Protein Refolding *via* Immobilisation on Crystal Surfaces

UNIVERSITY
of
GLASGOW

Katrina Ann Davidson

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Abstract

Is it possible to find an easy, generic method for protein refolding? The preparation of functionally active protein molecules from the unfolded state can be a difficult task. Although there are many well-established techniques for protein refolding, such as dilution, dialysis, chromatography and others, in many instances these methods can be time consuming and inefficient. A rapid, inexpensive and simple method for protein folding is a much sought after technique.

Proteins in the unfolded state (either inclusion bodies or unfolded by chemical or physical means) are generally solubilised in solutions containing urea or guanidine hydrochloride. The removal of these molecules from the protein environment is commonly utilised as a method for triggering refolding.

A new method for the refolding of biomolecular species has been developed *via* the formation of Protein Coated Micro-crystals (PCMC). The formation of PCMC is a recently developed method for the immobilisation protein upon the surface of a water-soluble excipient (salt, amino acid or sugar) *via* a co-precipitation reaction in a water miscible organic solvent. These proteins can then be used as immobilised biocatalysts in both the aqueous and organic phase.

In the immobilisation of unfolded, solubilised protein, the solubilising agents (e.g. urea or guanidine hydrochloride) are removed from the protein environment as they are soluble in the organic phase. The removal of these molecules initiates protein folding during the co-precipitation process.

In the course of this project, a number of proteins were studied in order to observe their behaviour in this immobilisation and simultaneous folding process. Lysozyme was utilised as it is an enzyme which is relatively simple to refold from the chemically unfolded state by conventional methods such as dilution. Upon immobilisation of lysozyme from the chemically unfolded state, up to 92% of the activity of the native protein was regained. The enzyme lipase, which is notoriously difficult to fold, was also used to determine the efficiency of this method under more challenging conditions. Lipase immobilised from the chemically unfolded state was seen to regain up to 36 % of the activity of the native protein.

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Definitions

Protein Coated Micro-Crystals (PCMC) - Protein immobilised upon the surface of a water soluble carrier material (excipient) via a co-precipitation reaction in organic solvent.

Excipient - An inactive water soluble material used as a carrier in the formation of PCMC

Measured Solids Recovery (%) - A measure of the percentage of the solids (protein and carrier) precipitated into organic solvent which is retrieved at the end of the process.

Theoretical Protein Loading (TPL) (%) - The ratio of protein to the total mass of solids used in the precipitation process (e.g. 6 mg of protein upon 54 mg carrier material would have a % TPL of (6/(54+6)) x 100 % = 10 %).

Measure Protein Loading (MPL) (%) - The actual protein loading based upon the measured protein concentration of the PCMC upon reconstitution (e.g. if 10 mg of reconstituted PCMC (in 1 ml) have a protein concentration of 0.99 mg/ml, then the % MPL of the samples is (0.99/10) x 100 % = 9.9 %).

Specific Activity of Enzyme - A measure of the change in absorbance of a substrate solution with respect to time per milligram of enzyme present per millilitre of solution (i.e. dA min^{-1} mg^{-1} ml^{-1}) during an activity assay.

% Activity of Native Protein - A comparison of the activity of the treated samples with the native protein. Calculated by dividing the specific activity of the treated protein by that of the native form.

Chapter One - Introduction

1.1 Proteins

Proteins are the very fabric of life. The number of different kinds of proteins in the human body approaches 100,000 and these molecules are involved in various processes upon which our lives depend.[1] In fact, every molecule in a living organism is essentially either a protein or formed as a result of the action of proteins.[2] Proteins are extremely versatile molecules which are involved in a number of different biochemical processes. These include (but are not limited to) the control of gene expression, catalysis, immune protection, mechanical support, transportation, growth initiation and nerve impulses.[3, 4]

Proteins are of great scientific interest because they carry out important functions. However, they are also structurally interesting. One of the great mysteries of modern biochemistry is the ability of these biomolecules to fold spontaneously to form a compact three-dimensional structure with all of the intricate detail required for functionality [5]

1.1.1 Proteins: From Amino Acid Sequence to Three- Dimensional Structure

The wide diversity of protein molecules would suggest the presence of fundamental differences in their synthesis. However, these are a homogenous class of molecules manufactured using the same cellular machinery. The basic building blocks of protein molecules are amino acids, of which there are twenty different types. These monomeric units consist of 3 parts: a carboxylic acidl group, an amino group and a side chain.

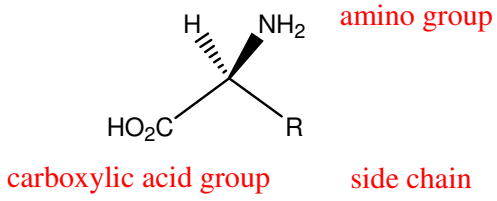


Figure 1: The Generic Structure of Amino Acids [6]

Variations in the functionality of the amino acid side chains include thiols, carboxylic acids, alcohols and other groups. The side chain functionality and the sequence of the amino acid chain are responsible for the structural and functional diversity of protein molecules.[7]

Amino acids can adopt one of two isomeric forms; either D or L. However, it is only the L form of amino acids which are found as constituents of protein molecules.[8]

The side chains of the 20 amino acids are depicted in Figure 2.

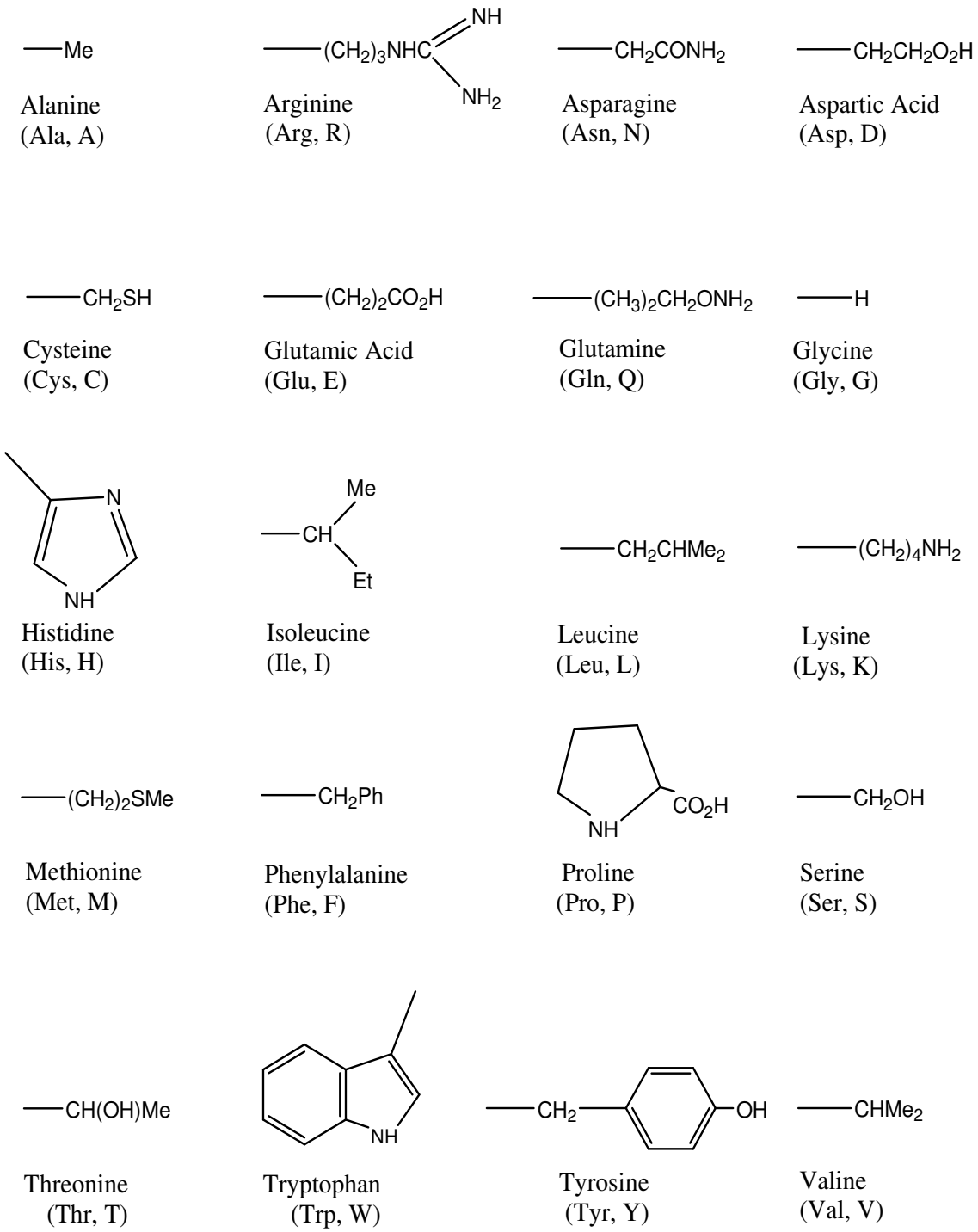


Figure 2: The Molecular Structure of the Sidechains of the Twenty Amino Acids Including Three-Letter Abbreviations and Single Letter Codes [9]

Amino acid residues are covalently linked by peptide bonds in order to form specific linear chains. These resulting strands fold to form three dimensional, active biomolecules.

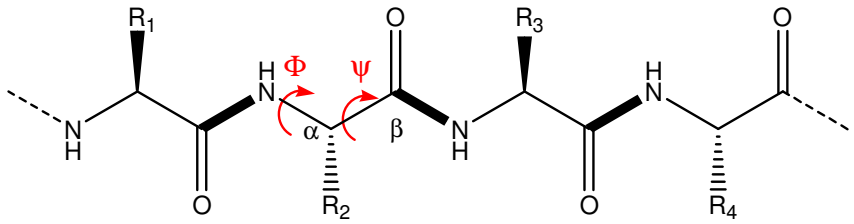


Figure 3: The Peptide Bond. The bonds shown in bold are the planar peptide bonds [6]

The peptide bonds shown are planar and usually adopt a *trans* conformation.[9] Rotation is possible around the bond between the nitrogen and α-carbon. This angle is denoted φ (*phi*). Similarly, the angle of the bond between the α-carbon and β-carbon is denoted ψ (*psi*). Obviously this means that there are a large number of possible combinations of angles, of which only one subset is correct to form the native structure.

The hierarchy of biomolecular structure describes the structural characteristics of protein molecules. From a single chain of amino acid residues, secondary structural characteristics can be formed including alpha helices, beta sheets and random coils.

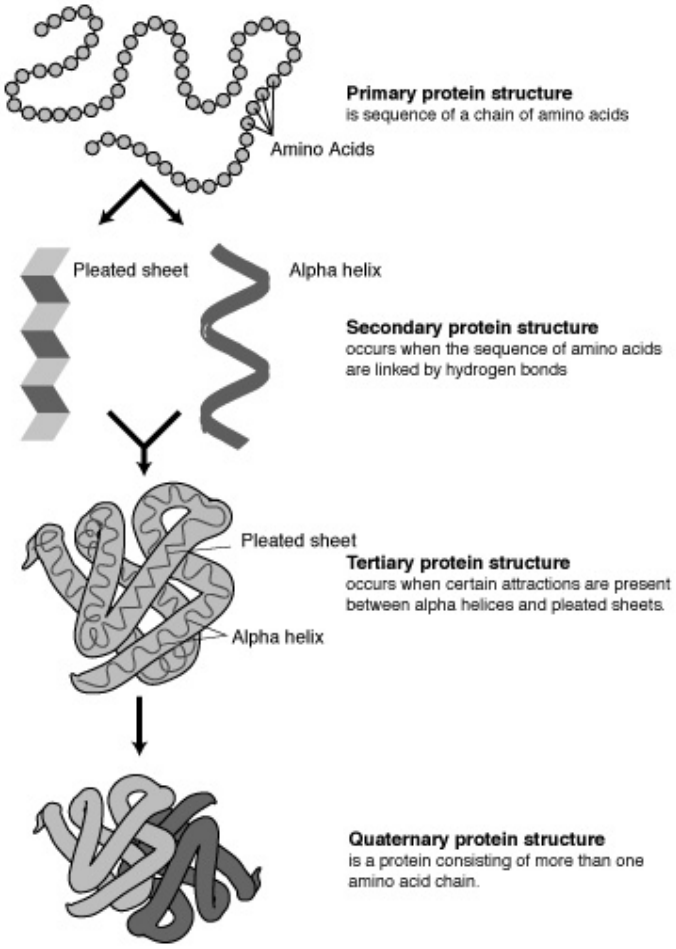


Figure 4: The Hierarchy of Protein Structure [10]

These secondary structural elements can themselves interact to form the tertiary structure of proteins. Multimeric structures can be formed when two or more of these folded amino acid chains interact giving rise to protein quaternary structure.[11]

1.1.1.1 Interactions Involved in the Maintenance of Protein Three-Dimensional Structure

The three dimensional structure of protein molecules is maintained by both covalent and non-covalent interactions.[12] Covalent bonds are formed by the sharing of a pair of electrons by two atoms, and example of which are disulfide bonds. Non-covalent interactions are however, the overwhelming forces involved in the stabilisation of the native, folded structure.

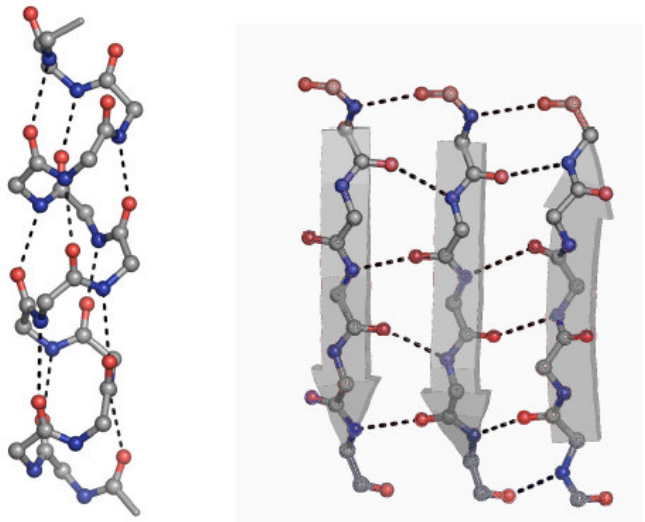
Non-covalent interactions are important in the formation of the organised structure of proteins. These non-covalent interactions are involved in the secondary and higher order

structures within protein molecules. These include electrostatic interactions, van der Waals forces, hydrogen bonding and hydrophobic interactions.[13]

Electrostatic interactions occur between oppositely charged groups within protein molecules. Examples of electrostatic interactions found in proteins include salt bridges, ionic bonds and ion pairs.[13]

Van der Waals interactions occur due to the fact that at any one time, the distribution of charge surrounding an atom is asymmetrical. This fluctuating dipole subsequently influences the electronic distribution of the neighbouring atoms *via* induced dipole interactions. These interactions are relatively short range (3-4 Å apart) and low energy (approximately 1 kcal mol^{-1}).[13]

Hydrogen bonding is at the boundary of covalent and non-covalent interactions. These interactions involve the sharing of a proton between two atoms of which one is hydrogen donor and the other a hydrogen acceptor.[14] Within protein molecules, the hydrogen donor is an oxygen or nitrogen atom which has a proton attached; the acceptor is either oxygen or nitrogen. Hydrogen bonding is vital for the maintenance of protein secondary structural characteristics such as helices and sheets.



(a) **(b)**

Figure 5: Hydrogen Bonding in the Maintenance of Protein Secondary Structural Characteristics [15]

The hydrogen bonds (>C=O····H–N–) are shown with dashed lines. The hydrogen atoms are not shown

(a) a-helical segment from Helicobacter pylori (b) fragment of a b-sheet from antithrombin. The first and second polypeptide chains are parallel in orientation; the second and the third are antiparallel.

The addition of hydrogen bonds to protein molecules confer increased stability to the structure, with the average hydrogen bond having energy of between 3 and 7 kcal mol^{-1}.[13]

Hydrophobic interactions are the repulsive forces which occur between non-polar groups and water.[16] Non-polar groups of protein molecules will tend to form clusters due to their repulsion of water and are therefore commonly found buried within protein structure, separated from the polar solvent.[16] Hydrophobic interactions are some of the most important in driving protein folding.

1.1.2 Proteins: Structural and Functional Variety

In general, protein molecules can be divided into four different types: globular proteins, fibrous proteins, membrane proteins and natively unfolded proteins.

Globular proteins are the most abundant protein structure and are generally found to be soluble. Many enzyme molecules are found to have globular structures. Fibrous proteins are often found in connective tissue and are generally mechanically strong structures. Many fibrous proteins are found to be extremely regular in conformation, large in size and insoluble under aqueous conditions.[17] Membrane proteins are found either connected to or transecting cell membranes and can act as receptors or transporters, although insolubility in conventional media has proven to be an obstacle for widespread characterisation.[18] Natively unfolded proteins are found to be important in transcriptional and translational regulation in eukaryotic organisms.[19] These protein molecules are found to have a lack of specific tertiary structure which confers an ability to bind to a range of ligands for functionality. Binding of a ligand generally results in increased order or folding of the disordered structure.[19]

1.1.3 Enzymes

1.1.3.1 Introduction to Enzymes

Enzyme molecules are proteins which display catalytic activity, usually upon a specific substrate or group of substrates.[20] Enzymatic action has been utilised for thousands of years, although the molecules responsible were unknown. Many different processes used in the preparation of alcohol, cheese and other everyday products were catalysed by enzymes. Early in the 19th century, studies were carried out upon the activity of yeast in sugar fermentation and the digestion of meat by stomach secretions. In the latter stages of that century, Eduard Buchnar discovered that it was possible to ferment sugar using yeast

extract, where no living cells were present.[21] He named the enzyme responsible for this zymase and for this discovery; he was awarded the Nobel Prize in Chemistry in 1907.[22] This was the birth of enzymatic research, leading to biochemical studies, structural biology and genomics.

1.1.3.2 Enzyme Structure

Understanding the complex function of enzyme molecules can be extremely difficult. Enzymes act as catalysts for chemical reactions and as such there is much interest in how these three-dimensional structures function with a view to industrial implementation.

Generally, within the three dimensional structure of an enzyme, there is a region that includes residues working together to produce this catalytic activity. This is commonly referred to as the active site of the enzyme.[23] Here, the enzymatic substrate will bind *via* highly specific non-covalent interactions so that the catalytic function of the enzyme can be carried out. Examples of such interactions include; hydrogen bonding, van der Waals, electrostatic and hydrophobic interactions.[24]

1.1.3.3 Catalysis

A catalytic species is one which accelerates the rate of a chemical reaction whilst remaining unchanged upon reaction completion. Catalysts are retrievable from the reaction media so that they might be used in subsequent chemical transformations.[25] Catalytic species and their effect upon a chemical transformation can be visualised using a reaction profile.

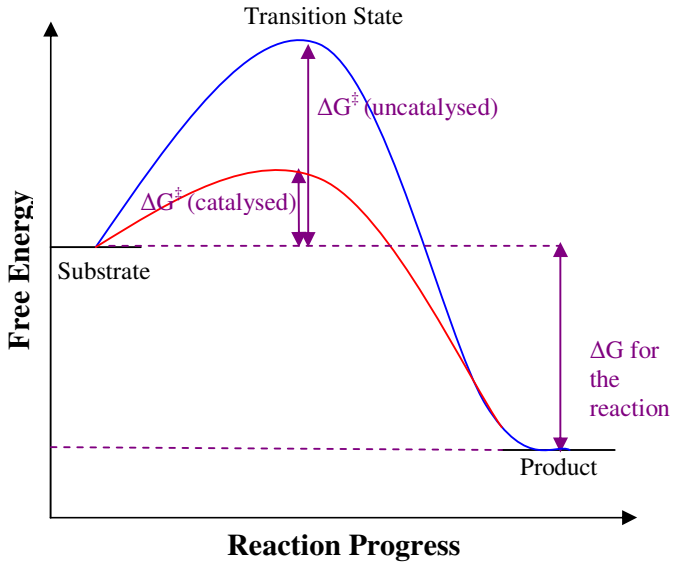


Figure 6: The Effect of Catalysts upon the Reaction Profile [26]

Catalysts, such as enzymes, lower the activation energy of chemical reactions, such that the reaction pathway from reactants to products has changed. The energy required to reach the transition state is reduced. Although the enthalpy change for the overall reaction process remains the same, the energy barrier to complete this process has been reduced resulting in an increased rate of reaction. However, although the activation energy of the reaction is changed, the free energy of both the reactants and products remains the same.[27]

1.1.3.4 Industrial use of Enzymes

Enzymes play a major role in many industrial processes due to their specificity and the large scope of reactions in which they can be involved. The biocatalytic fermentation of sugar to form ethanol is a reaction carried out by enzymes present in yeast. This, as a process, has been recognised for thousands of years in the production of alcohols such as wine and beer. Enzyme action is also noted in the oxidation of ethanol to form acetic acid. This reaction has been used in the manufacture of vinegar for thousands of years. In fact, there is a plethora of everyday products, from detergents to cheese, where manufacture relies upon the action of enzymes.[28]

Enzymes are able to carry out a broad range of highly specific reactions and also those which appear impossible by any other route. However, inherent problems which limit the use of enzymes as industrial catalysts are biomolecular instability in the desired reaction media and difficulties encountered in recycling.[29] Ambient temperatures (15-50 °C), pH (5-9) and use in aqueous media are generally required for enzymes to function efficiently if at all, hampering the use of these catalysts in an industrial setting.[30, 31]

1.2 Protein Folding, Misfolding and Aggregation

The human body contains approximately 100,000 different types of protein, all of which are involved in day to day running of many processes upon which we are reliant.[5] Upon synthesis of linear chains of amino acids *in vivo* upon the ribosome, these then are converted into three-dimensionally packed structures with biological activity.[5]

There is extensive interest in the process of protein folding with regards to the level of intricacy and efficiency with which this can be carried out in living cells. A greater knowledge of the processes by which protein molecules fold has implications in medicine, structural biology and materials science.[32]

1.2.1 Introduction to Protein Folding

As described previously, protein molecules are extremely complex three-dimensional structures. But how exactly do they obtain a native conformation from the unfolded state? This is one of the greatest mysteries in protein biochemistry, but at present there is no definitive answer.

Protein folding is understandably a phenomenally popular area of research. Researchers are attempting to gain an insight into the methods by which protein molecules fold with a view to utilising this knowledge in a number of different areas of science including molecular biology, biophysical chemistry and health and disease among others.

1.2.2 The History of Protein Folding Research

Over the last hundred or so years, there has been extensive research carried out in an effort to determine the physical and structural properties of protein molecules. It was noted by Ramsden in 1910 that "A dead frog placed in a saturated urea solution becomes translucent and falls to pieces in a few hours".[33] Ramsden noted that urea was responsible for the decomposition of the frog and this serendipitous finding illustrates some of the earliest studies of protein unfolding. These early studies have lead to an entire branch of science devoted to understanding, both physically and mechanistically, just how protein molecules achieve their native three-dimensional structure.

There have been many theories conveyed in an attempt to explain how protein molecules fold. One such influential theory was that of Cyrus Levinthal in the 1960s who speculated

that there must be specific pathways (both energetic and kinetic) by which protein folding occurs.[34] In the unfolded state, a polypeptide chain has a large number of degrees of freedom due to the rotation around the φ and ψ angles of the peptide backbone. As such there is an astronomical number of structural possibilities for the native state.[35] An example of this would be a polypeptide of approximately 100 residues. If there were only 3 possible torsion angles for each bond, there would be 9^{100} possible conformations. If all of these conformations were explored sequentially, even at picosecond speed, it could still take longer than the age of the known universe to find the correct conformation. He concluded therefore that there must be specific pathways through which proteins fold to find the correct native structure.[34]

Throughout the 1960s and 1970s, Christian Anfinsen was carrying out research on protein folding. At the time Anfinsen was carrying out this work, it was only possible to detect gross changes in protein structure, due to a lack of spectroscopic and hydrodynamic methods.[32] As such, many biomolecular folding experiments were carried out using enzymes for which there were known activity assays readily available. Protein folding was observed by the regained activity of the treated species compared with a native sample.

Anfinsen carried out a number of studies with the enzyme ribonuclease. This work showed that upon oxidative refolding, of the 105 possible disulfide combinations of the 8 half-cystines, only one is formed; the correct one.[36] This led Anfinsen to speculate that, at least in the case of small globular proteins, under a given set of environmental conditions (pH, solvent, temperature etc.), the spontaneous folding of protein molecules into a native state was governed by the amino acid sequence.[36] This native state would also occur at a minimum of free energy. Anfinsen also speculated that other proteins such as "shuffling enzymes" may be responsible for the overcoming of the rate limiting steps in cellular protein folding by acting as catalysts.[37] It was for his work with ribonuclease that, in 1972, Anfinsen received the Nobel Prize in Chemistry.[38]

1.2.3 The Nature of the Unfolded State

To understand the nature of the unfolded state, it is first necessary to consider the native structure. The native state is usually the biologically active form of the protein which consists of a number of regions of secondary structure held together by non-covalent interactions such as hydrogen bonds. However, in the case of the unfolded state, the parameters are less well defined.[16]

Under the generic heading of 'unfolded protein', there are a number of subsets which can be considered. Protein molecules can be misfolded, aggregated, in a molten globule or in a random coil conformation.

Misfolded structures are generally incorrectly or partially folded, but not entirely unlike the native structure. These structures can form as a result of a number of different chemical modifications including disulfide rearrangement or isomerization of proline residues.[16]

Aggregated protein can form as a result of thermal unfolding and generally contains regions of unfolded polypeptide. There are possibly regions of native structure present, but these combine with the unfolded regions to form dense aggregated species.

The molten globule state is a compact intermediate in the pathway of protein folding.[39] Within the molten globule, there is often found to be regions which correlate well with the peptide backbone of the native state (in terms of secondary structural characteristics). However, there is found to be a high level of disorder within the amino acid side chains.[16]

Randomly coiled structures can be considered to be the most irregular structure. Here, there is no correlation of structure from one amino acid to the next in the sequence. Every possible conformation is just as likely, therefore the amino acid chain is considered to have an entirely random conformation.[16]

In the context of this study, we are mostly concerned with the native, misfolded and aggregated state.

1.2.4 Current View of Protein Folding

Many analytical techniques have been used in order to determine the path embarked upon by an amino acid sequence to gain three-dimensional structure. The use of stopped flow techniques has described the formation of intermediates in a staged folding process.[32] It is also known that the hydrophobic effect is one of the major driving forces in protein folding.[39] The hydrophobic effect occurs due to the fact that water molecules have a high affinity for other water molecules *via* the formation of hydrogen bonds. Non-polar molecules do not have the ability to form such H-bonds, and therefore they are excluded from the network of water molecules. In the case of protein molecules in solution, the result of this is the burial of amino acids containing hydrophobic side chains in the interior of the structure, thus excluding water. [40]

The processes by which proteins fold to form native structure can be imagined by considering an energy landscape or folding funnel.[32, 39]

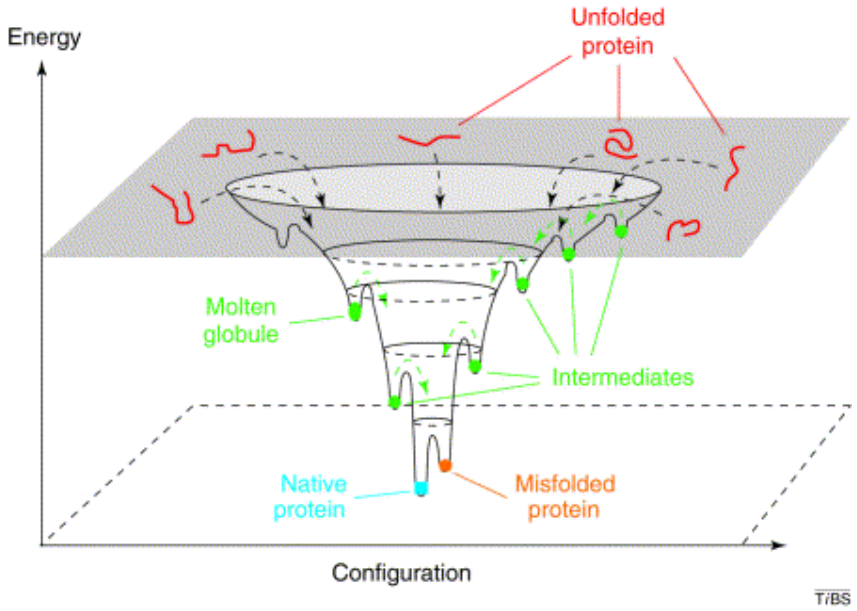


Figure 7: Schematic Diagram of a Folding Energy Landscape [32, 39]

Unfolded protein is found at the top of the folding funnel. This is energetically high and as the protein molecules decrease in energy, a decrease in entropy is also seen (i.e. an increase in order). As the energy of the protein molecules decrease, partially folded protein is seen to populate local energy minima. This results in the formation of stabilised molten globules of protein. The energy barrier of these minima is not sufficiently high to trap the partially folded protein; instead these serve as energy stops on the route to native structure.[32]

Although research has given an insight as to how protein molecules might actually fold *in vivo*, an efficient method for the folding of synthetic polymers and protein from the unfolded state is still proving an elusive target.

1.2.5 Protein Synthesis and Folding in the Cell

The wonder of protein biosynthesis cannot be underestimated. The manufacture of such large molecules with precise sequence and structural characteristics is no mean feat. The synthesis and folding of protein molecules in the cell is a highly complicated and intricate process for which the instruction is all detailed in the genetic material of the cell.

DNA molecules contain all of the information required to biosynthesise every protein in the body. The particular area of a DNA molecule which codes for a specific protein is

called a gene. The genes are accessed *via* unwinding of the DNA double helix close to the site of the required gene. A complementary strand of messenger RNA (mRNA) is transcribed from the gene where three nucleic acids code for each amino acid. This group of three nucleic acids which determine the amino acids used to synthesise the protein molecule are called codons. As each of the codons is transcribed, the DNA helix begins to recoil, forming the well known double helical structure. The mRNA molecule vacates the cellular nucleus and enters the cytoplasm to undergo translation upon the ribosome. In order for translation to occur, transfer RNA (tRNA) molecules (which are specific for types of amino acids) read for the encoded amino acid and subsequently present this to the ribosome so that it may become part of the growing polypeptide chain.[41] The truncated amino acid chain folds into the lowest energy conformation. However, this often leads to obtaining a non-native three-dimensional structure. Similarly, the high concentration of macromolecules in the cellular environment can lead to crowding and therefore aggregation. Protein molecules, such as molecular chaperones, resident in the cytoplasm have the ability to prevent or reverse this misfolding.[5]

Molecular chaperones are proteins whose functions include allowing incorrectly folded protein a second chance to find the correct fold. These chaperones act co-translationally to find misfolded protein and subsequently allow it to fold into a native conformation.[32]

1.2.6 The Importance of Understanding Protein Folding

The understanding of protein folding has wide implications for a number of different scientific genres. This is evident upon considering the number of scientific papers which have been published annually over the last forty or so years.

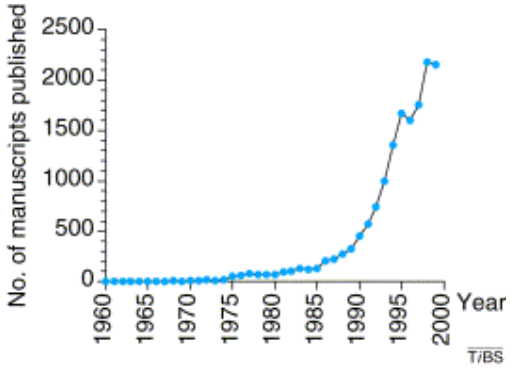


Figure 8: Annual Publication Numbers of Manuscripts (1960-1999) with the Words 'Protein' and 'Folding' in the Title or Abstract [32]

These publications included interests from molecular biology, biophysical characterisation, protein therapeutics, health and disease and method development and analysis.

1.2.6.1 Protein Folding in Health and Disease

Erroneous protein folding is a fundamental problem associated with the onset of many diseases. There are a number of procedures that protein molecules go through in the cell to ensure that they have the correct fold and to detect misfolding. However, despite these checks, errors occasionally occur giving rise to the manufacture of misfolded or aggregated species.[42]

The diverse functionality of protein molecules in an organism cannot be underestimated. These biomolecules are involved in everything from immune response to signalling pathways and therefore if a protein molecule is unable to carry out its desired function, these essential processes cease to occur.[5]

There are around twenty diseases which can be attributed to protein misfolding and/or the impairment of cellular machinery involved in the synthesis of correctly folded protein.[43]

A number of these diseases are documented in Table 1.

Disease	Protein Involved	Molecular Phenotype
Cystic Fibrosis	CFTR	Misfolding
Marfan Syndrome	Fibrillin	Misfolding
Scurvy	Collagen	Misfolding
Cancer	p53	Misfolding
Creutzfeldt-Jakob Disease	Prion	Aggregation
Alzheimer's Disease	β-Amyloid	Aggregation
Cataracts	Crystallins	Aggregation
Familial Amyloidosis	Transthyretin/Lysozyme	Aggregation

Table 1: Diseases Attributed to Protein Misfolding or Aggregation [43]

Seemingly insignificant errors in protein synthesis can therefore lead to serious medical conditions or impairments. The understanding of the methods by which protein molecules fold is important in medical research for drug discovery, disease prevention and intervention.

1.2.6.2 Inclusion Bodies

The advent of molecular biology has led to the ability to express recombinant genes in bacterial species such as *E.coli*. One of the problems commonly encountered upon using this technique is the formation of insoluble aggregated species called inclusion bodies.[44] Inclusion bodies are formed when bacteria are encouraged to produce large quantities of protein. Before these unfolded species can fold into a native conformation, some form of nucleation occurs to form aggregates.[45] The exact mechanism of inclusion body formation is unknown, but is thought to arise from the relative insolubility of unfolded polypeptides which aggregate before forming their native, soluble form.[46]

A kinetic competition exists between protein folding and aggregation. Protein folding is a zero order process and is therefore independent of protein concentration. Aggregation however is an intermolecular higher order process and as such has a rate which is raised to the power of the concentration of the protein present.[47] Therefore, if the concentration of protein is high, then the likelihood of aggregation occurring is high also.

Although there is no generic relationship between the types of proteins expressed and the ability to form inclusion bodies; net charge, solubility and rate of folding of intermediates appear to play a role.[48] However, for functional protein to be isolated from these insoluble species, they must first be solubilised in some way and then subjected to a laboratory protein folding technique.

1.2.7 Practical Methods for Laboratory Protein Folding

There are a number of methods which are currently employed for the folding of protein from aggregated species; dialysis, dilution, and chromatographic methods are the most common. All of these methods rely upon the solubilisation and (partial) unfolding of the protein before any folding can occur and subsequently, the gradual removal of these solubilisation agents from the protein environment to trigger folding.

1.2.7.1 Solubilisation Agents

Solubilising agents are molecules which are used commonly to increase the solubility of protein molecules in solution.[49] Generally solutions of urea and guanidine hydrochloride are used to this end.

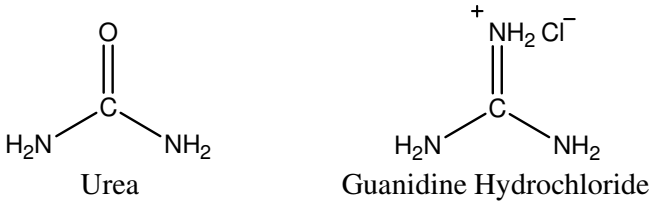


Figure 9: The Chemical Structure of Solubilising Molecules [50]

The ability of these molecules to solubilise protein is dependent upon interactions with the surface of the biomolecule and the surrounding solvent. Urea has the ability to form hydrogen bonds with water, therefore reducing the hydrophobic effect. This leads to an increase in the solubility of the protein in the unfolded state. Other solubilising agents include molecules such as polyethylene glycol which inhibits aggregation and cyclydextrins which form host-guest complexes with the hydrophobic exposed side chains and therefore reduce aggregation.

Dithiothreitol (DTT) and mercaptoethanol are reducing agents commonly used to break incorrectly (and correctly) matched disulfides which have formed in the aggregation process, ensuring that the protein is entirely solubilised.[49]

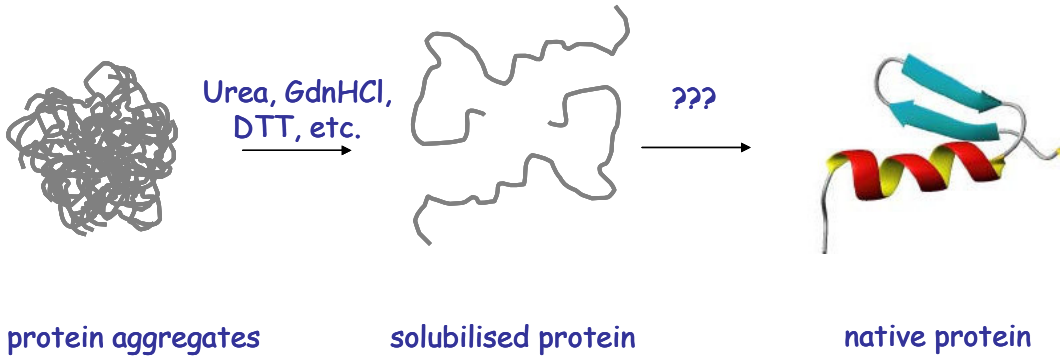


Figure 10: The Solubilisation of Aggregated Protein *en Route* to Native Structure

The solubilising molecules essentially prevent the individual protein molecules being drawn together in the formation of aggregated species.[50] The unfolded biomolecules are therefore held apart in solution, ready to undergo the folding process.

