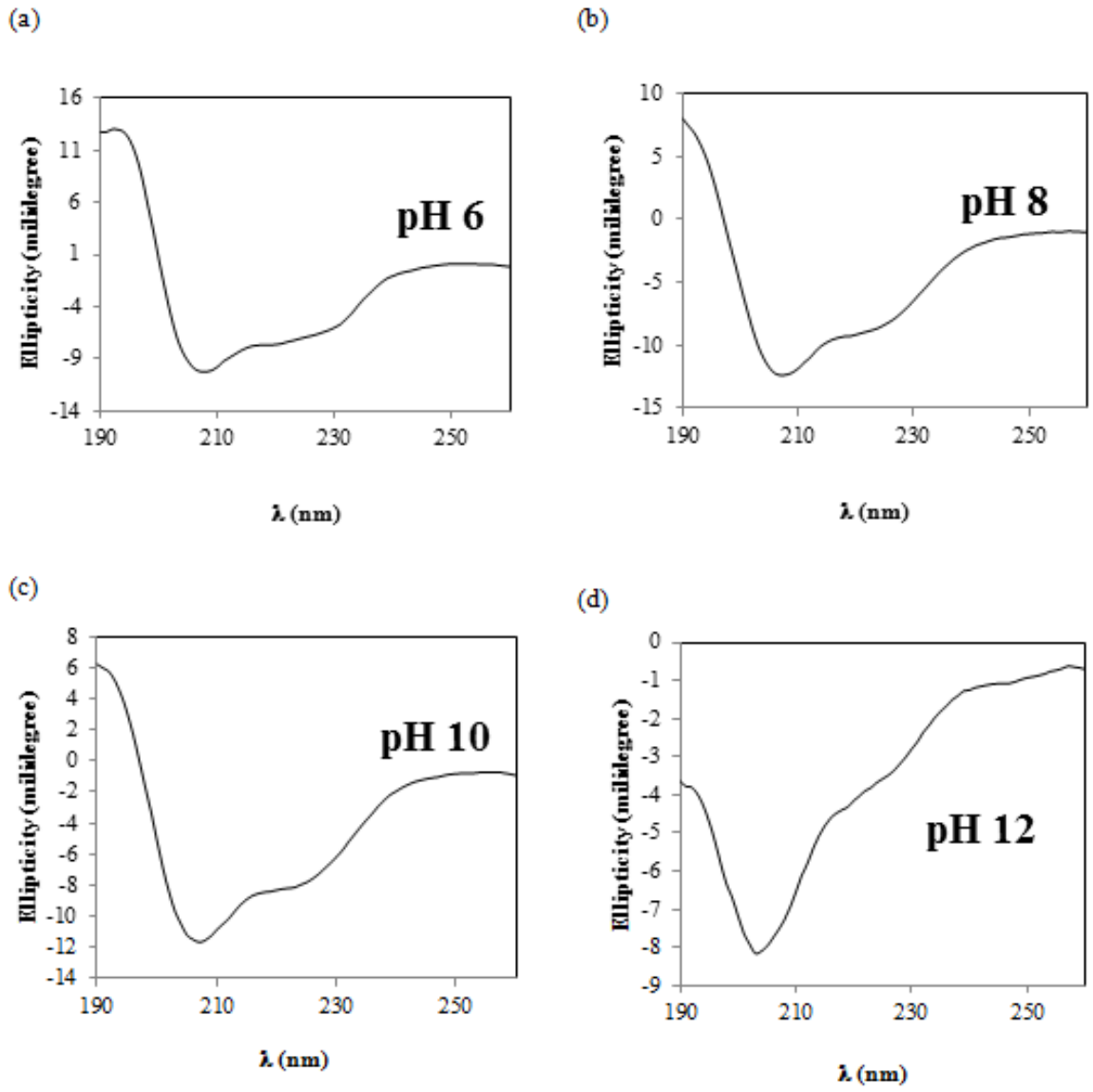


Figure 4.8 **Far-UV CD spectra of the remaining lysozyme in the supernatant after precipitation by MES at R_p = 16, for the pH range of 6.23 to 12.**

After scrutinizing the results, it was clear that the interaction between MES and the protein was mainly governed by electrostatic interactions that were required to bring both molecules closer to form a solid-like complex. However, if such a condition was not met, it seems that any interaction between them including the hydrophobic interaction which was apparently encountered in the AOT-lysozyme system was not very favourable for MES-lysozyme. Thus, this study highlighted another advantage of MES over AOT, in the sense that MES is more specific in interacting with lysozyme and exhibited significantly less denaturing hydrophobic interactions with lysozyme. Basically, this demonstrates that MES is a more appealing choice for a surfactant ligand for protein precipitation.

Table 4.2 **Secondary structure content of the non-precipitated lysozyme at pH 12, and its relative difference (%) with regards to native lysozyme structure.**

Secondary Structure (%)	Sample/ R_p		
	Native	pH 12	Difference (%)[a]
α-helix	30.9 ± 0.4	26.3 ± 2.1	-14.9
β-sheet	24.0 ± 0.1	32.1 ± 2.2	+33.8
β-turn	18.5 ± 0.1	18.8 ± 0.2	+1.8
Coil	26.7 ± 0.4	22.7 ± 0.7	-15.0
Average Difference (%)[b]			16.4

[a]The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis (Chen et al., 1977). In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10, as suggested by Chang et al. (1997). [b]The average difference for all of the secondary structure.

4.3.4 RECOVERY OF LYSOZYME FROM A LYSOZYME-MES COMPLEX

All of the recovery studies were performed from optimally (R_p = 16) precipitated lysozyme. The precipitate was recovered by means of both organic solvent addition and counter-ionic surfactant, TOMAC. Apart from the parameter of overall protein/activity recovery, protein recovery efficiency and activity recovery efficiency were also determined to examine the effectiveness of the recovery method applied to separate the lysozyme from the precipitate. The efficiency of protein recovery was calculated from:

$$\text{Protein Recovery Efficiency (\%)} = \frac{C_f \cdot V_f}{C_i \cdot V_i - C_e \cdot V_e - C_w \cdot V_w} \times 100\%$$

where C_e and C_w refer to the concentration of lysozyme at equilibrium after the formation of the precipitate, and the concentration of lysozyme in the wash fraction, respectively. Meanwhile, V_e and V_w refer to the volume of the equilibrium fraction after precipitation, and the washing fraction, respectively. On the other hand, the efficiency of the activity recovery was calculated as:

$$\text{Activity Recovery Efficiency (\%)} = \frac{a_f \cdot V_f}{a_i \cdot V_i - a_e \cdot V_e - a_w \cdot V_w} \times 100\%$$

where a_e and a_w refer to lysozyme activity in the equilibrium fraction after precipitation, and in the washing fraction, respectively.

4.3.4.1 The Use of Solvent in Lysozyme Recovery

The effect of 6 types of solvent, namely; acetone, ethanol, acetone-ethanol (50% v/v), methanol, chloroform and dimethyl sulfoxide on recovery were examined. After addition of the solvent, the surfactant (MES) dissolves into the organic phase, while the lysozyme remains as a precipitate. The precipitate was finally dissolved in a fresh phosphate buffer at pH 6.2 to obtain a surfactant-free lysozyme solution. Filtration through 0.45 μm pore size was used to remove any undissolved precipitate that may remain in the final buffer.

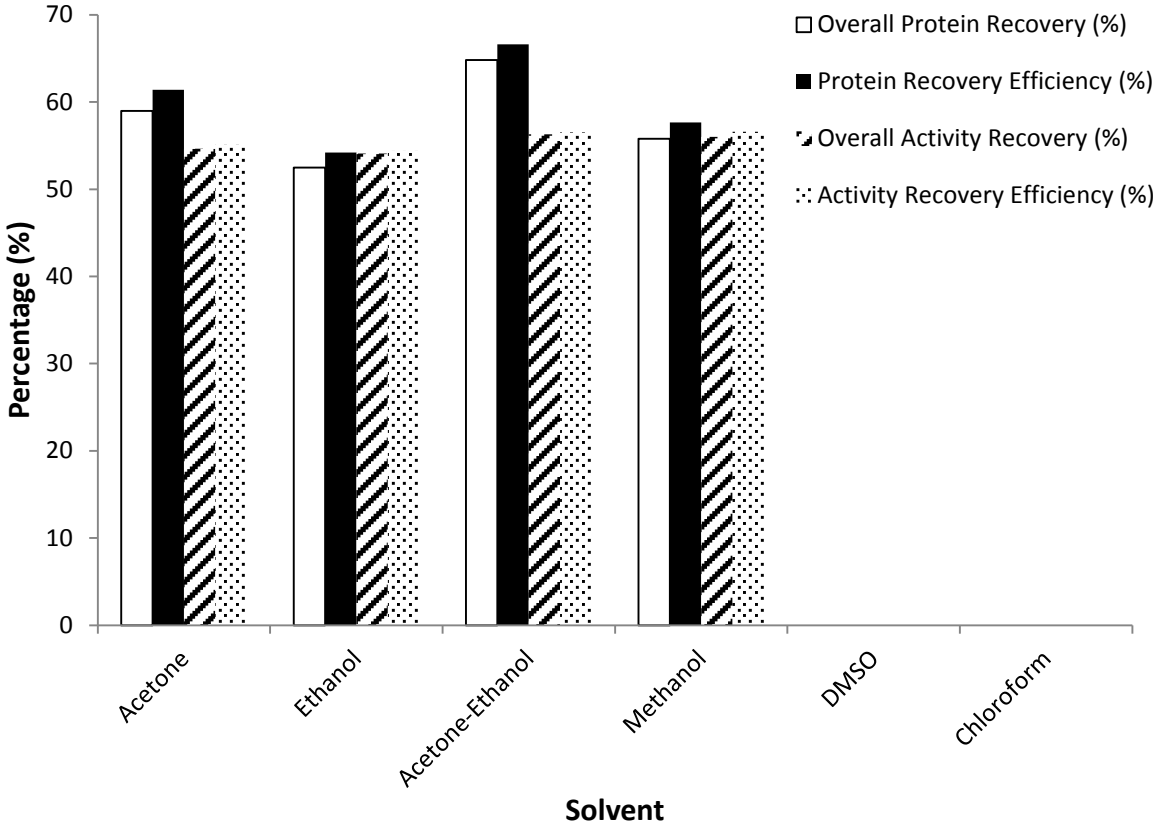


Figure 4.9 The effect of organic solvent type on the recovery of lysozyme from precipitate.

Figure 4.9 shows the performance of the solvents in terms of overall protein recovery (%), protein recovery efficiency (%), overall activity recovery (%) and activity recovery efficiency (%). Basically, comparable performances were obtained for all the solvents tested for both protein and activity recovery, except chloroform and DMSO. Overall activity recoveries of about 54-56% were obtained for acetone, ethanol, acetone-ethanol (50% v/v) and methanol. However, as can be seen from Figure 4.10, slightly higher protein recoveries were obtained for acetone (59%), either being used alone or in 50% mix with ethanol. This can be explained by the strong absorption of traces of acetone at 280 nm

wavelength that caused an over-estimation in the quantification of protein concentration in the final phase. Traces of acetone remained after the addition of phosphate buffer at pH 6.2 in the final stage, even though a precautionary step was taken to ensure its complete removal by air drying in the fume hood for 3 hours, instead of the conventional 30 min to 1 hour period. The timeframe was also not increased further in order not to compromise the lysozyme stability factor. In addition, such circumstances were also encountered by other researcher (Shin and Stuckey, 2011), in which it was reported that protein recovery as high as 157.3 % was obtained for acetone recovery.

It was reported earlier that lysozyme was not denatured by acetone, alcohol, ether, chloroform or toluene, even when they have acted for a considerable time (Fleming, 1932). In fact, these solvent can be used for the preservation of lysozyme-containing materials. Thus, the discussion on the recovery of lysozyme by organic solvents was made based on lysozyme's solubility in organic solvent, rather than denaturation. The more the protein dissolves in them, the less the amount precipitated out, and the lower the recovery yield obtained. For instance, the solubility of lysozyme was >100 mg/mL and 0.25 mg/mL, in DMSO and chloroform, respectively, as compared to 0.01 mg/mL and 0.02 mg/mL, in methanol and ethanol, respectively (Houen, 1996). This fact explains the generally comparable results of all of the solvents studied (i.e. 54 to 59%), except DMSO and chloroform where none of the lysozyme was recovered.

As for the CD analysis of the recovered lysozyme, only minor changes in the α-helix content of the lysozyme sample recovered by acetone and ethanol were detected, as indicated by the relative percentage difference shown (Table 4.3), and through t-test analysis. The α-helix content of the acetone and ethanol-recovered lysozyme increased slightly (33.6% and 32.9%, respectively) compared to the native form (30.9%). Meanwhile the random coil fraction in acetone-recovered lysozyme reduced slightly to 24% from 26.7%. Nevertheless, based on the activity results and CD profile presented, it is clear that the major conformation of the recovered lysozyme was still retained.