1.2.7.2 Dilution and Dialysis

Dilution is one of the most commonly utilised methods for refolding protein due to its simplicity.[51] This method triggers the folding of solubilised protein by gradually separating the solubilising agents from the environment of the unfolded biomolecule. This is generally carried out by adding a refolding buffer to the denatured protein in order to dilute the salt concentration of the agent (unfortunately, the protein concentration is reduced also).[48] Dialysis is an alternative to the dilution method in that there is still a gradual removal of the small molecules from the protein environment, without the inconvenience of a more dilute protein sample.[52]

A refolding buffer is an aqueous solution containing low concentrations of solubilising agents (such as urea at 1-2 M or GdnHCl at 0.5-1.5 M).[53] Also a redox couple such as cysteine/cystine or glutathione/reduced glutathione (usually at a 10:1 molar ratio) is commonly added to simultaneously reduce and re-oxidise mismatched (and correctly matched) disulfide bonds until the correct, energetically favourable combination is found.[54] In some cases, it has also been found that the presence of arginine or detergents can promote protein refolding.[55]

The unfolded protein can be diluted directly into or dialysed against the refolding buffer, which results in the concentration of the solubilising molecules being reduced and refolding initiated. Refolding yields of 65 % can be achieved by 85-fold dilution of denatured/reduced lysozyme, but due to the nature of this dilution very low protein concentrations are typically obtained (as an illustration; 15 mg/ml subjected to 85 fold dilution is equivalent to a final protein concentration of 0.18 mg/ml).[56] This method is therefore mainly used in small-scale research studies requiring low protein concentrations.[53]

1.2.7.3 Chromatography

Protein refolding can also be achieved using a number of chromatographic techniques. Chromatographic refolding is an attractive prospect as it can be easily automated and again results in the gradual removal of solubilising agents from the environment of the protein.[53]

1.2.7.3.1 Ion Exchange Chromatography

For the refolding of protein using ion exchange chromatography (IEC), initially the column is equilibrated with high concentrations of urea (8 M) and the unfolded protein in buffer is reversibly adsorbed to the ion exchanger helping to minimise aggregation.[57] The concentration of urea is gradually decreased by passing a refolding buffer through the

column. This triggers the refolding of the protein molecules, which are prevented from aggregating by the spatial constraints placed by the ion exchange matrix. Once the solubilising agents are removed and the protein is potentially refolded, it is left to equilibrate on the column for up to 40 hours for refolding to be completed. The refolded protein is subsequently eluted upon the implementation of a NaCl gradient.[57]

Although large volumes of refolding buffer are passed through the column, this is not used to dilute the protein to the same extent as dilution methods and therefore higher protein concentrations can be obtained using IEC.

In a particular example, bovine serum albumin (BSA) unfolded in urea was refolded on an IE column. Recovery yields of 55% refolded protein were obtained with a dilution of approximately 10-fold.[57]

1.2.7.3.2 Size Exclusion Chromatography

Size exclusion chromatography (SEC) is a technique commonly employed for the separation of molecules on the basis of their size.[58] In the case of unfolded, solubilised protein, SEC can be used to gradually separate small molecules such as urea and DTT from the protein environment.[59] The solubilising molecules are of the size to enter the pores of the beaded matrix and are therefore separated from the larger biomolecules.[51] This separation of unfolded and partially folded structures serves to reduce aggregation of the protein.[59] The newly folded protein essentially runs straight through the size exclusion column for collection, while the solubilisation molecules are subsequently washed from the matrix.

Utilising this method, refolding of hen egg white lysozyme has led to yields of 60% refolded protein after approximately 100-fold dilution of the protein with the refolding buffer.[51]

1.2.7.4 Three-Phase Partitioning

Three-Phase Partitioning (TPP) is a recent advancement in methodology design for the refolding of protein. Unfolded, solubilised protein is saturated with a common salting out reagent such as ammonium sulfate. Tertiary butanol is added to this mixture and is left to incubate at 40°C for 4 hours. At this stage, both aqueous and organic phases can be seen separated by an interfacial precipitate.[60, 61] The solubilising agents are found in the organic layer (as here, they have high solubility) and are therefore separated from the aqueous protein.

A practical example of this methodology is the refolding of the enzyme xylanase from urea.[60] Here the refolded protein was purified 21-fold and the recovered protein had 93% of the activity of the native protein. Unfortunately, only 5% of the total protein put into this refolding process was actually found to be present in the aqueous layer, with the remaining 95% in the interfacial precipitate. This protein was found to be irretrievable, presumably denatured and aggregated as a result of exposure to a denaturing interface.[60]

1.3 Protein Immobilisation

Enzymes are highly specific catalytic species and for this reason they are commonly utilised in chemical transformations.[62] Enzyme use under process conditions can be hampered by the lack of stability of protein under such conditions. Protein immobilisation is a method which is used in order to circumvent the problems associated with enzymatic stability and ease of handling.[63]

Over the past 30 years, great effort has been devoted to developing protein immobilisation techniques with a view to improving enzyme operational performance, ease of handling and recovery of the biocatalytic species.[31]

1.3.1 Advantages of Protein Immobilisation

There are a number of reasons why the immobilisation of protein molecules is carried out. Generally, immobilisation confers increased stability of the biomolecule and removes the need for delicate handling.[31] In biocatalysis, the immobilised enzyme has increased stability, is more easily retrieved from reaction media and therefore offers a prospect where the cost of product manufacture is decreased.[29, 64] For biomedical applications, it is advantageous to remove the need for refrigeration from protein based drugs. This can be achieved *via* immobilisation. Similarly, the immobilisation matrix may be used as a drug delivery vehicle when therapeutic proteins are immobilised. Immobilisation is also an attractive method for the controlled release of protein therapeutics. It is also possible to immobilise more than one protein together therefore allowing "cascade processes" or a series of chemical reactions to occur in one pot.[31]

1.3.2 Protein Immobilisation Methods

Generally, protein immobilisation occurs *via* one of three main processes; entrapment within an organic or inorganic polymer, chemical cross linking of protein molecules and immobilisation upon a carrier material.[31]

These immobilisation methods all have their advantages and disadvantages, some of which will be discussed.

1.3.2.1 Protein Immobilisation *via* Polymer Entrapment

It is possible to immobilise proteins within sol-gel matrices.[31] Sol-gel encapsulation is a facile and efficient method for the immobilisation of protein molecules, particularly enzymes for use in industrial applications such as synthetic organic chemistry.[65]

This method requires the acid or base catalysed hydrolysis of tetraalkoxysilanes in the presence of the protein of interest giving rise to a SiO_2 matrix for the encapsulation of the biomolecule.[65]

There are a number of examples of the use of this methodology in the literature[65-67] producing stable, highly active preparations for enzymatic catalysis.

1.3.2.2 Protein Immobilisation *via* Chemical Cross Linking

1.3.2.2.1 Cross-Linked Enzyme Crystals (CLECs)

Chemical cross-linking of crystallised proteins using glutaraldehyde was initially developed in 1964 by Quiocho and co-workers as a method for increasing the stability of delicate protein crystals for x-ray diffraction studies.[68] The researchers noted that these newly created cross-linked enzyme crystals (CLECs) retained (and in some cases improved upon) catalytic activity.

Glutaraldehyde is a bifunctional cross-linking agent which reacts with the amine functionality of lysine residues available upon the surface of protein molecules.[69] The homobifunctionality of glutaraldehyde means that it can form crosslinks between lysine residues either inter or intra molecularly.

Enzymes which have undergone chemical cross-linking to form CLECs have been shown to have increased activity, stability and enantioselectivity in organic solvents and water/solvent mixtures.[70] These immobilised enzymes can be stored at room temperature due to this increased stability which is generated as a result of the stabilisation of the protein crystal matrix.[69] These crystals also show greater ease of recycling when compared with their untreated counterparts due to their increased mechanical resistance.[69] CLECs have become commercially available as immobilised enzyme preparations and this has lead to their increased use. CLECs are commonly utilised in biotransformations, in x-ray studies and as microporus materials.[71]

A particular example of the stability conferred upon protein *via* CLEC immobilisation is illustrated. CLECs of chloroperoxidase cross-liked using glutaraldehyde retain most of

their catalytic activity up to 70 °C compared to their non-immobilised counterparts which only retain activity to 50 °C.

One of the main advantages of the use of CLECs over other immobilised enzymes, is the fact that there is no immobilisation matrix as such and therefore the entire mass of the CLECs are active species. Generally, some kind of matrix is required for immobilisation to take place and therefore gram per gram, CLECs have higher specific activity than other biocatalysts.[72] However, in order to manufacture CLECs, crystals of protein are used (which require high purity to form) and this can be a major stumbling block limiting the proteins available.[31]

1.3.2.2.2 Cross-Linked Enzyme Aggregates (CLEAs)

Chemical crosslinking of proteins using glutaraldehyde can also be used in the manufacture of CLEAs. This technique involves the precipitation of an aqueous solution of protein before subsequent cross-linking of the enzyme aggregates *via* reaction with glutaraldehyde or dextran polyaldehyde.[73] Precipitation gives rise to protein aggregates, but also acts as a purification method and therefore, small molecules are removed from the immediate environment of the protein.

CLEAs of penicillin G acylase were found to retain up to 90 % activity when immobilised in this way using dextran polyaldehyde as the cross-linking agent.[74]

1.3.2.3 Protein Immobilisation *via* the use of a Carrier Material

1.3.2.3.1 Protein Coated Micro-Crystals (PCMC)

The formation of Protein Coated Micro-Crystals is a recently developed method for the temporary immobilisation of protein. These crystals are formed from a water soluble excipient material, an aqueous solution of protein and organic solvent. This is therefore an inexpensive method of protein immobilisation.[75]

The core of these crystals can be made from a number of different materials including salts (e.g. K_2SO_4, KCl), amino acids (D,L-valine, L-glutamine) sugars (mannitol) and solid state buffers. (Na-AMPSO).[76]

PCMC can be stored as dry powders on the bench or as a suspension in organic solvent. The immobilised protein shows increased stability with no need for refrigeration. These crystals also display high activity and morphology which can be predicted, indicating that this method for protein immobilisation could therefore have widespread applications.[75]

1.4 Protein Coated Micro-Crystals (PCMC)

PCMC production is a novel method for the manufacture of immobilised proteins. The protein and crystal-forming component are mixed in an aqueous solution before being subsequently added drop-wise to a stirring water-miscible organic solvent. The protein molecules are rapidly dehydrated and are immobilised upon the crystal surface. Once immobilised, the protein is functional in either organic or aqueous media.[75]

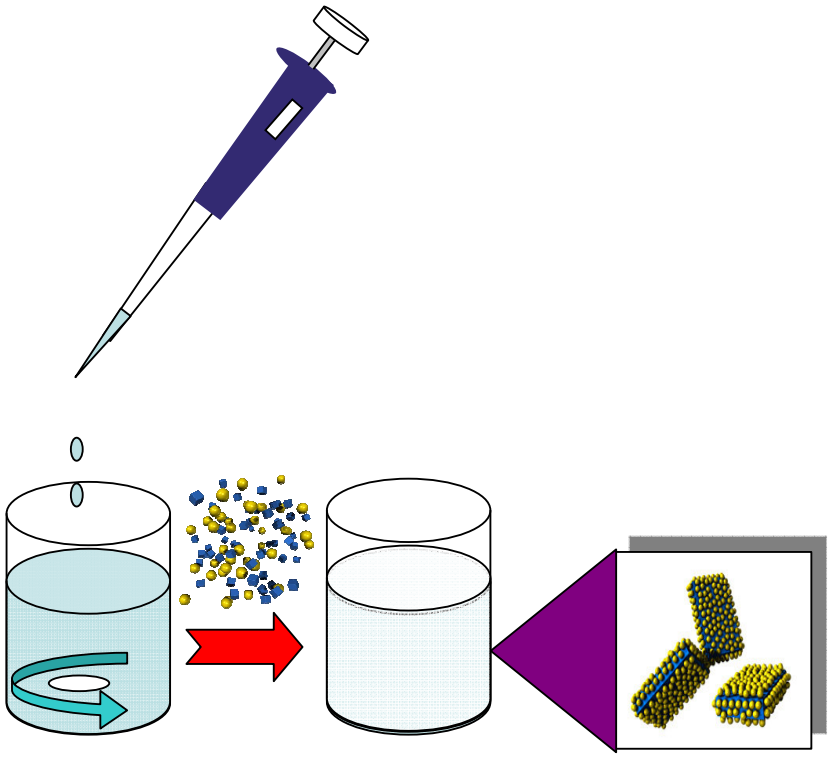


Figure 11: Schematic of PCMC Preparation Process

This rapid, one-step dehydration permits the majority of immobilised biomolecules to retain activity and minimise denaturation.[31]

The resultant particles are composed of a crystalline core of excipient material coated in the desired biomolecule.

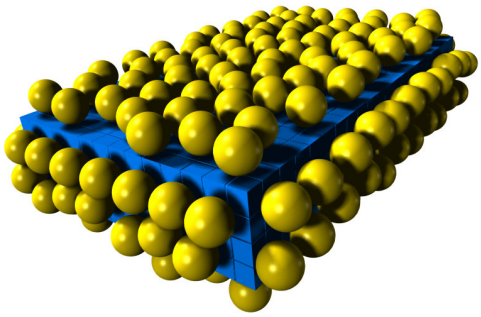


Figure 12: Schematic Representation of PCMC Structure (Image used with the permission of Dr. Marie Claire Parker, XstalBio Ltd.)

A schematic representation of PCMC structure is shown. The blue core is the crystal of salt, amino acid or sugar and the green molecules on the surface are dehydrated immobilised protein. The biomolecules which coat the crystal act to arrest crystal growth and therefore define particle size and morphology. These immobilised protein preparations have shown increased activity in organic solvent and are also water soluble and can therefore can be used for aqueous applications also.[77]

In the initial PCMC studies, the salt K_2SO_4 (which is insoluble in polar organic solvents and non hygroscopic) was mixed with protein. This mixture was subsequently dehydrated in a water miscible organic solvent. The carrier material is therefore prepared in situ *via* this dehydration process and the protein molecules associate with the support. Enzyme immobilisation and rapid dehydration have previously been demonstrated as a method for the production of enzyme preparations with enhanced activity in organic media.[78]

Further investigation indicated that this material was crystalline and the size of which could be tuned by varying the excipient material and protein loading. Transmission Electron Microscopy (TEM) of the immobilised protein preparation indicated that these crystals were 5 - 7 µm in size.

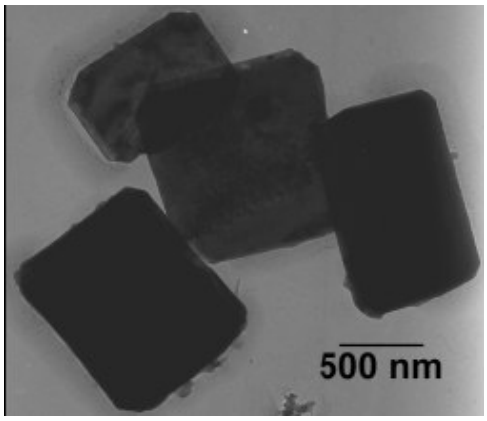
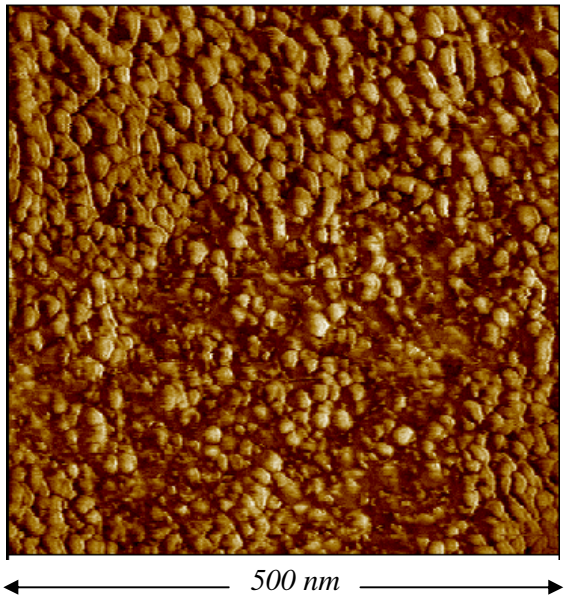


Figure 13: Transmission Electron Microscopy (TEM) Image of Subtilisin Coated K_2SO_4 Crystals [75]

In the absence of protein, the crystal size is greater indicating that the protein acts to limit the growth of the crystals during the precipitation process.[75]

The products of co-precipitation could be either immobilised protein contained within or upon the surface of the crystals. Atomic force microscopy was carried out in order to determine the surface morphology of the immobilised species.



The z-height is 15 nm and the image is 500 nm x 500 nm on the crystal surface.

Figure 14: Atomic Force Microscopy (AFM) Image of Subtilisin Coated K_2SO_4 Crystals (Image used with the permission of Dr. Marie Claire Parker, XstalBio Ltd.)

Atomic force microscopy (AFM) of the surface of subtilisin Carlsberg coated K_2SO_4 indicated that rather than a smooth surface (evidence of K_2SO_4 on its own) an uneven

surface was present which had presumably been coated with protein. The protein molecules upon the surface of the PCMC were resident as small clusters of around 15 nm (subtilisin Carlsberg is approximately 5 nm in size).

1.4.1.1 Preparation of PCMC - Selection of Excipient

The choice of excipient for protein immobilisation to form PCMC is very important and entirely depends upon the protein and its uses. In the case of therapeutic proteins for patient use, it is important to select a crystal-forming material which has no known hazards to human health. Examples of excipients which can be used for therapeutic protein immobilisation are glutamine, valine and a number of sugars.[79]

If the purpose of immobilisation is for biocatalyst preparation, it is important to ensure that the immobilisation medium does not interfere with any future chemical transformations which the enzyme will carry out. Salts such as potassium sulfate (K_2SO_4) and potassium chloride (KCl) are good for this purpose.[75, 77] Solid state buffers can also be used as the immobilisation matrix in the preparation of biocatalysts. This can lead to increased activity in the organic phase. Compounds such as Na-AMPSO, Na_2CO_3 and $NaHCO_3$ can be used.[76]

For research studies, it is not only important to select an inert material for immobilisation, but also one which will not interfere with further analysis of the sample (e.g. circular dichroism (CD), fluorescence, UV/vis spectroscopy). To carry out CD studies on immobilised samples, it is important that the immobilisation medium is either achiral or a racemate, and as such does not interfere with the spectroscopic method. Excipients such as K_2SO_4 and D,L-valine have these pre-requisites.

1.4.1.2 Preparation of PCMC - Selection of Solvent

Another important consideration when preparing PCMC is the choice of solvent. Although generally removed from the environment of the PCMC, traces of the solvent may remain within the crystals. When immobilising proteins which have therapeutic properties, it is important to select the solvent carefully to ensure that it is not detrimental to health to have traces of the solvent within an administrable drug.

In general, solvents such as propan-1-ol, propan-2-ol and ethanol are used in PCMC preparation. These solvents are used for a number of different reasons including their water miscibility properties and their ease of obtainment.

1.4.1.3 PCMC Isolation

PCMC can either be stored as a suspension in organic solvent or collected as a dry powder. For storage as a suspension, the crystals can be kept in their precipitating solvent or this solvent decanted and replaced with another. To obtain a dry powder sample, it is necessary to separate these crystals from the solvent medium. This can be achieved by vacuum filtration through the use of filter membranes. The dry powders which are obtained can be subsequently stored upon the bench. In the course of this research project, all PCMC samples were isolated as dry powders.

1.4.2 Process of PCMC Formation

The mechanism of PCMC formation is not entirely understood. As the aqueous mixture is added to stirring organic solvent, both precipitation of protein and crystallisation of excipient material are occurring. There are a number of possibilities with regards the possible structures which can form from the aqueous mixture of protein and excipient.

The excipient material, although soluble in water, has limited (or no) solubility in polar organic solvent. This causes rapid super-saturation of the solvent, beyond which, the material crystallises.

1.4.2.1 Crystal Lattice Mediated Self Assembly (CLAMS)

Co-precipitation can occur conventionally by one of two methods; both the protein and crystal forming component remain separate or the protein and excipient form a crystalline material with protein held within the crystal randomly.

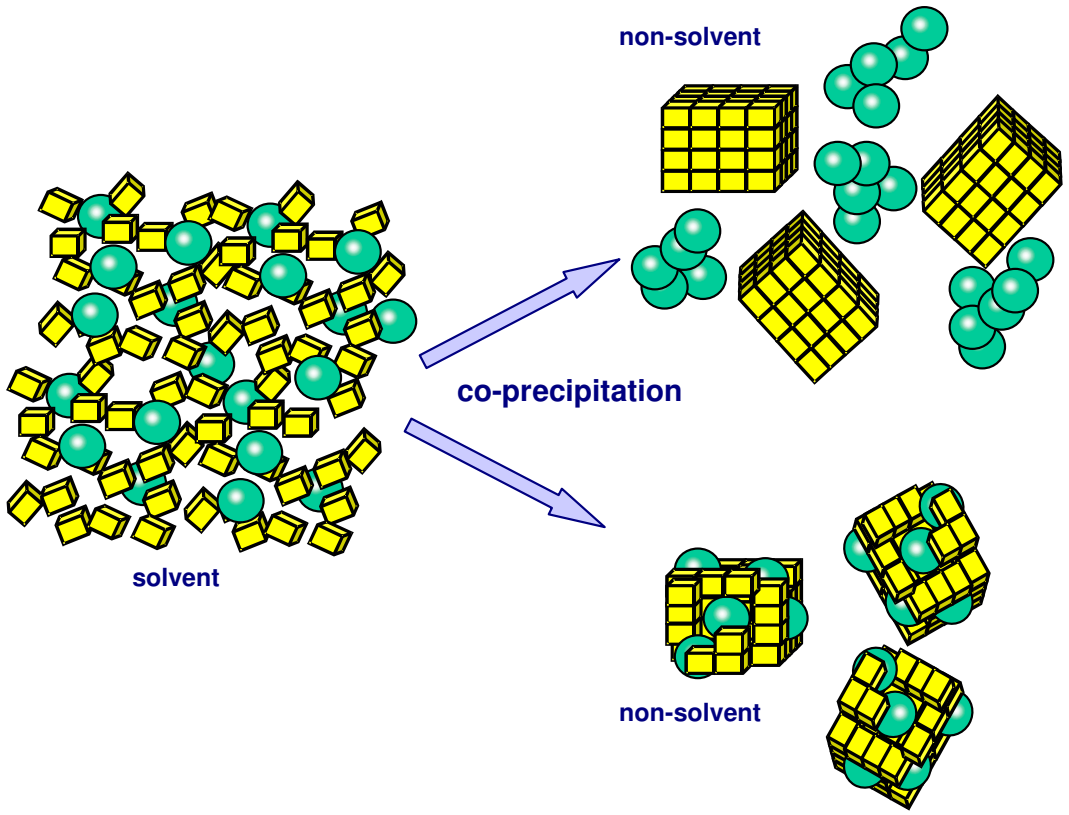


Figure 15: The Conventional Co-Precipitation Process (Image used with the permission of Dr. Barry D. Moore, WestChem Department of Pure and Applied Chemistry, University of Strathclyde)

However, in the case of PCMC formation, the co-precipitation process is thought to be driven by the crystal lattice energies, forcing the protein molecules to form a layer upon the surface of the crystals.

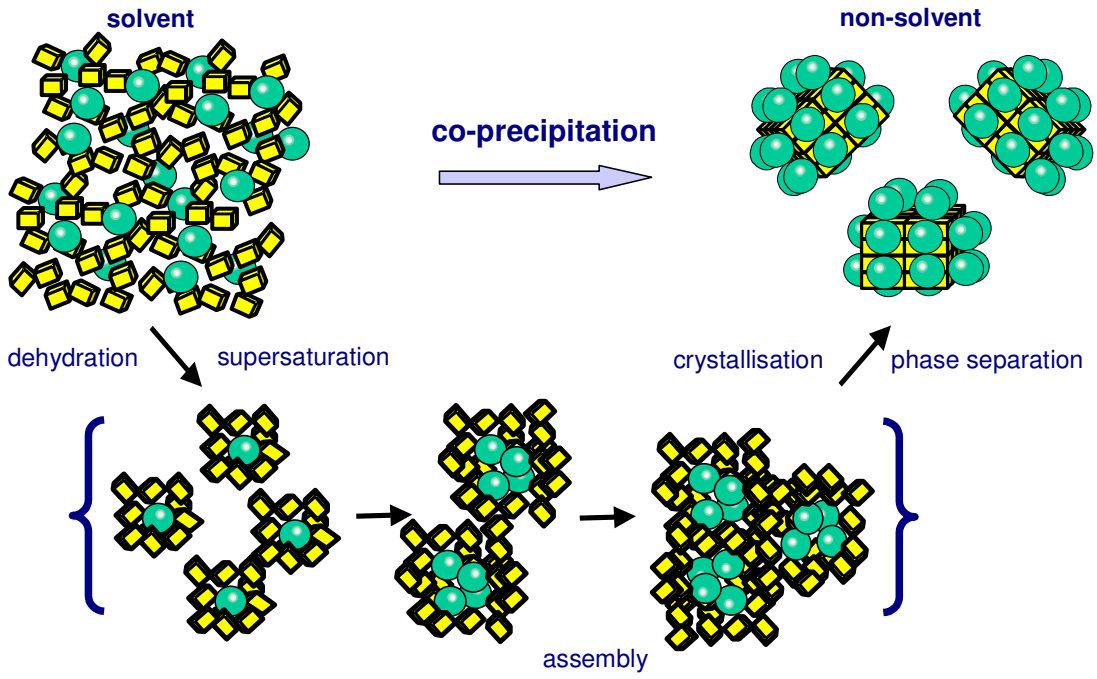


Figure 16: CLAMS Crystal Lattice Mediated Self-assembly (Image used with the permission of Dr. Barry D. Moore, WestChem Department of Pure and Applied Chemistry, University of Strathclyde)

It is generally accepted that under conditions of high organic solvent content (50:50 aqueous/organic), that protein is denatured.[78] However, in the formation of PCMC, rapid crystallisation results in the protein molecules passing through the denaturing conditions extremely quickly and as such become fortuitously trapped in the native state. [75, 80]

In PCMC formation, protein molecules act as a 'poison'[75] to the crystallisation of excipient and are therefore driven out to the surface of the crystal which is still growing. Due to the insoluble nature of protein molecules in organic solvent, they remain at the crystal surface. This process has been termed Crystal Lattice Mediated Self-assembly (CLAMS).[80]

1.4.3 Applications of PCMC Technology

The immobilisation of biomolecules to form PCMC has proven to be a very versatile method for the production of bioactive species. PCMC technology can be used for the immobilisation of enzymatic species, vaccines, therapeutic proteins, DNA and nanoparticles.

1.4.3.1 Immobilisation of Enzymes

One of the major applications of PCMC technology is in the manufacture of biocatalysts. The potential use of enzymes in organic synthesis is huge, but one of the major problems is the need to be able to carry out these reactions in organic solvent.[77] There is therefore an ever expanding need to find a generally applicable method for the immobilisation of enzymes for use in biotransformations.[29]

The immobilisation of enzymes as PCMC produces a protein coated powder which is soluble under aqueous conditions. In the organic phase, PCMC are insoluble and can therefore be employed in chemical transformations.[75] The enzyme remains active (and in a number of cases, proves to be more active than their non-immobilised counterpart)[77, 81] and can be easily retrieved from this medium for reuse.[76]

1.4.3.2 Immobilisation of Vaccines and Therapeutic Proteins

A major stumbling block in the supply of vaccine and therapeutic protein products is lack of thermal stability and maintenance of the cold chain.[82] Immobilisation of vaccines upon the surface of PCMC results in the formation of a dry powder formulation with increased stability and no need for refrigeration.[79, 83] Examples of vaccines which have been immobilised in this way include diphtheria toxoid, anthrax vaccine and the adenylate cyclase toxin of Bordetella pertussis.[79, 83] Therapeutic proteins such as insulin have also been immobilised. The particles are approximately 5 μm in size (depending upon the excipient material) and therefore are of suitable size for inhalation therapies.[84]

1.4.3.3 Immobilisation of DNA

There is an example in the literature of the use of PCMC technology to immobilise DNA molecules.[85] In exactly the same way as proteins, nucleic acids can be immobilised on to the surface of crystals prior to use as a primer in the polymerase chain reaction (PCR). The bioactivity of an immobilised oligonucleotide was retained, resulting in the correct gene product being amplified and no bases were altered or truncated.[85] It is possible therefore to immobilise DNA using this method with little or no detrimental effect to the activity of the nucleic acid which can be used in subsequent transfections.[85]

1.4.3.4 Immobilisation of Nanoparticles

The self assembly of nanoparticles upon the surface of crystalline materials can result in the manufacture of products with interesting optical, magnetic, electronic and catalytic properties.[86-88] Rapid co-precipitation of an excipient material with gold nanoparticles

results in the formation of immobilised species which can be easily stored in the solid state without aggregation. The resultant particles are also amenable to further chemical modification and polymer inclusion.[89]

1.5 Protein Folding *via* Immobilisation on Crystal Surfaces - Aims and Outline of Work

The formation of Protein Coated Micro-Crystals (PCMC) is a novel method for the immobilisation of aqueous protein solutions upon the surface of a water-soluble excipient (salt, amino acid or sugar) *via* a co-precipitation reaction in a water miscible organic solvent.[75]

It is the aim of this project to establish if it is possible to reversibly adsorb functionally active proteins from the unfolded state *via* immobilisation onto the surface of water-soluble crystals of an amino acid, sugar or salt with the simultaneous removal of denaturant molecules. This method of protein immobilisation from the native state has been used successfully with a number of different proteins.[75, 76, 81] Can this method of immobilisation provide an alternative, faster, more convenient, more economic way to refold stable, readily-refoldable proteins such as lysozyme? Also can this method provide a more successful method for the refolding of more intractable proteins such as lipase?

Unfolded or solubilised protein molecules are generally present in a solution containing additives such as urea, guanidine hydrochloride or dithiothreitol (DTT).

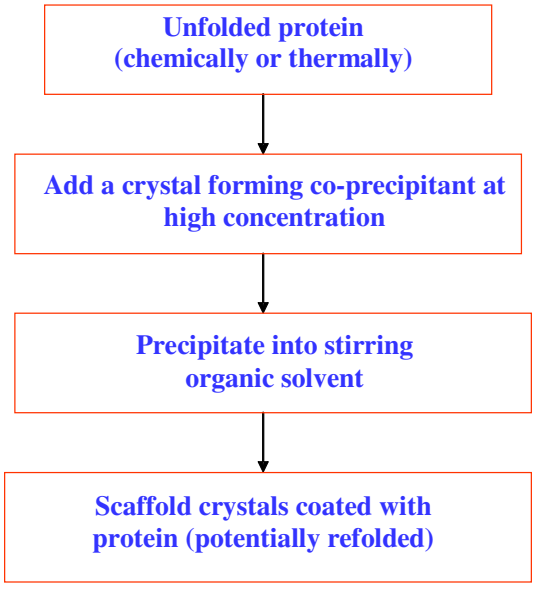


Figure 17: Preparation of PCMC of Potentially Refolded Protein

Upon mixing with a crystal forming component and subsequent precipitation, these solubilising molecules are removed from the protein environment due to the fact that they

are soluble in polar organic solvent. The protein and excipient remain insoluble in the organic phase and therefore are free to associate to form PCMC with the immobilisation of protein from the unfolded state.

An important consideration in the adoption of this approach for protein folding is at which stage of the manufacture of PCMC does the possible refolding of protein molecules occur? There are three possibilities: in the mixing of the excipient material with the denatured protein; the coprecipitation step; and upon the reconstitution of these dry powders into aqueous solution. Each of these steps will be considered.

In order to assess the feasibility of this approach, a number of physical methods including fluorescence, circular dichroism and UV/Vis spectroscopy, will be employed. The success and limitations of this approach will be described utilising lysozyme and lipase.

Chapter Two - Materials and Methods

2 Materials and Methods

2.1 Materials

Lysozyme (EC 3.2.1.17) from hen egg white (≥90 % purity), Lipase (EC 3.1.1.3, type I) from wheat germ (≥80 % purity), micrococcus lysodeikticus (ATCC No. 4698), urea (MW = 60.06; ≥ 99.5 % purity), guanidine hydrochloride (MW = 95.53; 98 % purity), potassium sulfate (MW = 174.26; ≥ 99 % purity), D,L-valine (MW = 117.15; ≥ 99.5 % purity), L-glutamine (MW = 146.14; ≥ 99.5 % purity), L-cystine (MW = 240.30; 99.5 % purity), L-cysteine (MW = 121.16; ≥ 99.5 % purity), *para*-nitrophenyl acetate (MW = 181.15; 99.5 % purity), sodium phosphate (MW =119.98; ≥ 99.0 % purity), trizma hydrochloride (MW = 157.60; ≥ 99.5 % purity), D,L-dithiothreitol (MW = 154.25; ≥ 99.5 % purity) were purchased from the Sigma-Aldrich company (Dorset, UK). Potassium chloride (MW = 74.55; ≥ 99.5 % purity) was sourced from BDH though VWR International, Lutterworth, UK. Propan-2-ol (≥ 99.5 % purity, HPLC grade) was purchased form Reidel-de-Haën. Ethanol (absolute purity) and Millipore filtration equipment (flask, filter and funnel) and filter membranes (47 mm diameter, 0.45 μm pore size) were purchased from Fisher Scientific. Dialysis cassettes of 3 ml volume and molecular weight cut off (MWCO) of 10000 Da were purchased from Pierce. All materials were used as received, with no further purification or modification.

2.1.1 Lysozyme

Lysozyme is an enzyme for which the function has been known for many years. Discovered in 1921 by Sir Alexander Fleming,[90-92] it has adopted a role as one of the most researched proteins in terms of its structural and functional characteristics and is used extensively for protein unfolding and folding studies.[93] Lysozyme was the first enzyme to successfully undergo x-ray diffraction in order to obtain a three-dimensional structure.[94, 95] This research has lead to some understanding of the actual protein folding process and how errors in this process can lead to problems of misfolding, aggregation and disease.[96]

Lysozyme is a small, single chain globular protein which is present in many mammalian secretions and tissues.[97, 98] The function of the enzyme *in vivo* is to selectively hydrolyse the β-(1,4) glycosidic linkage which is present in the cell walls of many micro-organisms and as a consequence of this, lysozyme is known to have antimicrobial properties.[99] The

most researched source of lysozyme is that from hen egg white, the structure of which is shown (Figure 1):

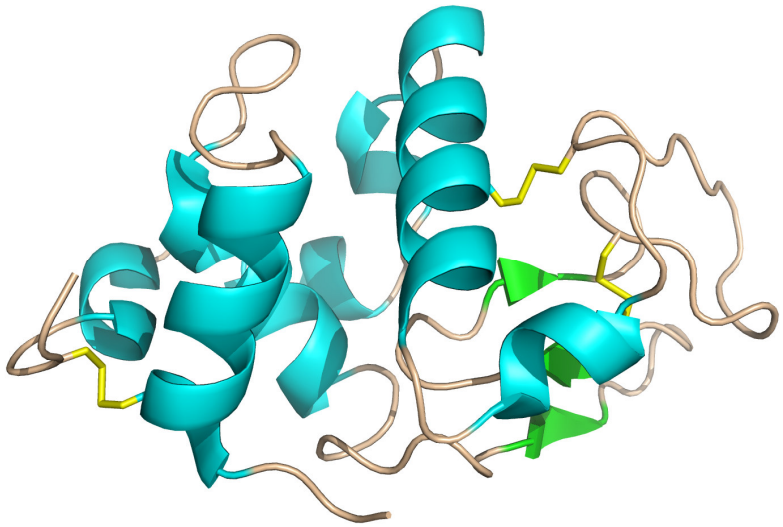


Figure 18: A Ribbon Structural Representation of Lysozyme (Image used with permission of Dr. A. McEwan, Department of Chemistry, University of Glasgow)

The protein is made up of 129 amino acid residues and physical properties including a molecular mass of 14.2 kDa. It is also known to have an isoelectric point at pH 11 and an optimum pH of 9.2.[98] There are four disulfide bonds present in lysozyme.[100]

2.1.2 Lipase

Lipase is an esterase belonging to the family of serine proteases.[101] It is used routinely in organic synthesis as a catalyst for the hydrolysis of ester bonds including triglycerides and carboxylic acid esters.[102] The enzyme also has a number of uses in food industries and can be used for the manufacture of baked goods, cheeses and even biofuels.[103]

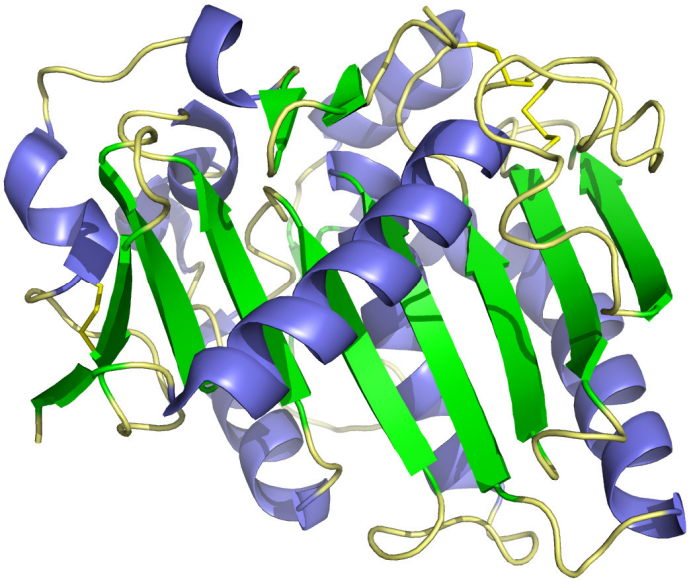


Figure 19: A Ribbon Structural Representation of Lipase (Image used with permission of Dr. A. McEwan, Department of Chemistry, University of Glasgow)

Lipase from wheat germ (Triticum aestivim) contains 350 amino acid residues and has a molecular weight of approximately 42 kDa and a pH optimum of 8.0.[101, 104] The enzyme is comprised of 20 % α-helix, 40 % β-sheet and the rest is of aperiodic structure.[101]

2.2 Methods

2.2.1 Determination of Protein Concentration

2.2.1.1 UV/Visible Absorption Spectroscopy

Ultra-violet absorption spectroscopy has been shown to be an invaluable tool for the determination of protein concentration[105, 106] UV absorption in the region of 250 - 300 nm gives information regarding the aromatic amino acid residues, whilst down towards 200 nm, the amide group of the peptide backbone is found. These are the chromophores present in protein which each have a characteristic transition or signals in these regions.[107] UV-visible spectrophotometers are used for this purpose, measuring the intensity of a light beam passing through a sample as a function of wavelength.[108] Absorption spectrophotometers can be either single beam or double beam instruments. The optical path of a single (split) beam instrument such as the one used for this study is shown.

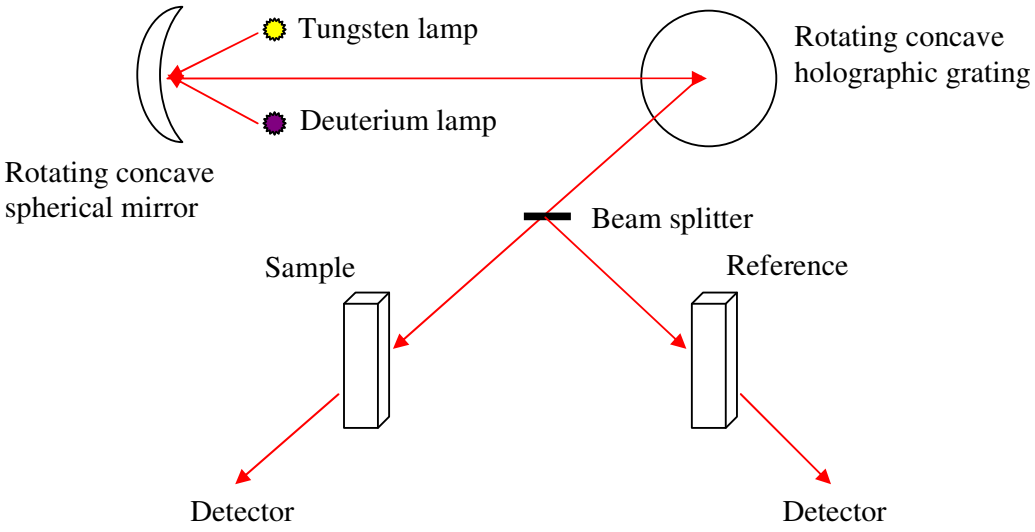


Figure 20: The Optical Path of a Single (Split) Beam Spectrophotometer [108]

In order to measure the absorbance of a sample at different wavelengths, tungsten and deuterium lamps are used to provide light in the visible (340-800 nm) and UV(200-350 nm) ranges, respectively. The light is reflected to a rotating concave holographic grating, which splits the light into its constituent wavelengths so that the absorbance at each of these wavelengths can be measured simultaneously by the detectors. The beam splitter allows the absorbance of a sample and a reference to be measured at the same time.[108]

2.2.1.1.1 Molar Extinction Coefficient and the Beer-Lambert Law

In order to determine protein concentration, it is first necessary to obtain the molar extinction coefficient. The molar extinction coefficient is a measure of the ability of a protein sample to absorb light at a particular wavelength.[109] In the case of proteins, the wavelength of interest is 280 nm. This is where the aromatic amino acids such as phenylalanine, tyrosine and in particular tryptophan absorb light and give rise to measurable absorbance. This absorbance can be related to the concentration of protein present by the Beer-Lambert law;[110]

$$A(\lambda) = \log (I_0/I) = \varepsilon(\lambda)cl$$

Equation 1: The Beer-Lambert Law for the Determination of Protein Concentration [111]

Where A is the absorbance at a specific wavelength, I and I_0 are the transmitted and incident light respectively, ε is the molar extinction coefficient at a specific wavelength, c is the protein concentration and l is the path length of the cuvette.[111]

A UV spectrum of the protein at different concentrations (by weight) is obtained and a calibration graph prepared. The gradient of this calibration plot is equal to the extinction coefficient of the desired protein. This experimentally determined coefficient can then be used to determine the concentration of protein in future samples. The experimentally derived extinction coefficient can be different from published values. This difference can be attributed to impurities present in the preparation received from the supplier or simply experimental error.

The extinction coefficient can also be estimated for proteins of known sequence based upon the number of tryptophan and tyrosine residues present. These aromatic amino acids are the primary chromophore when measuring the absorbance of a protein sample at 280 nm.

To determine the extinction coefficient of the desired proteins and further determine the protein concentration of samples, Shimadzu UV-160A and Thermo Biomate 5 spectrophotometers were used.

2.2.1.2 Determination of Lysozyme Concentration

In order to determine the concentration of lysozyme present in samples, a calibration graph was prepared of the absorbance at λ = 280 nm with respect to the concentration (by weight). The data was obtained in triplicate and the graph shown is an average of the data.

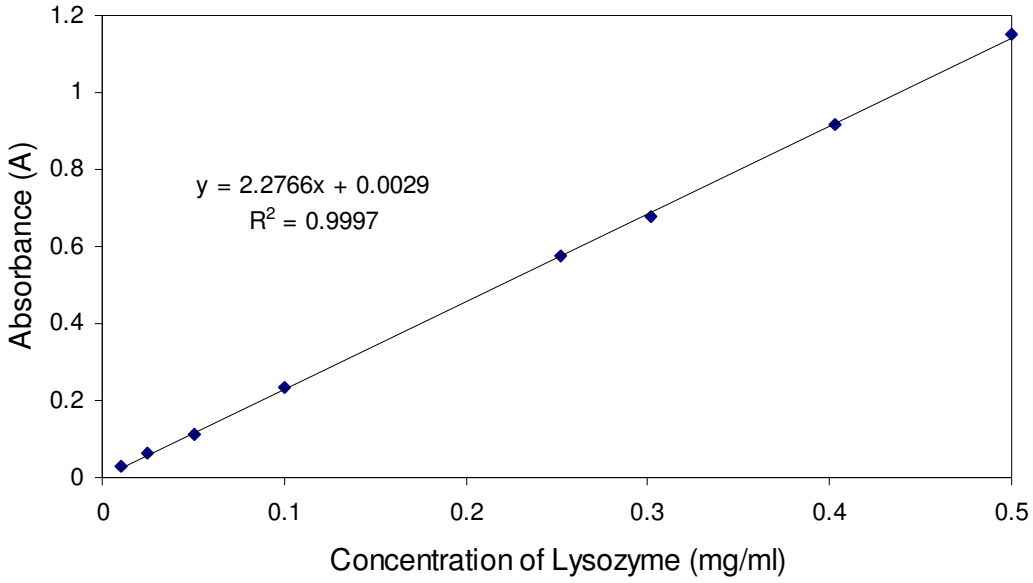


Figure 21: Calibration Graph of Lysozyme Concentration

The extinction coefficient of lysozyme determined from this calibration graph is 2.28 mL mg^{-1} cm^{-1} which was used to calculate the concentration of lysozyme present in samples prepared. The published extinction coefficient for lysozyme from hen egg white is 2.64 mL mg^{-1} cm^{-1}.

2.2.1.3 Determination of Lipase Concentration

To determine the concentration of lipase present in samples, a calibration graph was prepared of the absorbance at λ = 280 nm with respect to the concentration (by weight). The data was obtained in triplicate and the graph shown is an average of the data.

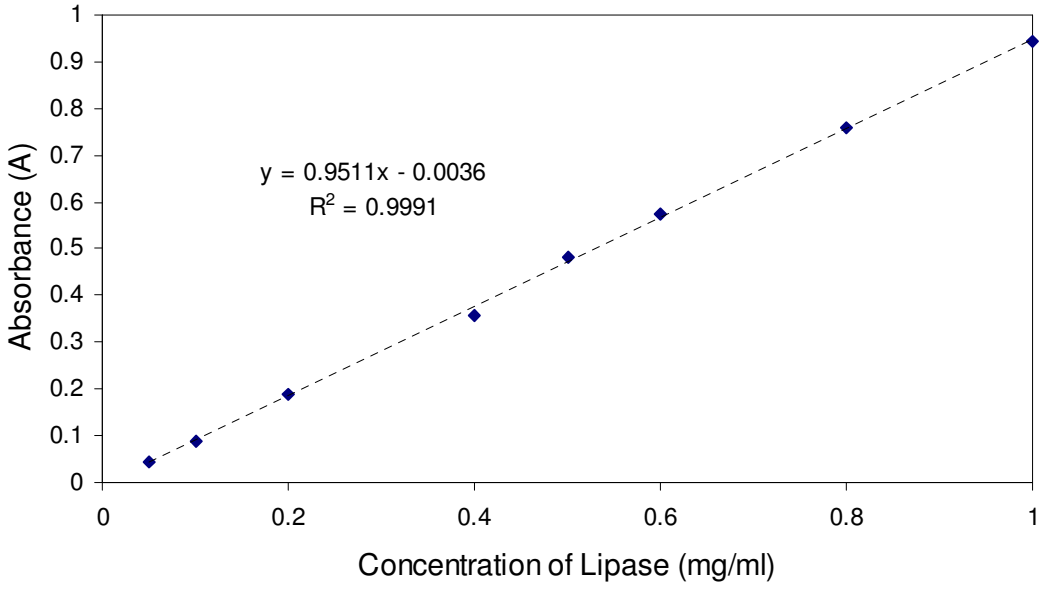


Figure 22: Calibration Graph of Lipase Concentration

The extinction coefficient of lipase determined from this calibration graph is 0.95 mL mg^{-1} cm^{-1} and this was used to calculate the concentration of lipase present in samples prepared. The published extinction coefficient of lipase from wheat germ is 1.75 mL mg^{-1} cm^{-1}.

2.2.2 Determination of Enzymatic Activity

The activity of an enzymatic sample can be routinely determined by carrying out an activity assay. This involves measuring spectrophotometrically the changes in the absorption characteristics of enzymatic substrates during chemical transformations.[112] Monitoring this observed change in absorbance with respect to time allows a calibration graph to be produced, from which the activity of enzyme samples can be determined.

The specific activity of an enzyme is a measure of the change in absorbance of the reaction mixture per unit time, per milligram of protein present. Activity assays used for lysozyme and lipase are described.

To determine the activity of the enzymes lysozyme and lipase, activity assays were employed using either a Shimadzu UV-160A or Thermo Biomate 5 spectrophotometers.

2.2.2.1 Determination of Activity of Lysozyme

Native lysozyme has antimicrobial properties and therefore has the ability to break down the cell walls of certain bacteria. This attribute is used in order to determine the activity of the enzyme. Micrococcus lysodeikticus cells are the most commonly utilised substrates in measuring the activity of lysozyme.

The enzyme breaks down the bacterial cell wall, giving rise to a change in the turbidity of the suspension as the cell contents spill into the supernatant. This change in turbidity can be seen spectrophotometrically by monitoring the absorbance at 450 nm at 25 °C. The rate of change of the absorbance of the suspension is indicative of the activity of the enzyme.

To carry out the assay, a suspension of Micrococcus lysodeikticus (9 mg in 30 ml 0.1 M potassium phosphate buffer, pH 7.0) and solutions of lysozyme of known concentration are required.[113]

Reference Cuvette:

1.45 ml Micrococcus lysodeikticus cells

0.05 ml H_2O

Sample Cuvette:

1.45 ml Micrococcus lysodeikticus cells

0.05 ml lysozyme of various concentrations in H_2O

The activity of each concentration of lysozyme is measured over a 5 minute time frame and is obtained in triplicate in order to prepare a calibration graph. The change in absorbance is negative as the turbidity of the suspension decreases in the cuvette.

In the course of this project, 2 different bottles of lysozyme were used to prepare immobilised samples. Calibration graphs were prepared for each lot of enzyme.

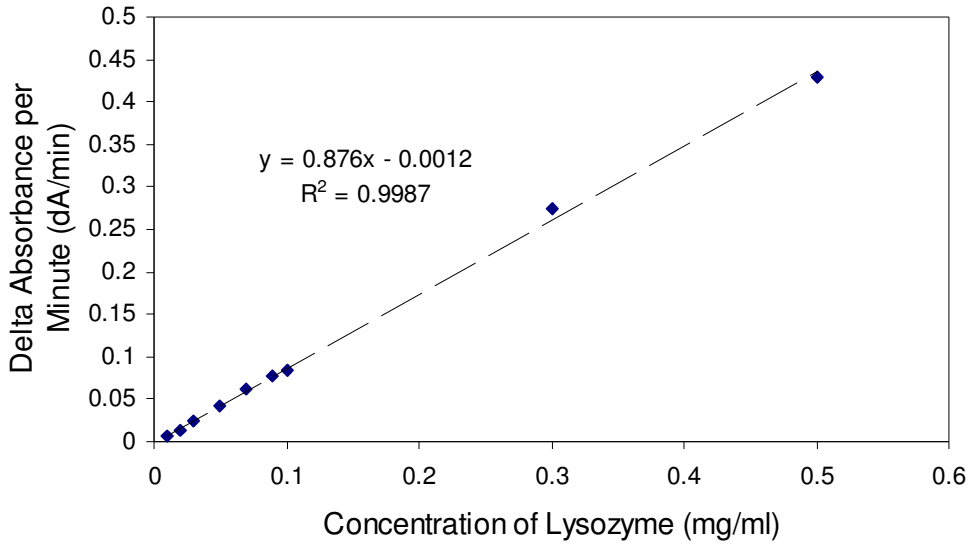


Figure 23: Calibration Graph of Lysozyme Activity

The specific activity of both lots of enzyme was determined by the method described above and was found to be 0.876 and 0.650 A $min^{-1}mg^{-1}ml^{-1}$ respectively. The specific activity of the lysozyme utilised will be quoted with each set of samples prepared.

2.2.2.2 Determination of Activity of Lipase

A simple method for the determination of the activity of lipase is the spectrophotometric monitoring of the hydrolysis of *para*-nitrophenyl acetate.[114]

Lipase is known to catalyse the hydrolysis of the aromatic ester *para*-nitrophenyl acetate (*p*-NPA) to the *para*-nitrophenoxide anion (*p*-NPO^-) in the presence of water.

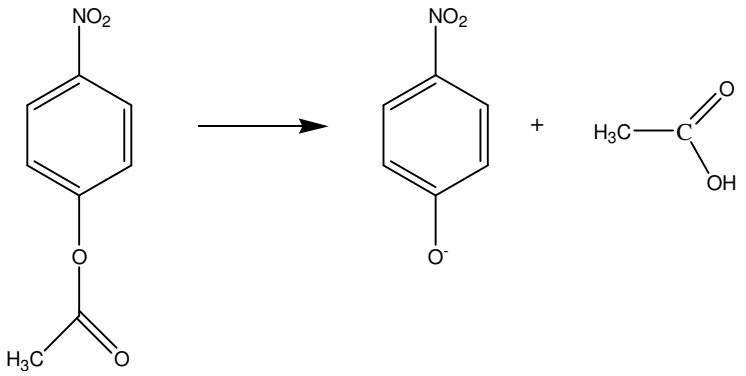


Figure 24: Hydrolysis of *p*-NPA catalysed by Lipase

The substrate *p*-NPA is insoluble in water and it is therefore initially dissolved in acetonitrile to carry out this assay. The *p*-NPA is a colourless solution, but the phenoxide anion is coloured yellow. This means that not only is the formation of product visible to the eye, but also by measuring the change in absorbance of the reaction mixture at 400 nm. If this change in absorbance is measured at 400 nm as the reaction proceeds, the rate of change of the absorbance is indicative of the rate of the hydrolysis reaction and can be measured in A $min^{-1}mg^{-1}$of protein (the specific activity).[114]

Reference Cuvette:

0.55 ml of 0.05 M sodium phosphate, pH 7.8

0.2 ml of 3 mM *p*-NPA in AcCN

Sample Cuvette:

0.5 ml of 0.05 M sodium phosphate, pH 7.8

0.2 ml of 3 mM *p*-NPA in AcCN

0.05 ml of lipase of various concentrations in 0.05 M sodium phosphate, pH 7.8

The activity of each concentration of lipase is measured over a three minute time frame and is obtained in triplicate to prepare a calibration graph.

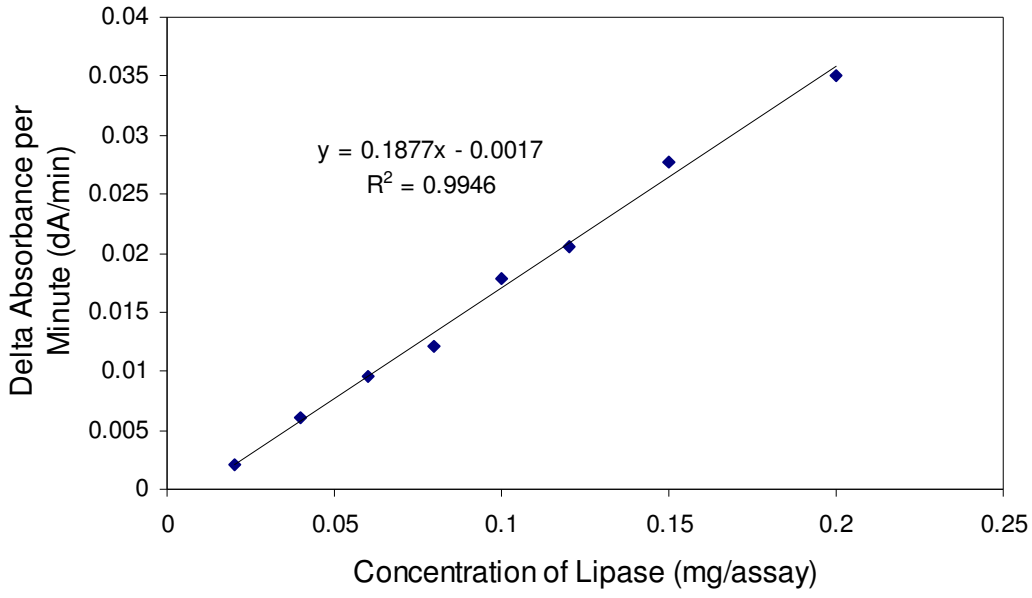


Figure 25: Calibration Graph of Lipase Activity

The specific activity of native lipase calculated from the calibration is 0.188 $Amin^{-1}mg^{-1}$.

2.2.3 Protein Unfolding

Protein molecules can be unfolded by employing both chemical and physical methods. Urea, GdnHCl and pH change are the most commonly used chemical methods for this purpose and physical methods include heating and pressure perturbation.

2.2.3.1 The Urea Unfolding of Proteins

The chemical unfolding of proteins by urea was carried out by dissolving the protein in a solution of 8 M urea in water or addition of the solubilising agent to an aqueous solution of protein. The unfolding of the protein was monitored by fluorescence and CD spectroscopy and activity assay where possible.

2.2.3.2 The Guanidine Hydrochloride Unfolding of Proteins

The chemical unfolding of proteins by GdnHCl was carried out by dissolving the protein in a solution of 6 M GdnHCl in water or addition of the salt to an aqueous solution of protein. The unfolding of the protein was monitored by fluorescence and CD spectroscopy and activity assay where possible

2.2.3.3 The Thermal Unfolding of Proteins

The thermal unfolding of proteins was carried out by heating the protein to 100 °C and then allowing the protein to cool to room temperature. Upon cooling, the aggregates which form are solubilised upon the addition of 6 M GdnHCl or 8 M Urea. This process was monitored by DSC, fluorescence and CD spectroscopy and activity assay where possible.

2.2.4 Preparation and Isolation of PCMC

2.2.4.1 Preparation of PCMC Samples - Standard Method

PCMC are prepared using a standard protocol. This involves the mixing of protein molecules (in either the native or unfolded state) and the excipient material (salt or amino acid), before adding this mixture drop-wise to stirring organic solvent.

The theoretical protein loading (TPL) of the PCMC describes the percentage by mass of protein present to excipient material. In order to investigate the effect of loading of the biomolecule on to the crystal surface, different ratios of protein to excipient were used. This allowed for the preparation of PCMC samples of different protein loadings.

During the precipitation of the protein/excipient mixture in the stirring organic solvent, the water content of this solvent was kept constant (between four and five percent by volume).

2.2.4.2 Preparation of PCMC - Continuous Method

A further method was employed for the preparation of PCMC. A continuous method was developed which allowed the mixing of excipient and protein (either unfolded or native) to occur over a period of time before subsequent addition to the solvent. This was achieved using syringe pumps and generally a flow rate of 0.01 ml/min of excipient into protein was used before precipitation in organic solvent. The hypothesis that led to the design of this method was that the mixing of protein and excipient solutions over time would lead to the gradual dilution of the solubilising agents, therefore triggering refolding.

2.2.5 Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy is a technique which is commonly employed in determining the structural characteristics of proteins.[115] CD occurs when the left and right handed components of circularly polarized light are absorbed to differing extents by a chiral chromophore.[116, 117] The chirality of the chromophore can be intrinsic or *via* the linkage of a chiral centre or placing in an asymmetric environment.[115] When CD is measured over a range of wavelengths, this gives rise to a CD spectrum. The unit commonly utilised in CD measurement is the *mean residue ellipticity* denoted $[\theta]_{mrw},\lambda$ at a given wavelength is $deg.cm^2.dmol^{-1}$.[118]

There are 2 main classes of chromophore in protein molecules; the peptide backbone and the amino acid side chains. The peptide backbone can be analysed in the far UV region (240-180 nm) to determine the helical or sheet content of the protein.[115]

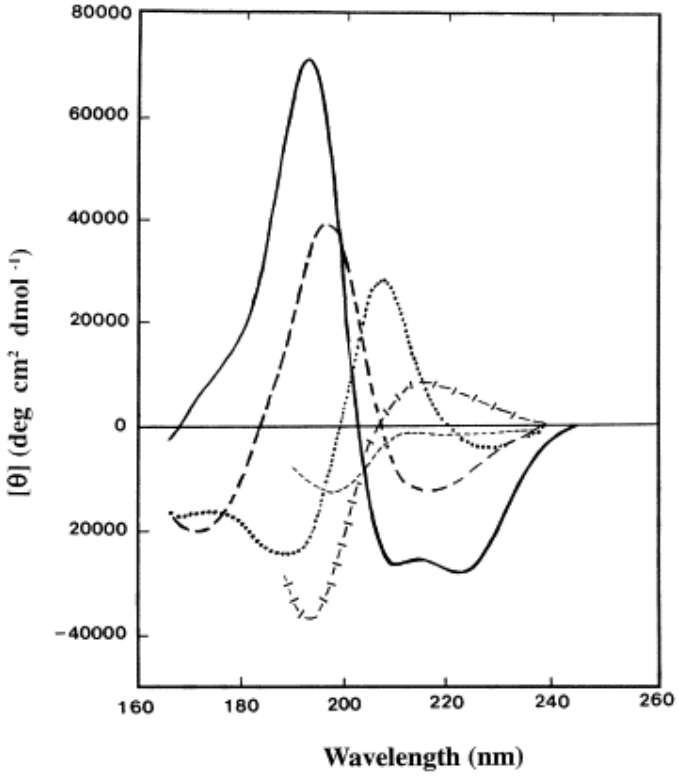


Figure 26: Far UV CD Spectrum Associated with Protein Secondary Structural Elements. Solid line, α-helix; long dashed line, antiparallel β-sheet; dotted line, type I β-turn; cross dashed line, extended 3_{10}-helix or poly (Pro) II helix; short dashed line, irregular structure.[118]

The CD of the aromatic amino acid side chains can be observed in the near UV region (320-260 nm) giving information about the environment of the side chains and the tertiary structure.[115, 118] This means that CD spectroscopy is therefore a useful tool in monitoring protein folding events and structural changes.[32]

2.2.5.1 CD Spectroscopy in the Aqueous Phase

CD spectroscopy is most commonly used upon aqueous samples of protein, free of any dust or other suspended particles. This is due to the fact that if there are suspended particles present in the CD sample, then this can cause scattering of the light and lead to a red-shifted CD spectrum.[119] It is therefore important to ensure that samples are free of such artefacts.

2.2.5.1.1 CD Spectroscopy in the Aqueous Phase - Protocol

All CD measurements were made with the guidance of Dr. Sharon M. Kelly of the Protein Characterisation Facility at the University of Glasgow and were made on a Jasco J-810 spectropolarimeter with model number J-810-150S.

Near UV measurements were made between 320 nm and 250 nm. A data pitch of 0.2 nm (distance between data points) was used with the instrument was in continuous scanning mode. A scanning speed of 10 nm/min, response time of 2 seconds and a bandwidth of 1 nm were also employed. Accumulations of 3 scans were recorded of the protein at approximately 1 mg/ml. The cell path length used for carrying out these measurements was 0.5 cm.

Far UV data was collected between 260 and 180 nm. A data pitch of 0.2 nm was used with the instrument in continuous scanning mode. A scan speed of 50 nm/min, response time of 0.5 seconds and a bandwidth of 1 nm were also employed. The CD spectrum of each sample was collected 4 times and subsequently accumulated to give a smooth spectrum using a cylindrical cell of path length 0.02 cm and a protein concentration of approximately 0.5 mg/ml.

2.2.5.2 CD Spectroscopy in the Solid Phase

There is great interest in the development of a spectroscopic method which allows the structure of immobilised protein and other particulate suspensions to be probed. Spectroscopic investigations of immobilised proteins usually involve the protein being desorbed before the measurements take place in an effort to avoid light scattering.[120]

CD spectroscopy can now be carried out on proteins immobilised upon hydrophobic and hydrophilic surfaces, although light absorption and scattering by the immobilisation matrices can lead to errors.[121]

A recent development in this field allows the collection of CD spectra of protein immobilised on the surface of PCMC, alhydrogel or silica.[122] This method involves incorporating a motorised rotating cell holder into the spectropolarimeter, which allows the immobilised sample to remain suspended during the time of measurement.

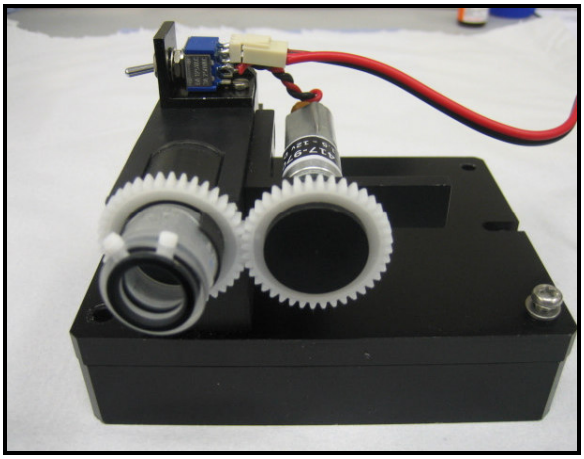


Figure 27: The Rotating Cell Holder (Image used with permission of Dr. A. Ganesan, XstalBio Ltd.)

The use of a rotating cell holder avoids the sedimentation process which is a major problem associated with CD measurement of particulate suspensions.

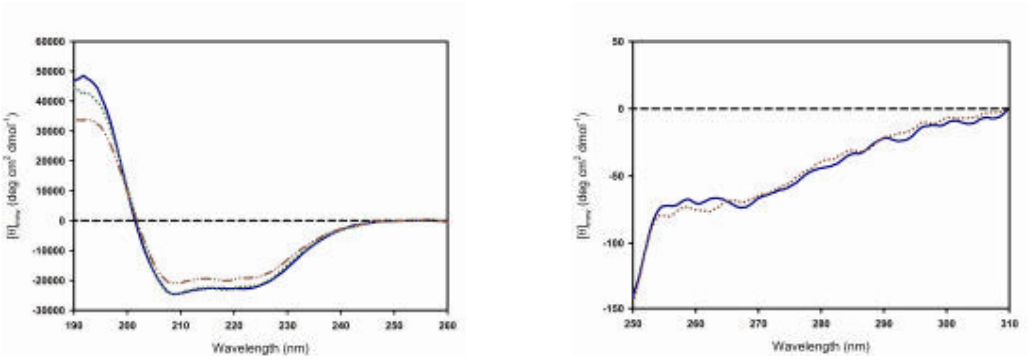


Figure 28: Far and Near UV CD Spectra of Immobilised BSA. Soluble BSA - solid line; BSA on alhydrogel (uncorrected), dot-dash; BSA on alhydrogel (5% w/w corrected) dotted [122]

In the figure above, it can be seen that the CD spectra obtained from the corrected alhydrogel immobilised protein is similar to that obtained from the soluble protein. This indicates that the structure of BSA immobilised upon the surface of the particles is very similar to the native form.

The rotation speed (usually 40-100 rpm) and cell path length must be optimised in order to maintain the uniformity of the suspension and as such avoid the uneven distribution of the immobilised protein particles in the light beam.[122]

The use of a rotating cell holder circumvents the errors associated with sedimentation and light scattering and therefore can be used to obtain CD spectra of particulate suspensions in UV transparent solvents such as water and ethanol.[122]

2.2.5.2.1 CD Spectroscopy in the Solid Phase - Protocol

All CD measurements were made on a Jasco J-810 Spectropolarimeter with model number J-810-150S.

Near UV measurements were made between 320 and 250 nm. The rotating cell holder was employed using a regulator setting of 4.5 V (this determines the speed of rotation). A data pitch of 0.2 nm was used and the instrument was in continuous scanning mode. A scanning speed of 10 nm/min, response time of 2 seconds and a bandwidth of 1 nm were also employed. Accumulations of 6 scans were obtained using a cell of path-length 0.05 cm with approximate protein concentration of 1 mg/ml.

Far UV data was collected between 260 and 180 nm. The rotating cell holder was employed using a regulator setting of 3.0 V. A data pitch of 0.2 was used with the instrument in scanning mode. A scan speed of 50 nm/min, response time of 0.5 seconds and a bandwidth of 1 nm were also employed. The CD spectrum of each sample was collected 6 times and subsequently accumulated to give a smooth spectrum using a cylindrical cell of path length 0.02 cm with a protein concentration of approximately 0.5 mg/ml.

2.2.6 Fluorescence Spectroscopy

Fluorescence spectroscopy is one of the most widely utilised tools in monitoring structural perturbations and environmental changes in protein molecules.[123] Fluorescence is a phenomenon which occurs when excited molecules return to the electronic ground state. Generally, when molecules de-excite, they return to the ground state non-radiatively. In fluorescence, the molecules undergo the process of vibrational relaxation (due to intermolecular collisions) which reduces the energy of the excited species.[124] The molecule therefore returns to the ground state from a lower energy excited state and therefore emits light of a lower energy (higher wavelength) than that used to excite the molecule.

The wavelength at which the maximum fluorescence intensity occurs (λ_{max}) is determined by the difference in energy between the ground state and the excited state. The polarity of

the molecular environment (solvent) can affect this. Upon excitation, an electron (which is negatively charged) is separated from the positive nucleus. This leads to a change in the dipole moment of the molecule. The energy required to induce this dipole depends upon the polarity (or dielectric constant) of the environment. If a molecule is present in a polar solvent, the energy required for excitation is lower than that for a non-polar medium. This means that as the polarity of the surroundings increases, the energy required for excitation decreases and therefore λ_{max} increases. [40]

Fluorescence of protein molecules occurs due the intrinsic properties of the aromatic amino acid building blocks.[123] The technique of fluorescence spectroscopy makes use of this natural phenomenon, although in some instances, fluorescent probes can be used in order to introduce fluorescent properties into molecules.[123]

A spectrofluorimeter is comprised of a source of light and an excitation monochomator. This allows different excitation wavelengths to be selected. Once the light has passed through the sample, the emitted fluorescence is measured at right angles to the incident light by a detector. A schematic representation of this is shown.

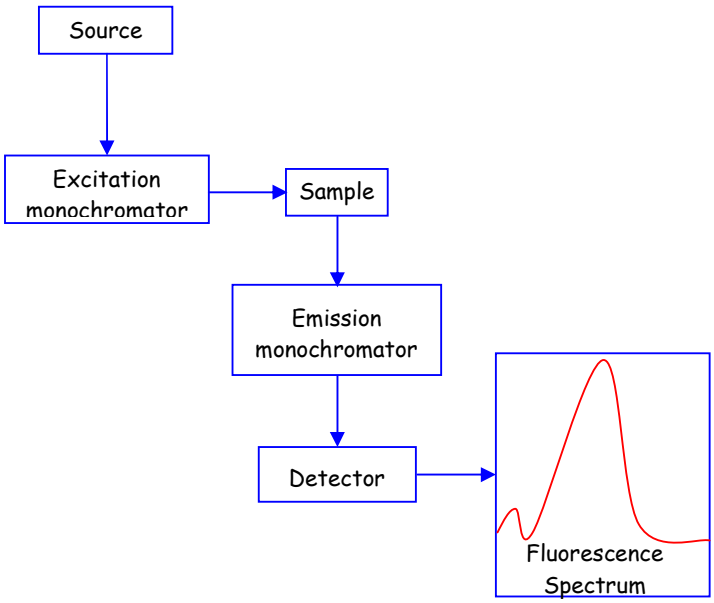


Figure 29: A Schematic Representation of the Path of Light Through a Fluorimeter [125]

Tryptophan fluorescence is commonly used in protein folding studies. Tryptophan residues give rise to the largest fluorescence intensity with relatively few of these residues being present in the protein.[126]

Irradiating protein samples with light of 290 nm excites the electrons of tryptophan residues and they therefore emit fluorescence. The value of λ_{max} is indicative of the microenvironment of the tryptophan residues.[127] Low wavelengths suggest that the tryptophan residues are buried within the hydrophobic, non-polar structure of the protein molecules. However, a shift towards higher wavelengths suggests that the tryptophan residues are no longer buried and are therefore able to interact with the surrounding polar solvent, changing the nature of their environment from the hydrophobic core of the protein, to the exterior solvent.[128]

2.2.6.1 Fluorescence Spectroscopy - Protocol

To carry out fluorescence measurements, a Spex FluoroMax spectrofluorimeter was employed. Samples were excited at 290 nm and fluorescence emission was measured between 280 nm and 460 nm. In the case of each sample, relevant baselines were subtracted from the sample spectrum (e.g. buffers, salts etc.). All spectra were collected at 25 °C.

2.2.7 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) is a technique used to study the energetics of transitional changes in protein molecules. This is a direct method to measure the heat energy uptake in a sample of known concentration as the temperature is being increased or decreased in a controlled fashion.[129] The energy required in order to keep both the sample and the reference cells at the same temperature is measured and hence DSC data is obtained.[130] A schematic representation of a differential scanning calorimeter is shown.

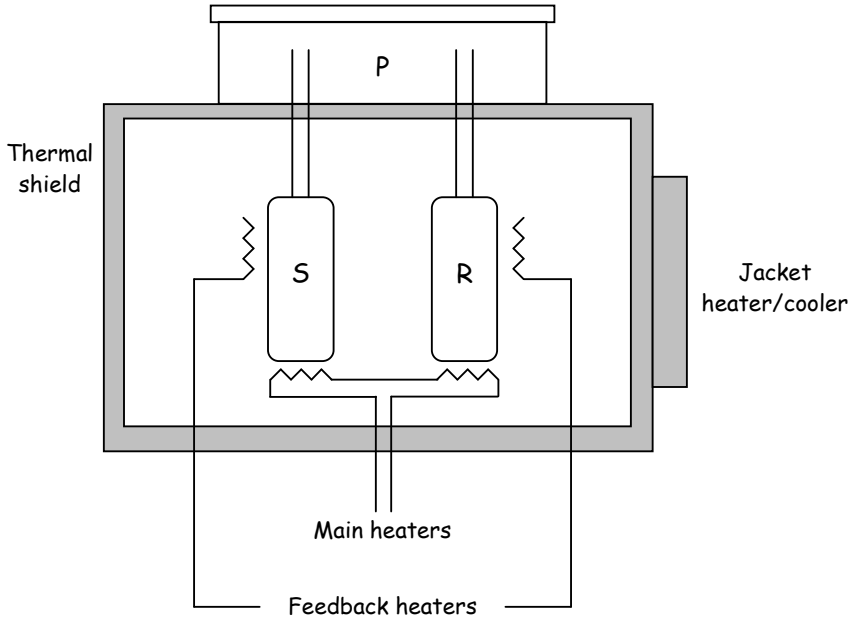


Figure 30: A Schematic Representation of a DSC Instrument. S, sample; R, reference; P, small pressure applied to the system.[129]

In a DSC instrument, the main heaters increase the temperature of both the sample and reference cells. The temperature of both cells is increased and monitored carefully until there is a difference between the sample and reference (due to energy being used to carry out a thermal transition).[131] The instrument recognises this difference and supplies more heat energy, *via* the feedback heaters, to the cell at the lower temperature. This allows the system to maintain the zero temperature difference between the sample and reference.[132]

The temperature at which the most heat energy is required for the sample and reference to maintain that temperature, gives rise to a transition in the DSC thermogram. This is the T_m, the mid-point temperature of the transition from the folded to the unfolded state.[129]

2.2.7.1 DSC Measurement - Protocol

In the context of this project, the information required from the DSC experiments was simply the temperature at which the thermal unfolding of the proteins of interest takes place and the reversibility of this process.