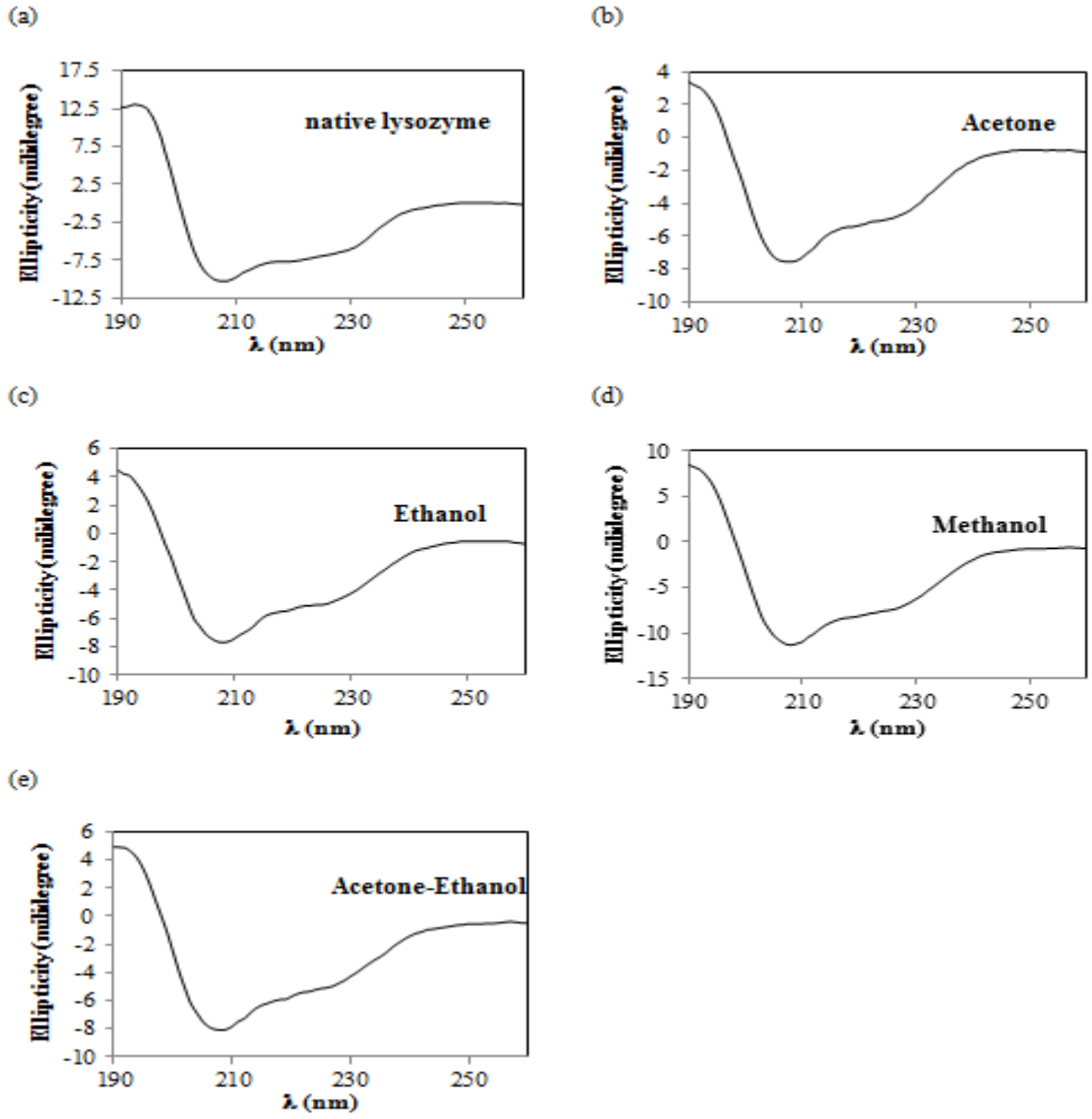


Figure 4.10 **Far-UV CD spectra of lysozyme recovered with various types of solvent followed by dissolution in 20 mM phosphate buffer, pH 6.23. Precipitation was performed at an R_p = 16, at pH 6.23.**

In addition, the results also highlighted that the overall protein/activity recovery and their recovery efficiency was comparable for all solvents, since the concentration of lysozyme in the supernatant after precipitation (C_e) and the washing fraction (C_w) were not significant. MES was able to completely precipitate the lysozyme in the sample, and there was no protein lost during the washing step (phosphate buffer) in the recovery stage. The recovery/extraction effect was solely achieved by the addition of an organic solvent, to dissolve the surfactant (and hence remove it from the protein) while precipitating the target protein. Again, this pointed to the effectiveness of the precipitation method overall.

Table 4.3 **Fraction of secondary structure element of the lysozyme recovered by various types of solvent.**

Sample	Percentage of Secondary Structure (%)				Average Difference (%)[b]
	α-helix	β-sheet	β-turn	Coil	
Native	30.9 ± 0.4	24.0 ± 0.1	18.5 ± 0.1	26.7 ± 0.4	-
Acetone	33.6 ± 1.5	23.5 ± 2.0	19.0 ± 0.1	24.0 ± 0.7	
Difference (%)[a]	+8.8	-2.3	+2.4	-10.2	5.93
Ethanol	32.9 ± 2.1	23.1 ± 0.5	18.8 ± 0.3	25.2 ± 1.9	
Difference (%)	+6.6	-3.6	+1.5	-5.7	4.35
Acetone-Ethanol	31.6 ± 0.1	23.6 ± 0.5	18.6 ± 0.2	26.2 ± 0.8	
Difference (%)	+2.4	-1.8	+0.5	-1.9	1.65
Methanol	31.7 ± 1.0	22.7 ± 1.2	18.4 ± 0.1	27.0 ± 0.1	
Difference (%)	+2.6	-5.2	-0.3	+1.0	2.28

[a]The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis (Chen et al., 1977). In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10, as suggested by Chang et al. (1997). [b]The average difference for the four kinds of secondary structure.

4.3.4.2 THE USE OF A COUNTER-IONIC SURFACTANT IN LYSOZYME RECOVERY

In order to recover lysozyme from the lysozyme-MES complex (precipitate), trioctyl-methyl ammonium chloride (TOMAC) was used. Essentially TOMAC is a quaternary ammonium salt, and thus possesses a permanent positive charge which can form salts with anions over a wide pH range. The precipitate recovered from the optimum R_p (16) was added to 20 mM phosphate buffer, pH 6.2, followed by the addition of 1 mL of TOMAC solution (dissolved in ethanol) to make up the molar ratio (R_r) of TOMAC to the MES range between 0 to 3.0 (0-3.05 mM). In principle, the ionic interactions between TOMAC and MES are stronger than between TOMAC and lysozyme, and this facilitates the release of lysozyme from the neutral complex into solution while leaving the simultaneously formed TOMAC-MES salt as a precipitate (Cheng and Stuckey, 2011).

As illustrated in Figure 4.11, it can be seen that there is an increasing trend of protein and activity recovery in conjunction with the recovery efficiency with an increment in R_r. By taking into account the protein/activity recovery and the concentration of surfactants (MES and TOMAC), it can be postulated that there are 3 stages in the counter-ionic surfactant recovery. Initially, at $R_r = 0$ (negative control), MES was detected, i.e., 0.0181 mM (standard deviation = ±0.0023) as it was released from the complex possibly due to the stripping effect of ethanol. However, the amount of protein released may still be too low to be detected at $R_r = 0$. Then, in the intermediate stage (between an R_r of 1 to 2), there was an increase in the activity recovery from about 70% ($R_r = 1$) to 83% ($R_r = 1.5$), and finally this reached a maximum of 90% (at $R_r = 2.0$); the activity recoveries were found to accord with protein recovery. At an R_r below 2, there was also an insufficient amount of the counter-ionic surfactant, TOMAC to interact with MES from the MES-protein complex. The concentration of MES was also found to decrease from 0.0139 mM ($R_r = 1$) to 0.0059 mM ($R_r = 2$). Meanwhile, TOMAC was starting to be detected at $R_r = 1.5$ at about 0.0567 mM, and this increased to 0.2188 mM at $R_r = 2.0$. At $R_r = 1.5$, near ideal/complete interaction between both surfactants occurred, in which the concentration of both surfactants was relatively low (0.0567 mM and 0.0059 mM, respectively, for TOMAC and MES). In the final stage (beyond $R_r = 2.0$), a possible maximum release of protein was achieved i.e. 90%, and this level extends until $R_r = 3.0$. At this stage, any addition of TOMAC accumulated in solution, for example at around 7.2% ($R_r = 2$) to 16% ($R_r = 3$). Hence, it can be suggested that most of the TOMAC added still bound with the lysozyme-MES complex, but did not release the remaining precipitated lysozyme fraction.

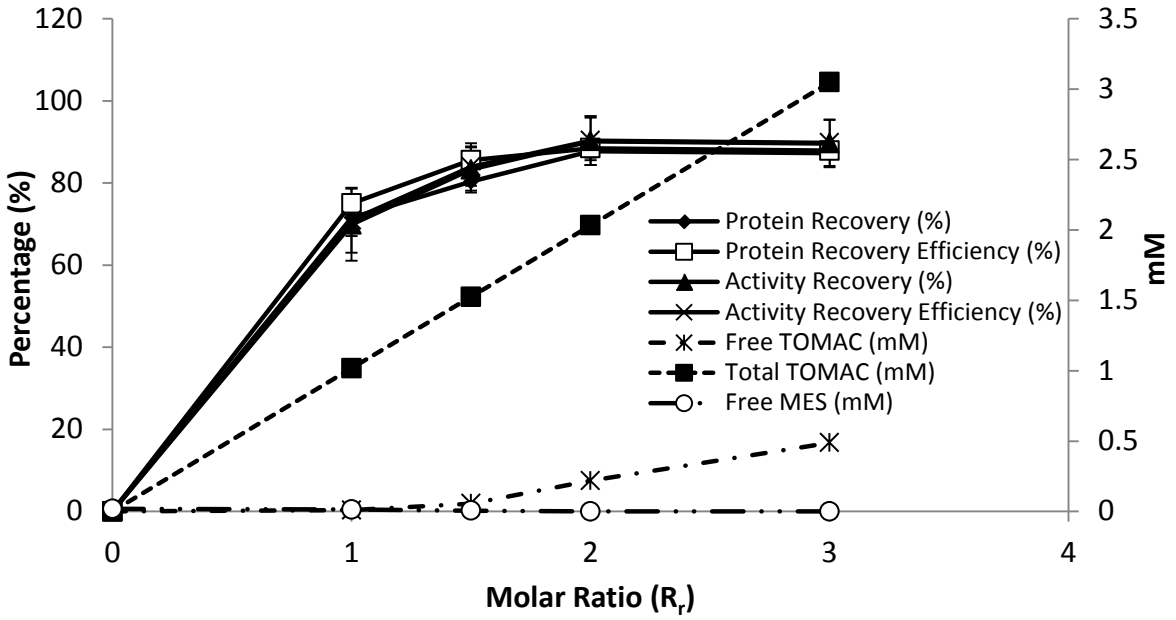


Figure 4.11 The effect of the counter-ionic surfactant, TOMAC to MES molar ratio (R_r) on the recovery of lysozyme.

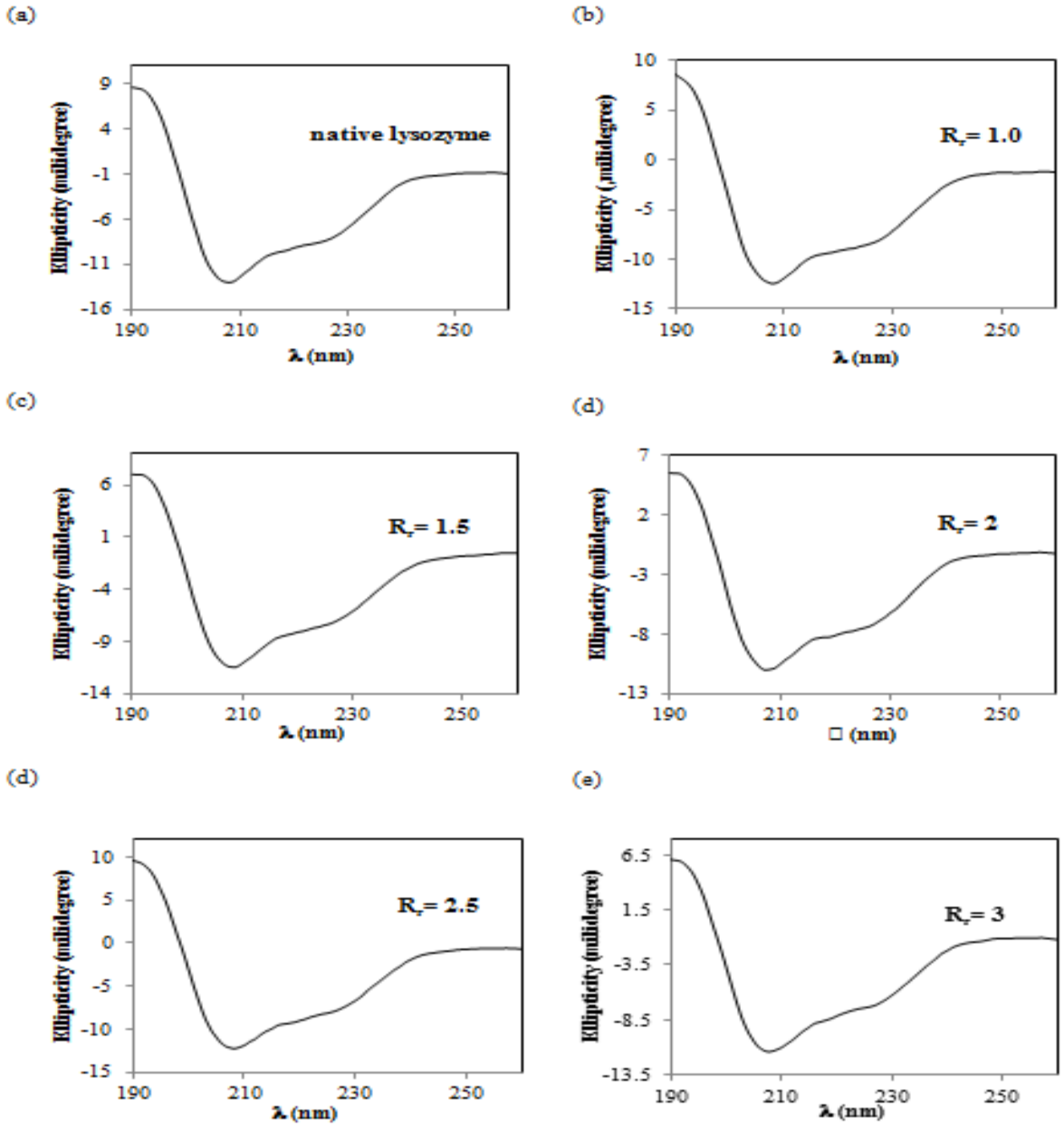


Figure 4.12 **Far-UV CD spectra of the recovered lysozyme by counter-ionic surfactant – TOMAC ranging from R_r = 1.0 to 3.0, with the respective native lysozyme, in 20 mM phosphate buffer and a protein concentration range of 0.09 to 0.1 mg/mL.**