DSC measurements were carried out on a MicroCal VP-DSC microcalorimeter and all buffers were centrifuged and degassed. Sample protein concentrations were determined using the previously described molar extinction coefficients and the scan rate if the instrument was set to 1 °Cmin^{-1}. For each sample, a thermogram of the buffer in which the protein was present was carried out to check for any artefacts and to use as a baseline. A

DSC thermogram of the sample was then obtained followed by a rescan to assess the reversibility of the unfolding process.

2.2.8 Dialysis

Dialysis is a purification method which allows proteins to be separated from small molecules by use of a semi permeable membrane, usually made from cellulose.[133] This membrane contains pores of a defined size so that there is a Molecular Weight Cut-Off (MWCO). This ensures that small molecules will be free to pass through the membrane into the surrounding medium, whilst keeping larger macromolecules within.[133]

Dialysis as a method can be used in order to exchange buffers or remove salts or other small molecules from the environment of proteins. The dialysis membrane containing protein and small molecules is immersed in an aqueous solution into which the latter can diffuse *via* the pores. Once equilibrium is reached, the dialysate is replaced until most of the small molecules have been removed from the environment of the protein and the concentration is at an acceptable level.

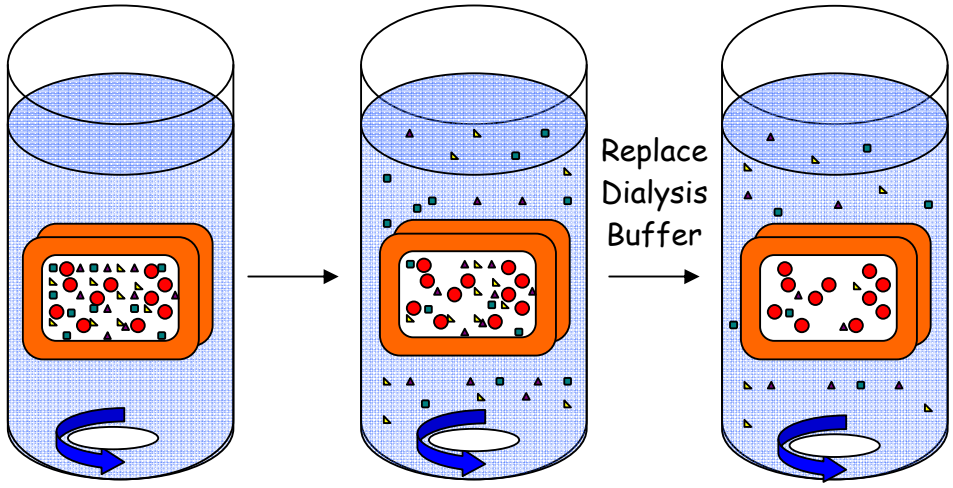


Figure 31: Dialysis - Gradual Removal of Small Molecules from Protein Environment

Dialysis is also a method commonly employed in protein folding methodology. The gradual removal of agents such as GdnHCl or urea from the protein is known to trigger the folding process and therefore dialysis is a simple method that may be used for protein folding experiments.

2.2.8.1 Dialysis - Protocol

Dialysis was carried out using dialysis cassettes with a 3 ml volume and molecular weight cut-off dependent upon the enzyme being used.

The solubilising agents were removed from the protein environment by dialysing against buffer in the absence of these additives. Unfolded lysozyme was dialysed against 50 mM Tris/HCl, pH 8.1. Lipase was dialysed against 50 mM Tris/HCl, pH 7.8.

Chapter Three - Unfolding, Immobilisation and Potential Refolding of Lysozyme

3 Unfolding, Immobilisation and Potential Refolding of Lysozyme

Techniques employed for the unfolding of protein molecules are generally found to take advantage of intrinsic properties of the biomolecules. Solutions of urea or guanidine hydrochloride are commonly utilised as they have the ability to increase protein solubility and interact at the protein surface to exclude solvent water (therefore dehydrating the protein).[134] These additives also have the capacity to interact with the hydrophobic core of protein molecules, therefore essentially pulling them apart from the inside out.[134] The physical nature of the interactions which hold protein molecules together can be easily exploited by these chaotropic agents.

A number of different methods can be used to unfold proteins such as lysozyme. Chemical and thermal unfolding are the most routinely practised due to their ease of implementation and success.

3.1 Chemical Unfolding of Lysozyme

3.1.1 Urea Unfolding of Lysozyme

In carrying out the urea unfolding of proteins, a number of things must be taken into consideration. It is necessary to prepare fresh urea solutions for each unfolding experiment. This is due to the fact that urea can form amidates with the amino acid residues The chemical unfolding of lysozyme can be monitored utilising using a number of different analytical techniques including activity assays, fluorescence and CD spectroscopy.[32]

3.1.1.1 Urea Unfolding of Lysozyme - Activity With Respect to Time.

In order to determine the optimal conditions for unfolding of lysozyme using urea, unfolding curves were prepared by determining the enzymatic activity with respect to time while varying the concentration of urea and reducing agent.

Lysozyme was dissolved in 50 mM Tris/HCl, pH 8.1 containing either 6 M or 8 M urea with 32 mM DTT. The activity of each of these samples was determined at set time intervals and an unfolding curve prepared.

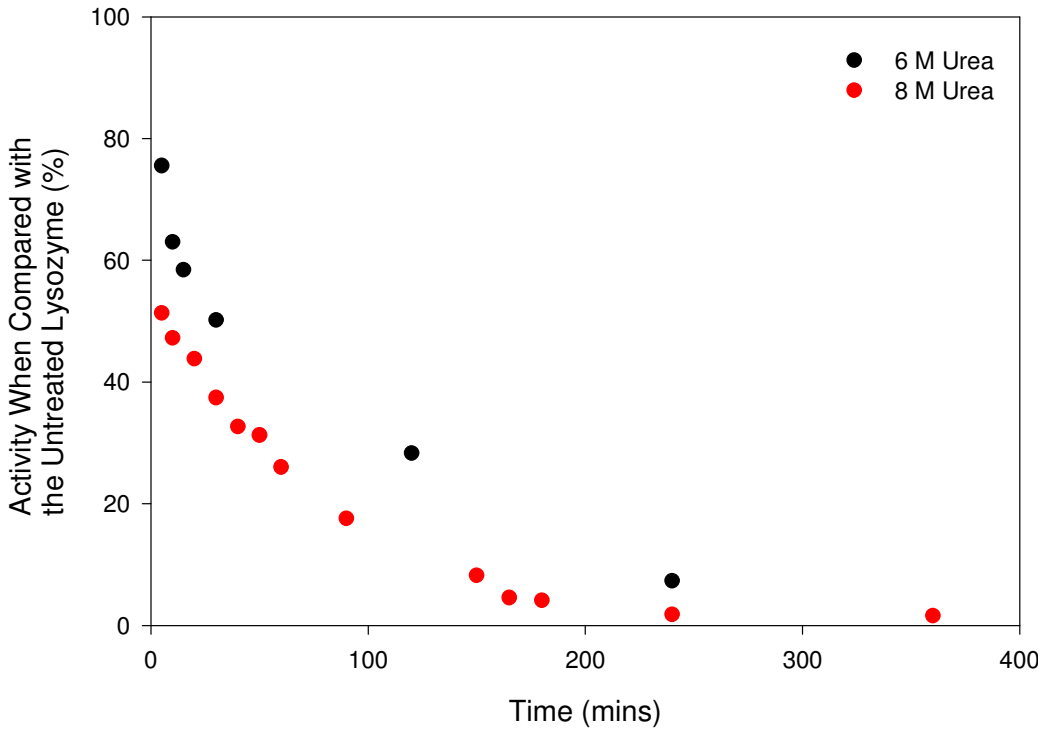


Figure 32: Urea Unfolding of Lysozyme with Respect to Time - Effect of Urea Concentration

As is expected, as the concentration of the chaotropic agent increases, the time which the protein takes to unfold is decreased. Unfolding in 8 M urea with 32 mM DTT allows the lysozyme to be unfolded after approximately four hours.

The activity of lysozyme incubated in 8 M urea containing different concentrations of DTT was also measured with respect to time. This was completed in order to see what effect (if any) the concentration of DTT had upon the time taken for the irreversible inactivation of lysozyme to occur.

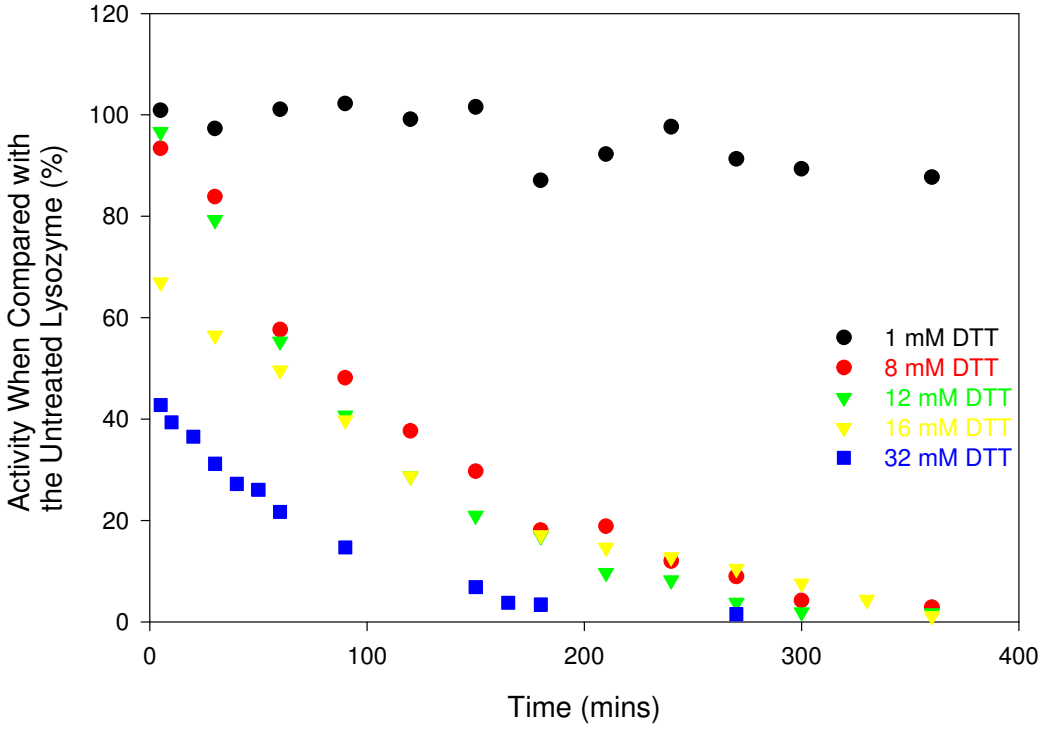


Figure 33: Urea Unfolding of Lysozyme with Respect to Time - Effect of DTT Concentration

DTT concentration plays an important role in the rate of lysozyme unfolding. The higher the concentration of DTT present, the faster the enzyme appears to unfold. As the concentration of DTT is increased, the ratio of the reducing agent with respect to protein increases. This results in rapid reduction of the disulfide bridges present in the protein. From the studies carried out here, optimal conditions for unfolding of lysozyme are 8 M urea and 32 mM DTT.

Incubation of lysozyme in 8 M urea in the absence of DTT does not have any significant effect upon the activity of the protein. Further studies were required in order to determine if this was due to the dilution of the denaturant from the environment of the protein upon

carrying out the activity assay (and hence refolding taking place to some extent) or if the protein was simply not being unfolded under these conditions.

3.1.1.2 Urea Unfolding of Lysozyme - Fluorescence Spectroscopy

The intrinsic fluorescent properties of lysozyme can be used in order to probe the enzyme structure both in the native and urea-incubated conformations.

Fluorescence spectra of lysozyme (0.1 mg/ml) incubated in 8 M urea (in the absence of DTT) were obtained with respect to time with a view to determining what was happening to the enzyme under these conditions.

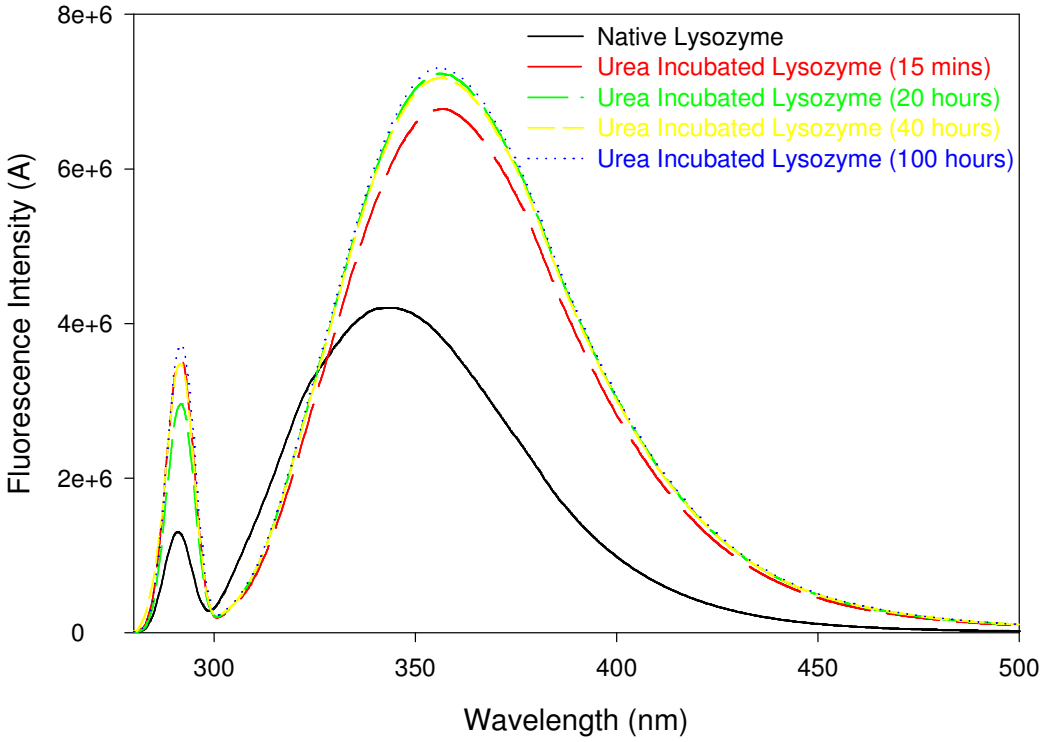


Figure 34: Urea Unfolding of Lysozyme with Respect to Time - Fluorescence Spectroscopy

In the case of native lysozyme, the wavelength at which the maximum fluorescence occurs is at 345 nm. This is typical for a globular protein in which the tryptophan residues are buried within the structure.[40]

Upon incubation of the protein in urea, the once buried tryptophan residues begin to interact with the external environment leading to a change in the fluorescent properties of the molecule. A red shift in the wavelength at which the maximum fluorescence intensity occurs is seen; therefore the maximum fluorescence intensity occurs at a longer wavelength. Incubation of lysozyme in urea alters the wavelength at which maximum

fluorescence occurred to 357 nm. This indicates that the tryptophan residues are no longer buried and are therefore free to interact with the surrounding solvent.

3.1.1.3 Urea Unfolding of Lysozyme - CD Spectroscopy

For protein folding and unfolding studies, CD spectroscopy is used routinely as it can provide a clear insight into structural changes with greater effectiveness than other spectroscopic methods.[116]

Circular dichroism spectroscopy of both the native and urea-incubated enzyme in the presence and absence of DTT were obtained. This was to ascertain the effect of the urea and DTT upon the structure of the protein and gain an insight into the effect upon the protein at a molecular level.

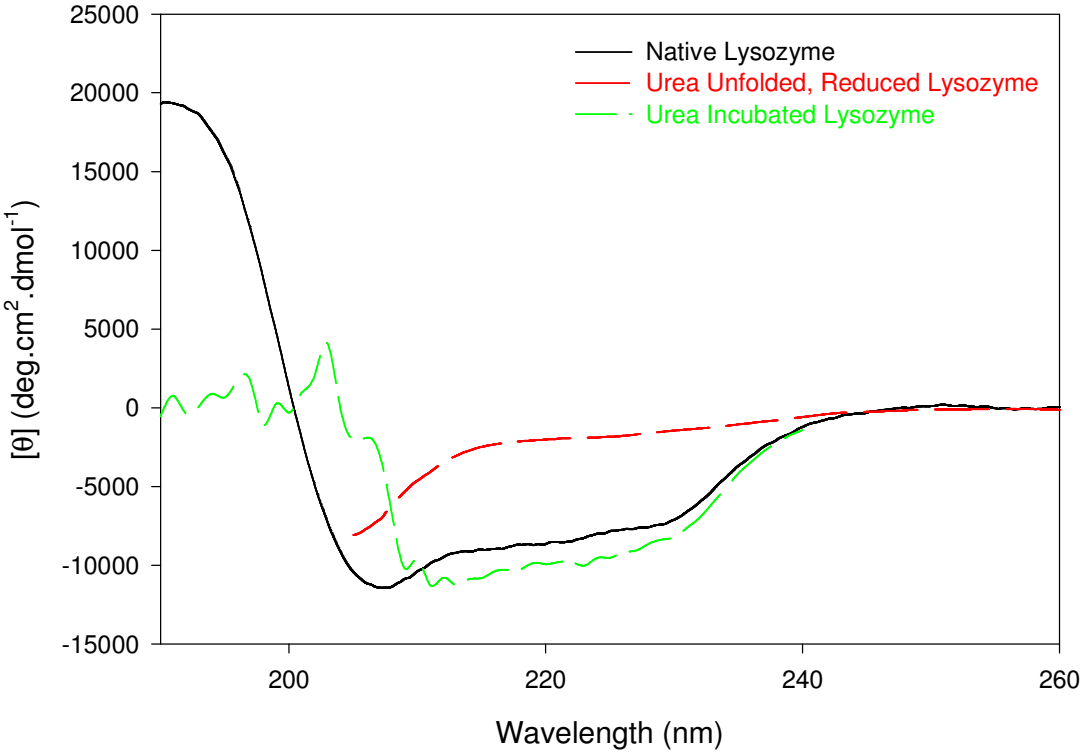


Figure 35: Far UV CD Spectrum of Lysozyme in the Native and Urea-Unfolded, Reduced State

The far UV CD spectrum of native lysozyme indicates that the protein is mainly composed of alpha helices. Incubation of lysozyme in 8 M urea in the absence of DTT appears to have some effect upon the secondary structure of the protein. Upon incubation of the enzyme in 8 M urea containing 32 mM DTT, the structure has changed. The secondary structure of the protein is no longer native, but appears to be unfolded. The presence of

DTT in the incubation buffer results in noise in the CD spectrum below 205 nm and therefore when measuring the spectrum of the urea-unfolded, reduced lysozyme, the spectrum is not recorded below that wavelength.

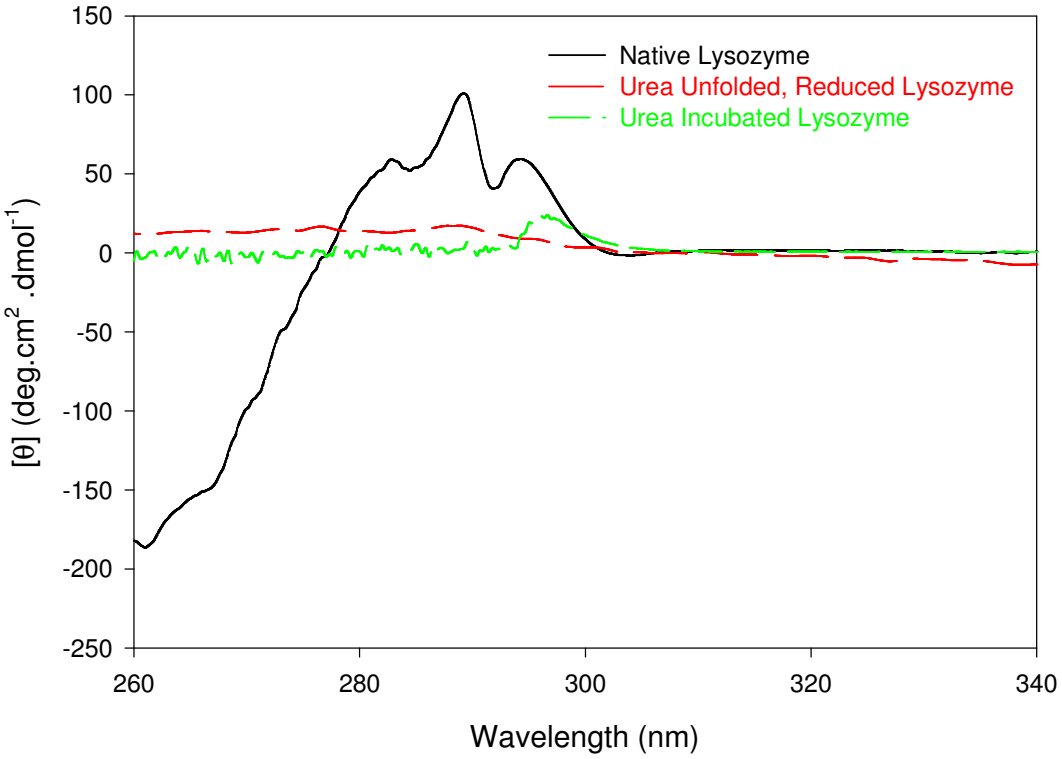


Figure 36: Near UV CD Spectrum of Lysozyme in the Native and Urea-Unfolded, Reduced State

The near UV CD spectrum of native lysozyme indicates that there are contributions from the aromatic amino acid residues present in the native structure. Incubation of the protein in 8 M urea results in the absence of near UV CD signal. This is due to the fact that urea has entered the charged, hydrophobic protein interior and has therefore affected the chirality of the amino acid environment which is required to give rise to CD.[50] In the urea-unfolded, reduced protein the urea molecules are not only able to permeate the protein interior, but the DTT present reduces the disulfide bonds present. This therefore results in a lack of CD signal in the near UV region.

3.1.1.4 Urea Unfolding of Lysozyme - Discussion

Lysozyme is routinely used for protein folding and unfolding studies due to the ease with which the enzyme will refold upon removal of the denaturing molecules from its environment.[135, 136] Activity measurements of urea-incubated and urea-incubated, reduced lysozyme indicate that in the absence of DTT, it is not possible to completely unfold

lysozyme. However, the observed activity could simply be an effect of the dilution of the urea from the lysozyme environment upon carrying out the aqueous activity assay. The observation that lysozyme activity decreases with respect to incubation time (rather than instantaneously) could equally be due to the effect of chemical modification of the amino acid residues by urea (amidate formation) or isomerization of proline.

Fluorescence measurements of lysozyme incubated in 8 M urea indicate that there is evidence of some kind of unfolding. A red-shift in the wavelength at which the maximum absorbance occurs is generally indicative of aromatic amino acids being exposed to the polar solvent, water (i.e. unfolding).[127] The shift in fluorescence maximum occurs almost immediately upon incubation of the protein in 8 M urea and can be attributed to the interaction of newly exposed aromatic amino acid residues, residing close to the surface of the protein, with solvent rather than those buried deep within the non-polar protein interior.[137]

Lysozyme incubated in 8 M urea containing 32 mM DTT for 4 hours showed no activity and the CD spectrum indicated that the protein was unfolded. This was due to the presence of DTT which reduced the disulfide bonds of the protein resulting in enhanced unfolding. The removal of disulfide bonds acts to decrease the overall stability of protein molecules. Specific examples of the removal of one of the disulfide bridges present in lysozyme (Cys6 - Cys127) results in various thermodynamic and kinetic consequences. Although the enzyme retains the same secondary and tertiary structure, its Tm is 25 °C lower than the untreated protein and the unfolded state becomes significantly more favourable (70 J K^{-1} mol^{-1})[100, 138]

High concentrations of urea are not in themselves sufficient to induce complete lysozyme unfolding, but may lead to chemical modification of the amino acid residues (hence we see a decrease in activity with respect to incubation time) or areas of localised unfolding. Spectroscopic evidence suggests that a reducing agent such as DTT is required to cause the complete collapse of lysozyme structure *via* the reduction of disulfide bonds.

3.1.2 Guanidine Hydrochloride Unfolding of Lysozyme

The chemical unfolding of lysozyme by salts such as 6 M guanidine hydrochloride was monitored using a number of spectroscopic methods including fluorescence and CD spectroscopy.[32]

3.1.2.1 Guanidine Hydrochloride Unfolding of Lysozyme - Fluorescence Spectroscopy

The GdnHCl unfolding of lysozyme was observed utilising fluorescence spectroscopy. Lysozyme (0.1 mg/ml) was dissolved in 50 mM Tris/HCl, pH 8.1 either in the presence or absence of GdnHCl. The GdnHCl sample was incubated for two hours at room temperature prior to carrying out any measurements.

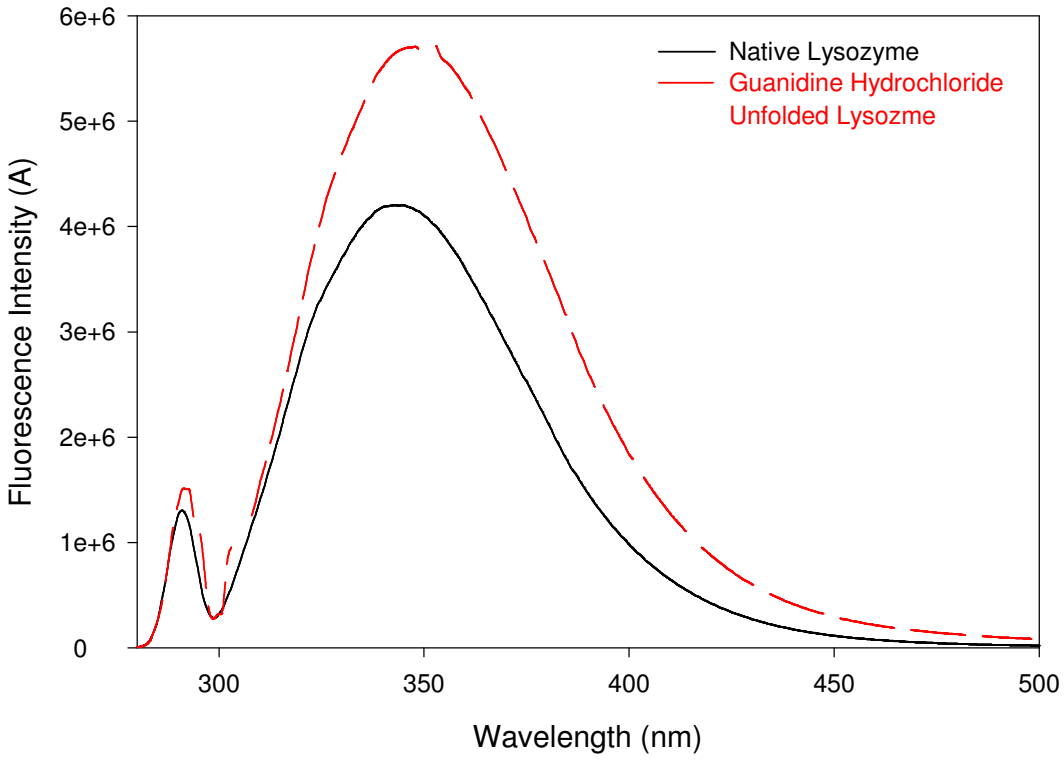


Figure 37: Guanidine Hydrochloride Unfolding of Lysozyme - Fluorescence Spectroscopy

Upon incubation for two hours in 6 M GdnHCl, lysozyme appears to be unfolded. The maximum fluorescence in the native sample is at 345 nm and in the case of the GdnHCl incubated sample; the maximum is at 352 nm. As the molecule begins to unfold, the buried aromatic residues begin to interact with the surrounding solvent. This subsequently changes the wavelength at which the maximum fluorescence occurs. This shift towards higher wavelength indicates that structural changes have occurred within the molecule and therefore the lysozyme is no longer in a native conformation

3.1.2.2 Guanidine Hydrochloride Unfolding of Lysozyme - CD Spectroscopy

Circular dichroism spectroscopy of both the native and guanidine hydrochloride incubated enzyme was obtained. This was to ascertain the effect of the salt upon the structure of the protein and gain an insight into the effect upon the protein at a molecular level.

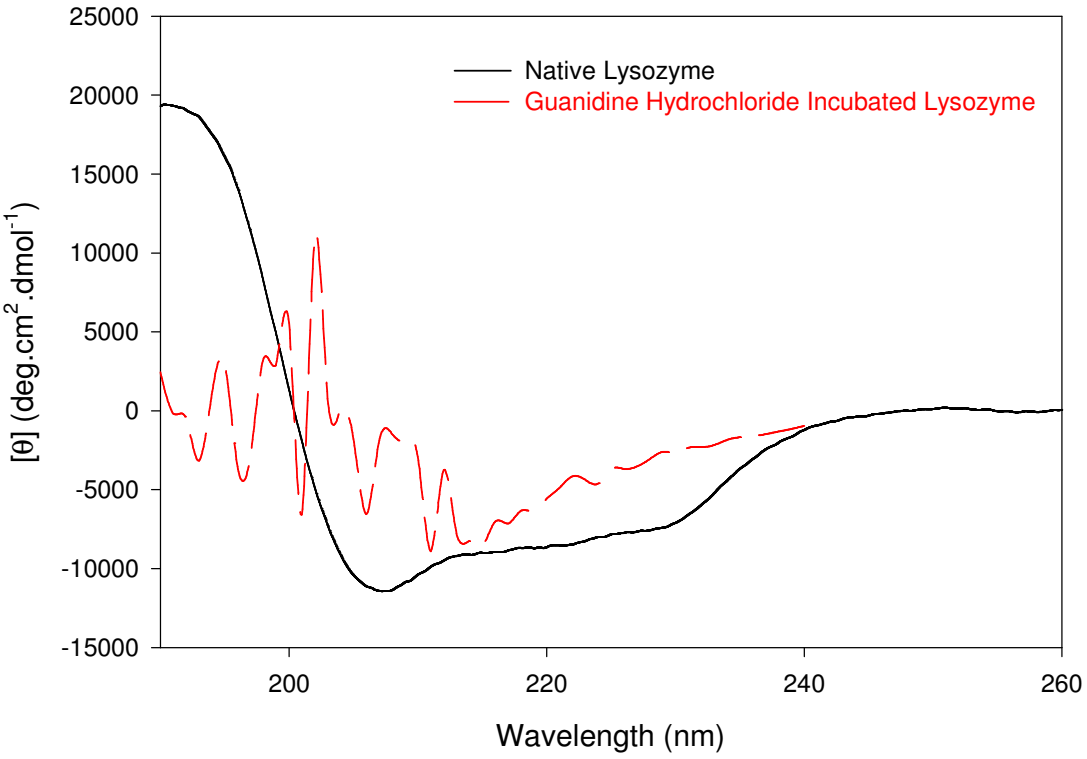


Figure 38: Far UV CD Spectra of Native and Guanidine Hydrochloride Incubated Lysozyme

The far UV CD spectrum of native lysozyme (as described previously), indicates that the protein contains a large alpha helical content. Upon incubation of native lysozyme in GdnHCl for two hours, the protein begins to loose that helical content as the tertiary and secondary structural characteristics associated with the protein are removed. The presence of GdnHCl results in a lot of noise appearing in the CD signal below 210 nm or so. This is due to the presence of the chloride ions at such high concentrations (6 M GdnHCl).

Near UV CD spectroscopy is routinely used to probe the secondary structure of protein molecules and gain an insight into the chirality of the environment of individual aromatic amino acids. The near UV CD spectra of native and GdnHCl incubated lysozyme are shown.

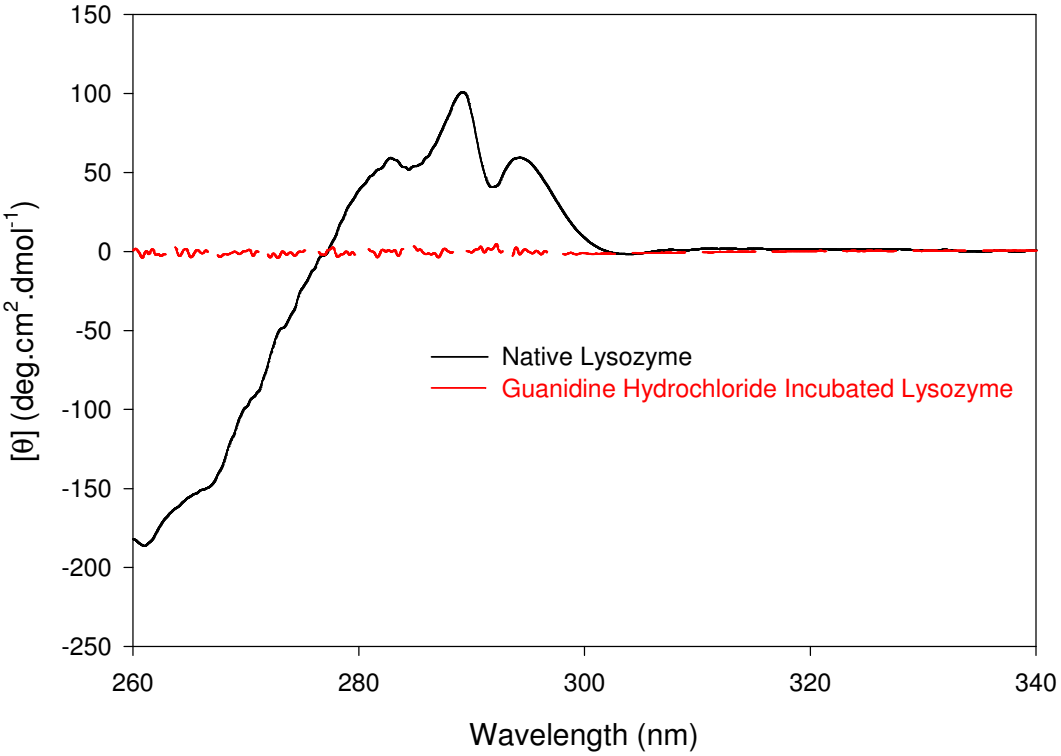


Figure 39: Near UV CD Spectra of Native and Guanidine Hydrochloride Incubated Lysozyme

Upon incubation of lysozyme in the salt GdnHCl, there is a complete loss of CD signal. This is a result of the GdnHCl unfolding the protein, therefore disrupting the structure leading to a change in the chirality of the amino acid environment.

3.1.2.3 Guanidine Hydrochloride Unfolding of Lysozyme - Discussion

From the studies carried out, it is evident that incubation of lysozyme in the chaotropic agent GdnHCl results in the disruption of the protein molecule so that it is no longer in a folded/native conformation. Fluorescence and CD spectroscopy indicated that upon incubation in the salt, the buried aromatic amino acids were no longer so. GdnHCl can therefore be considered a suitable denaturing salt for the enzyme lysozyme.

3.2 Thermal Unfolding of Lysozyme

For the preparation of thermally-unfolded lysozyme, it is first necessary to assess how the protein responds to heat and at which temperature unfolding takes place.

3.2.1 Differential Scanning Calorimetry of Lysozyme

Differential Scanning Calorimetry (DSC) was carried out on lysozyme to assess protein response to increasing temperature. This involves the controlled heating of the protein to determine at which temperature the protein is unfolded and if there is any refolding of the protein upon cooling. The DSC thermogram and rescan of lysozyme at 1 mg/ml is shown:

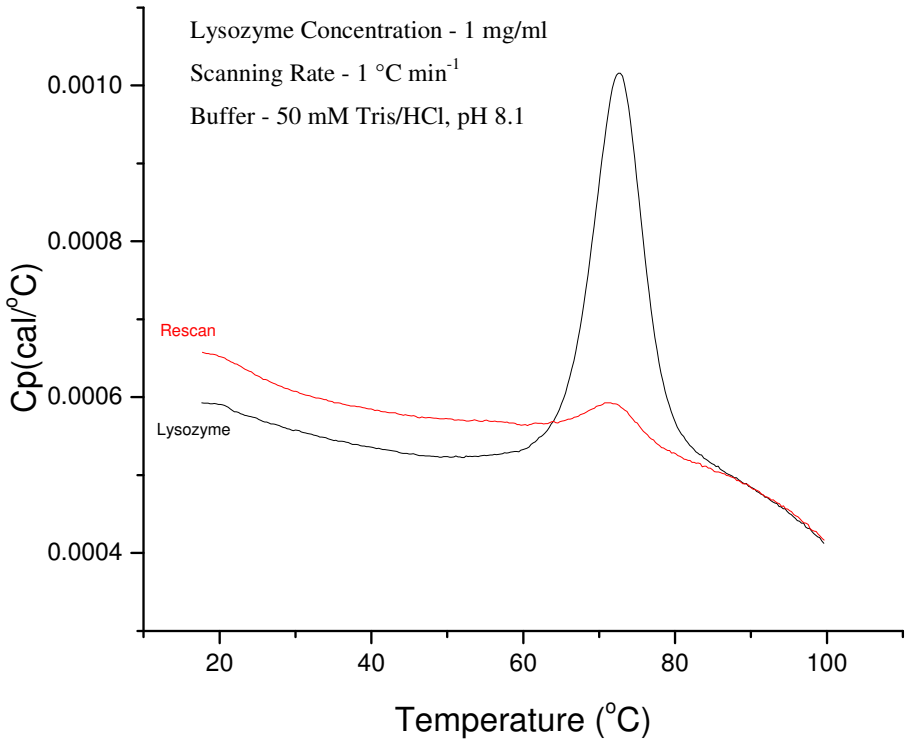


Figure 40: DSC Thermogram of Lysozyme

The DSC thermogram indicates that in order to unfold lysozyme thermally, the protein must be heated to a minimum of 72.8 °C (the measured T*m*). The rescan shows that upon cooling, thermally-unfolded lysozyme either regains some structure or some of the protein is able to refold into a native conformation and therefore a thermal transition is seen on the DSC rescan. However, the majority of protein remains apparently unfolded after heating to 100 ºC and subsequently cooled under these conditions.

3.2.2 Thermal Unfolding of Lysozyme - Fluorescence Spectroscopy

Lysozyme (0.1 mg/ml) was thermally unfolded by heating to 90 °C and then allowed to cool to room temperature. Once cooled, aggregates were seen to form (hence large intensity of scatter peak in the fluorescence spectrum). The thermally-unfolded lysozyme was solubilised upon addition of 6 M GdnHCl and the aggregates were seen to dissolve (hence reduction in the intensity of the scattering peak in spectrum). The fluorescence spectra of both the thermally-unfolded and solubilised proteins were obtained.

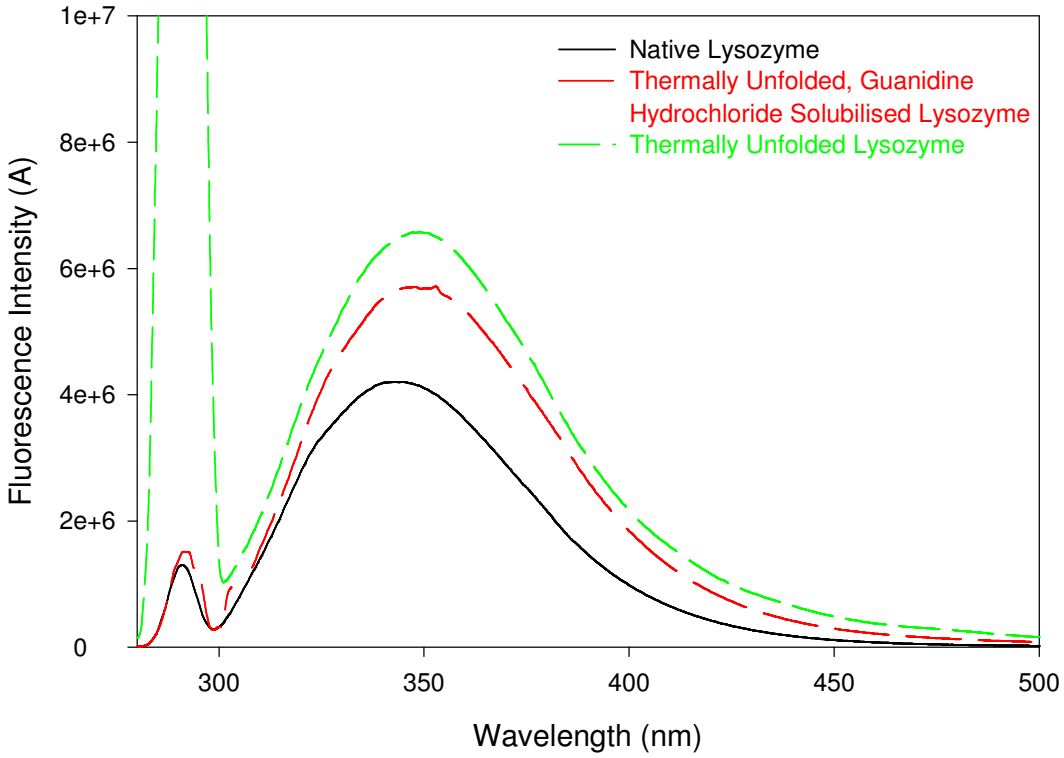


Figure 41: Thermal Unfolding and Guanidine Hydrochloride Solubilisation of Lysozyme

As can be seen from the data shown, upon thermal unfolding, the fluorescence spectrum of lysozyme changes (as expected). The presence of a high intensity scattering peak at 290 nm indicates that there are aggregates present in the protein solution. Upon addition of the salt GdnHCl, the scattering peak is removed.

3.2.3 Thermal Unfolding of Lysozyme - CD Spectroscopy

A CD spectrum of thermally-unfolded lysozyme was obtained upon solubilisation of the aggregates in GdnHCl.

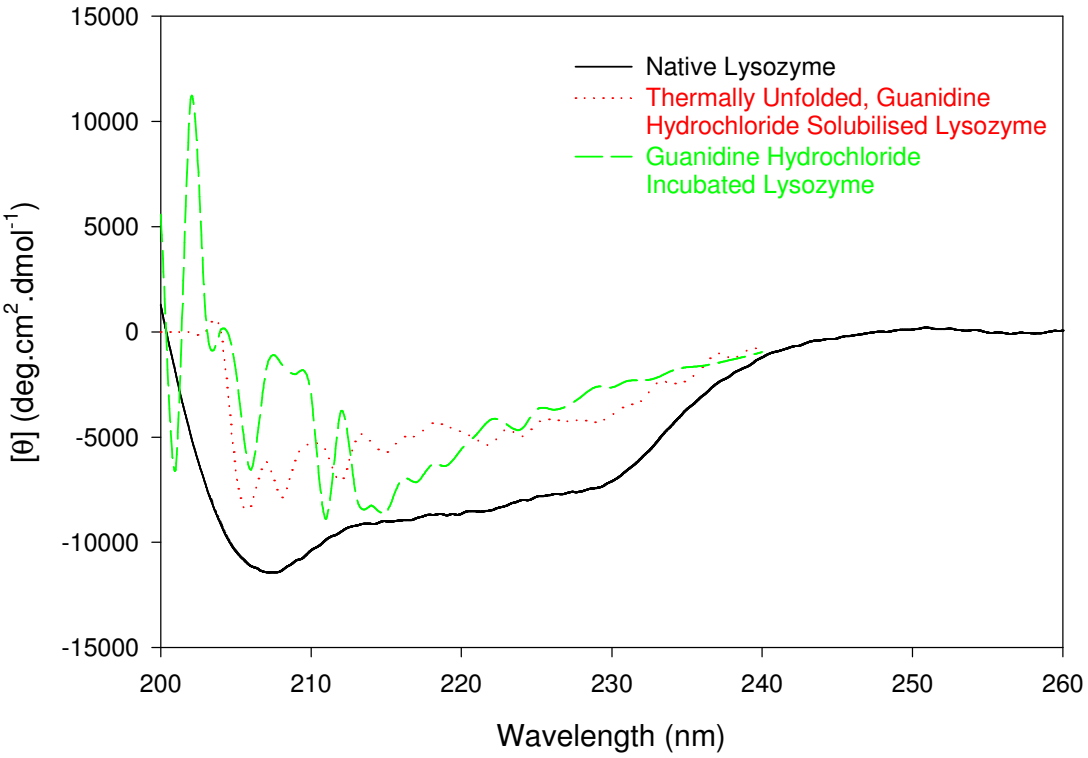


Figure 42: Far UV CD Spectra of Lysozyme in the Native, Thermally-Unfolded and Guanidine Hydrochloride Incubated State

Thermal unfolding and GdnHCl solubilisation of lysozyme, removes the secondary structural elements associated with the enzyme. Near UV CD spectroscopy was carried out to determine the effect of thermal unfolding and subsequent solubilisation upon the amino acid residues of lysozyme.

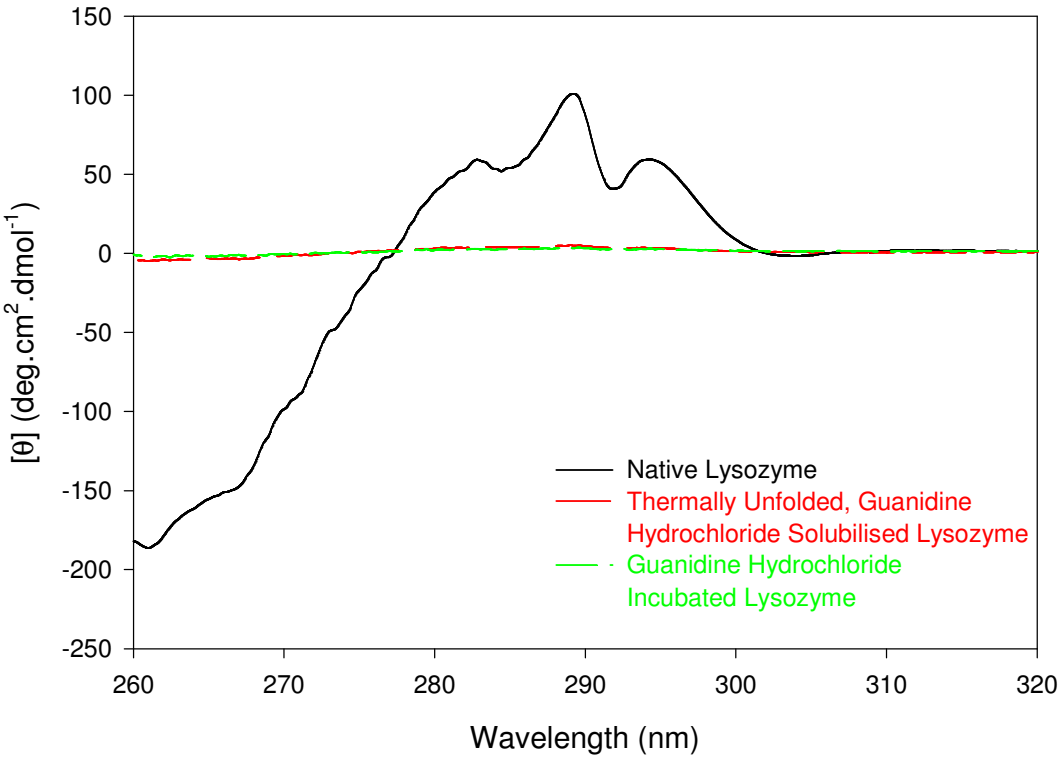


Figure 43: Near UV CD Spectra of Native, Thermally-Unfolded and Guanidine Hydrochloride Incubated Lysozyme

Near UV CD spectroscopy indicates unsurprisingly that upon thermal unfolding and subsequent solubilisation of lysozyme with GdnHCl, there is loss of CD signal due to complete unfolding.

3.3 Immobilisation of Lysozyme from the Native and Unfolded State

PCMC of native and unfolded lysozyme were prepared to determine the efficiency of this method for both the immobilisation and potential folding of the enzyme.

To find the optimum conditions for the immobilisation and potential folding steps, various excipients, solvents, protein loadings and concentrations were employed. Additives such as a redox couple (for example cystine and cysteine) were also used to assess what effect, if any, they had upon the potential refolding of lysozyme. Different methods were also employed in the manufacture of PCMC.

3.3.1 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State

3.3.1.1 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State

Lysozyme was unfolded upon incubation of the protein in 50 mM Tris/HCl, pH 8.1 containing 6 M GdnHCl. The unfolded protein was subsequently immobilised *via* the formation of PCMC.

3.3.1.1.1 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State - Results

PCMC of both native and GdnHCl unfolded lysozyme were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and specific activity (0.876 $Amin^{-1}mg^{-1}ml^{-1}$ for untreated protein preparation)

Details of PCMC sample preparation can be found in Appendix 1.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC158_01	Lysozyme on D,L-valine, 15 % load in 2-PrOH	98.3	16.3	0.832 (± 0.017)	94.9 (± 1.9)
KC158_02	Lysozyme on D,L-valine, 20 % load in 2-PrOH	87.5	20.4	0.843	96.2
KC158_03	Lysozyme on D,L-valine, 25 % load in 2-PrOH	97.9	25.9	0.812	92.7
KC162_01	Lysozyme in GdnHCl on D,L-valine, 12.5 % load in 2-PrOH	84.3	11.3	0.673 (± 0.021)	77.0 (± 2.4)
KC162_02	Lysozyme in GdnHCl on D,L-valine, 15 % load in 2-PrOH	76.1	15.9	0.642	73.4
KC162_03	Lysozyme in GdnHCl on D,L-valine, 17.5 % load in 2-PrOH	83.7	18.6	0.713	81.5
KC162_04	Lysozyme in GdnHCl on D,L-valine, 20 % load in 2-PrOH	79.4	21.8	0.673 (± 0.026)	76.8 (± 3.0)
KC162_05	Lysozyme in GdnHCl on D,L-valine, 22.5 % load in 2-PrOH	67.4	24.1	0.703	80.3
KC162_06	Lysozyme in GdnHCl on D,L-valine, 25 % load in 2-PrOH	67.8	28.6	0.694	79.3

Table 2: Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State - Results

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

3.3.1.1.1.1 Immobilisation of Lysozyme from the Guanidine Hydrochloride Unfolded State - CD Spectroscopy

CD spectroscopy was carried out of the immobilised protein samples upon reconstitution. The sample shown is KC162_03, GdnHCl unfolded lysozyme subjected to immobilisation on D,L-valine in propan-2-ol.

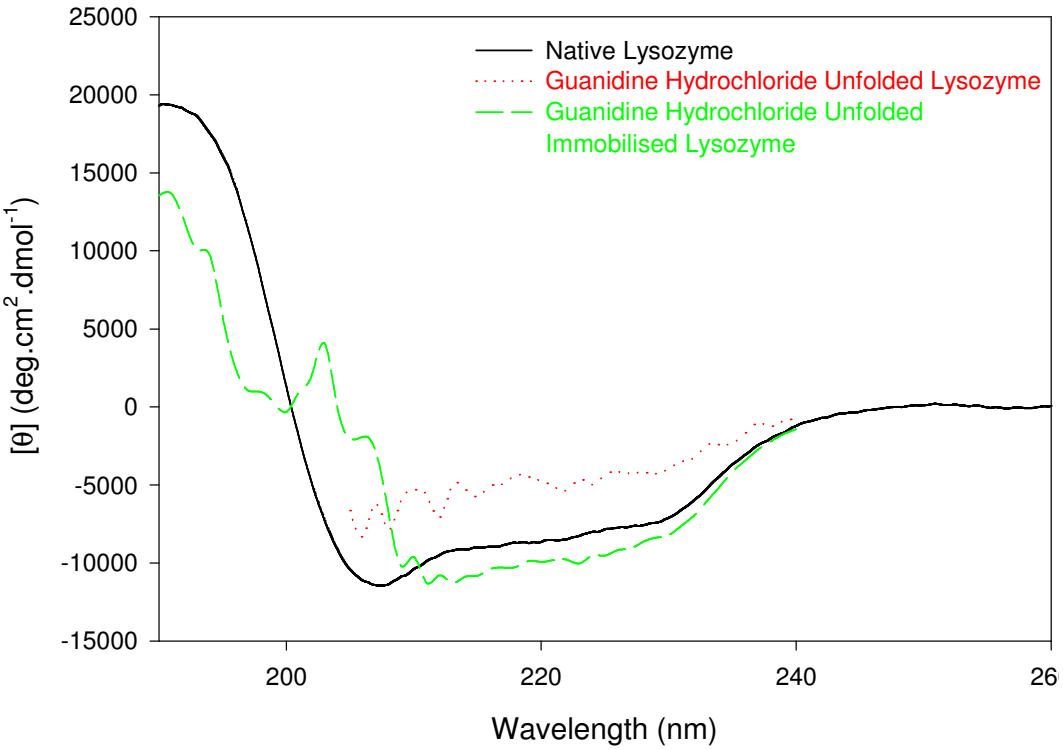


Figure 44: Immobilisation of Lysozyme from the Guanidine Hydrochloride Unfolded State - CD Spectroscopy

3.3.1.1.2 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State - Discussion

The measured solids recovery of the PCMC suspensions suggest that immobilised native lysozyme samples can be prepared using D,L-valine as an excipient with very little of the solid or protein being lost to the solvent.

In order to ascertain the effect of the immobilisation on the activity of both the native and the GdnHCl unfolded protein, it is necessary to compare the observed activity measured for these samples.

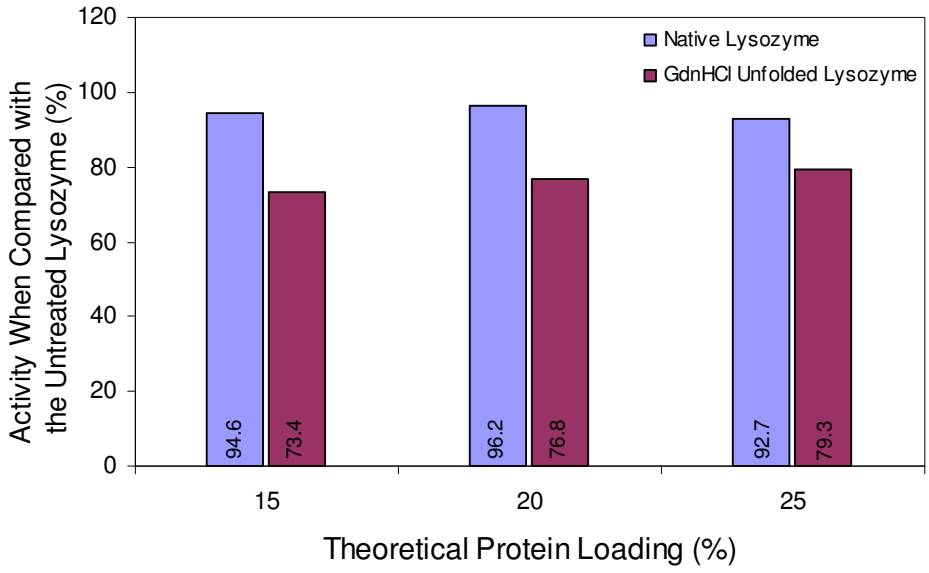


Figure 45: Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State - Comparison of Retained Activity

Immobilisation of the native protein on D,L-valine at theoretical protein loadings ranging from 15 - 25 % can retain up to 96.2 % of activity when compared with the untreated lysozyme. When lysozyme is unfolded in GdnHCl and subsequently subjected to immobilisation, the protein regains up to 81.5 % of the activity of the untreated lysozyme. This suggests that the protein, although experimentally shown to be unfolded by GdnHCl (using fluorescence and CD spectroscopy), can regain activity upon removal of the salt from the protein environment.

CD spectroscopy of the GdnHCl unfolded immobilised PCMC preparation indicates that upon immobilisation and exclusion of the solubilising agent, lysozyme can regain secondary structure and activity akin to the native state.

3.3.1.2 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State at Lower Protein Loadings

In order to determine if lysozyme could be immobilised and refolded successfully at lower protein loadings, samples were prepared of native and GdnHCl unfolded lysozyme (6 mg/ml) at protein loadings ranging from 0.6 - 20 %.

3.3.1.2.1 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State at Lower Protein Loadings - Results

PCMC of both native and GdnHCl unfolded lysozyme were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination

of the % MPL (Measured Protein Loading) and specific activity (0.6502 $Amin^{-1}mg^{-1}ml^{-1}$ for untreated protein preparation).

Details of PCMC sample preparation can be found in Appendix 2.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity ($A\ min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC198_01	Lysozyme on D,L-valine, 0.6 % load in 2-PrOH	90.7	0.9	0.592	91.0
KC198_02	Lysozyme on D,L-valine, 5 % load in 2-PrOH	99.7	4.9	0.605	93.0
KC198_03	Lysozyme on D,L-valine, 10 % load in 2-PrOH	86.8	10.6	0.604	92.9
KC198_04	Lysozyme on D,L-valine, 15 % load in 2-PrOH	85.8	14.2	0.613	94.3
KC198_05	Lysozyme on D,L-valine, 20 % load in 2-PrOH	88.4	20.7	0.544 (± 0.035)	83.7 (± 5.4)
KC187_05	Lysozyme in GdnHCl on D,L-valine, 0.6 % load in 2-PrOH	87.7	0.8	0.435	66.9
KC187_06	Lysozyme in GdnHCl on D,L-valine, 5 % load in 2-PrOH	89.5	5.3	0.398	61.2
KC187_07	Lysozyme in GdnHCl on D,L-valine, 10 % load in 2-PrOH	77.5	9.8	0.489	75.2
KC187_08	Lysozyme in GdnHCl on D,L-valine, 15 % load in 2-PrOH	75.8	16.2	0.546	84.0
KC187_09	Lysozyme in GdnHCl on D,L-valine, 20 % load in 2-PrOH	95.8	21.2	0.521 (± 0.022)	80.1 (± 3.4)

Table 3: Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State at Lower Protein Loadings - Results

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

3.3.1.2.1.1 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State - CD Spectroscopy

Samples KC198_04 and KC187_08 were subjected to CD spectroscopy both as powders in suspension and upon reconstitution.

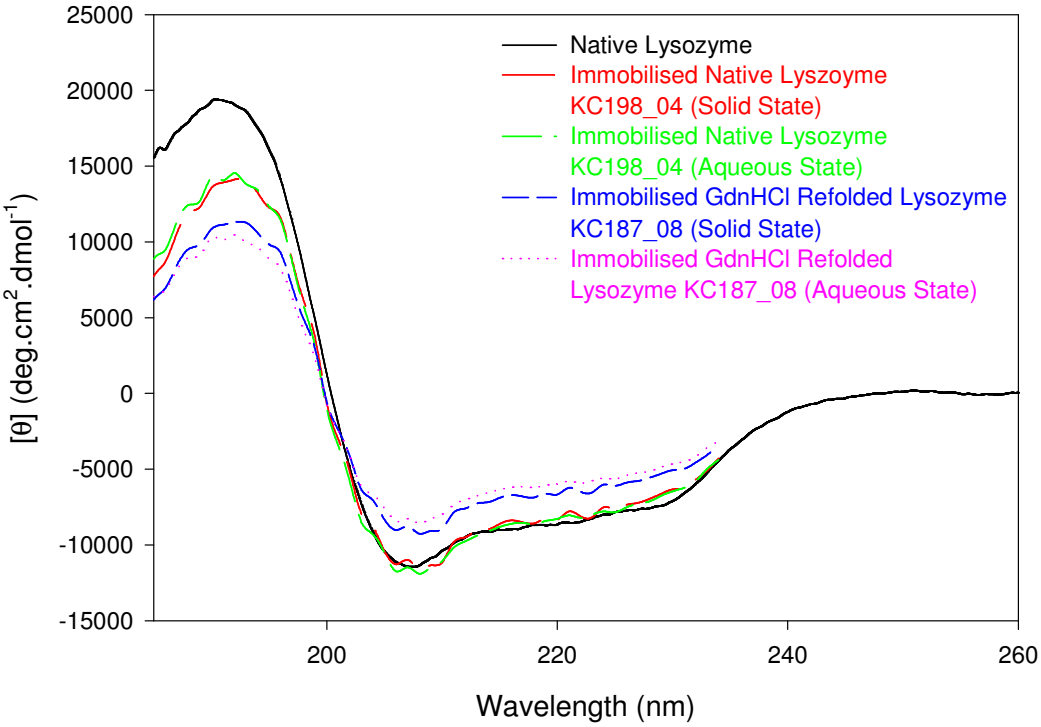


Figure 46: Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State - CD Spectroscopy (The difference in intensities of bands is due to variations in sample protein concentration)

3.3.1.2.2 Immobilisation of Native and Guanidine Hydrochloride Unfolded Lysozyme at Lower Protein Loadings - Discussion

The PCMC prepared from native and GdnHCl unfolded lysozyme at lower loadings indicate that lysozyme can be immobilised on D,L-valine with high measured solid recovery.

To determine the effect of immobilisation on the lysozyme, the observed activity is compared for PCMC of both native and GdnHCl unfolded protein of identical formulations.

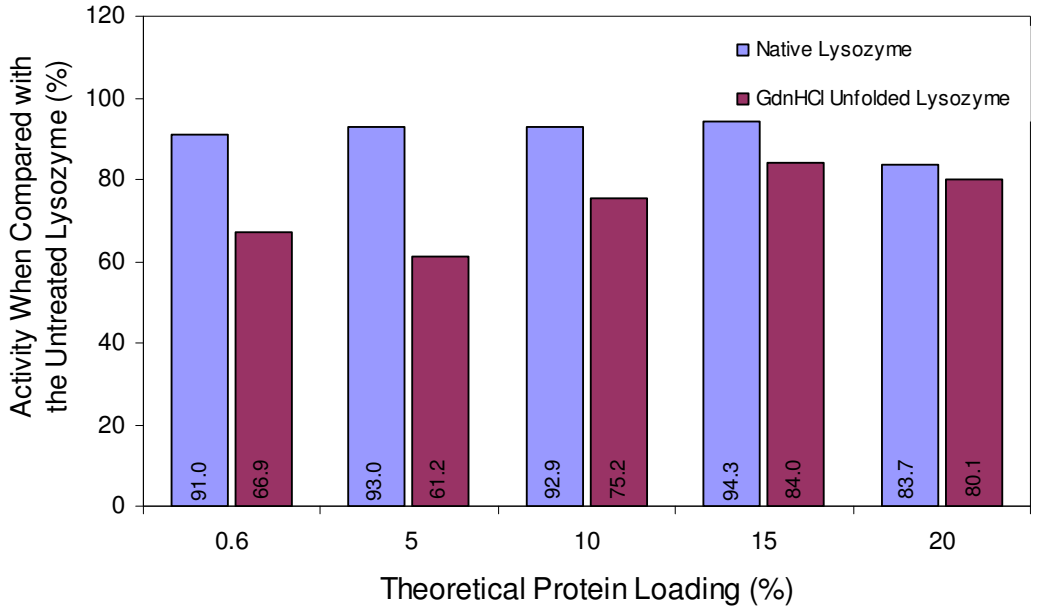


Figure 47: Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State at Lower Protein Loadings - Comparison of Retained Activity

Immobilisation of the native protein on D,L-valine at theoretical protein loadings ranging from 0.6 - 20 % can retain up to 94.3 % of activity when compared with the untreated protein.

When lysozyme is unfolded in GdnHCl and subsequently subjected to immobilisation at these loadings, the enzyme regains up to 84.0 % of the activity of the untreated protein. This suggests that, although experimentally shown to be unfolded by GdnHCl (by fluorescence and CD spectroscopy), the protein can regain activity upon removal of the salt from the protein environment, by employing loadings of 0.6 - 20 %.

CD spectroscopy of the immobilised native lysozyme indicates that the protein is held in its native state upon the surface of the excipient material. Upon reconstitution, the protein molecule is released from the surface and is present in the aqueous phase as a native structure. CD spectroscopy of lysozyme immobilised from the GdnHCl unfolded state results in protein which appears to be in a conformation similar to that of the native enzyme.

3.3.1.3 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump

In order to determine if increased enzymatic activity could be regained by the gradual mixing of the protein solution and excipient as opposed to the abrupt change in immediate

mixing; a syringe pump was employed. This allowed the excipient to be added to the protein containing solution at a slow rate (0.01 ml/min) to allow gradual dilution of the denaturant before the immobilisation step occurs.

3.3.1.3.1 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump - Results

PCMC of native and GdnHCl unfolded lysozyme manufactured using a syringe pump, were analysed *via* the reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and the specific activity (0.876A $min^{-1}mg^{-1}ml^{-1}$ for untreated protein).

Details of PCMC sample preparation can be found in Appendix 3.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC258_16	Lysozyme, on D,L-valine, 5 % loading in 2-PrOH	81.2	5.6	0.544	62.1
KC258_19	Lysozyme in GdnHCl on D,L-valine 5 % loading in 2-PrOH	95.7	6.1	0.128	14.6

Table 4: Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump - Results

3.3.1.3.2 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump - Discussion

Native lysozyme, when mixed gradually with excipient using a syringe pump before precipitation, shows a reduction in retained activity when compared with identical formulations conventionally prepared.

The PCMC prepared *via* the use of a syringe pump can be compared with two samples with identical formulations; KC198_02 (native lysozyme) and KC187_07 (GdnHCl unfolded lysozyme).

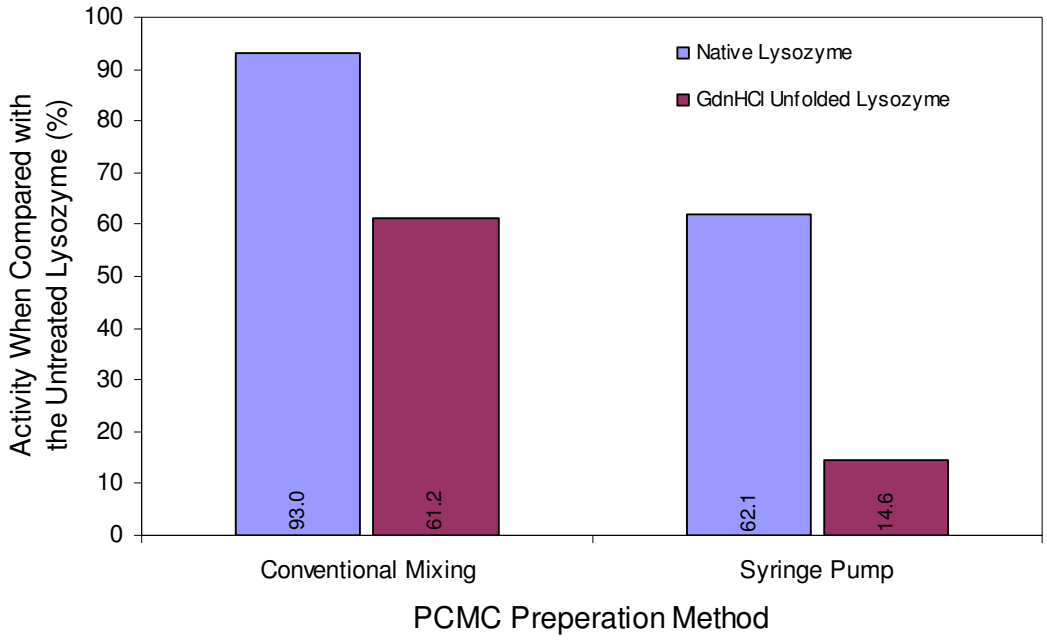


Figure 48: Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State at 5 % Loading - Effect of Using a Syringe Pump

When compared with identical PCMC formulations, those prepared using syringe pumps for gradual protein-excipient mixing tend to have lower activity than their conventionally mixed counterparts.

Native lysozyme can be immobilised at 5 % loading with the retention of up to 93 % enzymatic activity. However, when a syringe pump is employed to mix the protein and excipient before precipitation, this activity is reduced to 62 %. This could be due to the time the aqueous solution of protein is kept at room temperature to carry out the slow mixing, when compared with the immediate mixing and precipitation of the conventional PCMC preparation method.

PCMC of GdnHCl unfolded lysozyme when prepared by immediate mixing and precipitation can regain up to 61 % of the activity of the untreated protein. However, when gradual mixing is employed, this regained activity is reduced to 14.6 %.

3.3.1.4 Dialysis Refolding of Lysozyme from the Guanidine Hydrochloride Unfolded State - Comparison with Immobilisation

Dialysis is a common method used for protein folding which involves the gradual controlled removal of denaturing molecules from the environment of the protein.

Dialysis, as a method for refolding was compared with immobilisation for GdnHCl unfolded lysozyme. Lysozyme (6 mg/ml in 50 mM Tris/HCl, pH 8.1) which had been chemically unfolded *via* addition of 6 M GdnHCl was subjected to dialysis against buffer containing no GdnHCl salt. The protein was incubated for 2 hours in the salt before being subjected to dialysis (2 x 3 hours plus overnight).

Upon dialysis, protein aggregation was seen to occur to some extent, although not all protein had aggregated. Upon removal of the protein from the dialysis cassette, the enzyme was subjected to fluorescence spectroscopy and the activity assay described for the protein.

Fluorescence spectra of the GdnHCl unfolded and dialysed proteins were obtained. The wavelength at which the maximum fluorescence intensity occurred for the native protein was the same as had been previously described (345 nm).

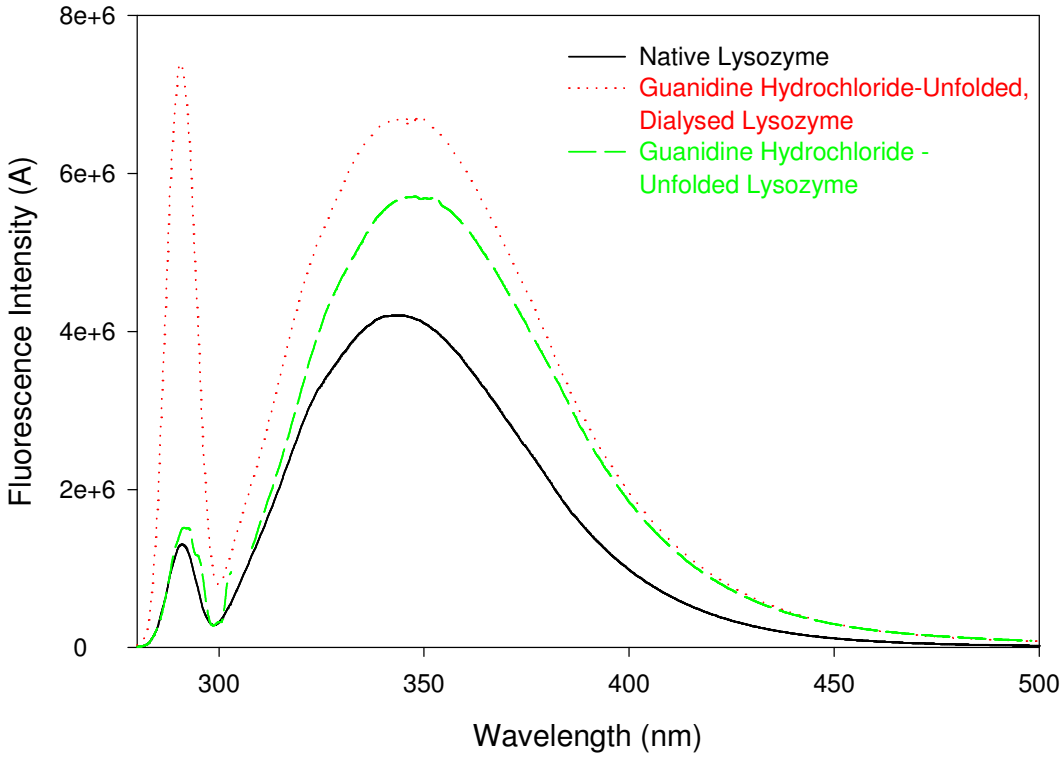


Figure 49: Dialysis Refolding of Lysozyme from the Guanidine Hydrochloride Unfolded State – Fluorescence Spectroscopy

When the lysozyme had been subjected to GdnHCl unfolding, this wavelength increased (353 nm) as expected. Upon subjecting the unfolded protein to dialysis, the wavelength at which the maximum fluorescence intensity occurs decreased slightly to 349 nm. This indicated that the protein was no longer in an unfolded, solubilised state, but perhaps was partially refolded or aggregated.

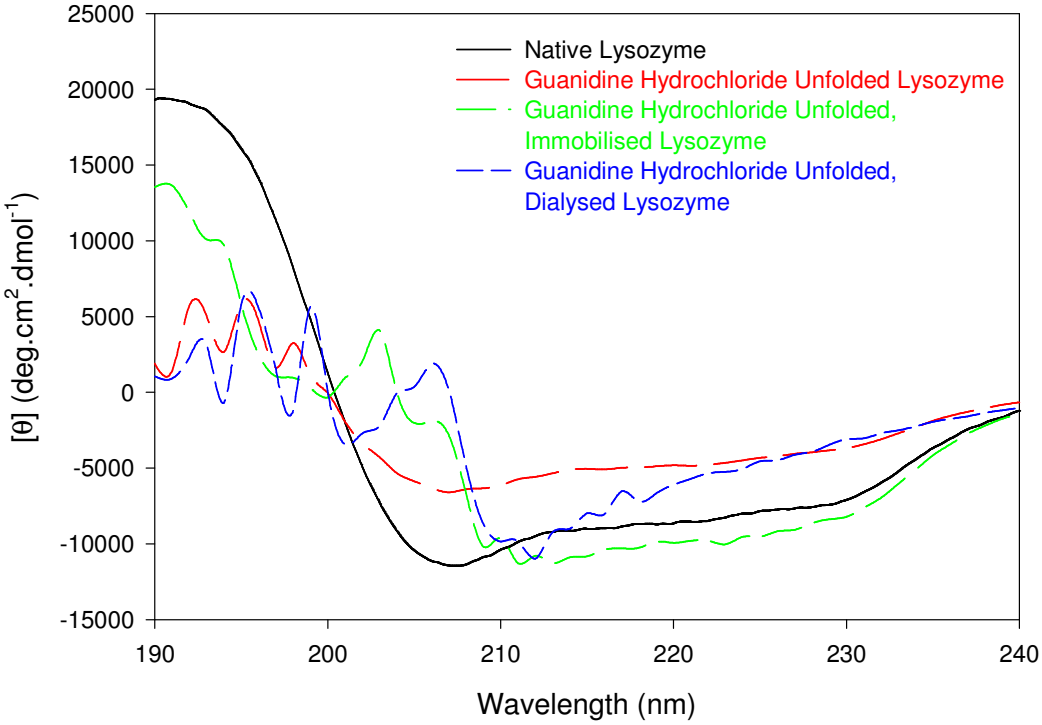


Figure 50: Dialysis Refolding of Lysozyme from the Guanidine Hydrochloride Unfolded State – CD Spectroscopy

CD spectroscopy of the guanidine hydrochloride unfolded and dialysed protein shows that upon implementation of dialysis as a refolding method, some regain of secondary structure is seen

The enzymatic activity assay indicated that GdnHCl unfolded lysozyme at a concentration of 6 mg/ml could regain 19.8 % of the activity of the native, untreated protein. This compares unfavourably with the immobilisation method which can regain 61.2 % activity from lysozyme under the same conditions.