Table 4.4 **Fractions of α–helix, β-sheet, β-turn, and random coil for the native and recovered lysozyme by counter-ionic surfactant – TOMAC, from R_r = 1.0 to 3.0, respectively.**

Sample/ R_r	Percentage of Secondary Structure (%)				Average Difference (%)[b]
	α-helix	β-sheet	β-turn	Coil	
Native	30.9 ± 0.4	24.0 ± 0.1	18.5 ± 0.1	26.7 ± 0.4	-
1.0	29.9 ± 0.2	24.8 ± 0.2	18.5 ± 0.1	26.6 ± 0.3	
Difference (%)[a]	-4.1	+3.3	-0.1	+0.3	1.95
1.5	29.6 ± 0.8	24.9 ± 0.5	18.4 ± 0.1	27.2 ± 0.4	
Difference (%)	-5.1	+3.7	-0.6	+2.5	2.98
2.0	28.8 ± 0.7	25.8 ± 1.0	18.4 ± 0.0	27.0 ± 0.2	
Difference (%)	-7.4	+7.1	-0.6	+1.7	4.20
2.5	30.3 ± 0.4	24.1 ± 0.3	18.4 ± 0.1	27.2 ± 0.3	
Difference (%)	+2.7	+0.8	-0.5	+2.4	1.60
3.0	30.5 ± 1.1	24.6 ± 1.1	18.5 ± 0.1	26.4 ± 0.2	
Difference (%)	+2.1	+2.5	+0.1	-0.3	1.25

[a]The difference in the percentages of secondary structure between the protein sample and the native state, determined by computational analysis (Chen et al., 1977). In general, it is accepted that there is a major deviation from the native state when the difference (%) is larger than 10, as suggested by Chang et al. (1997). [b]The average difference for the four kinds of secondary structure.

The CD results, together with the deconvolution of the spectra obtained across the range of R_r s examined (Figure 4.12 and Table 4.4), highlighted the fact that there were no significant changes in the conformation of the lysozyme recovered by using the counter-ionic surfactant – TOMAC. Hence, based on the activity and CD results, it was proven that MES can work similarly to AOT as a precipitant for this surfactant precipitation technique without causing any denaturation of the target protein.

Previously, Cheng and Stuckey (2012) claimed to have successfully recovered lysozyme from the lysozyme-AOT complex using the counter-ionic surfactant, TOMAC, where 100% recovery was

obtained at an R_r of 2. However, they did not address the problem of TOMAC accumulation in solution after its addition up to R_r =3 (final concentration of 3.07 mM). They only performed a methylene blue (cationic dye) assay on the recovered lysozyme solution which can only detect anionic surfactants (AOT and MES). In our work, a methyl orange (anionic dye) assay was performed to detect any remaining TOMAC in solution according to the method of Wang & Langley (1975), with a slight modification in terms of volume. Hence, this allowed for a better monitoring of any carry-over of both surfactants in the recovered phase, and optimization of the recovery process based on counter-ionic surfactant. Cheng and Stuckey (2012) previously reported the use of several cationic surfactants: TOMAC, DTAB and DODMAC to recover lysozyme from a lysozyme-AOT complex. TOMAC/DTAB were reported to have achieved full recovery, but for DODMAC, however, the highest recovery achieved was only about 80%; in addition to this, the recovery dropped as the concentration exceeded that point. They have put this down to the effect of the DODMAC structure, in which it is highly hydrophobic due to the presence of long alkyl chain.

In this work, we accept that the optimum R_r was 1.5 where about 83% recovery was obtained with a TOMAC carry-over of 10.86%. Nonetheless, compromise on protein purity after protein precipitation depends on the downstream separations units that follow. Surfactant removal from protein samples can be very important; for example, in mass spectrometry the protein sample must be free from any detergent prior to the analysis, because even at low concentrations it can contaminate instruments and interfere with column binding, elution and ionization.

Thus, in this work, an attempt was also made to improve this result in terms of yield and addressing the carry-over/bleeding of TOMAC by performing a 2-stage recovery process which has never been done before. In the first recovery step, counter-ionic recovery at R_r =1 was performed that yielded about 69.6% lysozyme. After the first stage recovery, the MES-TOMAC complex formed was present together with the remaining lysozyme-MES complex precipitate. During the second stage recovery, the amount of TOMAC added to the second stage was calculated based on the molar ratio of TOMAC to the released lysozyme in the earlier first stage, i.e. around 16, rather than based on the amount of MES as the amount of lysozyme-bound MES was unknown. About 8.8% of lysozyme was recovered in the second stage to make up a total recovery of 78.4%. Although the surfactant carry-over detected was low in both of the stages (0.0043 mM and 0.0397 mM, for the first and second stage, respectively), this 2-stage recovery approach proved to not significantly improve the result of single stage recovery, as reported earlier. This was probably down to the difficulty in recovering the lysozyme-MES precipitate after the extensive mixing-centrifugation steps, and overall some lysozyme might have been lost during these steps. For instance, although the centrifugation speed was increased from 7,500 to 12,500 rpm, this did not seem sufficient to completely separate the precipitate from the supernatant. The recovered solution was again filtered (0.45 μm pore size) to completely remove the

suspended particulates, and this may have contributed to the inefficiency of the second stage recovery process.

However, surfactant carry-over should not be the bottleneck for surfactant precipitation because in order to remove the surfactant, a physical method can simply be employed after the counter-ionic recovery such as chromatography, addition of an adsorbent, or filtration. For instance, in this study, the lysozyme recovered at R=2 (where 90% recovery was achieved) can be further purified by additional filtration to remove the TOMAC carried over.

4.4 CONCLUSION:

Overall, it can be concluded that the precipitation level of lysozyme by MES was a strong function of R_p and pH. The level of precipitation increased proportionally with an increase in R up to an optimum point i.e. $R_p = 16$, but this full precipitation region extends up to a higher R_p (38). From the pH study it was clear that electrostatic interactions were the main driving force behind the MES precipitation technique, and a sufficient amount of net electrical charge neutralisation was required in order for the insoluble complex or precipitate to form.

In the recovery of lysozyme precipitate by solvent, all of the solvents used: acetone, ethanol, 50% mixture of acetone/ethanol along with methanol generally showed comparable performance (about 54 – 56% activity recoveries), except for chloroform and DMSO where none of the lysozyme was recovered due to the high solubility of lysozyme in both of the solvents, rather than it being denatured. In addition, the lysozyme precipitate was also successfully recovered by counter-ionic surfactant – TOMAC at $R_r = 1.5$, where the estimated protein recovery of 80% was achieved with a 10.86% "carry-over" of TOMAC. Although higher recovery was achieved at higher Rs in this work, the optimum R was set at 1.5 as a compromise to minimise the amount of surfactant carried over after only a single recovery step. Most significantly, the structural integrity of all the lysozyme recovered, either through solvent extraction or counter-ionic surfactant, was maintained which was confirmed by the CD spectra and deconvolution results.

Interestingly, in contrast to AOT, MES did not demonstrate the phenomenon of major structural transformation at higher surfactant concentrations during precipitation which was reportedly responsible for the drop in the level of precipitation with AOT. Furthermore, MES only exhibited minimal hydrophobic interaction with the protein molecules once the favourable electrostatic interactions between the surfactant's anionic group and the protein's positively charged groups was absent (pH>pI). It interacts with lysozyme mainly through electrostatic interactions over hydrophobic interactions. Therefore, MES exhibits better selectivity than AOT, especially for the handling of samples with high impurities. In conclusion, along with its economic benefits and environmental

features, this work has highlighted the exciting potential of using MES as the precipitant in the surfactant precipitation technique.

CHAPTER 5 BACTERIOCIN EXTRACTION BY THE SURFACTANT PRECIPITATION

5.1 INTRODUCTION

Fundamental studies on the surfactant precipitation technique based on model protein systems i.e. a single protein in buffer, has been carried out quite extensively done to date, and basic insights have been gained. Its feasibility of use and potential benefits have also been highlighted (Shin et al., 2004; Cheng and Stuckey, 2012). Nevertheless, to our knowledge, there are only 2 reports in the literature on the application of this precipitation technique to a "real world" complex sample: (1) Lysozyme from chicken egg white, by Shin et al. (2003b), and (2) Xylanase from industrial cellulase product of *Trichorderma*, by Shin et al. (2004). Thereby, so as to prove the viability of this technique to downstream protein processing, it is very important to widen its application to other realistic samples.

Bacteriocin is an extracellular secondary metabolite produced by lactic acid bacteria, and generally it is a ribosomally synthesized, small (<10 kDa) cationic peptide (Pingitore, 2007). It is used as a biopreservative in food products as it is active against pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus* and *Salmonella spp.* (Pingitore et al., 2007; Masuda et al., 2011). In addition, it has been granted with a GRAS (generally recognized as safe) status (Ahern et al., 2003; Sharma et al., 2011), and interest in its production is driven by the market need to have a natural antimicrobial agent as an alternative to the chemical additives that may cause adverse effects (Martin-Visscher et al., 2008).

Previously, Abbasiliasi et al. (2012) has isolated a bacteriocin-producing lactic acid bacteria, *Pediococcus acidilactici* Kp10, from dried curds derived from Iranian milk. The bacteriocin-like inhibitory substances (BLIS) activity was shown to be active from pH 2 to 9 against the pathogenic bacteria, *Listeria monocytogenes*, and it was also reported that its activity was stable in the presence of pepsin, α-amylase and catalase, but not to proteinase K or trypsin.

In the typical downstream processing of bacteriocin, conventional multi-step methods such as precipitation by ammonium sulphate or ethanol, ion-exchange chromatography, molecular filtration chromatography and hydrophobic interaction chromatography were mainly employed (Pingitore et al., 2007; Saint-Hubert et al., 2009). Hence, alternative separation methods are always needed due to the limitations that are commonly associated with the aforementioned conventional methods: tedious and expensive (Carolissen-Mackay et al., 1997; Kelly et al., 2000). In this work, a first attempt was made

to employ the surfactant precipitation technique with AOT to separate bacteriocin from a real complex sample of *Pediococcus* sp. in M17 fermentation broth. The influence of pH and AOT concentration on the precipitation and overall recovery of bacteriocin were investigated. In addition, a comparison was also made with the competing technique of reverse micellar extraction.

5.2 MATERIALS AND METHODS

Materials

M17 broth used for the fermentation of *Pediococcus* was purchased from Merck (Darmstadt, Germany). Isooctane used in reverse micellar extraction was from Aldrich Chemistry, USA.

Instruments

All chemicals were weighed by using an analytical balance (ALPS-AL204, Mettler Toledo, UK). A pH meter (S47-K, Mettler Toledo, UK) was used for all pH measurements. An ultracentrifuge (Sorvall Evolution RC) and Sorvall SLA-1500 rotor, (Thermo Electron Corporation, USA) was used to separate the biomass. A UV-Vis Spectrophotometer (Lambda 25, PerkinElmer, UK) and 1 cm quartz cuvettes were used to measure the protein concentration. An end-over-end mixer, Reax 2 (Heidolph. Germany) was used to mix the surfactant and protein mixture.

5.2.1 MICROORGANISM AND BLIS PRODUCTION

The BLIS producing strain, *Pediococcus acidilactici* Kp10, was isolated from dried curd and the details have been described in the previous study (Abbasiliasi et al., 2012). The primary culture was prepared by taking a single bacterial colony from an agar plate and growing it in a 50 mL tube containing 10 mL of M17 broth, followed by incubation at 37°C for 24 h without shaking. The inoculum was prepared by inoculating 1% (v/v) of the primary culture into a 50 mL tube containing 10 mL of M17 medium, and incubated at 37°C on a shaker agitated at 100 rpm for 24 h.

5.2.2 PREPARATION OF BACTERIOCIN CRUDE EXTRACT

The cell free culture supernatant was prepared by ultracentrifuge (Sorvall Evolution RC) with Sorvall SLA-1500 Rotor, (Thermo Electron Corporation, USA) for 30 min, 30000 ×g, 4°C.

5.2.3 PREPARATION OF AOT PHASE

The AOT solution was prepared by dissolving it in distilled water with a concentration range of 2.89 to 43.23 mM. At the moment of AOT addition to the sample, the final AOT concentration ranged from 0.26 to 3.93 mM. Therefore, AOT was present in the monomer form at these concentrations as the CMC of AOT in water is 4.1 mM in water at 25°C (Linfield, 1976).

5.2.4 PRECIPITATION METHOD

1 mL volume of AOT solution in water was added to 10 mL of bacteriocin-containing fermentation broth, and the mixtures were mixed for 5 min with an end-to-end mixer. The samples were then centrifuged at 14000 ×g for 5 min. Acetone was added to the precipitates to dissolve the AOT in the precipitate, and this was followed by a second centrifugation step to recover the protein precipitate. Finally, the precipitates were dissolved in a fresh phosphate buffer at pH 7.