3.3.2 Immobilisation of Lysozyme from the Native and Urea-Unfolded State

3.3.2.1 Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced State

Urea cannot induce complete unfolding of lysozyme from the native state on its own. It requires the assistance of a reducing agent such as DTT (dithiothreitol) to break the disulfide bonds involved in holding the protein molecule in a native conformation.

PCMC of urea-incubated and urea-unfolded, reduced lysozyme (12 mg/ml) was manufactured in order to ascertain the effect of the addition of the reducing agent had upon the prepared immobilised samples. These PCMC were prepared at the same theoretical protein loading in different precipitating solvent systems.

3.3.2.1.1 Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced State - Results

PCMC of urea-incubated and urea-unfolded, reduced lysozyme were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and specific activity (0.876 $Amin^{-1}mg^{-1}ml^{-1}$ for untreated protein preparation).

Details of PCMC sample preparation can be found in Appendix 4.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC111_01	Lysozyme in urea + DTT on D,L-valine, 16.5 % load in EtOH	57.2	**	**	**
KC111_02	Lysozyme in urea + DTT on D,L-valine, 16.5 % load in 2-PrOH	76.3	15.1	0.083 (± 0.019)	9.0 (± 2.2)
KC111_03	Lysozyme in urea + DTT on D,L-valine, 16.5 % load in 1-PrOH	40.1	18.7	0.019	1.1
KC111_04	Lysozyme in urea on D,L-valine, 16.5 % load in EtOH	63.6	18.3	0.715	81.6
KC111_05	Lysozyme in urea on D,L-valine, 16.5 % load in 2-PrOH	84.5	17.9	0.804	91.7
KC111_06	Lysozyme in urea on D,L-valine, 16.5 % load in 1-PrOH	56.2	19.8	0.730 (± 0.041)	83.3 (± 4.7)

Table 5: Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced State Using Various Solvents - Results (** The sample was insoluble upon attempted reconstitution)

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

3.3.2.1.1.1 Immobilisation of Lysozyme from the Urea-Incubated State - CD Spectroscopy

CD spectroscopy was carried out of the immobilised protein samples upon reconstitution. The sample shown is KC111_05, lysozyme incubated in urea subjected to immobilisation on D,L-valine in propan-2-ol.

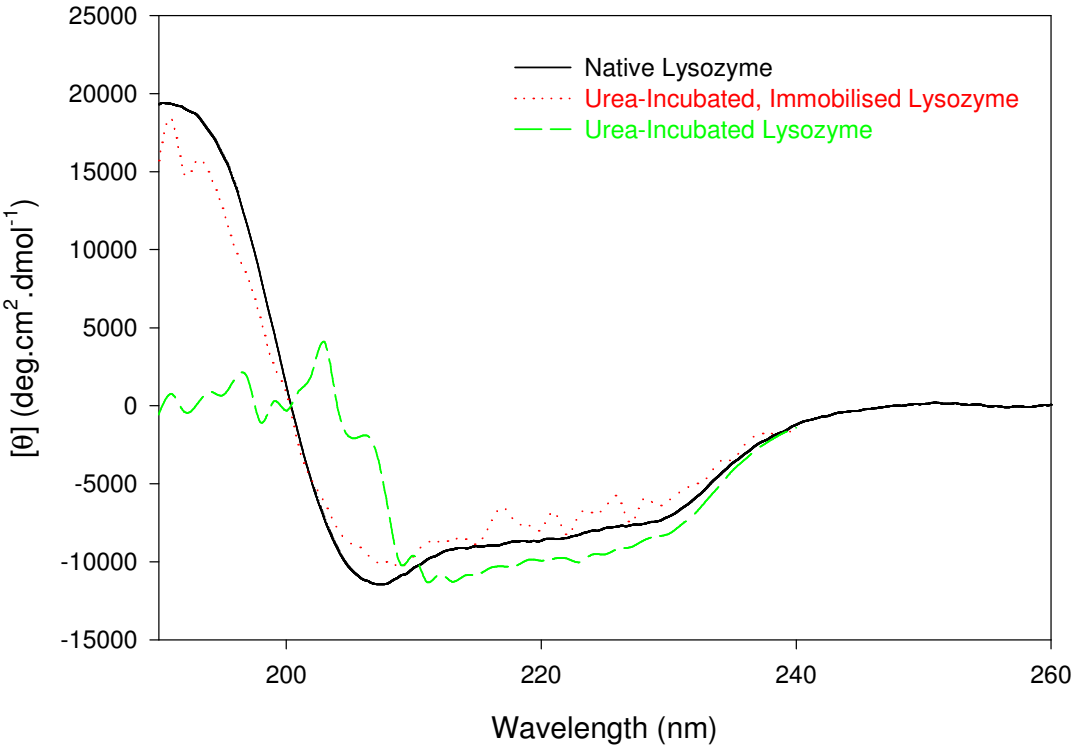


Figure 51: Immobilisation of Lysozyme from the Urea-Incubated State - CD Spectroscopy

3.3.2.1.2 Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded Reduced State - Discussion

From this set of experiments, it is evident that propan-2-ol is the best solvent for the precipitation of lysozyme at this particular loading with D,L-valine as an excipient with regards to the measured solid recovery and the observed activity.

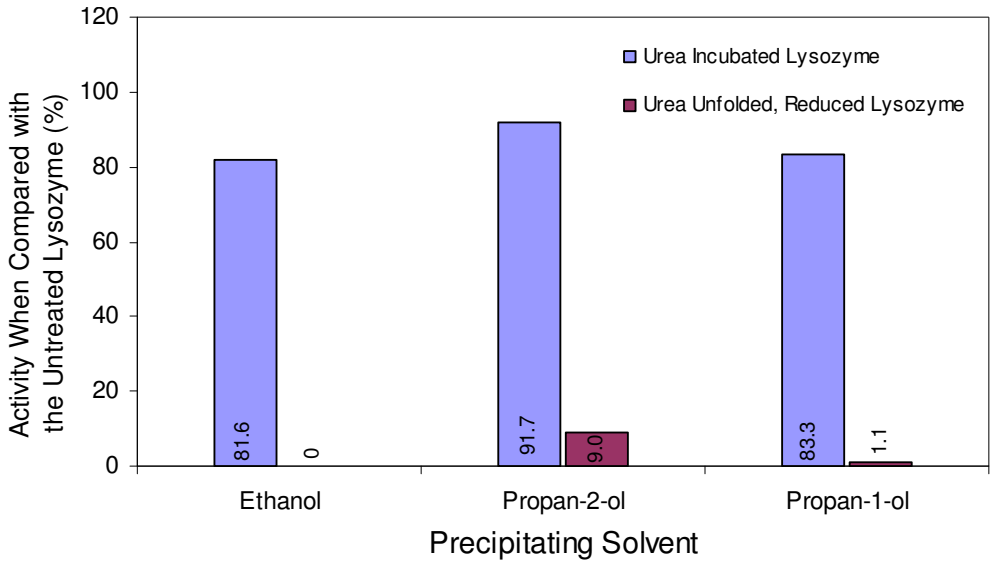


Figure 52: Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced State - Observed Activity

It is possible to prepare PCMC of urea-incubated lysozyme at 16.5 % loading in various solvents with little overall loss of activity. Previous studies indicate that lysozyme is reversibly unfolded in 8 M urea, so little loss of activity would be expected, although it is interesting to note that urea can be removed from the environment of this protein without significant loss of enzymatic activity.

The PCMC prepared from urea-unfolded, reduced lysozyme *via* precipitation in ethanol yielded insoluble protein and as such the measured protein loading and activity could not be determined. However, the PCMC prepared in propan-2-ol and propan-1-ol were soluble and show some regain of enzymatic activity. There are a number of reasons this could be possible.

At the immobilisation stage, the amino acid carrier material takes longer to precipitate in ethanol than in propanol. This could result in the enzyme being held in the unfolded state in the organic solvent for longer. The presence of alcohols has been known to stabilise the unfolded state in many studies with lysozyme.[139, 140] It is therefore possible to suggest that in the case of immobilisation in ethanol, rather than the enzyme regaining native structure *via* immobilisation, the protein is held in a non-native conformational state (possibly as an aggregate) which is then immobilised. This could explain the low solubility of the immobilised protein upon reconstitution under aqueous conditions.

The CD spectra indicate that it is possible to reversibly adsorb protein on to the surface of an amino acid (such as D,L-valine) with the exclusion of additives such as urea. Upon

reconstitution, the biomolecule appears to retain most (if not all) of the secondary structural elements associated with the native state.

3.3.2.2 Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced State Using L-Glutamine as an Excipient

PCMC of urea-incubated lysozyme and urea-unfolded, reduced lysozyme (12 mg/ml) were manufactured in order to see the effect of the addition of reducing agent upon the prepared immobilised samples. These PCMC were prepared at the same theoretical protein loading in different precipitating solvents.

3.3.2.2.1 Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced State Using L-Glutamine as an Excipient - Results

PCMC of urea-incubated and urea-unfolded, reduced lysozyme were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and specific activity (0.876 $Amin^{-1}mg^{-1}ml^{-1}$ for untreated protein preparation).

Details of PCMC sample preparation can be found in Appendix 5.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC111_07	Lysozyme in urea + DTT on L-glutamine, 37.5 % load in EtOH	60.3	**	**	**
KC111_08	Lysozyme in urea + DTT on L-glutamine, 37.5 % load in 2-PrOH	84.1	**	**	**
KC111_09	Lysozyme in urea + DTT on L-glutamine, 37.5 % load in 1-PrOH	84.8	**	**	**
KC111_10	Lysozyme in urea on L-glutamine, 37.5 % load in EtOH	70.5	15.4	0.569	64.9
KC111_11	Lysozyme in urea on L-glutamine, 37.5 % load in 2-PrOH	86.3	11.1	0.382	43.6
KC111_12	Lysozyme in urea on L-glutamine, 37.5 % load in 1-PrOH	76.3	11.4	0.374 (± 0.023)	42.6 (± 2.6)

Table 6: Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced State Using L-Glutamine as an Excipient - Results (** The sample was insoluble upon attempted reconstitution)

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

3.3.2.2.2 Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced State Using L-Glutamine as an Excipient - Discussion

The use of the excipient L-glutamine renders all of the samples prepared from urea-unfolded, reduced lysozyme as insoluble and as such the protein loading and activity of these samples have not been determined.

In the case of PCMC prepared from urea-incubated lysozyme, the observed activity for these preparations is less than that seen for D,L-valine.

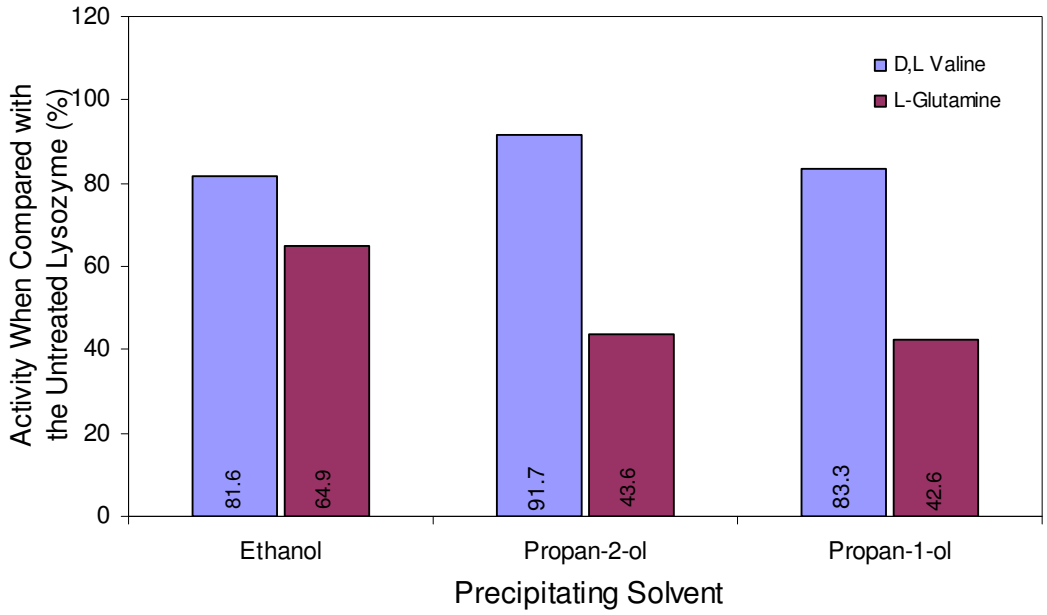


Figure 53: Comparison of D,L-Valine and L-Glutamine as Excipients for Urea-Incubated Lysozyme Immobilisation

From the data provided it is evident that D,L-valine is a better excipient for lysozyme under these conditions than L-glutamine. The PCMC prepared from L-glutamine have lower measured solid recovery and observed activity than their D,L-valine counterparts.

3.3.2.3 Immobilisation of Lysozyme from Urea-Incubated and Urea-Unfolded, Reduced state at Lower Protein Loadings

To determine if reducing the loading of the protein could have any positive effect upon the solubility (and observed activity) of the PCMC, immobilised protein samples were prepared with these objectives in mind.

Lysozyme 12 mg/ml in 50 mM Tris/HCl, pH 8.1 containing 8 M Urea either in the presence or absence of 32 mM DTT was used as a stock from which to prepare PCMC immobilised upon D,L-valine.

3.3.2.3.1 Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced state at Lower Protein Loadings - Results

PCMC of urea-incubated and urea-unfolded, reduced lysozyme were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and specific activity (0.876 $Amin^{-1}mg^{-1}ml^{-1}$ for untreated protein preparation)

Details of PCMC sample preparation can be found in Appendix 6.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC119_01	Lysozyme in urea + DTT on D,L-valine, 9 % load in EtOH	64.3	**	**	**
KC119_02	Lysozyme in urea + DTT on D,L-valine, 9 % load in 1-PrOH	35.2	**	**	**
KC119_03	Lysozyme in urea + DTT on D,L-valine, 9 % load in 2-PrOH	86.8	7.6	0.124 (± 0.036)	14.1 (±4.1)
KC119_07	Lysozyme in urea on D,L-valine, 9 % load in EtOH	70.5	8.9	0.664	75.8
KC119_08	Lysozyme in urea on D,L-valine, 9 % load in 1-PrOH	86.3	7.9	0.712	81.3
KC119_09	Lysozyme in urea on D,L-valine, 9 % load in 2-PrOH	89.3	9.3	0.772	88.1

Table 7: Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced state at Lower Protein Loadings - Results (** The sample was insoluble upon attempted reconstitution)

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

3.3.2.3.2 Immobilisation of Lysozyme from the Urea-Incubated and Urea-Unfolded, Reduced state at Lower Protein Loadings - Discussion

Reducing the protein loading has a significant effect upon the observed activity of the sample precipitated in 2-PrOH when compared to PCMC prepared at 16.5 % loading (KC111_02).

However, the PCMC of urea-unfolded, reduced protein precipitated in ethanol remains insoluble and therefore the measured protein loading and activity cannot be determined.

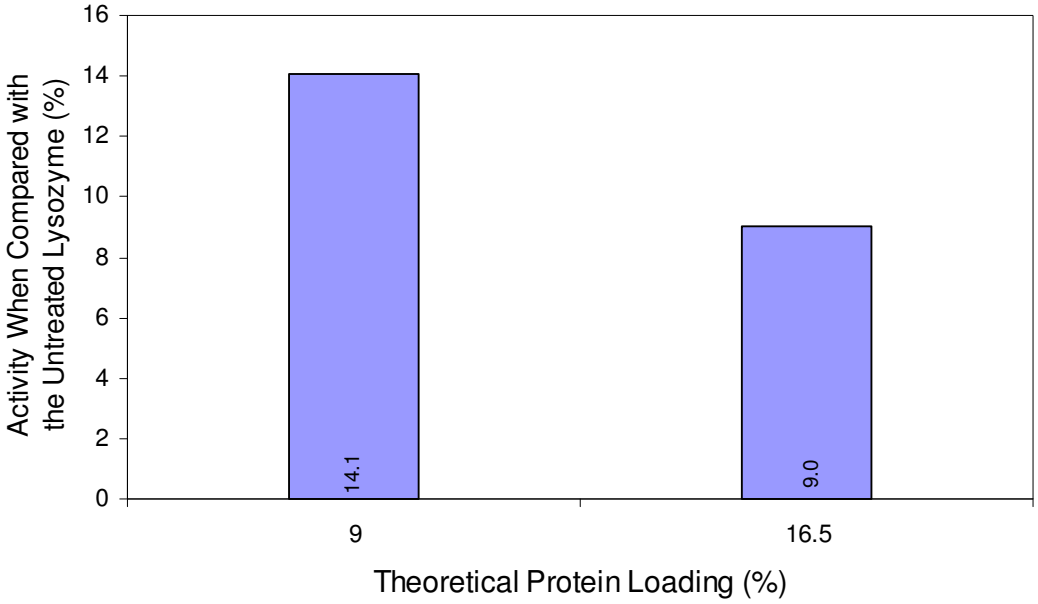


Figure 54: Immobilisation of Urea-Unfolded, Reduced Lysozyme on D,L-Valine - Effect of Protein Loading

The activity of this sample increases by over 50 % (with respect to KC111_02 which has a protein loading of 16.5 %). This is obviously a favourable effect upon the sample and further investigation was carried out in an attempt to improve upon this.

3.3.2.4 Immobilisation of Lysozyme from the Urea-Unfolded, Reduced State from Lower Stock Concentrations

The previously described PCMC were manufactured from various protein stocks at a concentration of 12 mg/ml lysozyme in 50 mM Tris/HCl, pH 8.1 containing 8 M Urea and 32 mM DTT. To determine if reducing the protein concentration of the stock from which samples were prepared would have any positive effect upon the solubility (and observed activity) of the PCMC, immobilised protein samples were prepared from lysozyme at a concentration of 6 mg/ml.

3.3.2.4.1 Immobilisation of Lysozyme from the Urea-Unfolded, Reduced State from Lower Stock Concentrations - Results

PCMC of urea-unfolded, reduced lysozyme were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and specific activity (0.876 $Amin^{-1}mg^{-1}ml^{-1}$ for untreated protein).

Details of PCMC sample preparation can be found in Appendix 7.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC77_01	Lysozyme in urea + DTT on D,L-valine, 9 % load in EtOH	62.0	**	**	**
KC77_02	Lysozyme in urea + DTT on D,L-valine, 9 % load in 2-PrOH	84.7	9.3	0.376 (± 0.021)	42.9 (± 2.4)
KC77_03	Lysozyme on D,L-valine, 9 % load in EtOH	77.9	9.8	0.717	81.8
KC77_04	Lysozyme on D,L-valine, 9 % load in 2-PrOH	89.6	8.6	0.825	94.2

Table 8: Immobilisation of Lysozyme from the Urea-Unfolded, Reduced State from Lower Stock Concentrations - Results (** The sample was insoluble upon attempted reconstitution)

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

3.3.2.4.1.1 Immobilisation of Lysozyme from the Urea-Unfolded, Reduced State - CD Spectroscopy

CD spectroscopy was carried out upon immobilised lysozyme which had undergone urea unfolding in the presence of DTT (KC77_02). The protein immobilised in this sample was subjected to 8 M urea and 32 mM DTT before subsequent precipitation with D,L-valine in propan-2-ol.

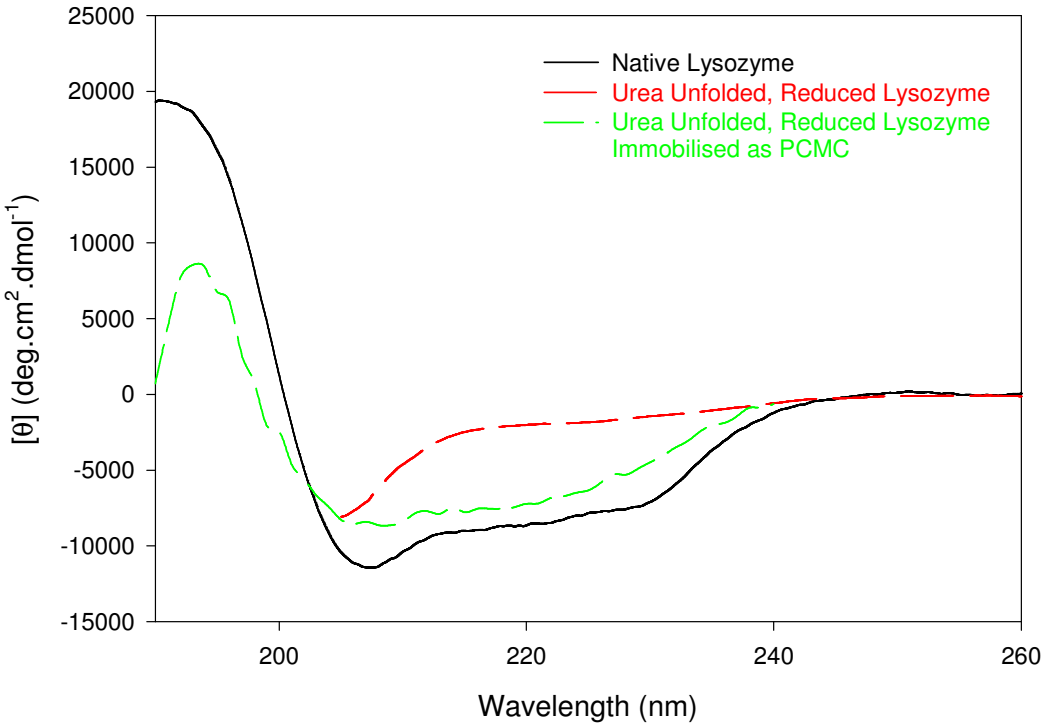


Figure 55: Far UV CD Spectrum of Native Lysozyme and Urea-Unfolded, Reduced Lysozyme Immobilised on D,L-valine in Propan-2-ol

CD spectroscopy was carried out upon the reconstituted sample in 50 mM Tris/HCl, pH 8.1. Upon coprecipitation, the urea and DTT were dissolved in the precipitating solvent; propan-2-ol.

To determine if the addition of a redox couple would have an effect upon the observed activity of the immobilised urea-unfolded, reduced lysozyme, further analysis was carried out on sample KC77_02.

After ascertaining that the redox couple of cystine/cysteine had no effect upon the spectrophotometric activity assay, the PCMC were reconstituted in a buffer containing the redox pair (50 mM Tris/HCl, pH 8.1 containing 0.8 mM L-cystine and 8 mM L-cysteine) and incubated for two hours with gentle stirring. The activity of KC77_02 was seen to increase to around 45 % when compared with the untreated lysozyme (an increase of 5 % when compared to KC77_02 in the absence of a redox couple.

3.3.2.4.2 Immobilisation of Lysozyme from the Urea-Unfolded, Reduced State from Lower Stock Concentrations - Discussion

Reducing the concentration of protein from which the PCMC preparation were made, has the effect of increasing the observed activity for samples prepared in propan-2-ol.

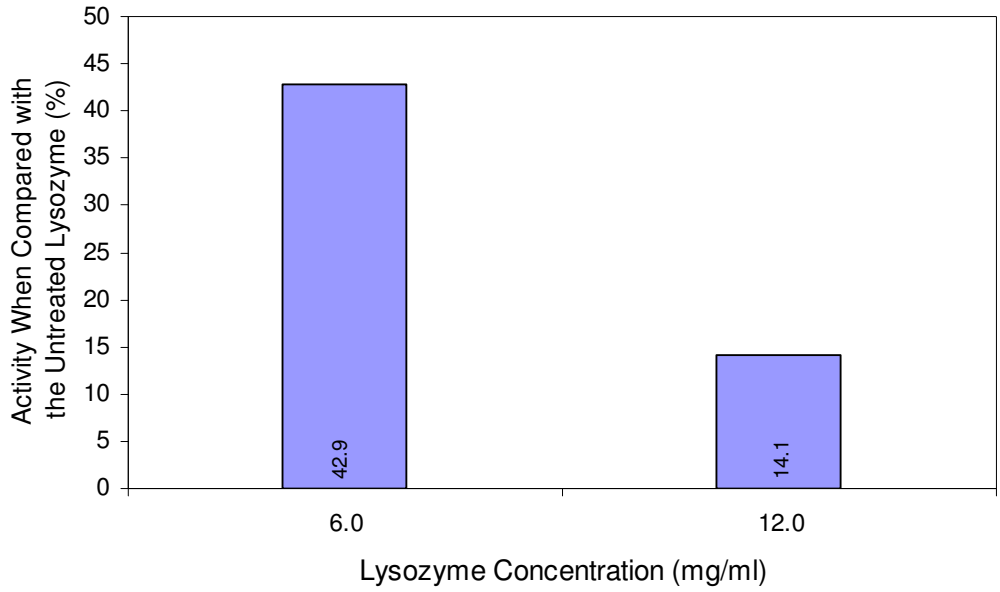


Figure 56: Immobilisation of Urea-Unfolded, Reduced Lysozyme on D,L-Valine - Effect of Protein Stock Concentration

A decrease in the concentration of the protein stock from which the PCMC were prepared (from 12 mg/ml to 6 mg/ml) resulted in a 300 % increase in the observed activity of the PCMC sample prepared in 2-PrOH.

As previously noted, the measured solid recovery is highest in propan-2-ol due to the slightly higher solubility of D,L-valine in ethanol than in 2-PrOH. There is also a delay in the addition of the lysozyme/ D,L-valine mixture and the appearance of precipitated material (PCMC).

The obtained CD spectrum of sample KC77_02 indicates that upon immobilisation of lysozyme upon the surface of D,L-valine, significant secondary structure can be regained. This could be a result of either a mixture of folded and unfolded populations or a partially folded population. Nonetheless, it is possible to say that the immobilisation of lysozyme from the unfolded, reduced state can give rise to either a partially folded or mixed product. It would have been interesting to carry out solid state CD spectroscopy on this formulation, but this could not be done due to inadequate quantities of the material.

The addition of a redox couple during reconstitution had little effect upon the observed activity. After two hours incubation of the PCMC in reconstitution buffer containing the redox couple, the activity of the protein increased by 5 % when compared with the non-incubated sample.

3.3.2.5 Immobilisation of Lysozyme from the Native and Urea-Unfolded, Reduced State Using a Syringe Pump

In order to determine if increased enzymatic activity could be regained by the gradual mixing of the protein solution and excipient; a syringe pump was employed. This allowed the excipient to be added to the protein containing solution at a slow rate (0.01 ml/min) to allow dilution of the denaturant before the immobilisation step occurs.

3.3.2.5.1 Immobilisation of Lysozyme from the Native and Urea-Unfolded, Reduced State Using a Syringe Pump - Results

PCMC of native and urea-unfolded, reduced lysozyme manufactured through the use of a syringe pump, were analysed *via* the reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and the specific activity (0.876A $min^{-1}mg^{-1}ml^{-1}$ for untreated protein).

Details of PCMC sample preparation can be found in Appendix 8.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC258_16	Lysozyme, on D,L-valine, 5 % load in 2-PrOH	81.2	5.6	0.544	62.1
KC258_20	Lysozyme in Urea + DTT on D,L-valine 5 % load in 2-PrOH	77.2	**	**	**

Table 9: Immobilisation of Lysozyme from the Native and Urea-Unfolded, Reduced State Using a Syringe Pump - Results (** The sample was insoluble upon attempted reconstitution)

3.3.2.5.2 Immobilisation of Lysozyme from the Native and Urea-Unfolded, Reduced State Using a Syringe Pump - Discussion

As previously noted, native lysozyme, when mixed gradually with excipient before precipitation using a syringe pump, shows a reduction in retained activity when compared with identical samples prepared in the conventional manner.

PCMC of urea-unfolded, reduced lysozyme when prepared by immediate mixing and precipitation can regain up to 43 % of the activity of the untreated protein. However, when gradual mixing is employed, the resultant PCMC are insoluble in the reconstitution buffer and as such, the protein loading and observed activity cannot be determined.

3.3.2.6 Dialysis Refolding of Lysozyme from the Urea-Unfolded, Reduced State - Comparison with Immobilisation

Dialysis, as a method for refolding was compared with immobilisation for urea-unfolded, reduced lysozyme. Lysozyme (6 mg/ml in 50 mM Tris/HCl, pH 8.1) which had been chemically unfolded *via* the addition 8 M Urea with 32 mM DTT was subjected to dialysis against buffer containing no additives. The protein was incubated for two hours before being subjected to dialysis (2 x three hours plus overnight).

Upon dialysis, protein aggregation was seen to occur to some extent, although not all protein had aggregated. Upon removal of the protein from the dialysis cassette, the enzyme was subjected to fluorescence spectroscopy and the activity assay described for the protein.

Fluorescence spectra of the urea-unfolded, reduced and dialysed proteins were obtained. The wavelength at which the maximum fluorescence intensity occurred for the native protein was the same as had been previously described (345 nm).

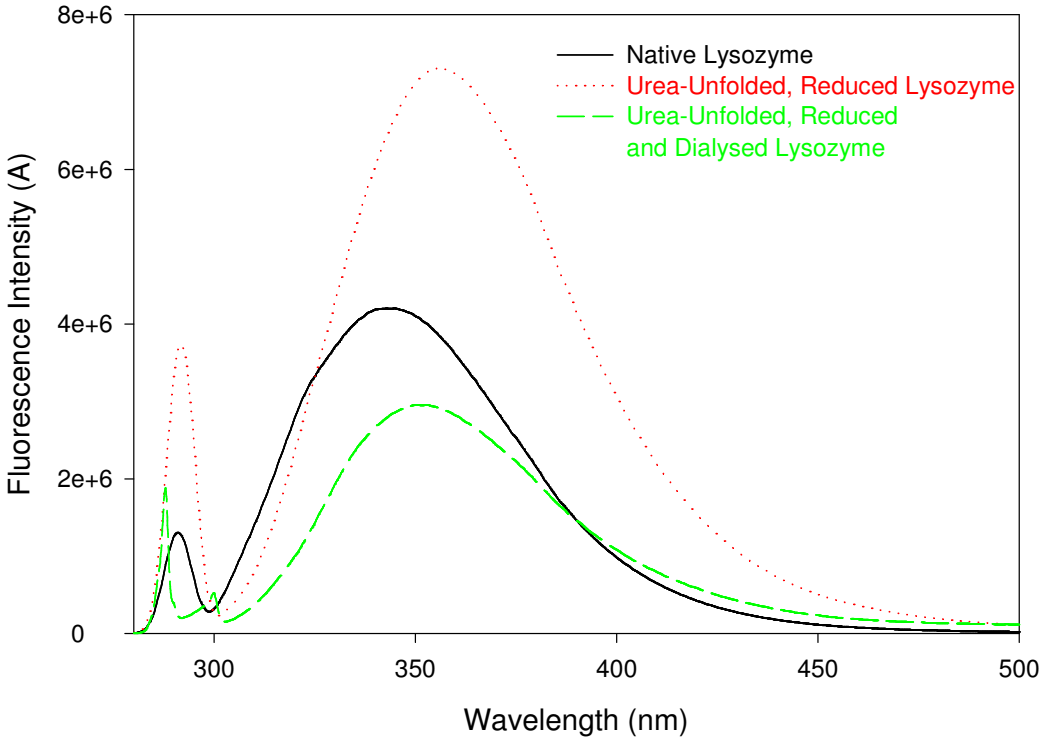


Figure 57: Dialysis of Urea-Unfolded, Reduced Lysozyme - Fluorescence Spectroscopy

When the lysozyme had been subjected to urea unfolding and reduction, this wavelength increased (356 nm) as expected. Upon subjecting the unfolded protein to dialysis, the wavelength at which the maximum fluorescence intensity occurs decreased slightly to 353 nm.

The enzymatic activity assay indicated that urea-unfolded, reduced lysozyme at a concentration of 6 mg/ml could regain 9.2 % of the activity of the native, untreated protein. This compares unfavourably with PCMC formation. Immobilisation of lysozyme from the urea-unfolded, reduced state can lead to the regain of up to 43 % activity compared with the untreated enzyme.

3.3.3 Immobilisation of Lysozyme from the Native and Thermally-Unfolded State

Thermal unfolding of proteins is a commonly used technique in protein denaturation studies. Upon heating, the protein molecules begin to unfold and interact with each other, resulting in the formation of aggregates. This aggregated/misfolded state may be a better model for inclusion bodies than lysozyme in the urea or guanidine hydrochloride unfolded state. In order to carry out any kind of refolding methodology on this aggregated protein,

these insoluble species must first be solubilised in the presence of an agent such as urea or GdnHCl.

PCMC of both native and thermally-unfolded GdnHCl (6 M) or urea (8 M) solubilised lysozyme were prepared.

3.3.3.1 Immobilisation of Lysozyme from the Native and Thermally-Unfolded, Guanidine Hydrochloride-Solubilised State

To determine if it is possible to immobilise thermally-unfolded, GdnHCl-solubilised lysozyme on to the surface of D,L-valine with the regain of enzymatic activity, immobilised protein samples were prepared. Lysozyme was thermally-unfolded and solubilised upon incubation of the aggregated protein in 6 M GdnHCl.

3.3.3.1.1 Immobilisation of Lysozyme from the Native and Thermally-Unfolded, Guanidine Hydrochloride-Solubilised State - Results

PCMC of thermally-unfolded, GdnHCl-solubilised lysozyme were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and specific activity (0.876 $Amin^{-1}mg^{-1}ml^{-1}$ for untreated protein).

Details of PCMC sample preparation can be found in Appendix 9.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC258_01	Lysozyme on D,L-valine, 1 % load in 2-PrOH	92.2	0.7	0.785	89.6
KC258_02	Lysozyme on D,L-valine, 5 % load in 2-PrOH	88.2	6.1	0.825	94.2
KC258_03	Lysozyme, heat unfolded on D,L-valine, 1 % load in 2-PrOH	88.0	**	**	**
KC258_04	Lysozyme, heat unfolded on D,L-valine, 5 % load in 2-PrOH	94.0	**	**	**

Table 10: Immobilisation of Lysozyme from the Native and Thermally-Unfolded Guanidine Hydrochloride-Solubilised State - Results (** The sample was insoluble upon attempted reconstitution)

3.3.3.1.2 Immobilisation of Lysozyme from the Native and Thermally-Unfolded, Guanidine Hydrochloride-Solubilised State - Discussion

Upon reconstitution, the thermally-unfolded GdnHCl-solubilised protein samples were insoluble in the aqueous phase. As such, the measured protein loading and specific activity of these samples could not be determined.

3.3.3.2 Immobilisation of Lysozyme from the Native and Thermally-Unfolded, Urea-Solubilised State

In order to determine if it is possible to immobilise thermally-unfolded, urea-solubilised lysozyme on to the surface of D,L-valine with the regain of enzymatic activity, immobilised protein samples were prepared. Lysozyme was thermally unfolded and solubilised upon incubation of the aggregated protein in 8 M urea.

3.3.3.2.1 Immobilisation of Lysozyme from the Native and Thermally-Unfolded, Urea-Solubilised State - Results

PCMC of thermally-unfolded lysozyme solubilised in urea were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and specific activity (0.876 $Amin^{-1}mg^{-1}ml^{-1}$ for the untreated protein).

Details of PCMC sample preparation can be found in Appendix 10.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC258_01	Lysozyme on D,L-valine, 1 % load in 2-PrOH	92.2	0.7	0.785	89.6
KC258_02	Lysozyme on D,L-valine, 5 % load in 2-PrOH	88.2	6.1	0.825	94.2
KC258_05	Lysozyme, heat unfolded on D,L-valine, 1 % load in 2-PrOH	82.8	**	**	**
KC258_06	Lysozyme, heat unfolded on D,L-valine, 5 % load in 2-PrOH	94.3	**	**	**

Table 11: Immobilisation of Lysozyme from the Native and Thermally-Unfolded, Urea-Solubilised State - Results (** The sample was insoluble upon attempted reconstitution)

3.3.3.2.2 Immobilisation of Lysozyme from the Native and Thermally-Unfolded, Urea-Solubilised State - Discussion

Upon reconstitution, the thermally-unfolded, urea-solubilised protein samples were insoluble in the aqueous phase. As such, the measured protein loading and specific activity of these samples could not be determined.

3.3.3.3 Immobilisation of Lysozyme from the Native and Thermally-Unfolded State Using a Syringe Pump

To determine if the gradual dilution of urea from the environment of the thermally-unfolded, solubilised protein would help to regain activity from the unfolded protein, PCMC samples were prepared using a syringe pump. This equipment allows the gradual dilution of GdnHCl or urea from the environment of the protein before precipitation begins (therefore triggering the refolding process *via* dilution).

3.3.3.3.1 Immobilisation of Lysozyme from the Native and Thermally-Unfolded State Using a Syringe Pump - Results

PCMC were prepared of lysozyme (from both the native and thermally-unfolded, solubilised state) using a syringe pump to mix the excipient and protein together gradually

(0.01 ml/min excipient into protein) before immediate precipitation of the mixture into 2-PrOH.

PCMC prepared from GdnHCl or urea-solubilised, thermally-unfolded lysozyme, were analysed *via* the reconstitution of the immobilised protein preparation in 50 mM Tris/HCl, pH 8.1 and subsequent determination of the % MPL (Measured Protein Loading) and the specific activity (0.876 $Amin^{-1}mg^{-1}ml^{-1}$ for the untreated protein).

Details of the PCMC sample preparation can be found in Appendix 11.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity ($A\ min^{-1}mg^{-1}ml^{-1}$)	% Activity of Native Protein
KC258_16	Lysozyme on D,L-valine, 5 % load in 2-PrOH	81.2	5.6	0.544	62.1
KC258_17	Lysozyme, heat unfolded, in GdnHCl on D,L-valine, 5 % load in 2-PrOH	88.7	**	**	**
KC258_18	Lysozyme, heat unfolded, in urea on D,L-valine, 5 % load in 2-PrOH	93.2	**	**	**

Table 12: Immobilisation of Lysozyme from the Native and Thermally-Unfolded State Using a Syringe Pump - Results (** The sample was insoluble upon attempted reconstitution)

3.3.3.3.2 Immobilisation of Lysozyme from the Native and Thermally-Unfolded State Using a Syringe Pump - Discussion

As previously noted, native lysozyme, when mixed gradually with excipient before precipitation using a syringe pump, shows a reduction in retained activity when compared with identical samples prepared without the use of a syringe pump.

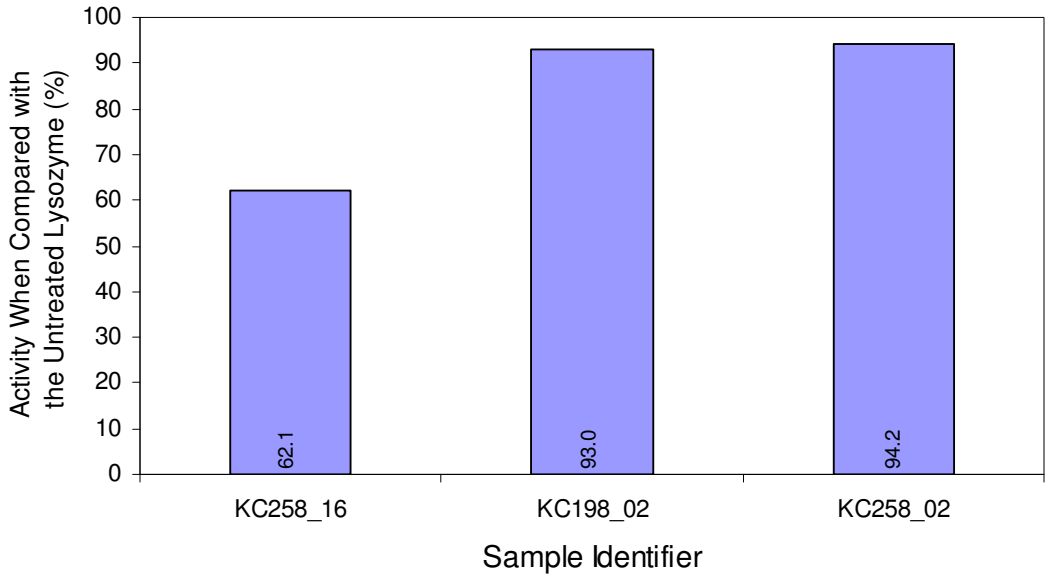


Figure 58: Immobilisation of Lysozyme from the Native State at 5 % Loading - Effect of Using a Syringe Pump

Identically prepared PCMC samples of native lysozyme immobilised on D,L-valine in propan-2-ol retain activity within 2 % of each other. However, when the excipient material and protein are gradually mixed as in the case of KC258_16, the activity retained decreases to approximately 62 %. This could be due to the time the aqueous solution of protein is kept at room temperature to carry out the slow mixing when compared with the immediate mixing and precipitation of the conventional PCMC preparation method.

When PCMC of thermally-unfolded lysozyme are prepared, the resulting immobilised protein is insoluble. Similarly when gradual mixing is employed to thermally-unfolded, urea or GdnHCl-solubilised lysozyme before precipitation, the resultant PCMC are insoluble in the reconstitution buffer and as such, the protein loading and activity cannot be determined.

3.4 Discussion and Conclusions

In the immobilisation and refolding of lysozyme, there are three different stages during which the refolding of the enzyme can occur: upon the addition of excipient to the unfolded protein (and subsequent dilution of the denaturant materials); during the actual PCMC coprecipitation process; or upon reconstitution of the powder under aqueous conditions.

Activity measurements show that lysozyme does not display catalytic activity upon dilution of the denaturing molecules from the protein environment. There is no evidence also that the presence of the excipient material D,L-valine has any triggering effect upon the refolding of lysozyme. Although amino acids such as arginine can be used in order to trigger protein folding, valine has no such effect.[141]

Solid State CD spectroscopy was carried out upon chemically unfolded lysozyme which had been subjected to PCMC formation. This indicated that the enzyme was present upon the surface of the microcrystal in a structure resembling the native state (section 3.3.1.2). CD spectroscopy of the reconstituted material showed very little change in the secondary structure of the protein when compared with the solid-state structure. The evidence obtained in the course of this study using lysozyme therefore indicates that the refolding of this particular enzyme occurs during the process of PCMC formation.

During PCMC formation, the ability of the surrounding solvent in maintaining native structure can be considered. Solvent conditions are important for protein folding and structure.[142] Under aqueous conditions, the interior of protein molecules are hydrophobic in character with the exterior being generally of a polar nature. In the presence of organic solvent, ionic and hydrogen bonding interactions are disrupted.[142] However, it has been shown from this study that lysozyme can be coprecipitated in organic solvent from both the native and the chemically-unfolded state with the retention (regain) of structure and function.

In the precipitation of lysozyme from the native state, the protein is seen to retain its structural characteristics. There is a body of evidence suggesting that in the presence of high concentrations of hydrophobic solvent, protein molecules such as lysozyme retain native structure due to “kinetic trapping which results in a more rigid structure in the absence of water”.[142] This is a result of the restrictions placed upon the conformational flexibility of the protein in organic solvent when compared with aqueous-organic

mixtures.[143] During precipitation of chemically-unfolded lysozyme, the denaturing molecules (urea or GdnHCl) are rapidly removed from the environment of the biomolecule, triggering folding.

From the data obtained, lysozyme can be chemically unfolded utilising urea (in the presence or absence of DTT) or the salt guanidine hydrochloride and subsequently refolded *via* the formation of PCMC from a range of conditions and solvents. Urea-incubated lysozyme can be immobilised upon the surface of DL-valine in 2-PrOH with the retention of up to 94.2 % of the activity compared to untreated lysozyme (KC77_04).

Upon addition of DTT to the unfolding conditions, the reduction of disulfide bonds leads to suppression of the regained activity. Immobilisation of urea-unfolded, reduced lysozyme can result in the regain of 42.9 % of the activity of the native, untreated protein. When compared with a commonly employed method for refolding (dialysis), the immobilisation method compares favourably. Dialysis of lysozyme from the urea-unfolded, reduced state regains 9.2 % of the activity of the native, untreated enzyme with formation of some aggregated species.

Lysozyme immobilised from the GdnHCl unfolded state can regain up to 84 % of the activity of the untreated enzyme depending upon the immobilisation excipient material and the precipitating solvent. Again, when compared with dialysis as a method for refolding, the PCMC immobilisation method excels. Dialysis of GdnHCl unfolded lysozyme regains 19.8 % of the activity of the untreated enzyme.

The immobilisation of lysozyme from the thermally-unfolded, solubilised state proved to be a greater challenge for this method than chemically unfolded species. Upon immobilisation, thermally-unfolded, solubilised lysozyme from various conditions yielded an insoluble enzyme preparation with little or no native structure. However, dialysis of the thermally unfolded, solubilised material also yielded an insoluble, aggregated product.

The refolding of lysozyme from the chemically or thermally-unfolded state *via* the formation of PCMC therefore outperforms or compares well with a generally applied method for protein refolding.

Chapter Four - Unfolding, Immobilisation and Potential Refolding of Lipase

4 Unfolding, Immobilisation and Potential Refolding of Lipase

Lipases have enormous potential as biocatalysts for biotransformations.[144, 145] These enzymes are utilised in a number of different hydrolysis reactions including ester and triglyceride hydrolysis.[104]

Lipase was subjected to chemical and thermal unfolding experiments and fluorescence and CD spectroscopy used to determine the conformational state of the protein. The enzyme was subsequently immobilised in both the native and unfolded state upon the surface of water soluble materials such as salts and amino acids for the formation of Protein Coated Micro-Crystals (PCMC).[77, 81 75]

The unfolding of lipase is typically irreversible; this presents a much greater challenge to the PCMC refolding technique than lysozyme.

4.1 Preparation of Chemically and Thermally-Unfolded Lipase

4.1.1 Preparation of Chemically-Unfolded Lipase

Chemically-unfolded lipase was prepared *via* the incubation of the protein in an aqueous solution of salts such as GdnHCl (6 M).

4.1.1.1 Guanidine Hydrochloride Unfolding of Lipase - Fluorescence Spectroscopy

Lipase (0.1 mg/ml) was incubated in 6 M GdnHCl for two hours at room temperature. Fluorescence measurements were made of the protein in both the native and the GdnHCl unfolded state.

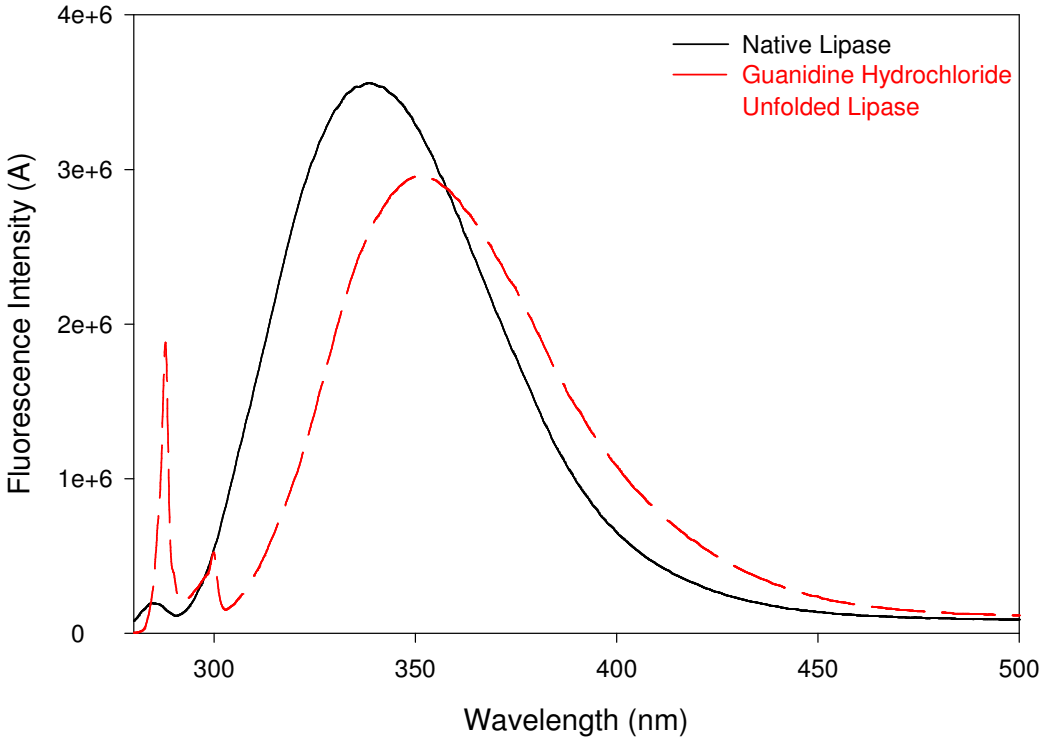


Figure 59: Guanidine Hydrochloride Unfolding if Lipase - Fluorescence Spectroscopy.

In the fluorescence spectrum of native lipase, the wavelength at which the maximum fluorescence occurs is 338 nm. This is in the range expected for a typical globular protein in which the tryptophan residues are buried within the structure.[40]

The aromatic amino acids in protein molecules give rise to the measured fluorescence. As protein unfolding occurs, the once buried tryptophan residues begin to interact with the

external environment leading to a change in the fluorescent properties of the biomolecule. As the molecule unfolds, a red shift in the wavelength at which the maximum fluorescence intensity is seen, resulting in a maximum fluorescence at a longer wavelength.

The wavelength at which the maximum fluorescence intensity occurs, changes upon incubation in GdnHCl to 350 nm. This indicates that these aromatic residues are no longer buried and are subsequently free to interact with the surrounding solvent, therefore suggesting that the protein is no longer in a folded state.

4.1.1.2 Guanidine Hydrochloride Unfolding of Lipase - CD Spectroscopy

Circular dichroism spectra of both the native and GdnHCl unfolded enzyme were obtained.

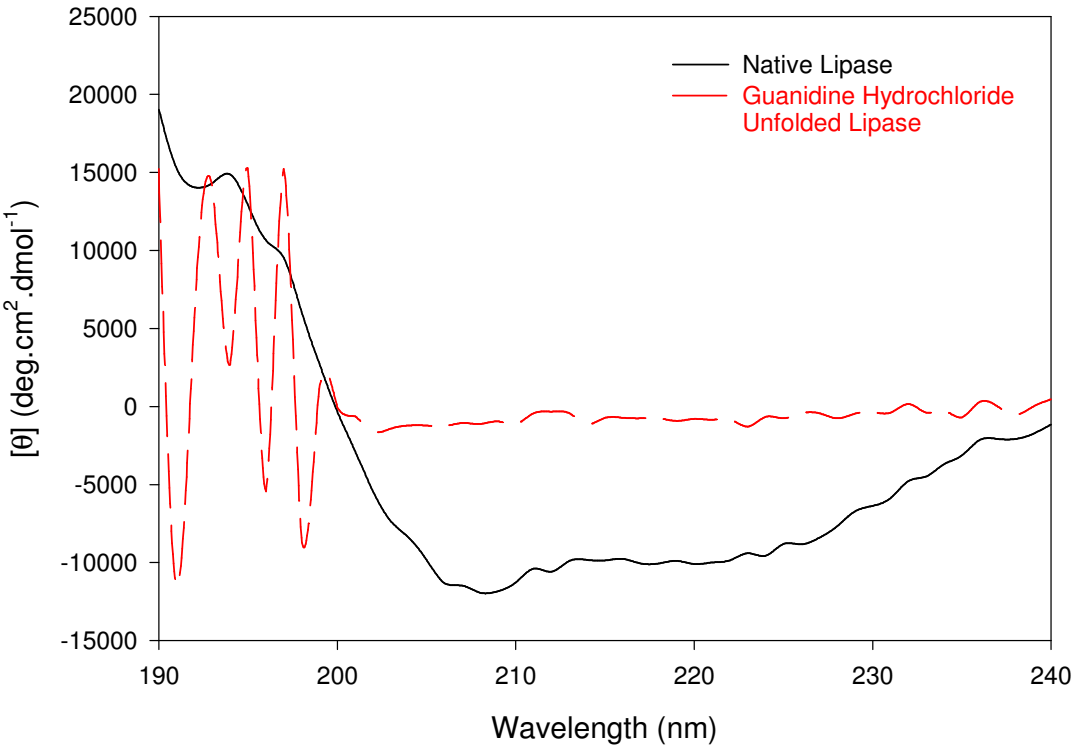


Figure 60: Guanidine Hydrochloride Unfolding of Lipase - Far UV CD Spectroscopy

CD spectroscopy of GdnHCl unfolded lipase indicates that upon incubation of lipase in 6 M GdnHCl for 2 hours, the protein is completely unfolded. The spectral noise obtained below 200 nm is due to the presence of the GdnHCl.

4.1.2 Preparation of Thermally-Unfolded Lipase

For the preparation of thermally-unfolded lipase, it is first necessary to assess how the protein responds to heat and at which temperature unfolding takes place. Differential Scanning Calorimetry can be used for this purpose.

4.1.2.1 Differential Scanning Calorimetry of Lipase

In order to assess the behaviour of lipase upon heating, Differential Scanning Calorimetry (DSC) was carried out on the protein. This involves controlled heating to determine at which temperature the protein is unfolded and if there is any refolding of the protein upon cooling. The DSC trace of lipase at 1 mg/ml is shown:

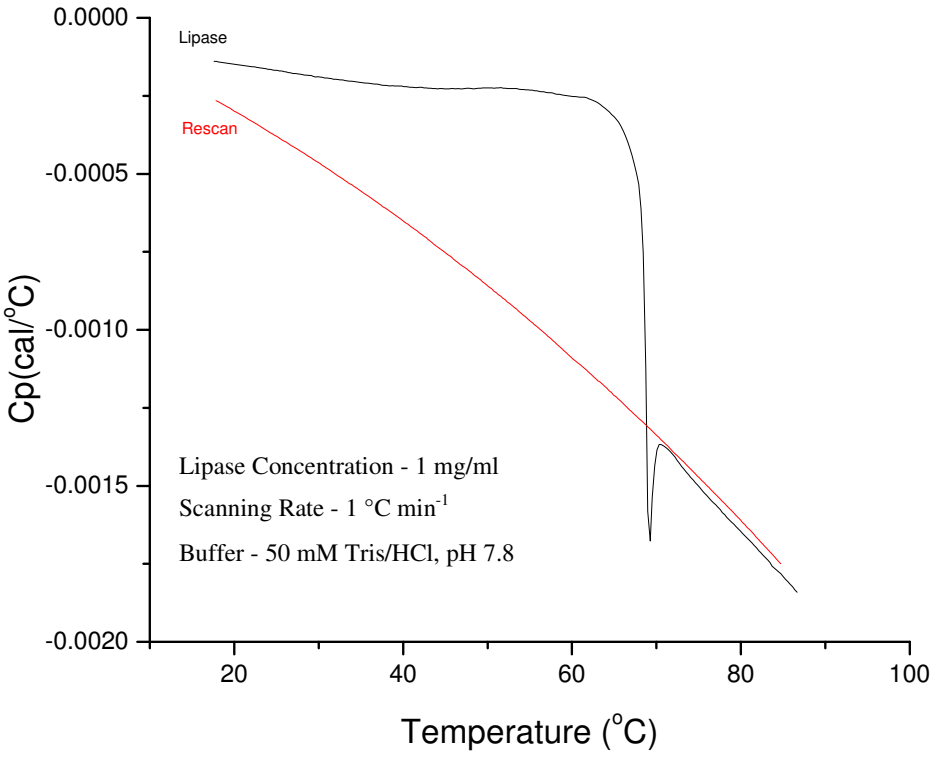


Figure 61: DSC Thermogram of Lipase

The DSC thermogram indicates that in order to unfold lipase thermally, the protein must be heated to a minimum of 64.3 °C (the measured Tm). The rescan shows that upon cooling, there is no evidence that thermally-unfolded lipase regains any native structure. This is a typical exothermic DSC signal which is typical of irreversible aggregation of unfolded protein.

4.1.2.2 Thermal Unfolding of Lipase - Fluorescence Spectroscopy

Lipase (0.1 mg/ml) was thermally unfolded by heating to a temperature of 90 °C and then allowed to cool to room temperature. Once cooled, visible aggregates were seen to form. The thermally-aggregated lipase was solubilised upon addition of 6 M GdnHCl and the aggregates were seen to dissolve to produce heat unfolded, solubilised lipase. The fluorescence spectrum of both this aggregated and solubilised protein is presented.

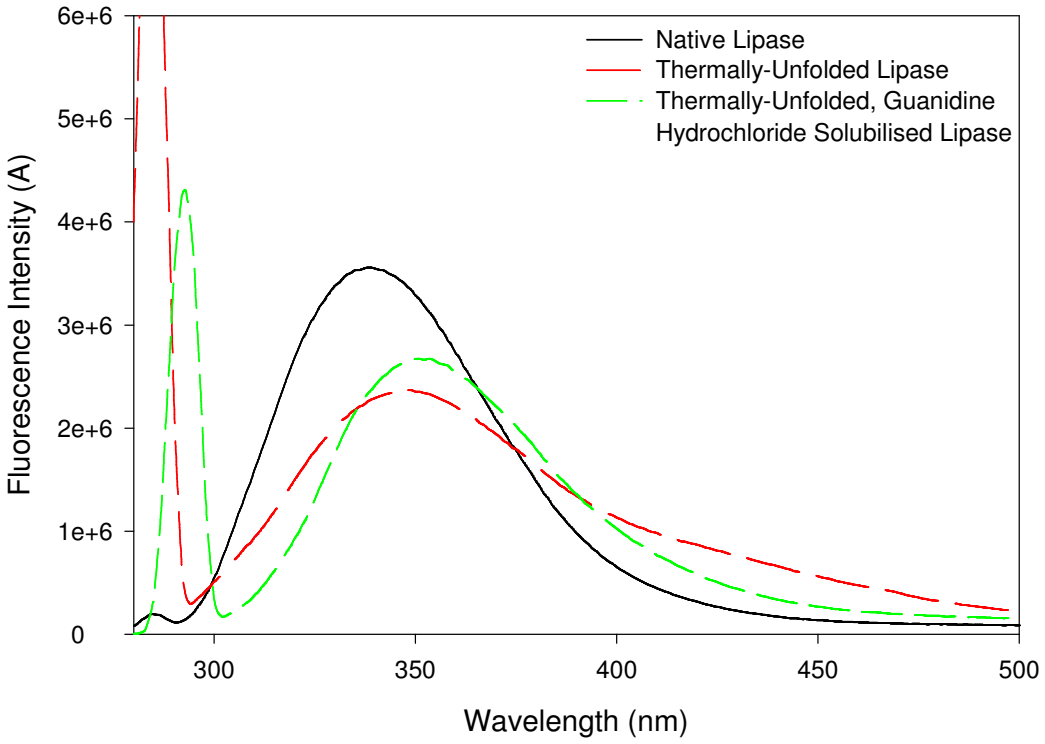


Figure 62: Thermal Unfolding of Lipase - Fluorescence Spectroscopy

Upon heating, the wavelength at which the maximum fluorescence intensity is seen increases from 338 nm for the native protein to 348 nm. This indicates that the protein is no longer in a native conformation.

This wavelength is lower than that of the chemically-unfolded lipase. Although the protein is no longer in a native state, the tryptophan residues which give rise to the fluorescence are confined within an aggregate and as such cannot interact with the surrounding solvent freely or in the same way as the native or chemically-unfolded protein.

Upon solubilisation with GdnHCl, this maximum fluorescence is seen to shift to a higher wavelength (352 nm). This indicates that the aggregates have been solubilised and the protein is no longer in an aggregated nor a native conformation.

4.1.2.3 Thermal Unfolding of Lipase - CD Spectroscopy

CD spectroscopy of the thermally-unfolded, GdnHCl-solubilised lipase was carried out in order to ascertain the effect of heating and subsequent solubilisation upon the enzyme structure

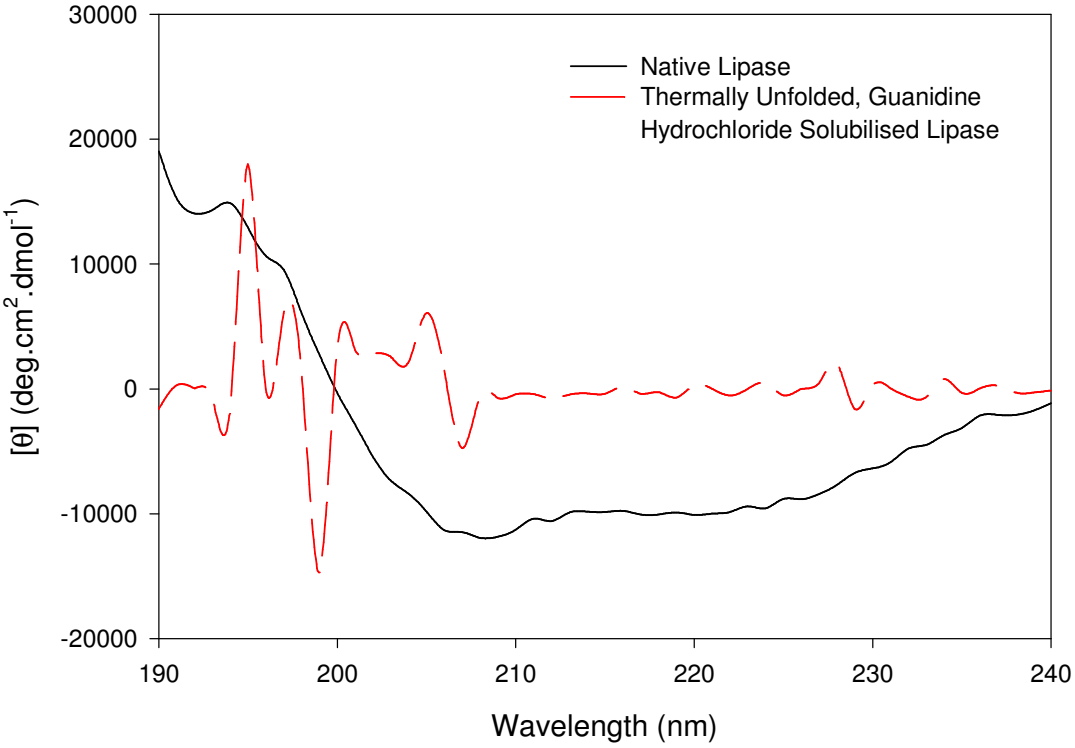


Figure 63: Thermal Unfolding of Lipase - CD Spectroscopy

Circular dichroism spectroscopy of the thermally-unfolded, GdnHCl-solubilised lipase indicates that upon heating and subsequent solubilisation, the enzyme is completely unfolded.

4.2 Immobilisation of Lipase from the Native and Unfolded State

PCMC of both native and unfolded protein were prepared in order to determine the efficacy of this method for both the immobilisation and potential folding of lipase.

To find the optimum conditions for the immobilisation and folding steps, various excipients, solvents and protein concentrations were employed. Additives such as a redox couple (for example cystine and cysteine) and catalytic substrate (10 molar percent of *p*-NPA) were also utilised in order to see if they had any effect upon the potential refolding of lipase. Different methods were also employed for the formation of the PCMC.

From previously published studies it is known that lipase from different sources can be immobilised upon the surface of PCMC with little or no loss of activity in both the aqueous and organic phases.[77] Techniques similar to these were adopted in this study and therefore little protein degradation or loss of activity is expected from the native state.

Once precipitated, the samples were filtered, weighed and stored as dry powders in glass vials. UV/vis spectroscopy and activity assays were carried out in order to determine the effect of both chemical and thermal unfolding, solubilisation and immobilisation upon lipase.

4.2.1 Immobilisation of Lipase from the Native and Chemically-Unfolded State

4.2.1.1 Immobilisation of Lipase from the Native and Guanidine Hydrochloride Unfolded State - Results

PCMC of both native and GdnHCl unfolded lipase were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 7.8 and subsequent determination of the %MPL (Measured Protein Loading) and the specific activity (0.1877 $Amin^{-1}mg^{-1}$ for untreated protein).

Details of PCMC sample preparation can be found in Appendix 12.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC214-01	Lipase on K_2SO_4, 1 % load in 2-PrOH	88.0	2.1	0.1614 (± 0.010)	86.0 (± 5.3)
KC214-02	Lipase on K_2SO_4, 5 % load in 2-PrOH	88.0	4.6	0.1472	78.4
KC214-03	Lipase on K_2SO_4, 10 % load in 2-PrOH	86.5	11.2	0.1747	93.1
KC214-04	Lipase on K_2SO_4, 15 % load in 2-PrOH	82.1	16.7	0.1779	94.8
KC214-05	Lipase on K_2SO_4, 20 % load in 2-PrOH	84.5	21.2	0.1451	77.3
KC214-06	Lipase on K_2SO_4, 50 % load in 2-PrOH	82.2	53.8	0.0944	75.2
KC214-07	Lipase in GdnHCl on K_2SO_4, 1 % load in 2-PrOH	96.4	1.8	0.0439 (± 0.012)	23.4 (± 6.4)
KC214-08	Lipase in GdnHCl on K_2SO_4, 5 % load in 2-PrOH	99.7	5.1	0.0358	19.1
KC214-09	Lipase in GdnHCl on K_2SO_4, 10 % load in 2-PrOH	96.0	10.6	0.0376	36.3
KC214-10	Lipase in GdnHCl on K_2SO_4, 15 % load in 2-PrOH	90.0	17.2	0.0681	34.2
KC214-11	Lipase in GdnHCl on K_2SO_4, 20 % load in 2-PrOH	89.3	22.1	0.0653	34.8
KC214-12	Lipase in GdnHCl on K_2SO_4, 50 % load in 2-PrOH	83.6	49.4	0.0143	7.6

Table 13: Immobilisation of Lipase from the Native and Guanidine Hydrochloride Unfolded State - Results

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

4.2.1.1.1 Immobilisation of Lipase from the Native and Guanidine Hydrochloride Unfolded State - CD Spectroscopy

CD spectroscopy was carried out on immobilised native (KC214_04) and GdnHCl unfolded lipase (KC214_09) upon reconstitution in order to gain an insight into the structure of the reconstituted protein sample.

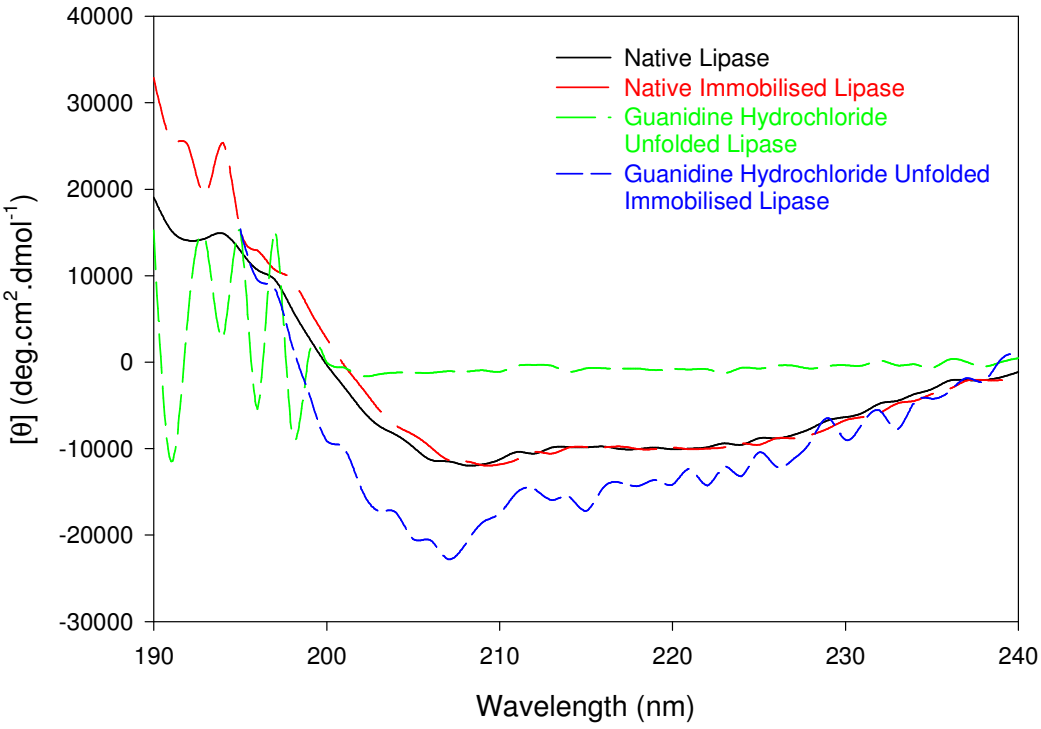


Figure 64: Immobilisation of Lipase from the Native and Guanidine Hydrochloride Unfolded State - CD Spectroscopy

4.2.1.2 Immobilisation of Lipase from the Native and Guanidine Hydrochloride-Unfolded State - Discussion

The measured solids recovery of the prepared PCMC suggest that immobilised lipase samples can be prepared using K_2SO_4 as an excipient with very little of the solid or protein being lost to the solvent.

In order to ascertain the effect of the immobilisation of both the native and the GdnHCl unfolded protein, it is necessary to compare the observed activity measured for these samples.

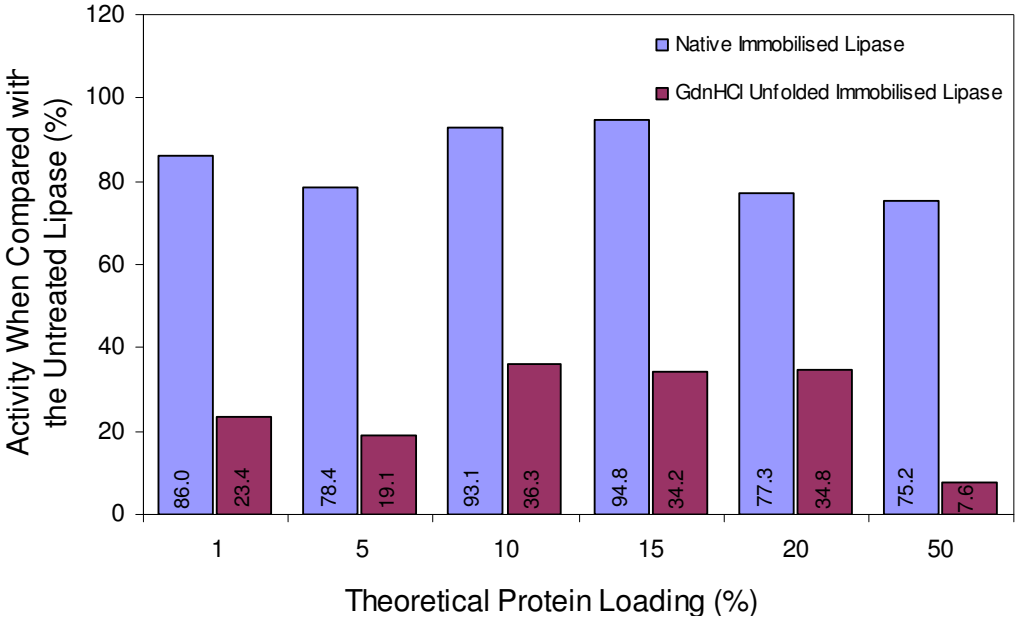


Figure 65: Immobilisation of Lipase from the Native and Guanidine Hydrochloride Unfolded State - Comparison of Regained Activity

Immobilisation of the native protein on K_2SO_4 at theoretical protein loadings ranging from 1 - 50 % can retain up to 94.8 % of activity when compared with the untreated protein.

When lipase is unfolded in GdnHCl and further subjected to immobilisation, the protein regains up to 36.3 % of the activity of the untreated lipase. This suggests that although experimentally shown to be unfolded using GdnHCl (by fluorescence and CD spectroscopy), lipase can regain a moderate level of activity upon removal of the salt from the protein environment.

CD spectroscopy shows that upon reconstitution, the native immobilised sample is almost identical in structure to the untreated protein. Immobilisation of the GdnHCl incubated protein confers some regain of structure from the unfolded state.

4.2.2 Immobilisation of Lipase from the Native and Chemically-Unfolded State Using a Syringe Pump

In order to determine if increased enzymatic activity could be regained by the gradual mixing of the protein solution and excipient; a syringe pump was employed. This allowed the excipient to be added to the protein containing solution at a slow rate (0.01 ml/min) to allow gradual dilution of the denaturant before the immobilisation step occurs.

4.2.2.1 Immobilisation of Lipase from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump- Results

PCMC of native and GdnHCl unfolded lipase manufactured using a syringe pump, were analysed *via* the reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 7.8 and subsequent determination of the %MPL (Measured Protein Loading) and the specific activity (0.1877A $min^{-1}mg^{-1}$ for untreated protein).

Details of PCMC sample preparation can be found in Appendix 13.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC255_01	Lipase, on K_2SO_4, 5 % load in 2-PrOH	84.7	6.04	0.127	67.9
KC255_03	Lipase in GdnHCl on K_2SO_4, 5 % load in 2-PrOH	83.3	**	**	**

Table 14: Immobilisation of Lipase from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump - Results (**The sample was insoluble upon attempted reconstitution)

4.2.2.2 Immobilisation of Lipase from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump - Discussion

Native lipase, when mixed gradually with excipient before precipitation using a syringe pump shows a reduction in retained activity when compared with an identical sample prepared in the conventional manner.