5.2.5 RECOVERY OF BACTERIOCIN FROM THE AOT-BACTERIOCIN COMPLEX

The bacteriocin was recovered from the AOT-bacteriocin complex by first washing it with 1 mL of distilled water to remove the remaining impurities attached. Following a centrifugation step (14,000 ×g, 5 min), the complex was separated and 1 mL of acetone was added. 10 µL of 0.1 M NaCl solution was later added to the solvent phase followed by a second centrifugation step. The protein precipitate was collected and washed with acetone to remove the remaining AOT. After centrifugation, the precipitate was dissolved in fresh 20 mM, pH 6 PBS buffer.

5.2.6 REVERSE MICELLAR EXTRACTION OF BACTERIOCIN

Forward extraction was carried out by mixing 5 mL of cell-free supernatant of bacteriocin crude with 5 mL of isooctane in presence of AOT. AOT was first dissolved in isooctane before the organic phase was added to the sample. The mixture was mixed in an axial-mixing mode in a 30 min contact time. Effect of AOT concentration (5-50 mM), pH (2-9), and NaCl concentration (0-0.3 mM) of the crude was studied. The two phases were allowed to be separated by gravity in room temperature, total protein and activity of bacteriocin in the remaining crude was analysed. The organic phase obtained from the forward extraction was subjected to backward extraction (i.e. stripping of bacteriocin from reverse micelle system). Stripping solution used during backward extraction for all experiments was 20 mM phosphate buffer with 2 M KCl (pH 9).

5.2.7 OPTIMISATION OF SEPARATION PARAMETERS

Optimisation of the experimental conditions was performed with regard to overall bacteriocin's activity recovery (%), protein recovery (%), and purification factor. These parameters were calculated using the following equations:

$$Activity\ recovery\ (\%) = \frac{Total\ activity\ in\ final\ solution}{Total\ activity\ in\ original\ crude} \times 100\% \qquad (1)$$

$$Protein\ recovery\ (\%) = \frac{Total\ protein\ in\ final\ solution}{Total\ protein\ in\ original\ crude} \times 100\% \quad (2)$$

$$Purification\ factor = \frac{Specific\ activity\ in\ final\ solution}{Specific\ activity\ in\ original\ crude} \quad (3)$$

5.2.8 ANALYSIS

5.2.8.1 BACTERIOCIN ACTIVITY

The antimicrobial activity of bacteriocin was determined by the agar well diffusion assay (Abbasiliasi et al., 2012) based on the size of the inhibition zone formed against the indicator bacteria, *Listeria monocytogenes* on an agar plate. Aliquots of the sample (100 µL) were placed in wells (6 mm diameter) in cooled soft agar plates (25 mL) which was previously seeded (1% v/v) with the actively growing test strain. The plates were incubated at 37°C for 24 h for the growth of the test strain. After 24 hours, the size of the growth inhibition zones was measured, and the activity unit (AU) is defined as the unit area of the inhibition zone per unit volume (mm^2/mL) using the following formula:

$$Activity\ Unit\ (mm^2/mL) = \frac{L_Z - L_S}{V}$$

L_z = clear zone area (mm^2)
L_S = well area (mm^2)
V = volume of sample (mL)

5.2.8.2 PROTEIN CONCENTRATION

The protein concentration in the initial crude bacteriocin extract and the remaining sample after the formation of precipitate was determined by UV spectrophotometer at an absorbance of 280 nm (A280nm). Distilled water was used as the blank, and all result were zeroed against it. A standard calibration curve was constructed based on BSA (rather than bacteriocin, due to non-availability of pure bacteriocin) with a concentration range of 0 to 1.0 mg/mL, to determine the total protein concentration in the sample (R^2= 0.9988). Any sample with an absorbance reading of above 0.60 was diluted.

5.3 RESULTS AND DISCUSSION

5.3.1 PRECIPITATION OF BACTERIOCIN AS A FUNCTION OF PH

Considering that charge is the driving force in surfactant precipitation, the experiment was started with pH variation looking for an optimum precipitation. It has been reported in the literature (Kelly et al., 2000) that generally bacteriocin has a pI point of around 8.5 and above. Thus, in order to perform the primary capture of bacteriocin using the anionic surfactant, AOT, as a precipitating ligand, the pH of the solution must be below 8.5 to allow for the formation of ionic pairs between AOT and the target protein, bacteriocin. Hence, the pH scouting was performed in the range of 2 to 6. Basically, the natural pH of the fermentation broth was 6, and concentrated HCl (5 M) was used to adjust the pH downwards. From the experimental results (Table 5.1), surprisingly, it was observed that essentially precipitation only started to occur at pH 4 (indicated by the formation of a white insoluble complex), which was well below the hypothetical pI point. This result implied that for bateriocin, a certain degree of ionization and therefore charge density must be acquired before any electrostatic interactions can take place, and then for precipitation to occur. Interestingly, it was also observed that at a pH higher than 4, the estimated specific activities of the non-precipitated bacteriocin were reduced to about half of the original activity (Table 5.1), where the comparisons were made based on the specific activity of the original crude broth at their respective pHs. This pointed to the effect of non-specific hydrophobic interactions that have taken place as the result of weak electrostatic interactions being present.

Table 5.1 **Fraction of non-precipitated bacteriocin in solution as a function of pH. Precipitation was performed with an AOT concentration of 11.58 mM. The standard deviation is shown in parenthesis. The original bacteriocin was most active at pH 3 to 6, with a specific activity averaging 53.9 (± 1.1); at pH 2 the original specific activity was 44.1 (±2.0).**

pH	Fraction of Activity (%)	Fraction of Protein (%)	Specific Activity (U/mg)
2	0.0 (± 0.0)	91.0 (± 3.3)	0.0 (± 0.0)
4	0.0 (± 0.0)	94.2 (± 1.1)	0.0 (± 0.0)
5	47.1 (± 5.2)	100 (± 0.0)	27.4 (± 5.0)
6	48.7 (± 8.9)	100 (± 0.0)	29.0 (± 2.1)

5.3.2 EFFECT OF AOT CONCENTRATION ON THE PRECIPITATION OF BACTERIOCIN

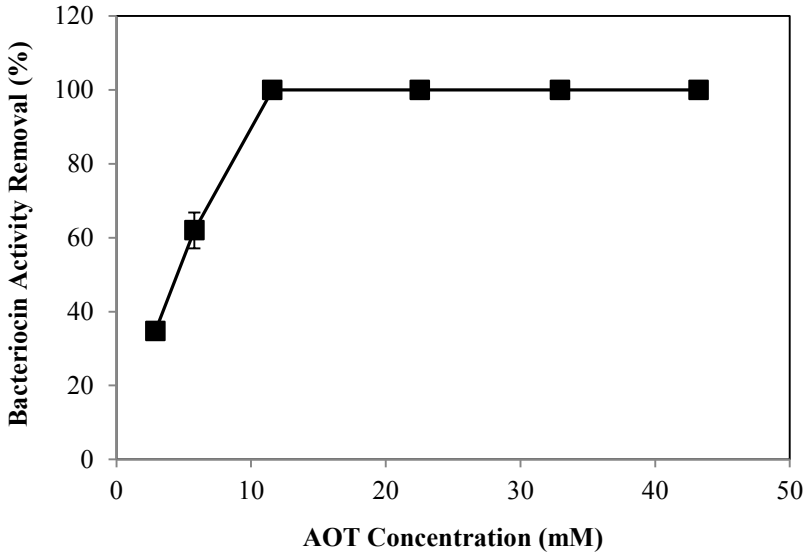


Figure 5.1 **The effect of AOT concentration on the bacteriocin's activity removal in the crude broth at pH 4.**

Figure 5.1 highlights the percentage of bacteriocin's activity removal as a function of AOT concentration employed during the precipitation. The effect of AOT concentration added ranged from 2.9 to 43.2 mM on precipitation, was conducted at pH 4. The AOT concentrations applied were in the range of 1.1 to 3.9 mM at the moment of addition, which was below its CMC value reported to be 4.1 mM in water at 25°C (Linfield, 1976); thus it was ensured that the AOT added was in a free monomeric form rather than in micellar structures. From Figure 5.1, apparently, more activity was removed as the concentration of AOT was increased. Complete removal of activity was achieved at the concentration of 11.27 mM, and this plateau level was maintained up to 43.2 mM, the highest concentration examined.

5.3.3 EFFECT OF AOT CONCENTRATION ON ACTIVITY RECOVERY

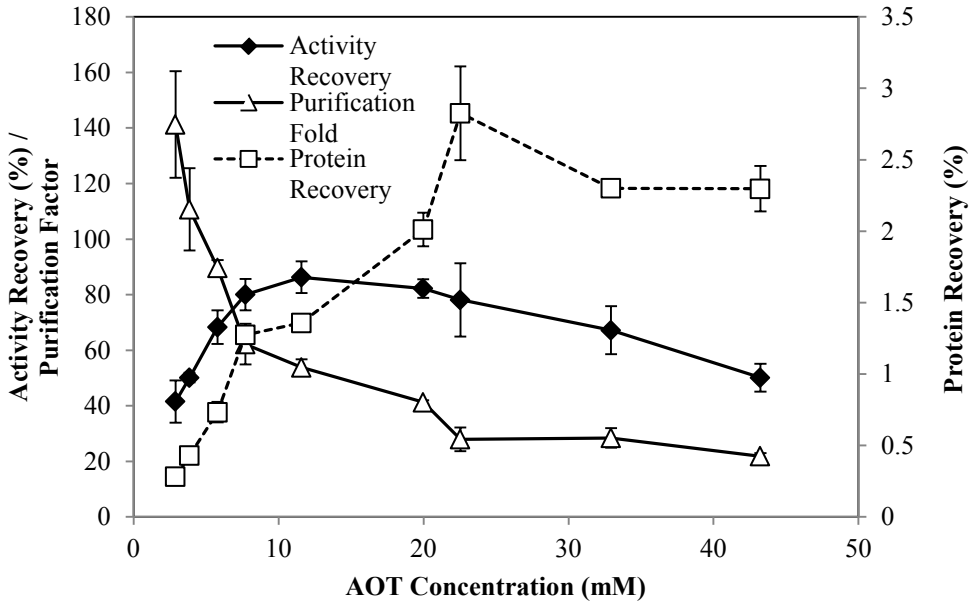


Figure 5.2 **Effect of AOT concentration added during precipitation on the recovery of bacteriocin at pH 4. The activity and concentration of the total protein in the original crude were about 2675.2±199.7 U/mL, and 53.9±0.1 mg/mL, respectively.**

The white precipitate obtained from the precipitation step earlier was treated with acetone containing 0.99 mM of NaCl to extract the bacteriocin from the protein-surfactant complex. The bacteriocin precipitate was then redissolved in 20 mM phosphate buffer, pH 7.0. In this part of the work, the examination of the recovery performance as a function of the AOT concentration, and the pH of the initial fermentation broth employed in the precipitation stage was performed. As the concentration of AOT was increased from 2.89 mM, the level of activity recovery increased correspondingly until it reached about 86.3%, at a concentration of 11.58 mM. Such an increasing trend was in accordance with the amount of bacteriocin's activity removal during the precipitation step (Figure 5.2). Nevertheless, beyond a concentration of 11.58 mM the level of recovery began to decline rather than remained stationary as encountered in the curve of bacteriocin's activity removal earlier. Although it was obvious that this reflected the excessive binding of AOT molecules on the neutral AOT-bacteriocin complex, at this point it was unclear what the main cause of this phenomenon was: (1) inactivation/denaturation of the bacteriocin that resulted in the resolubilisation of the precipitate, or; (2) the high degree of AOT binding that prevented the release (recovery) of the bacteriocin from the AOT-bacteriocin complex.

For the first situation, as the analysis of bacteriocin's activity removal (Figure 5.1) was done through a mass balance, the determination cannot discriminate between the removal of activity by precipitation or inactivation of bacteriocin at higher AOT concentrations, and thus the result might erroneously include the latter case. In comparison with the earlier studies (Chapter 3 and 4) where a single protein solution was used, it was almost impossible to determine if any inactive bacteriocin with structural changes was present in solution after the precipitation step in this study, due to the complex nature of the initial broth. In spite of that, more protein was precipitated as the amount of AOT added was increased. Thus, if the denatured/inactive form of bacteriocin was present in solution, overall protein reduction in the broth will likely be 'masking' its presence. Besides, based on previous experience with protein inactivation (resolubilisation) in surfactant precipitation, i.e. the study of precipitation of lysozyme by AOT (Cheng and Stuckey, 2012), the resolubilisation of lysozyme occurred at a molar ratio of R>23 in a stepwise fashion, as shown in the precipitation curve as a function R (AOT to lysozyme), in other words, the fractions of activity could supposedly still be detected in the remaining sample after precipitation at the earlier stage of the inactivation if it occurred. However, such a trend was not observed in this work (Figure 5.1).

Hence, it is postulated that the second reason might have contributed to the reduction effect in the recovery of the activity beyond the optimum AOT concentration. Possibly, this can be proven in the future if the recovery efficiency of the precipitated bacteriocin at higher AOT concentrations can be improved, so that is equal to the amount achieved at optimum concentration.

In terms of protein recovery, higher amounts of protein were recovered as the concentration of AOT was increased, clearly because of the higher amount of protein precipitated. On the other hand, the opposite trend was obtained for the purification factor. Hence, the optimum concentration of AOT was chosen to be 11.57 mM, where the maximum recovery of activity was achieved (86.3%), and a purification factor of 53.8% was recorded.

5.3.4 EFFECT OF PH ON ACTIVITY RECOVERY

Figure 5.3 illustrates the activity recovery of the bacteriocin in conjunction with the amount of protein recovered (%) as a function of the pH of the initial broth. As the pH was adjusted from 2 to 4, an almost constant recovery of activity was obtained (88 to 86%), while the amount of protein recovered decreased from 2.6 to 1.6% with the same increment of pH. This was in accordance with the results from the precipitation stage where as the concentration of AOT increased, more protein was co-precipitated with the target protein – bacteriocin, eventually affecting the purity of the recovered bacteriocin. No recovery was obtained at pH 5 and 6 as no precipitate was formed initially during the precipitation stage. From these results it was determined that pH 4 was the optimum pH, which was

the minimum threshold for precipitation to occur, and where the highest purity of bacteriocin was obtained.

On the other hand, Table 5.2 documents the specific activity of the recovered bacteriocin at various pHs in conjunction with its original value at that particular pH. Notably, there was marked increase in the specific activity of the recovered bacteriocin as compared to their original values in the fermentation broth. This result was in agreement with the literature (Saint-Hubert et al., 2009; Sharma et al., 2011), which indicated that the specific activity of bacteriocin increased with an increase in product purity. For instance, for the recovery at pH 3 and 4, an increment factor (which also represented the purification factor) of about 44.1 and 53.8% was determined, respectively.

While a satisfactory result (86% activity recovery) was obtained, complete recovery was not achieved, and it was believed that the recovery stage was the limiting factor rather than the precipitation step, as 100% precipitation of bacteriocin was reached earlier. It is worth highlighting the fact that further improvement in the recovery efficiency could still be achieved through further optimization of the solvent recovery step (e.g. type of solvent, or concentration of salt in the solvent), as well as the use of counter-ionic surfactants to extract the precipitated bacteriocin molecule from the solid precipitate.

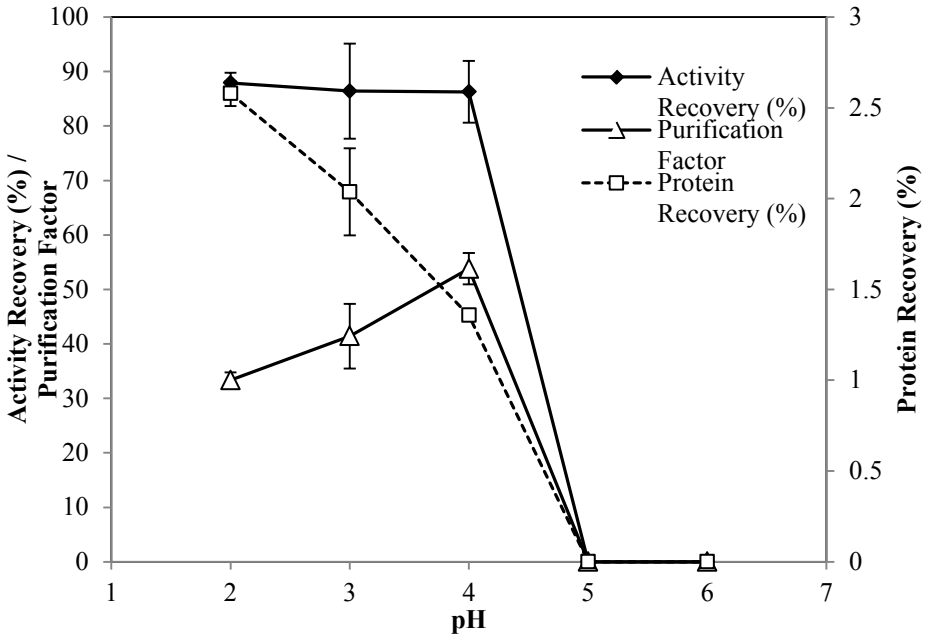


Figure 5.3 **Effect of pH of the initial fermentation broth on overall bacteriocin recovery. The concentration of AOT used was 11.57 mM.**

Table 5.2 **Specific activity of the recovered bacteriocin relative to its original value in the fermentation broth at pH 2 and 4.**

pH	Specific Activity (U/mg)	
	Final Product	**Initial**
2	1470.8 (± 63.9)	44.1 (±2.0)
3	2343.1 (± 275.7)	53.1 (± 0.2)
4	2878.9 (± 155.1)	53.5 (± 0.2)

5.3.5 REVERSE MICELLAR EXTRACTION OF BACTERIOCIN

An attempt was also made in this work to use another surfactant-based purification method, reverse micellar extraction, to separate the same bacteriocin peptide from the broth so that a comparison could be made; the overall results of the work are shown in Figure 5.4. The effect of various parameters (pH, AOT concentration and NaCl concentration) employed during the forward extraction on overall recovery were examined. At low pH (2), rather than being incorporated into the micelles, the cationic bacteriocin molecule tended to interact with the AOT molecules, and formed a precipitate at the interface between the organic and aqueous phase. The selectivity of bacteriocin solubilisation by the reverse micelles escalated as the pH increased (Figure 5.4a), suggesting that a combination of electrostatic and hydrophobic interactions were involved during the uptake of bacteriocin molecules by the organic phase. Hence, unlike the surfactant precipitation performed earlier, pH 7 was chosen as the optimum pH point for this technique.

In the plot of activity recovery against AOT concentration, an increasing trend was observed (Figure 5.4b). However, the recovered bacteriocin at the concentration of 30 mM and above unexpectedly showed poor stability, as the inhibition zone formed in the agar well diffusion assay was gradually outgrown by the *Listeria* bacteria after a 1 day period. This implied that at higher AOT concentrations, the bacteriocin might have interacted hydrophobically with the surfactant that resulted in structural changes. It has been reported in the literature that the bacteriocin peptide has hydrophobic characteristics (Saint-Hubert et al., 2009; Lappe et al., 2012), and that feature was widely exploited for its purification by hydrophobic interaction chromatography and the use of a synthetic hydrophobic adsorbent, Amberlite®-XAD® (Zendo et al., 2005; Martin-Visscher et al., 2008; Masuda et al., 2011). In addition to that, the above assumption may well be supported as such a stability problem was not encountered in the earlier study of direct precipitation by AOT where the concentrations employed were at least one order of magnitude smaller than for reverse micellar extraction (for instance, for the 30 mM AOT concentration used in the reverse micelle system, only

about 0.015 mol AOT interacting with 1 mg of protein in the sample for precipitation, rather than about 0.15 mol of AOT/mg protein for reverse micellar extraction). Lastly, in terms of salt (NaCl) concentration, the results suggested that the M17 broth has supplied the required electrolyte for the formation of reverse micelles, as further addition of salt seemed to reduce activity recovery. Overall, under the conditions specified above, an estimated activity recovery of 48% and a purification of about 20 was obtained for the reverse micellar extraction.

Therefore, it was observed that the reverse micellar technique was relatively complicated and less efficient in purifying bacteriocin, due to the limitations of using high surfactant concentrations. This result was consistent with the work of Shin et al. (2004), who pointed that the use of surfactant in a reverse micellar extraction method to purify enzymes was less effective than the direct precipitation method. In addition, Abbasiliasi et al. (2014) has also reported that the purification of the same bacteriocin using a polymer–salt aqueous two-phase system (ATPS) consisting of polyethylene–glycol (PEG) with sodium citrate resulted in an up to 8.43 fold concentration, with a yield of 81.18%. The optimum conditions of the ATPS were: 26.5% PEG (8000)/11% sodium citrate with a tie-line length of 46.38% (w/w), a volume ratio of 1.8, and 1.8% crude load at pH 7 without the presence of NaCl.

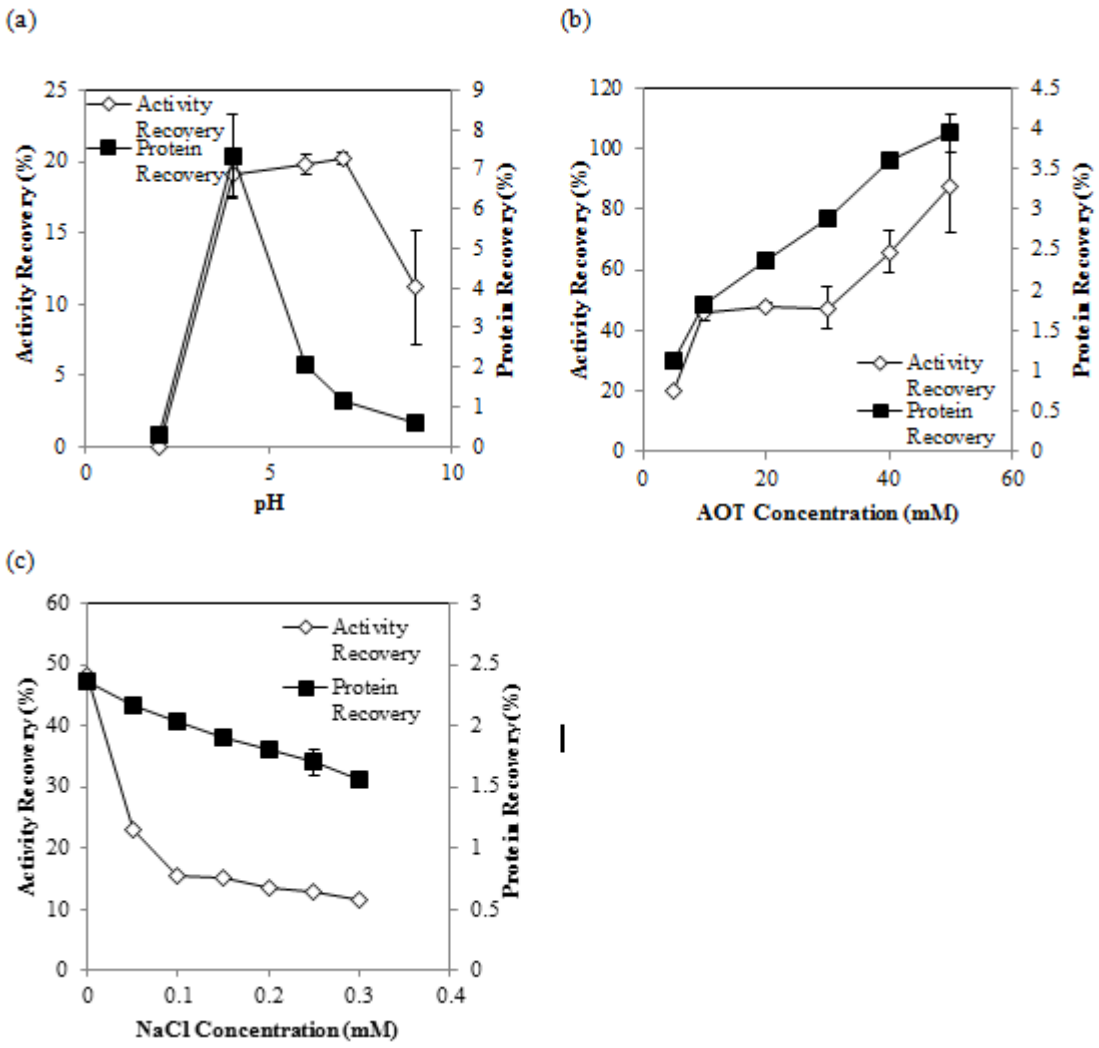


Figure 5.4 **Overall result of the preliminary reverse micellar extraction of the bacteriocin from *Pediococcus acidilactici* Kp10. Effect of several experimental parameters employed during forward extraction: (a) Effect of pH on extraction efficiency of bacteriocin; AOT concentration used was 5 mM, 0 mM NaCl concentration, (b) Effect of AOT concentration on extraction efficiency of bacteriocin; at pH 7, 0 mM NaCl concentration, (c) Effect of NaCl concentration on extraction efficiency of bacteriocin; pH 7, AOT concentration of 20 mM. Stripping solution used during backward extraction for all experiments was 20 mM phosphate buffer with 2 M KCl, pH 9 and the average protein concentration of crude protein was about 0.2 mg/mL.**

Hence, the proposed surfactant precipitation method was undoubtedly the more effective choice of separation techniques as compared to the above mentioned methods, an easier and cheaper alternative, not to mention that it is a well understood precipitation-based technique. Furthermore, the employment of a precipitation method (i.e. ammonium sulphate precipitation) in the initial stages of

the conventional purification scheme for bacteriocin was common, for concentration purposes, because normally many bacteriocins were not produced in high amounts by the producer strain (Pingitore, 2007; Sharma et al., 2011). From the work of the same researchers, only about 8 to 18.5% of activity recovery was achieved by that precipitation. It is predicted that it is very likely that the surfactant precipitation method can improve overall recovery, and also offers considerable potential to combine the initial concentration step with the primary capturing step.

5.3.6 CONCLUSION

The surfactant precipitation technique (using AOT) has been shown to be a very powerful and effective method to separate bacteriocin directly from its complex fermentation broth, resulted in an overall activity recovery of 86.3%, and a purification factor of about 53.8, as performed in just a single precipitation step with: 11.56 mM concentration of AOT, pH 4, precipitate recovery by acetone (0.99 mM NaCl) and a final recovery phase of 20 mM PBS, pH 7. It was also shown in this work that this separation performance was better than the results obtained by the other separation techniques (reverse micellar extraction, aqueous two-phase and possibly ammonium sulphate precipitation).

As expected, pH had a marked influence on this technique; it was found that a certain degree of ionization/charge density must be acquired by the bacteriocin molecule in order to interact electrostatically with AOT before any precipitation can take place. This was illustrated by precipitation only starting to occur well below the hypothetical pI (8.5 and above). On the other hand, if the net positive charge threshold cannot be reached, bacteriocin tended to interact with AOT through unfavourable hydrophobic interactions, most probably altering its conformation and eventually inactivated it. Moreover, the dependence of recovered bacteriocin purity on the pH of the initial fermentation broth was also highlighted in this work.

Finally, in terms of the effect of AOT concentration, the level of precipitation and therefore the activity recovery increased with increasing concentration until a particular optimum point (11.56 mM) was reached. Nevertheless, beyond this point the activity recovery reduced, presumably due to the extensive binding of more AOT molecules on the AOT-bacteriocin complex.

CHAPTER 6 CONCLUSION AND FUTURE WORK

6.1 INTRODUCTION

In this chapter, a summary of the research findings on a new alternative protein separation technique by surfactant precipitation was presented. The summary of the results obtained include: (1) Development of a surfactant precipitation method for low pI proteins based on cationic surfactants; (2) Feasibility of using a biocompatible surfactant - MES as a precipitating-ligand, and; (3) Application of the surfactant precipitation method to the purification of a target protein from a real complex fermentation broth sample. Recommendations for future work are presented at the end of this chapter.

6.2 SUMMARY OF THE RESULTS

6.2.1 DEVELOPMENT OF A SURFACTANT PRECIPITATION METHOD FOR LOW PI PROTEINS BASED ON CATIONIC SURFACTANTS

In order to develop a cationic surfactant (TOMAC)-based direct precipitation technique, the effect of experimental factors (molar ratio of surfactant to protein, and pH) on performance was studied. 3 model proteins: BSA, α-amylase and trypsin inhibitor, all low pI proteins varying in size, were used in the cationic surfactant (TOMAC)-based precipitation study. It was discovered that all of these proteins were effectively precipitated by TOMAC. R_p had a direct influence on the precipitation level, and under the range examined for these proteins, a similar precipitation trend was observed in the sense that the level of precipitation increased with a rise in R_p up to a maximum level, followed by a plateau stage. 100% precipitation efficiency was achieved at a stoichiometric R_p of 1 to 50, 25 and 7.7 (TOMAC:protein) respectively for BSA (pH 6.2), α-amylase (pH 6.0) and trypsin inhibitor (pH 7.6), and therefore was designated as the optimum R_p. In terms of the effect of pH, the amount of protein precipitated increased correspondingly with an increase in distance between the proteins' pI and the pH (towards the alkaline direction). Hence, it was shown that the electrostatic attraction between the cationic head group of TOMAC and the proteins' anionic groups were the driving force for the precipitation. It should be mentioned that α-amylase was unusual in this respect, the maximum

level of precipitation achieved at pH 6.0 dropped to about 89% and 92%, as the pH was increased to 7.6 and 9.19, respectively. By embracing the concept of binding isotherm shift with pH change and charge neutralisation in precipitation, it was predicted that this drop was a result of insufficient amounts of TOMAC monomer present to interact with the additional ionic binding sites.

The recoverability of the precipitated proteins from their TOMAC-protein insoluble complex was the crucial factor that eventually determines the viability of this separation technique. Two means of recovery; solvent solubilisation and counter-ionic surfactant dimerisation were examined in the recovery of these proteins from their precipitate. The first objective in this recovery section was **to evaluate the performance of different organic solvents in recovering the target protein from its surfactant-protein complex.** It was found that BSA was not recoverable by either method, indicating that BSA had denatured after aggregation due to the strong interactions between the surfactant monomer and BSA.

Beside the capability of solvent to extract the target protein from the precipitate, the outcome of solvent recovery also depended on the protein's stability and solubility in the particular solvent used. Obviously, any solvent that has a detrimental effect on enzyme activity and causes denaturation is not the right choice for recovery. It is also evident that the higher the solubility of a target protein in a particular solvent, the greater the amount of protein lost during the recovery step, and eventually the lower the final recovery percentage is, as observed in the experimental results for trypsin inhibitor recovery by ethanol where more than 80% of the protein was detected in the solvent phase. In the case of α-amylase, the recovery by organic phase (ethanol and acetone) was very poor overall, and even the addition of salt, i.e. NaCl, up to a concentration of 0.9 mM did not change that.

The next objective for the recovery study was to optimise the recovery of the target protein from its precipitate using the counter-ionic surfactant method. In contrast to solvent recovery, about 37% of α-amylase was recovered by using an anionic surfactant, AOT (at $R_r = 0.5$), proving that the poor recovery by solvent extraction was not due to the denatured state of α-amylase after precipitation, but was rather down to the solvent's properties. Moreover, the effectiveness of the counter-ionic surfactant recovery technique was further highlighted in the case of trypsin inhibitor, where 100% recovery was achieved at $R_r = 2.0$ (AOT to TOMAC). In conclusion, the counter-ionic surfactant recovery method offered the benefits of improved recovery, and possibly has less of a denaturing effect which can be especially useful for solvent sensitive proteins. However, one possible drawback for this type of recovery was the carry-over of surfactant at the end of the process. As a result, an additional separation unit may be required to remove the surfactant residue.

Not only is the final recovery yield important, it also matters that the structural integrity and the bioactivity of the recovered proteins is relatively intact. Therefore, **to examine the structural integrity of the protein recovered that was subjected to the surfactant precipitation method**, the CD determination and enzymes' activity (for α-amylase and trypsin inhibitor) of the final products were analysed. Based on the deconvolution of the CD spectra obtained, it was found that the secondary structure of these recovered proteins were not significantly different from their native structure. In addition, the recovered enzymes were fairly active with their substrate; RBB-starch for α-amylase, and trypsin for trypsin inhibitor, and no change in the specific activity for both enzymes was observed compared to their original native structure.

Finally, based on the experimental results obtained, it was clear that the cationic surfactant, TOMAC, has a more or less identical mechanism of protein precipitation to the anionic surfactant, AOT, as reported in literature.

One of the matters of great interest in this work was **to determine a universal and ubiquitous protein hydrophilicity/hydrophobicity parameter that can represent the overall efficiency of surfactant precipitation**, **and be used to predict its performance in precipitation in the future**. By combining the experimental results of previous researchers with ours, it was found that among the parameters examined, hydrophobicity (determination based on retention coefficients of amino acid residues by HPLC) and size (molecular weight) exhibited a good correlation with overall protein recovery, and consequently the efficacy of surfactant precipitation for a specific protein. In other words, although this technique might encounter difficulties when trying to directly precipitate and recover a target protein with a high hydrophobicity and molecular weight, it can still be employed indirectly by way of removing the impurities and leaving the target protein in the solution at the end.

6.2.2 FEASIBILITY OF USING A BIOCOMPATIBLE SURFACTANT - MES AS A PRECIPITATING-LIGAND.

The current focus in the detergent industry is evolving towards the use of biocompatible surfactants which have the advantage of being environmental friendly and offering better detergency performance. **Hence, the next objective was to study the feasibility of using a biosurfactant, MES, as a precipitating ligand for lysozyme with surfactant precipitation.** From the results, it was found that the amount of MES (studied as the molar ratio of MES to lysozyme) and pH have a strong influence on performance. The amount precipitated was directly related to R_p up until $R_p = 16$, when 100% precipitation was achieved, and the plateau stage was retained up to an $R_p = 38$. In terms of pH, the level of lysozyme precipitated reduced with increasing pH from pH 6.23 to 12 (where no

precipitation occurred), indicated that electrostatic interactions were the driving force behind precipitation. The optimum conditions for precipitation were chosen as R_p = 16 in 20 mM phosphate buffer, pH 6.2. Meanwhile, the precipitate was successfully recovered by either counter-ionic surfactant (83% at R_r = 1.5) or organic solvents (slightly less than 60%, but could possibly be improved with the addition of salt). In addition, most importantly, the activity and structural integrity of the protein recovered using both methods was maintained. Minimum carry-over of TOMAC was obtained under the counter-ionic recovery method employed (10.86%), not to mention the ease of removing the detergent completely by simply performing additional unit operation such as filtration right after the recovery step.

In the literature, research on the surfactant precipitation method has focused mainly on the use of the anionic surfactant, AOT, with lysozyme as the model protein. **Therefore, it was logical to compare the performance of MES to the conventional surfactant - AOT with a lysozyme precipitation.** The optimum R_p (complete precipitation of lysozyme) for MES was similar to AOT precipitation, i.e. 16. Meanwhile, in terms of the maximum recovery achieved, about 90% was recorded for MES with a counter-ionic surfactant, TOMAC, treatment at R_r = 2 (100% for AOT, at similar R). However, in comparison to AOT, MES exhibited minimum denaturing-causing hydrophobic interactions with the protein, particularly as evidenced during the precipitation stage at higher R_ps, and when favourable electrostatic interactions did not exist (pH was above the pI of lysozyme). As a result, the reduction in the percentage precipitated at higher R_ps (as encountered in AOT study at R>23), and unwanted destructive hydrophobic binding at pH 12 were not observed for MES. With these observations, taking into account that ionic interactions are more dominant than hydrophobic interactions for MES, it is clear that MES is a more selective precipitating ligand than AOT.

As a conclusion, in conjunction with its environment-friendly features, the appeal of better economic value (due to fact that it is locally produced in Malaysia), and a comparable performance to AOT, the use of MES as a precipitating-ligand is another step towards improving this precipitation technique.

6.2.3 APPLICATION OF THE SURFACTANT PRECIPITATION METHOD TO THE PURIFICATION OF A TARGET PROTEIN FROM A REAL COMPLEX FERMENTATION BROTH

Since the potential and functionality of the surfactant precipitation method has been demonstrated early on in this work for a single protein in buffer, there is obviously a need to test this method in a

real complex sample i.e. fermentation broth. **With this in mind, the next objective was to develop a separation method based on the surfactant precipitation technique for bacteriocin from lactic acid bacteria, *Pediococcus acidilactici* Kp10 using AOT.**

The bacteriocin was successfully purified by about 53.8 fold with 86.3% activity recovery under the following optimum conditions: 11.56 mM concentration of AOT, initial broth pH of 4, precipitate recovery by acetone (0.99 mM NaCl) and a final recovery phase of 20 mM PBS, pH 7. It was found that acquiring a certain degree of positive ionization for the bacteriocin molecule was the prerequisite for precipitation to occur using anionic AOT. This was illustrated by the experimental result where bacteriocin only began to precipitate at pH 4, although it has an estimated pI of 8 to 9. On the other hand, at a pH>4 it was postulated that bacteriocin interacted with AOT through unfavourable hydrophobic interactions which eventually inactivated it, and this was based on the activity analysis of the non-precipitating bacteriocin. Thus, it was clear that pH had a marked influence on the precipitation process.

In addition, activity removal during the precipitation stage and the concentration of AOT had a near direct relationship with their increment up to a concentration of 11.56 mM, where complete removal was achieved. In terms of its effect on overall activity recovery, a decreasing effect was observed beyond the optimum concentration. The activity recovery gradually decreased from 86.3% at 11.56 mM, to about 50.1% at 43.2 mM. This can be explained by the extensive binding of the AOT to the neutral AOT-bacteriocin, which seems to hinder the release of bacteriocin into solution, and thus the recovery.

The technique of surfactant precipitation evolved out of another surfactant-based method, reverse micellar extraction. The newer method mainly has the edge over the other in terms of the concentration of surfactant employed, which can be at least 1 order of magnitude smaller. **In order to have a bigger picture, the last objective of this work was to compare the performance of the surfactant precipitation method to another surfactant based separation technique, reverse micellar extraction.** Under the determined conditions (AOT concentration of 20 mM, pH 7, no NaCl addition), only about 48% activity recovery was achieved with an estimated purification factor of 20. Based on this result, the partitioning of the bacteriocin into the micelles clearly occurred through a different mechanism (pH 7 rather than at pH 4 as in precipitation method), and it was suggested that a combination of electrostatic and hydrophobic interactions was involved. An attempt to improve the result through an increase in AOT concentration was unsuccessful as it was found out that bacteriocin was sensitive to the amount of AOT used. Although a higher recovery was achieved at a concentration of 30 mM and above, the stability of the recovered bacteriocin was greatly reduced as indicated by the agar well diffusion assay. Considering that weak electrostatic interactions were present at pH 7 (as it was determined earlier that no precipitation occurred at this pH), and the fact that bacteriocin was a

similar amphiphilic molecule like the surfactant, it was postulated that a higher degree of hydrophobic interaction between AOT and the bacteriocin peptide might have contributed to this result.

From these observations, it was concluded that the proposed surfactant precipitation technique was a more effective means of recovery of the protein. It should be mentioned that even though the extent of the purification work done was not comprehensive, and there is still room for improvement (e.g. optimisation on the recovery efficiency by solvent and counter-ionic surfactant recovery), it was nonetheless a ground-breaking effort on the real application of the surfactant precipitation technique to the purification of a target protein from fermentation broth.

In summary, the technique of protein separation by surfactant precipitation epitomises what a desirable alternative bioseparation technique should be – simple, cheap as it is locally produced (MES), environmentally friendly (in the example of MES), and most importantly, an effective mainstream purification method.

6.3 FUTURE WORK:

1. In order to simplify the overall process, the bacteriocin precipitate was recovered only by treatment with acetone followed by dissolution of the final precipitate with a fresh buffer. Hence, a lot of room is still available for further study in terms of the optimisation on the recovery efficiency. **Further work can be continued to complete the bacteriocin purification work**, for instance by looking into the type of solvent and range of NaCl concentrations employed, recovery through the means of a counter-ionic surfactant, the possibility of performing an additional cycle of precipitation/recovery, and lastly by addressing the issue of surfactant carry-over which might be encountered.
2. The **application of MES as a precipitant** for a target protein from a real fermentation broth was examined after it was successfully applied to a single protein model system. In a complex sample, the degree of recovery in terms of overall yield and process selectivity in the presence of impurities such as the medium constituents and other hydrophobic proteins expressed by the host cell could pose real problems for this precipitation technique. If necessary, the extent of this work could also be extended to include an additional unit operation in the overall purification scheme to address the issue of surfactant carry-over.
3. There were claims in the literature that the shorter the alkyl chain of the surfactant, the lower the denaturing effect or structural changes during its interaction with a protein. Based on this hypothesis, it might be interesting to examine this situation with a different surfactant. For example, **DTAB (dodecyl trimethyl ammonium bromide), another type of cationic**

surfactant with a stronger charge density and shorter non-polar chain, may possibly improve this technique.

4. **The concept of adding a co-precipitant** (e.g. PEG, titanium oxide) to the system with an aim to shielding the non-polar group of the surfactant i.e. known to be unfolding-causing, and strengthen the ionic force of the surfactant head group should be examined.
5. Up to this point, the structural integrity of the final product (i.e. separated protein) was examined based on secondary structure assessment using circular dichroism. However, it was reported that the protein might undergo **tertiary structure changes** after interacting with surfactant while maintaining their secondary structure. Hence, this research area could be looked into, for example through the application of NMR spectroscopy.
6. Important **characteristics of protein precipitates** are the particle size distribution, density and mechanical strength. It was highlighted in the literature that protein precipitates that are small in size and low in density can lead to filtration or centrifugation problems, and can result in excessive bulk volumes of the final dried precipitate. Thus, a study should be carried out to investigate the significance of protein precipitate characteristics, and the influence of operating conditions on this characteristic.
7. As far as the application of this technique is concerned, it uses need to be expanded to **other proteins**, to establish the viability of this separation technique.

LIST OF REFERENCES

1) Abbasiliasi, S., Tan, J. S, Ibrahim, T. A. T., Ramanan, R. N., Vakkhshiteh, F., Mustafa, S., Ling, T.C., Rahim, R. A. and Ariff. A. B. 2012. Isolation of *Pediococcus acidilactici* Kp10 with ability to secrete bacteriocin-like inhibitory substance from milk products for applications in food industry. BMC Microbiology 12: 260.
2) Abbasiliasi, S., Tan, J. S., Ibrahim, T. A. T., Kadkhodaei, S., Ng, H. S., Vakhshiteh, F., Ajdari, Z., Mustafa, S., Ling, T. C., Rahim, R. A. and Ariff, A. B. 2014. Primary recovery of a bacteriocin-like inhibitory substance derived from *Pediococcus acidilactici* Kp10 by an aqueous two-phase system. Food Chemistry 151: 93-100.
3) Ahern, M., Verschueren, S., van Sinderen, D., 2003. Isolation and characterization of a novel bacteriocin produced by *Bacillus thuringiensis* strain B439, FEMS Microbiol. Lett. 220, 127-131.
4) Aires-Barros, M. R. and Cabral, J. M. S. 1991. Selective separation and purification of two lipases from *chromobacterium viscosum* using AOT reversed micelles. Biotechnology and Bioengineering 38, 1302-1307.
5) Andersen, K. K., Oliveira, C. L. P., Larsen, K. L., Poulsen, F. M., Callisen, T. H., Westh, P., Pedersen, J. S. Otzen, D. E. 2009. The role of decorated SDS micelles in sub-cmc protein denaturation and association. J. Mol. Biol. 391: 207–226.
6) Barlow, D. J. and Thornton, J. M. 1986. The distribution of charged groups in proteins. Biopolymers 25, 1717-1733.
7) Bigelow, C. C. and Channon, M. 1976 in Handbook of Biochemistry and Molecular Biology, Proteins (Fasman, G. D., Ed.) Vol. I, pp 209-243, CRC Press, Cleveland, OH.
8) Blinkhorn, C., and Jones, M. N. 1973. Effect of surfactant structure on ribonuclease - surfactant interactions. Biochemical Journal 135:547-549.
9) Bohm, G. 1997. CDNN: CD spectra deconvolution software version 2.1. University of Halle-Hettenberg: Halle
10) Boychyn, M., Doyle, W., Bulmer, M., More, J. and Hoare, M. 2000. Laboratory Scaledown of Protein Purification Processes Involving Fractional Precipitation and Centrifugal Recovery. Biotechnology and Bioengineering 69 (1): 1-10.
11) Canfield, R. E. and Liu, A. K. 1965. The disulfide bonds of egg white lysozyme (muramidase). J. Biol. Chem. 240, 2000.
12) Carolissen-Mackay, V., Arendse, G. and Hastings, J.W. 1997. Purification of bacteriocins of lactic acid bacteria: problems and pointers. International Journal of Food Microbiology 34 (1): 1–16.

13) Chang, Q-L., Chen, J-Y., Zhang, X-F. and Zhao, N-M. 1997. Effect of the Cosolvent Type on the Extraction of α-Amylase with Reversed Micelles: Circular Dichrosim Study. Enzyme and Microbial Technology 20: 87-92.
14) Chemithon Corporation. 2008?. Methyl ester sulphonate the new generation surfactant. Retrieved from http://www.chemithon.com/Resources/pdfs/MES%20-%20Next%Generation%20Surfactant.pdf. 13 March 2015.
15) Chen, G.C. and Yang, J.T. 1977. Two-point calibration of circular dichrometer with d-10-camphorsulfonic acid. Anal. Lett. 10:1195-1207.
16) Cheng, S.I and Stuckey, D.C. 2012. Protein precipitation using anionic surfactant. Process Biochemistry 47: 712-719
17) Cheng, S.I. and Stuckey, D. 2011b. Protein Separation Using Surfactant Precipitation [PhD Thesis]. Imperial College London. 75-183. Available from Imperial College London Library.
18) Cheng, S.I. and Stuckey, D.C. 2011a. Protein Recovery from Surfactant Precipitation. Biotechnology Progress 27(6): 1614-22.
19) Chertov, O., Biragyn, A., Kwak, L. W., Simpson, J. T., Boronina, T., Hoang, V. M. Prieto, D. A., Conrads, T. P., Veenstra, T. D. and Fisher, R. J. 2004. Organic solvent extraction of proteins and peptides from serum as an effective sample preparation for detection and identification of biomarkers by mass spectrometry. Proteomics 4(4): 1195-1203.
20) Chou, S-T. and Chiang, B.H. 1998. Reversed micellar extraction of hen egg lysozyme. Journal of Food Science 63 (3): 399-402.
21) F, A. M. J., Wall, M. J. and Doucette, A. A. 2013. Maximizing recovery of water-soluble proteins through acetone precipitation. Analytica Chimica Acta 796: 48-54.
22) Darby, N. J. and Creighton, T. E. 1993. Protein structure. Oxford University Press.
23) Davies, R.C., A. Neuberger, and B.M. Wilson. 1969. The dependence of lysozyme activity on pH and ionic strength. Biochimica et Biophysica Acta (BBA) – Enzymology 178:294-305.
24) Fan, K., Ouyang, P., Wu, X. and Lu, Z. 2001. Enhancement of the activity of papain in mixed reverse micellar systems in the presence of Tween 80. Journal of Chemical Technology & Biotechnology 76(1), 27-34.
25) Fleming, A. 1932. Proceedings of the Royal Society of Medicine 26 (1), 71-84.
26) Frattali, V. 1969. Soybean Inhibitors III. Properties of a Low Molecular Weight Soybean Proteinase Inhibitor. The Journal of Biological Chemistry 244 (2): 274-280.
27) Fresta, M., Guccione, S., Beccari, A. R., Furneri, P. M. and Puglisi, G. 2002. Combining molecular modelling with experimental methodologies: mechanism of membrane

permeation and accumulation of ofloxacin. Bioorganic & Medicinal Chemistry 10: 3871-3889.

28) Fuda, E., P. Jauregi, and D.L. Pyle. 2004. Recovery of lactoferrin and lactoperoxidase from sweet whey using colloidal gas aphrons (CGAs) generated from an anionic surfactant, AOT. Biotechnol Prog 20:514-525.

29) Gekko, K. and Hasegawa, Y. 1986. Compressibility-structure relationship of globular proteins. Biochemistry 25: 6563-6571.

30) George, D. A. and D. C. Stuckey. 2010. Extraction of monoclonal antibodies (IgG1) using anionic and anionic/nonionic reverse micelles. Biotechnology Progress 26: 1352-1360.

31) Goto, M., Ishikawa, Y., Ono, T., Nakashio, F. and Hatton, A. 1998. Extraction and activity of chymotrypsin using AOT-DOLPA mixed reversed micellar systems. Biotechnology Progress 14(5): 729-734.

32) Greenfield, N. 1996. Methods to estimate the conformation of proteins and polypeptides from circular dichroism data. Analytical Biochemistry 235:1-10.

33) Günther S.L. and Stuckey, D.C. 2011. Extraction of IgG4 Fab Fragments Using HDEHP-Isooctane and -Corn Oil Reverse Micelles. Separation Science and Technology 46 (5) 708-719.

34) Guo, D., Mant, C. T., Taneja, A. K., Parker, J. M. R. and Hodges, R. S. 1986. Prediction of peptide retention time in reversed-phase high performance liquid chromatography. I. Determination of retention coefficients of amino acid residues of model synthetic peptides. J. Chromatogr. 359: 499-517.

35) Guzman, R. Z., Kilpatrick, P. K. and Carbonell, R. G. 1990. Affinity precipitation of avidin by using ligand-modified surfactants. In Chapter 11, ACS Symposium Series 419. Downstream processing and bioseparation. Recovery and purification of biological products. Ed: Hamel, J-F. P., Hunter, J. B. and Sikdar, S. K. p212-236.

36) Hayes, D. G. 2009. Biobased Surfactants, Overview and Industrial State-of-the-Art, in Biobased Surfactants and Detergents: Synthesis, Properties, and Applications, edited by Hayes, D. G., Kitamoto, D., Solaiman, D.K.Y. and Ashby, R.D. Champaign, IL, AOCS Press,, pp. 3-25.

37) Hilbrig, F. and Freitag, R. 2003. Protein purification by affinity precipitation. Journal of Chromatography B 790 (1-2): 79-90.

38) Hönig, W. and Kula, M-R. 1976. Selectivity of protein precipitation with polyethylene glycol fractions of various molecular weights. Analytical Biochemistry 72(1-2): 502-512.

39) Horn, D. and Heuck, C. C. 1983. Charge determination of proteins with polyelectrolyte titration. The Journal of Biological Chemistry. 254 (3): 1665-1670.

40) Houen, G. 1996. The solubility of proteins in organic solvents. Acta Chemica Scandinavica 50: 68-70.

41) Jarudilokkul, S., Poppenborg L.H., and Stuckey, D.C. 1999. Backward extraction of reverse micellar encapsulated proteins using a counterionic surfactant. Biotechnology and Bioengineering 62:593-601.

42) Jauregi, P. and Varley, J. 1998. Colloidal gas aphrons: a novel approach to protein recovery. Biotechnology and Bioengineering 59: 471– 481.

43) Jones, M. N. 1975. A theoretical approach to the binding of amphipathic molecules to globular proteins. J. Biochem. 151: 109-114.

44) Jones, M .N. 1996. Protein–surfactant interactions, in: S. Magdassi (Ed.), Surface activity of proteins, Marcel Dekker, New York, pp. 237–284.

45) Jonsson, B. 1999. Surfactants and polymers in aqueous solution. John Wiley & Sons Ltd.

46) Kato, A., Matsuda, T., Matsudomi, N., and Kobayashi, K. 1984. Determination of Protein Hydrophobicity Using a Sodium Dodecyl Sulfate Binding Method. J. Agrlc. Food Chem 32 (2): 284-288

47) Kato, Y., Nakamura, K., Kitamura, T., Moriyama, H., Hasegawa, M. and Sasaki, H. 2002. Separation of proteins by hydrophobic interaction chromatography at low salt concentration. Journal of Chromatography A 971:143-149.

48) Kelly, N. A., Reuben, B. G., Rhoades, J. and Roller, S. J. 2000. Solvent extraction of bacteriocins from model solutions and fermentation broths. J. Chem. Technol. Biotechnol. 75:777-784.

49) Kelly, S. M., Jess, T. J. and Price, N. C. 2005.How to study proteins by circular dichroism. Biochimica et Biophysica Acta 1751: 119 – 139.

50) Kopaciewicz, W., Rounds, M. A. Fausnaugh, J. and Regnier, F. E. 1983. Retention model for high-performance ion-exchange chromatography. Journal of Chromatography 266: 3-21.

51) Kyte, J. and Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157: 105-132.

52) Lamb, S.B. and Stuckey, D.C. 1999. Enzyme immobilisation on colloidal liquid aphrons (CLAs): the influence of protein properties. Enzyme and Microbial Technology 24:541-548.

53) Langenhof, M., Leong, S. S. J., Pattenden, L. K. and Middelberg, A. P. J. 2005. Controlled oxidative protein refolding using an ion-exchange column. Journal of Chromatography A, 1069: 195–201.

54) Lappe, R., Sant'Anna, V. and Brandelli, A. 2012. Extraction of the antimicrobial peptide cerein 8A by aqueous two-phase systems and aqueous two-phase micellar systems. Natural Product Research 26 (23): 2259-2265.

55) Laskowski, M., and M. Laskowski, Jr. 1954. Naturally occurring trypsin inhibitors. Advances in protein chemistry 9:203-242.

56) Lienqueo, M. E., Mahn, A. and Asenjo, J. A. 2002. Mathematical correlations for predicting protein retention times in hydrophobic interaction chromatography. Journal of Chromatography A 978: 71–79.

57) Linfield, W.M. 1976. Sulfocarboxylic acid derivatives. Anionic surfactants Part II New York: Marcel Dekker.

58) Lion Eco Chemicals Sdn. Bhd. Mizulan. (n.d.). Methyl ester sulphonate (MES) for laundry detergent [Brochure]. Pasir Gudang, Johor, Malaysia: Author.

59) Lye, G. J., Asenjo J.A. and Pyle D. L. 1995. Extraction of lysozyme and ribonuclease A using reverse micelles: limits to protein solubilization. Biotechnology and Bioengineering 47: 509–519.

60) Mackie, A., and P. Wilde. 2005. The role of interactions in defining the structure of mixed

61) Mant, C. T., Zhou, N. E., Hodges, R. S. 1989. Correlation of protein retention times in reversed-phase chromatography with polypeptide chain length and hydrophobicity. Journal of Chromatography A 476: 363–375.

62) Martin-Visscher, L. A., Belkum, M. J. V. Garneau-Tsodikova, S., Whittal, R. M., Zheng, J., McMullen, L. M. and Vederas, J. C. 2008. Isolation and characterization of carnocyclin A, a novel circular bacteriocin produced by *carnobacterium maltaromaticum* UAL307. Applied and environmental microbiology 74 (15): 4756–4763.

63) Masuda, M., Odake, H., Miura, K. and Oba, K. 1993. Biodegradation of 2-Sulfonatofatty Acid Methyl Ester (α-SFMe). Journal of Japanese Oil Chemists' Society 42 (9), 643.

64) Masuda, Y., Ono, H., Kitagawa, H., Ito, H., Mu, F., Sawa, N., Zendo, T. and Sonomoto, K. 2011. Identification and characterization of leucocyclicin Q, a novel cyclic bacteriocin produced by *leuconostoc mesenteroides* TK41401. Applied and environmental microbiology 77(22): 8164–8170.

65) Mathew, D.S. and Juang, R-S. 2005. Improved back extraction of papain from AOT reverse micelles using alcohols and a counter-ionic surfactant. Biochemical Engineering Journal 25: 219–225.

66) McMurry, J. 1992. In Organic Chemistry, 3rd Ed. California: Brooks/Cole Publishing. 1042-1045.

67) Morén, A. K. and Khan. A. 1998. Surfactant Hydrophobic Effect on the Phase Behavior of Oppositely Charged Protein and Surfactant Mixtures: Lysozyme and Sodium Alkyl Sulfates. Langmuir 14, 6818-6826.

68) Muga, A., Arrondo, J. L. R., Bellon, T., Sancho, J. and Bernabeu, C. 1993. Structural and functional studies on the interaction of sodium dodecyl sulphate with galactosidase. Arch. Biochem. Biophys. 300, 451-457.

69) Myers, D. 1992. A brief history of surfactant science and technology, surfactant science and technology: an overview surfactant science and technology (2nd Ed.). VCH Publishers Inc. 3.

70) Naoe, K., Ura, O., Hattori, M., Kawagoe, M. and Imai, M. 1998. Protein extraction using non-ionic reverse micelles of Span 60. Biochemical Engineering Journal 2: 113-119.

71) Nikas, Y. J., Liu, C. L. Srivastava, T., Abbott, N. L. and Blankschtein, D. 1992. Protein partitioning in 2-phase aqueous non-ionic micellar solutions. Macromolecules 25: 4797-4806.

72) Nozaki, Y. and Tanford, C. 1971. The Solubility of Amino Acids and Two Glycine Peptides in Aqueous Ethanol and Dioxane Solutions. Establishment of a hydrophobicity scale. J. Biol. Chem. 246 (7), 2211-2217.

73) Nozaki, Y., Reynolds, J. A. and Tanford, C. 1974. The interaction of a cationic detergent with bovine serum albumin and other proteins. J. Biol. Chem. 249 (14): 4452-4459.

74) Otzen, D. 2011. Protein-surfactant interactions: a tale of many states. Biochimica et Biophysica Acta 1814: 562–591.

75) Otzen, D. E. 2002. Protein unfolding in detergents: effect of micelle structure, ionic strength, pH, and temperature. Biophys. J. 83: 2219–2230.

76) Otzen, D. E. and Oliveberg, M. 2002b. Burst-phase expansion of native protein prior to global unfolding in SDS. J. Mol. Biol. 315: 1231–1240.

77) Petsko, G. A. and Ringe, D. 2004. Protein structure and function. Primers in biology. New Science Press Ltd.

78) Pi, Y-Y., Shang, Y-Z., Peng, C-J., Liu, H-L., Hu, Y. and Jiang J-W. 2006. Interactions between Bovine Serum Albumin and Gemini Surfactant Alkanediyl-α,ω-Bis(Dimethyldodecyl-Ammonium Bromide) Biopolymers 83, 243–249.

79) Pingitore, E. V., Salvucci, E., Sesma, F. and Nader-Macía M. E. 2007. Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB), In: Communicating Current Research and Educational Topics and Trends in Applied Microbiology. Mendez-Vilas, A. [Editor]. FORMATEX Research Center, Badajoz, Spain. P557-568.

80) Pires, M. J., Aires-Barros, M. R. and Cabral, J. M. S. 1996. Liquid-liquid extraction of proteins with reversed micelles. Biotechnology Progress 12: 290-301.

81) Porter, M. R. 1994. Handbook of Surfactants. 2nd Edition. Blackie Academic and Professional, London. pp. 11.

82) Price, S. E., Jappar, D., Lorenzo, P., Saavedra, J. E., Hrabie, J. A. and Davies, K. M. 2003. Micellar catalysis of nitric oxide dissociation from diazeniumdiolates. Langmuir 19: 2096-2102.

83) Randolph, T. W. and Jones, L. S. 2002. Surfactant–protein interactions, in: J.F. Carpenter, M. Manning (Eds.), Rational design of stable protein formulations, Kluwer Academic/Plenum Publishers, New York, pp. 159–175.

84) Rangel-Yagui, C. O., Pessoa, A. and Tavares, L. C. 2005. Micellar solubilization of drugs. Journal of Pharmacy and Pharmaceutical Sciences 8: 147-163.

85) Reynolds, J. A. and Tanford, C. 1970. The gross conformation of protein–sodium dodecyl sulfate complexes. J. Biol. Chem. 245: 5161–5165.

86) Rinderknecht, H., Wilding, P. and Haverback, B.J. 1967. A new method for the determination of alpha-amylase. Experientia 23(10): 805.

87) Ryoo, W., Webber, S. E. and Johnston, K. P. 2003. Water-in-Carbon Dioxide Microemulsions with Methylated Branched Hydrocarbon Surfactants. Ind. Eng. Chem. Res. 42, 6348-6358

88) Sadama, A., and Beelaram, A. M. 1995. Efficiency and economics of bioseparation - some case-studies. Abstracts of Papers of the American Chemical Society 209:56-60.

89) Saint-Hubert, C., Durieux, A., Bodo, E. and Simon, J. P. 2009. Large scale purification protocol for carnocin KZ 213 from Carnobacterium piscicola. Biotechnol Lett: 31:519–523.

90) Salgado, J. C., Rapaport, I. and Asenjo, J. A. 2006. Predicting the behaviour of proteins in hydrophobic interaction chromatography 1: using the hydrophobic imbalance (HI) to describe their surface amino acid distribution. Journal of Chromatography A 1107: 110–119.

91) Satsuki, T. 1992. Applications of MES in detergents. INFORM 3 (10): 1099-1109.

92) Satsuki, T., Umehara, K. and Yoneyama, Y. 1992. Performance and Physicochemical Properties of α-Sulfo Fatty Acid Methyl Esters. Journal of American Oil Chemists' Society 69 (7), 672.

93) Scopes, R. 1994. Protein Purification: Principles and Practice. 3rd Ed. Springer p76-93.

94) Sharma, N., Kapoor, R., Gautam, N. and Kumari, R. 2011. Purification and characterization of bacteriocin produced by B*acillus subtilis* R75 isolated from fermented chunks of mung bean (*Phaseolus radiatus*). Food Technol. Biotechnol. 49 (2): 169–176.

95) Shen, R. 2005. Extraction of industrial lipase with CTAB based mixed reverse micelles. Guocheng Congcheng Xuebao 5(3): 255-259.

96) Shih, Y-C., John, M. Prausnitz, J. M. and Blanch, H. W. 1992. Some characteristics of protein precipitation by salts. Biotechnology and Bioengineering 40(10): 1155-1164

97) Shin, Y-O, Wahnon, D., Weber, M. E. and Vera, J. H. 2004. Selective precipitation and recovery of xylanase using surfactant and organic solvent. Biotechnology and Bioengineering 86 (6): 698-705.

98) Shin, Y-O, Weber, M. E. and Vera, J. H. 2003. Reverse micellar extraction and precipitation of lysozyme using sodium di(2-ethylhexyl) sulfosuccinate. Biotechnology Progress 19: 928-935.

99) Shin, Y-O. 2002. Extraction of lysozyme using surfactants [PhD Thesis]. McGill Univ., Montreal, Quebec. p 129-57. Available from National Library of Canada, Ottawa.

100) Shin, Y-O., Rodil, E. and Vera, J. H. 2003b. Selective precipitation of lysozyme from egg white using AOT. Food Engineering and Physical Properties 68 (2): 595-599.

101) Shin, Y-O., Rodil, E. and Vera, J. H. 2004. Precipitation and recovery of cytochrome c and hemoglobin using AOT and acetone. Separation Science and Technology 39: 1005-1019.

102) Shiomori, K., Ebuchi, N., Kawano, Y., Kuboi, R. and Komasawa, I. 1998. Extraction characteristic of bovine serum albumin using sodium bis(2-ethylhexyl) sulfosuccinate reverse micelles. Journal of Fermentation and Bioengineering 86 (6): 581-587.

103) Shugar, D. 1952. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. Biochimica et Biophysica Acta 8, 302-309.

104) Somasundaran, P. 2006. Encyclopedia of Surface and Colloid Science, Volume 2. CRC Press, Boca Raton. p6254.

105) Stadelman, W.J., and J.C. Owen. 1995. Egg Science and Technology. 4th Edition.Haworth Press, New York. pp 126.

106) Stein, E. A., Junge, J. M. and Fischer, E. H. 1960. The amino acid composition of α-amylase from Aspergillus oryzae. Journal of Biological Chemistry 235 (2), 1160, 371-378.

107) Stenstam, A. and Khan, A. and H. Wennerström. 2001. The Lysozyme-Dodecyl Sulfate System. An Example of Protein-Surfactant Aggregation. Langmuir 17, 7513-7520.

108) Stenstam, A., Topgaard, D. and Wennerström, H. 2003. Aggregation in a Protein-Surfactant System. The interplay between hydrophobic and electrostatic interactions. J. Phys. Chem. B 107: 7987-7992.

109) Takagi, T., K. Tsujii, and K. Shirahama. 1975. Binding isotherms of sodium dodecyl-sulfate to protein polypeptides with special reference to SDS-polyacylamide gelelectrophoresis. Journal of Biochemistry 77:939-947.

110) Takeda, K., Shigeta, M., and Aoki, K. 1987. Secondary structures of bovine serum albumin in anionic and cationic surfactant solutions. J. Colloid Interface Sci. 117(1): 120–126.

111) Turro, N.J., X.G. Lei, K.P. Ananthapadmanabhan, and M. Aronson. 1995. Spectroscopic probe analysis of protein-surfactant interactions - the BSA/SDS system. Langmuir 11:2525-2533.

112) Vaidya, A. A., Lele, B. S., Deshmukh, M. V. and Kulkarni, M. G. 2001. Design and evaluation of new ligands for lysozyme recovery by affinity thermoprecipitation. Chemical Engineering Science 56:5681-5692.

113) Valstar, A. 2000. Protein-Surfactant Interactions. Acta Universitatis Upsaliensis. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology 563. 50 pp. Uppsala. ISBN 91-554-4795-3

114) Valstar, A., Almgren, M. and Brown, W. 2000. The Interaction of Bovine Serum Albumin with Surfactants Studied by Light Scattering. Langmuir 2000, 16, 922-927

115) Vasudevan, M. and Wiencek, J. M. 1996. Mechanism of the extraction of proteins into Tween 85 nonionic microemulsions. Industrial and Engineering Chemistry Research 35: 1085-1089.

116) Wang, L and Langley, D. 1975. Determining Cationic Surfactant Concentration Ind. Eng. Chem. Prod. Res. Dev. 14 (3), 210–212.

117) Ward, K. and Stuckey, D. 2011. Protein precipitation and recovery using cationic and non-ionic surfactants [Master Thesis]. Imperial College London, London.

118) Wenk, M.R. and Fernandis, A.Z. 2007. Manuals in biomedical research: a manual for biochemistry protocols Vol. 3, World Scientific Publishing Co. Pte. Ltd., Singapore, Pp 2-7.

119) Yano, K., Hasegawa, T., Kuboi, R., Komasawa, I. and Tsuchido, T. 1994. Characterization of surface properties of heat shock proteins for the separation using aqueous two-phase systems. Journal of Chemical Engineering of Japan 27: 808-814.

120) Yao, P. and Gao, G. 2008. Structure and activity transition of lysozyme on interacting with and releasing from polyelectrolyte with different hydrophobicity. Journal of Polymer Science Part A, Polymer chemistry 46:4681-4690.

121) Zendo, T., Eungruttanagorn, N., Fujioka, S., Tashiro, Y., Nomura, K., Sera, Y., Kobayashi, G., Nakayama, J., Ishizaki, A. and Sonomoto, K. 2005. Identification and production of a bacteriocin from *enterococcus mundtii* QU 2 isolated from soybean. Journal of Applied Microbiology 99 (5): 1181-1190.