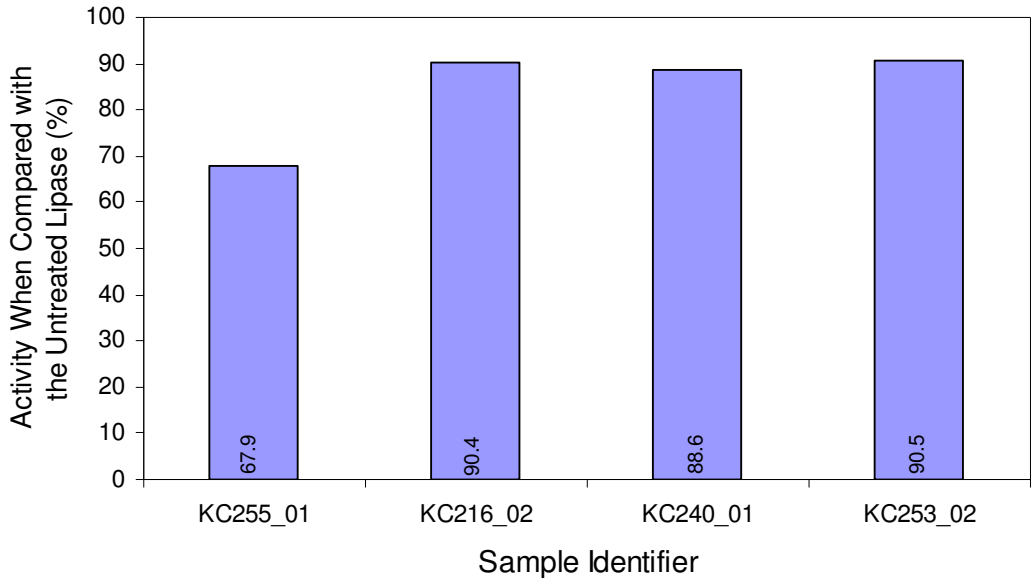


Figure 66: Immobilisation of Native Lipase at 5 % Loading - Effect of Using a Syringe Pump

Identical PCMC formulations of native lipase immobilised on K_2SO_4 in propan-2-ol all retain activity within 2 % of each other. However, when the excipient material and protein are gradually mixed as in the case of KC255_01, the retained activity decreases to approximately 68 %. This could be due to the time the aqueous solution of protein is kept at room temperature to carry out the slow mixing when compared with the immediate mixing and precipitation of the conventional PCMC preparation method.

4.2.2.3 Dialysis Refolding of Lipase in the Guanidine Hydrochloride Unfolded State- Comparison with Immobilisation

Dialysis is a common method used for protein folding which involves the gradual controlled removal of denaturing molecules from the environment of proteins.

Dialysis, as a method for refolding was compared with the immobilisation method for GdnHCl unfolded lipase. Lipase (6 mg/ml in 50 mM Tris/HCl, pH 7.8) which had been chemically-unfolded *via* the addition 6 M GdnHCl was subjected to dialysis against buffer containing no GdnHCl salt. The protein was incubated for two hours before being subjected to dialysis (two x three hours plus overnight).

Upon dialysis, protein aggregation was seen to occur to some extent, although not all of the protein present had aggregated. Upon removal of the protein from the dialysis cassette, the enzyme was subjected to fluorescence spectroscopy and the activity assay described for the protein.

Fluorescence spectra of the GdnHCl unfolded and dialysed protein were obtained. The wavelength at which the maximum fluorescence intensity occurred for the native protein was the same as had been previously described (338 nm).

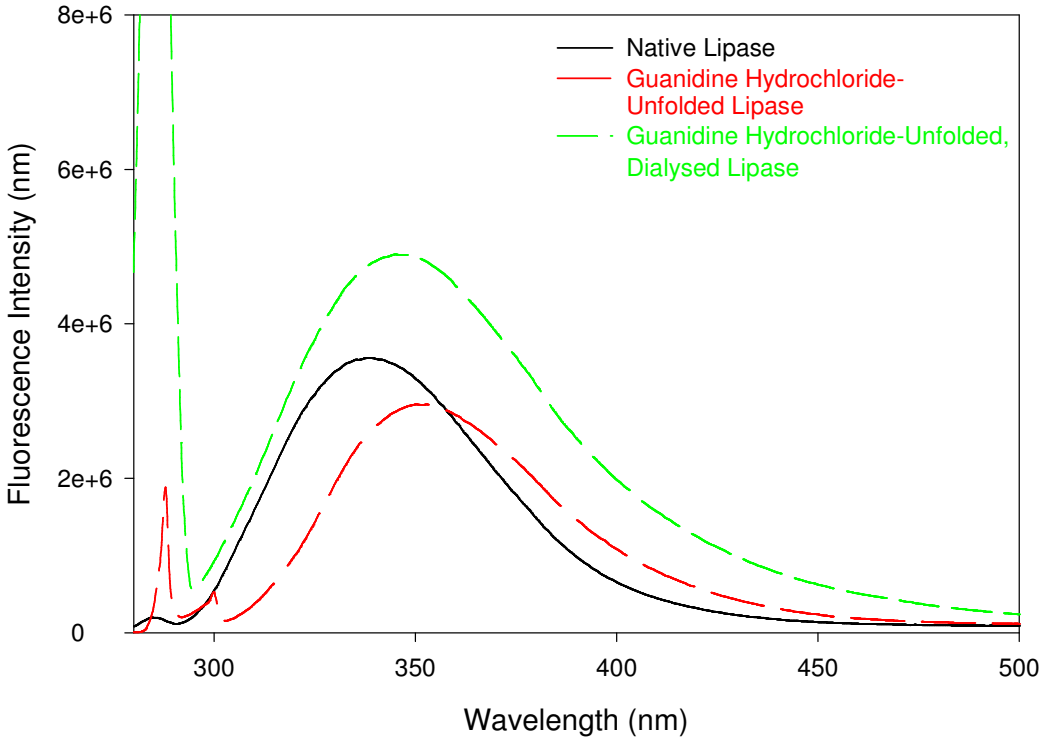


Figure 67: Dialysis Refolding of Lipase from the Guanidine Hydrochloride Unfolded State – Fluorescence Spectroscopy

When the lipase had been subjected to GdnHCl unfolding, this wavelength increased (350 nm) as expected. Upon subjecting the unfolded protein to dialysis, the wavelength at which the maximum fluorescence intensity occurs decreased slightly to 346 nm. This indicated that the protein was no longer in an unfolded, solubilised state.

The enzymatic activity assay indicated that chemically-unfolded lipase at a concentration of 6 mg/ml could regain 11.37 % of the activity of the native, untreated protein.

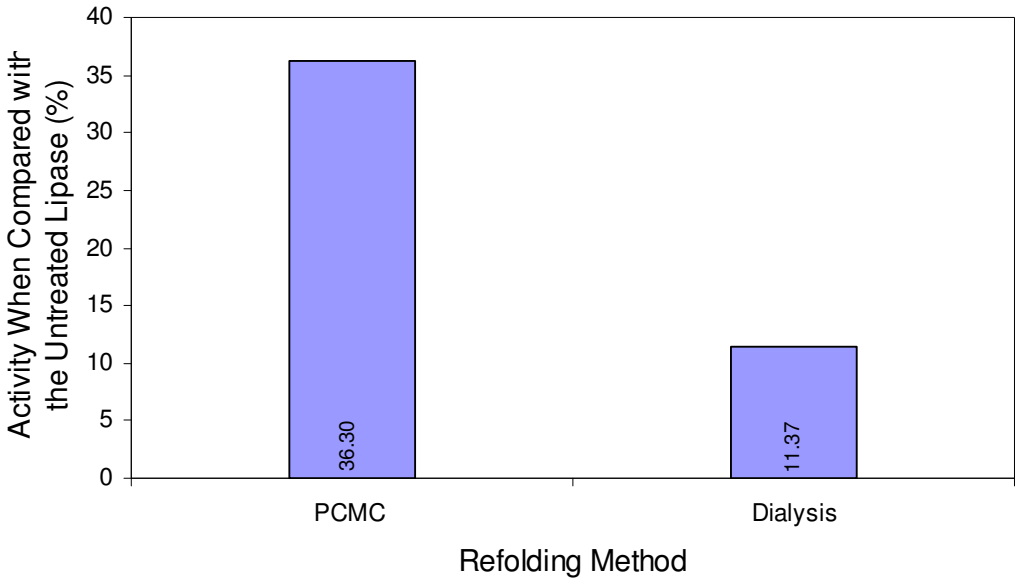


Figure 68: Comparison of Dialysis and PCMC Preparation as Refolding Methods

The data obtained indicated that lipase unfolded in GdnHCl regained activity to a greater extent *via* immobilisation using PCMC technology when compared with simple dialysis as a refolding method.

Immobilisation by the formation of PCMC can therefore be described as a more efficient method for the refolding of GdnHCl unfolded lipase than simple dialysis refolding.

4.2.3 Immobilisation of Lipase from the Native and Thermally-Unfolded State

PCMC were prepared of both native and thermally-unfolded lipase, solubilised in the presence of 6 M GdnHCl

4.2.3.1 Immobilisation of Lipase from the Native and Thermally-Unfolded, Solubilised State - Results

PCMC of native and thermally-unfolded lipase were analysed *via* the reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 7.8 and subsequent determination of the %MPL (Measured Protein Loading) and the specific activity (0.1877A $min^{-1}mg^{-1}$ for untreated protein).

Details of PCMC sample preparation can be found in Appendix 14.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC216_01	Lipase on K_2SO_4, 1 % load in 2-PrOH	97.4	1.3	0.1798 (± 0.014)	95.8 (± 7.5)
KC216_02	Lipase on K_2SO_4, 5 % load in 2-PrOH	94.5	5.1	0.1697	90.4
KC216_03	Lipase on K_2SO_4, 10 % load in 2-PrOH	93.6	11.7	0.1599	85.2
KC216_04	Lipase on K_2SO_4, 15 % load in 2-PrOH	95.6	15.3	0.1757	93.6
KC216_05	Lipase on K_2SO_4, 20 % load in 2-PrOH	90.5	21.3	0.1440	76.7
KC216_06	Lipase on K_2SO_4, 50 % load in 2-PrOH	80.6	53.2	0.1630 (± 0.012)	86.9 (± 6.4)
KC216_07	Lipase on D,L-valine, 1 % load in 2-PrOH	83.2	1.4	*	*
KC216_08	Lipase on D,L-valine, 5 % load in 2-PrOH	84.9	4.8	*	*
KC216_09	Lipase on D,L-valine, 10 % load in 2-PrOH	83.0	10.7	*	*
KC216_10	Lipase on D,L-valine, 15 % load in 2-PrOH	74.6	16.1	*	*
KC216_11	Lipase on D,L-valine, 20 % load in 2-PrOH	66.2	21.7	*	*
KC216_12	Lipase on D,L-valine, 50 % load in 2-PrOH	72.2	51.6	*	*

Table 15: Immobilisation of Lipase from the Native State Using K_2SO_4 and D,L-Valine as Excipients - Results (*The data could not be determined for this sample)

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC226_01	Lipase, heat unfolded on K_2SO_4, 1 % load in 2-PrOH	92.4	**	**	**
KC226_02	Lipase, heat unfolded on K_2SO_4, 5 % load in 2-PrOH	98.9	**	**	**
KC226_03	Lipase, heat unfolded on K_2SO_4, 10 % load in 2-PrOH	98.8	**	**	**
KC226_04	Lipase, heat unfolded on K_2SO_4, 15 % load in 2-PrOH	97.7	**	**	**
KC226_05	Lipase, heat unfolded on K_2SO_4, 20 % load in 2-PrOH	87.5	**	**	**
KC226_06	Lipase, heat unfolded on K_2SO_4, 50 % load in 2-PrOH	90.5	**	**	**
KC226_07	Lipase, heat unfolded on D,L-valine, 1 % load in 2-PrOH	80.8	**	**	**
KC226_08	Lipase, heat unfolded on D,L-valine, 5 % load in 2-PrOH	60.0	**	**	**
KC226_09	Lipase, heat unfolded on D,L-valine, 10 % load in 2-PrOH	75.4	**	**	**
KC226_10	Lipase, heat unfolded on D,L-valine, 15 % load in 2-PrOH	84.3	**	**	**
KC226_11	Lipase, heat unfolded on D,L-valine, 20 % load in 2-PrOH	87.3	**	**	**
KC226_12	Lipase, heat unfolded on D,L-valine, 50 % load in 2-PrOH	80.5	**	**	**

Table 16: Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State - Results

(**The sample was insoluble upon attempted reconstitution)

4.2.3.2 Immobilisation of Lipase from the Native and Thermally-Unfolded, Solubilised State - Discussion

When lipase is thermally-unfolded with subsequent solubilisation by GdnHCl, the aggregates which form are seen to dissolve. When this protein mixture is further subjected to immobilisation *via* the formation of PCMC, the resultant material is insoluble under the aqueous conditions required for the activity assay to be carried out. Filtration of these re-constituted samples results in removal of these insoluble particles, but also removal of virtually all protein from the buffer solution as the protein is held in these insoluble species. The MPL and specific activity of these samples was therefore undeterminable.

The measured solids recovery of the PCMC prepared using D,L-valine as an excipient was lower than those which were prepared from K_2SO_4. This could be due to the fact that although the precipitating solvent is saturated with the excipient before precipitation occurs, some of the amino acid has been lost to the solvent due to the increasing water content as the protein/excipient mixture is added.

4.2.3.2.1 Lipase coated K_2SO_4

Native lipase, when immobilised on K_2SO_4 using theoretical protein loadings of 1 - 50 %, retained up to 95.8 % of the activity of the untreated protein. This indicates that lipase in the native state can be immobilised upon this excipient using the PCMC preparation method with very little detrimental effect on the activity of the protein.

4.2.3.2.2 Lipase coated D,L-Valine

Upon analysis of lipase coated D,L-valine samples, both native and heat unfolded, there seemed to be an anomaly with the activity assay. Activity of up to 200 % was seen for each sample, and this was obviously unexpected.

The assay was subsequently carried out in the presence of uncoated crystals (no lipase present). This demonstrated that the excipient had a kinetic effect upon the rate of hydrolysis of *p*-NPA (the substrate used in the activity assay). This therefore resulted in the activity of the immobilised enzyme being masked by the effect of the D,L-valine upon the reaction.

Subsequent investigation proved that this effect was not due to any pH change occurring upon addition of the amino acid to the substrate solution, and it appeared therefore that the D,L-valine was also able to hydrolyse the ester.

There is precedent for this reaction. *p*-NPA is insoluble in water and therefore acetonitrile is used as a solvent for the substrate. The ester, in the presence of an organic solvent, is susceptible to hydrolysis by the amino acids.[146, 147] D,L-valine therefore has the ability to hydrolyse the *p*-NPA ester under these conditions.

PCMC of thermally-unfolded lipase immobilised upon D,L-valine showed activity upon reconstitution even although there were aggregates present. This could be attributed to the fact that the D,L-valine was soluble in the buffer (even although the protein was not) and was therefore able to hydrolyse the ester substrate under these conditions.

D,L-valine is therefore not a suitable excipient for the immobilisation and potential refolding of lipase as the enzymatic activity of the samples one of the primary analysis techniques being carried out, could not be determined for any of these powders.

4.2.3.3 Dialysis Refolding of Thermally-Unfolded, Solubilised Lipase - Comparison with Immobilisation

Dialysis, as a method for refolding was compared with the immobilisation method for thermally-unfolded, solubilised lipase. Lipase (6 mg/ml in 50 mM Tris/HCl, pH 7.8) which had been thermally unfolded *via* heating to 90 ºC and subsequently solubilised by incubation in 6 M GdnHCl, was subjected to dialysis against buffer containing no GdnHCl salt. The protein was incubated for two hours before being subjected to dialysis (two x three hours plus overnight).

Upon dialysis, protein aggregation was seen to occur immediately upon gradual removal of the denaturing salts from the protein environment. Upon removal of the protein from the dialysis cassette, the enzyme was subjected to fluorescence spectroscopy and the activity assay described for the protein.

Fluorescence spectra of the GdnHCl unfolded and dialysed protein was obtained. The wavelength at which the maximum fluorescence intensity occurred for the native protein was the same as had been previously described (338 nm).

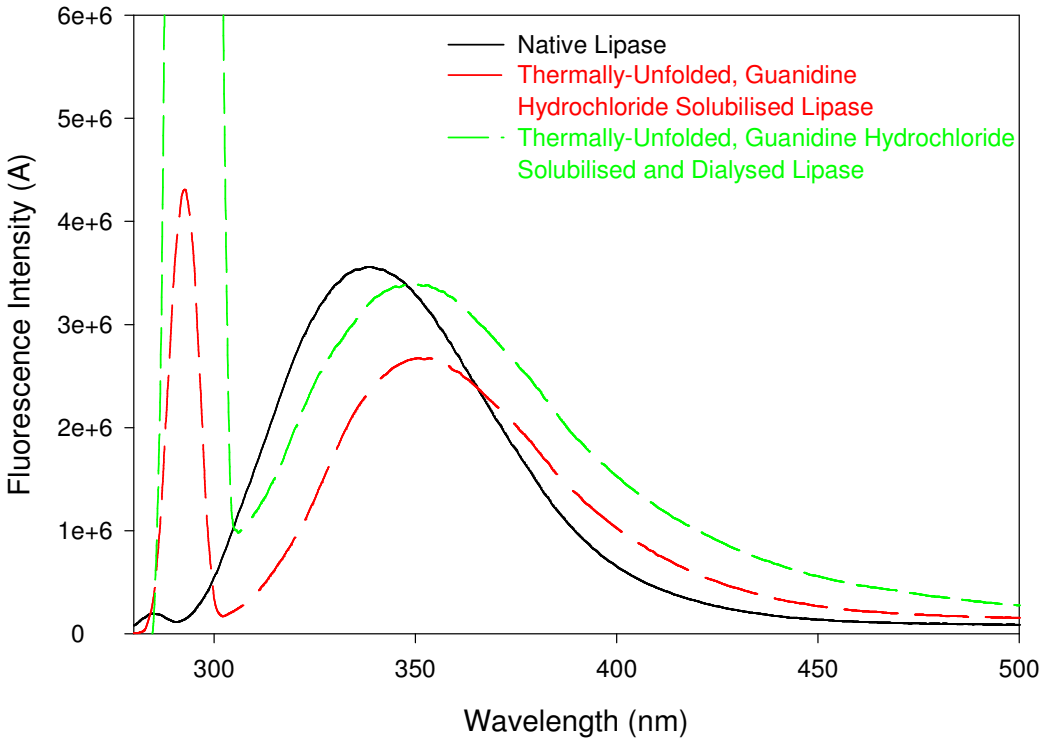


Figure 69: Dialysis Refolding of Thermally-Unfolded, Solubilised Lipase – Fluorescence Spectroscopy

When the lipase had been subjected to thermal unfolding and solubilisation, this wavelength increased (351 nm) as expected. Upon being subjected to dialysis, the wavelength at which the maximum fluorescence intensity occurs decreased slightly to 350 nm.

CD spectroscopy was carried out upon the thermally-unfolded lipase and the dialysed lipase.

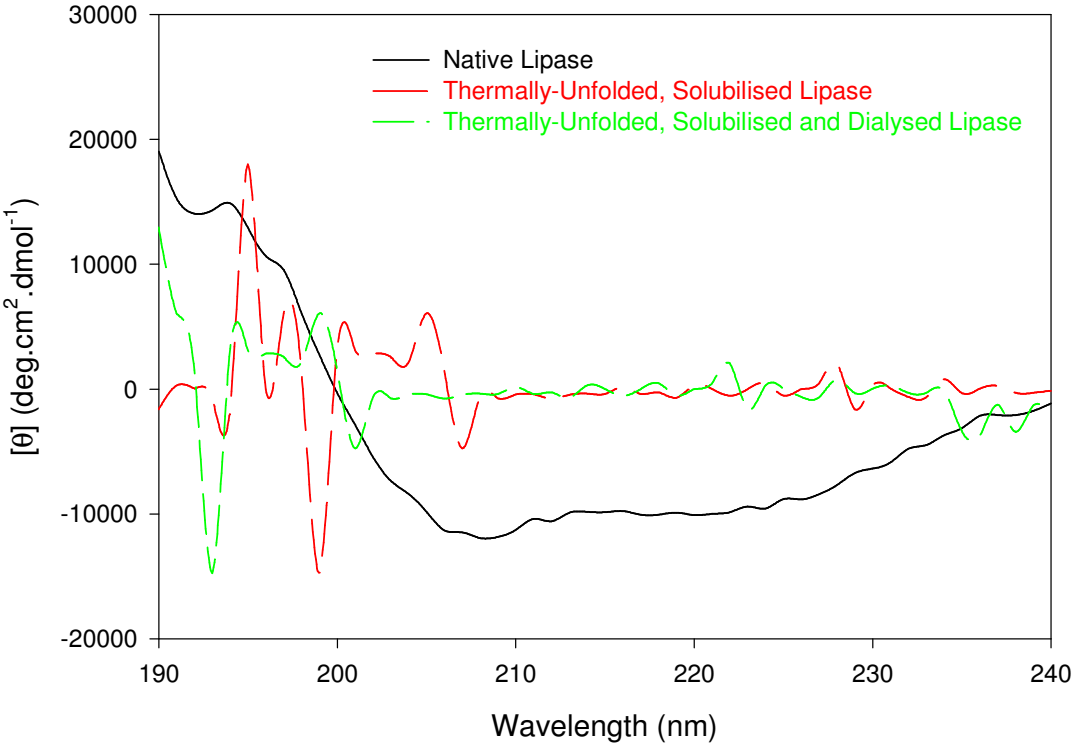


Figure 70: Dialysis Refolding of Thermally-Unfolded, Solubilised Lipase - CD Spectroscopy

The CD spectrum of the dialysed protein shows that there has been no regain in secondary structure towards the native state upon dialysis.

The previously described activity assay was carried out on the filtered dialysed protein with the result that no activity was regained upon dialysis. The PCMC prepared from the thermally-unfolded, solubilised lipase also showed no regain of activity upon immobilisation and therefore, neither method was appropriate for the refolding of thermally-unfolded lipase at this concentration.

4.2.4 Immobilisation of Lipase from the Native and Thermally-Unfolded State with the Introduction of a Redox Couple

The immobilisation of thermally-unfolded lipase had previously formed insoluble aggregates upon reconstitution and no further analysis could therefore be carried out. This could be attributed to the fact that the protein molecules immobilised upon the surface of the excipient were not folded in an active conformation or had interacted with each other to form insoluble species immediately prior to immobilisation.

Many studies have been carried out employing redox couples to potentially form the correct disulfide pairing and aid the folding process.[148-150] Upon incubation of the thermally-unfolded, solubilised lipase with a redox couple such as cystine/cysteine, the disulfides which have been broken can be allowed to reform and break again until the most energetically favourable correct combination is found.

4.2.4.1 Immobilisation of Lipase from the Native State Utilising K_2SO_4 and KCl as Excipient Materials - Results

PCMC of native lipase were analysed *via* reconstitution of the immobilised protein sample in 50 mM Tris/HCl, pH 7.8 and subsequent determination of the %MPL (Measured Protein Loading) and the specific activity (0.1877A $min^{-1}mg^{-1}$ for untreated protein).

Details of PCMC sample preparation can be found in Appendix 15.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC240_01	Lipase on K_2SO_4, 5 % load in 2-PrOH	91.0	5.3	0.1663 (± 0.013)	88.6 (± 6.9)
KC240_02	Lipase on K_2SO_4, 10 % load in 2-PrOH	88.0	10.9	0.1386	73.9
KC240_03	Lipase on K_2SO_4, 15 % load in 2-PrOH	89.3	15.7	0.1636	87.2
KC240_04	Lipase on K_2SO_4, 20 % load in 2-PrOH	86.7	21.3	0.1373	73.2
KC240_05	Lipase on KCl, 5 % load in 2-PrOH	69.8	5.5	0.1590 (± 0.015)	84.5 (± 6.9)
KC240_06	Lipase on KCl, 10 % load in 2-PrOH	82.0	9.2	0.1220	65.0
KC240_07	Lipase on KCl, 15 % load in 2-PrOH	82.3	14.6	0.1655	88.2
KC240_08	Lipase on KCl, 20 % load in 2-PrOH	82.7	20.8	0.1254	67.0

Table 17: Immobilisation of Lipase from the Native State Utilising K_2SO_4 and KCl as Excipient Materials - Results

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

4.2.4.2 Immobilisation of Lipase from the Native State Utilising K_2SO_4 and KCl as Excipient Materials - Discussion

Analysis of the PCMC of native lipase indicated that the enzyme could be immobilised on the surface of K_2SO_4 crystals with the retention of up to 89 % of the activity of the untreated enzyme.

Similarly, immobilisation of the native protein on the surface of KCl prepares immobilised enzyme with the retention of up to 88 % of the activity of untreated lipase.

The measured solids yield of the KCl samples is significantly less than that of the K_2SO_4 preparations. This could be due to some of the KCl dissolving in the precipitating solvent rather than being involved in PCMC co-precipitation.

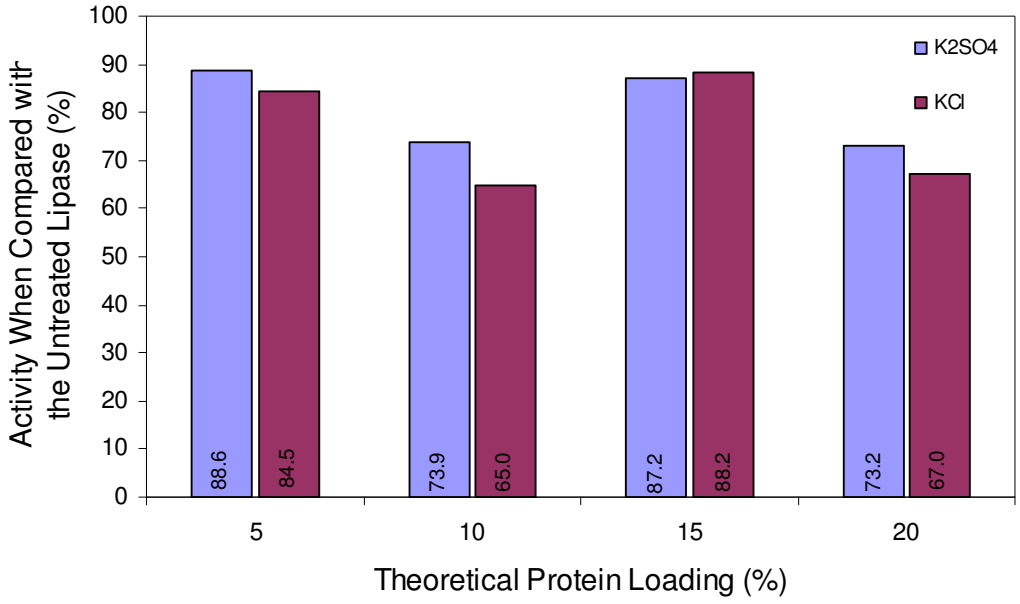


Figure 71: Comparison of K_2SO_4 and KCl as Excipients for Lipase Immobilisation

From the data provided it is evident that K_2SO_4 is a marginally better excipient for lipase immobilisation at these protein loadings than KCl. The PCMC prepared using KCl as an excipient in general have lower solid recovery and retain less activity than their K_2SO_4 counterparts.

4.2.4.3 Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State - Results

PCMC of thermally-unfolded lipase (prepared from 6 mg/ml lipase) were analysed *via* reconstitution of the immobilised protein preparation in 50 mM Tris/HCl, pH 7.8 with a view to subsequent determination of the %MPL (Measured Protein Loading) and the specific activity (0.1877A $min^{-1}mg^{-1}$ for untreated protein). However the PCMC samples proved to be insoluble.

Details of PCMC sample preparation can be found in Appendix 16.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC240_17	Lipase, heat unfolded on K_2SO_4, 5 % load in 2-PrOH	96.3	**	**	**
KC240_18	Lipase, heat unfolded on K_2SO_4, 10 % load in 2-PrOH	95.8	**	**	**
KC240_19	Lipase, heat unfolded on K_2SO_4, 15 % load in 2-PrOH	99.5	**	**	**
KC240_20	Lipase, heat unfolded on K_2SO_4, 20 % load in 2-PrOH	97.0	**	**	**
KC240_21	Lipase, heat unfolded on KCl, 5 % load in 2-PrOH	86.5	**	**	**
KC240_22	Lipase, heat unfolded on KCl, 10 % load in 2-PrOH	71.8	**	**	**
KC240_23	Lipase, heat unfolded on KCl, 15 % load in 2-PrOH	86.2	**	**	**
KC240_24	Lipase, heat unfolded on KCl, 20 % load in 2-PrOH	79.3	**	**	**

Table 18: Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State - Results
(**The sample was insoluble upon attempted reconstitution)

4.2.4.4 Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State - Discussion

The PCMC prepared using lipase which had been thermally-unfolded were insoluble in the reconstitution buffer and as such, further physical measurements of the immobilised enzyme could not be carried out.

4.2.4.5 Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State with the Introduction of a Redox Couple - Results

In an attempt to aid refolding (and therefore the solubility of the prepared PCMC), a redox couple was added to the unfolded protein during the GdnHCl solubilisation step in an effort to allow the correct reformation of disulfide bonds formed from the 9 cystine residues present in wheat germ lipase.

PCMC of thermally-unfolded lipase with the addition of a redox couple (prepared from 6 mg/ml lipase) were analysed *via* reconstitution of the immobilised protein preparation in 50 mM Tris/HCl, pH 7.8 with a view to subsequent determination of the %MPL (Measured Protein Loading) and the specific activity (0.1877A $min^{-1}mg^{-1}$ for untreated protein). However the PCMC samples proved to be insoluble.

Details of PCMC sample preparation can be found in Appendix 17.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC240_09	Lipase, heat unfolded with redox pair on K_2SO_4, 5 % load in 2-PrOH	93.5	**	**	**
KC240_10	Lipase, heat unfolded with redox pair on K_2SO_4, 10 % load in 2-PrOH	97.8	**	**	**
KC240_11	Lipase, heat unfolded with redox pair on K_2SO_4, 15 % load in 2-PrOH	96.7	**	**	**
KC240_12	Lipase, heat unfolded with redox pair on K_2SO_4, 20 % load in 2-PrOH	98.2	**	**	**
KC240_13	Lipase, heat unfolded with redox pair on KCl, 5 % load in 2-PrOH	83.8	**	**	**
KC240_14	Lipase, heat unfolded with redox pair on KCl, 10 % load in 2-PrOH	84.1	**	**	**
KC240_15	Lipase, heat unfolded with redox pair on KCl, 15 % load in 2-PrOH	85.3	**	**	**
KC240_16	Lipase, heat unfolded with redox pair on KCl, 20 % load in 2-PrOH	87.0	**	**	**

Table 19: Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State with the Introduction of a Redox Couple - Results (**The sample was insoluble upon attempted reconstitution)

4.2.4.6 Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State with the Introduction of a Redox Couple - Discussion

The addition of cystine/cysteine as a redox couple appeared to have little or no effect upon the solubility of the PCMC samples prepared and as such the sample remained insoluble.

Again, the protein concentration before immobilisation for these samples was high (6 mg/ml) and further investigation was required to see if a lower concentration of protein would provide more favourable results.

Another consideration is the loading of the protein on the surface of the crystals. If the protein molecules are beginning to fold *via* immobilisation, the close proximity of these biomolecules to one another may lead to interactions between them, and hence, to the formation of protein aggregates.

4.2.4.7 Dialysis Refolding of Lipase from the Thermally-Unfolded, Solubilised State with the Addition of a Redox Couple - Comparison with Immobilisation

The process of immobilisation of unfolded protein and the rapid removal of the denaturing salts from the environment of the protein did not yield soluble crystals. It can be assumed therefore that the immobilisation method for potential lipase refolding is unsuccessful.

Lipase (6 mg/ml) which had been thermally-unfolded and allowed to cool was solubilised in the presence of 6 M GdnHCl containing the redox couple of cystine and cysteine. The protein was incubated for 2 hours before being subjected to dialysis (two x three hours plus overnight).

The protein in the dialysis cassette was seen to aggregate almost immediately upon dialysis. This observation can be attributed to the solubilising agent GdnHCl being removed from the environment of the protein and therefore resulting in the interaction of the protein molecules with each other, forming aggregates.

4.2.5 Immobilisation of Lipase from the Native and Thermally-Unfolded State at Low Concentrations and Loadings with the Introduction of a Redox Couple.

PCMC were prepared of lipase from the native and thermally-unfolded state. These samples were prepared both in the presence and absence of a redox couple at low loadings from protein at a concentration of 1 mg/ml in the appropriate buffer.

4.2.5.1 Immobilisation of Lipase from the Native State at Low Concentrations and Loadings - Results

PCMC of native lipase were analysed *via* reconstitution of the immobilised protein preparation in 50 mM Tris/HCl, pH 7.8 with a view to subsequent determination of the %MPL (Measured Protein Loading) and the specific activity (0.1877A $min^{-1}mg^{-1}$ for untreated protein).

Details of PCMC sample preparation can be found in Appendix 18.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC245_01	Lipase on K_2SO_4, 0.01 % load in 2-PrOH	85.3	0.018	*	*
KC245_02	Lipase on K_2SO_4, 0.1 % load in 2-PrOH	84.5	0.21	*	*
KC245_03	Lipase on K_2SO_4, 0.5 % load in 2-PrOH	84.8	0.59	*	*
KC245_04	Lipase on KCl, 0.01 % loading in 2-PrOH	78.1	0.024	*	*
KC245_05	Lipase on KCl, 0.1 % load in 2-PrOH	80.1	0.19	*	*
KC245_06	Lipase on KCl, 0.5 % load in 2-PrOH	77.7	0.61	*	*

Table 20: Immobilisation of Lipase from the Native State at Low Concentrations and Loadings - Results (*The data could not be determined for this sample)

4.2.5.2 Immobilisation of Lipase from the Native State at Low Concentrations and Loadings - Discussion

The assay used to determine the activity of lipase was not sensitive enough to be used with protein at such low concentrations; therefore, the specific activity if the immobilised protein samples could not be determined.

In order to gain some kind of insight into the effect that immobilisation confers on to the protein, the fluorescence spectrum of each of the PCMC samples was obtained. There was no evidence of refolding having taken place from the fluorescence spectra.

4.2.5.3 Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State at Low Concentration and Loading - Results

PCMC of thermally-unfolded lipase were analysed *via* reconstitution of the immobilised protein in 50 mM Tris/HCl, pH 7.8 with a view to subsequent determination of the %MPL (Measured Protein Loading) and the specific activity (0.1877A $min^{-1}mg^{-1}$ for untreated protein).

Details of the PCMC sample preparation can be found in Appendix 19.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC245_07	Lipase, heat unfolded on K_2SO_4, 0.01 % load in 2-PrOH	86.4	**	**	**
KC245_08	Lipase, heat unfolded on K_2SO_4, 0.1 % load in 2-PrOH	84.8	**	**	**
KC245_09	Lipase, heat unfolded on K_2SO_4, 0.5 % load in 2-PrOH	87.8	**	**	**
KC245_10	Lipase, heat unfolded on KCl, 0.01 % load in 2-PrOH	76.9	**	**	**
KC245_11	Lipase, heat unfolded on KCl, 0.1 % load in 2-PrOH	76.6	**	**	**
KC245_12	Lipase, heat unfolded on KCl, 0.5 % load in 2-PrOH	70.2	**	**	**

Table 21: Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State at Low Concentrations and Loadings - Results (**The sample was insoluble upon attempted reconstitution)

4.2.5.4 Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State at Low Concentration and Loading - Discussion

Upon reconstitution, the thermally-unfolded immobilised samples were again shown to be insoluble in the aqueous phase, although appeared to have reconstituted to a greater extent. To the naked eye, there appeared to be fewer, smaller aggregates.

4.2.5.5 Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State at Low Concentration and Loading with the Introduction of a Redox Couple - Results

PCMC of thermally-unfolded lipase with the addition of a redox couple were analysed *via* reconstitution of the immobilised protein in 50 mM Tris/HCl, pH 7.8 with a view to subsequent determination of the %MPL (Measured Protein Loading) and the specific activity (0.1877A $min^{-1}mg^{-1}$ for untreated protein).

Details of the PCMC sample preparation can be found in Appendix 20.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC245_13	Lipase, heat unfolded with redox pair on K_2SO_4, 0.01 % load in 2-PrOH	83.5	**	**	**
KC245_14	Lipase, heat unfolded with redox pair on K_2SO_4, 0.1 % load in 2-PrOH	82.7	**	**	**
KC245_15	Lipase, heat unfolded with redox pair on K_2SO_4, 0.5 % load in 2-PrOH	85.5	**	**	**
KC245_16	Lipase, heat unfolded with redox pair on KCl, 0.01 % load in 2-PrOH	83.0	**	**	**
KC245_17	Lipase, heat unfolded with redox pair on KCl, 0.1 % load in 2-PrOH	79.9	**	**	**
KC245_18	Lipase, heat unfolded with redox pair on KCl, 0.5 % load in 2-PrOH	83.1	**	**	**

Table 22: Immobilisation of Lipase from the Thermally-Unfolded, Solubilised State with the Addition of a Redox Couple - Results (**The sample was insoluble upon attempted reconstitution)

4.2.5.6 Immobilisation of Thermally-Unfolded Lipase at Low Concentration and Loading with the Introduction of a Redox Couple - Discussion

Upon lowering of both the stock protein concentration and the theoretical protein loading, the PCMC appeared to have marginally greater solubility in the aqueous phase. The addition of the redox couple however showed no improvement in the solubility of the immobilised protein

The PCMC appear to the eye to have reconstituted to a greater extent than previously prepared samples.

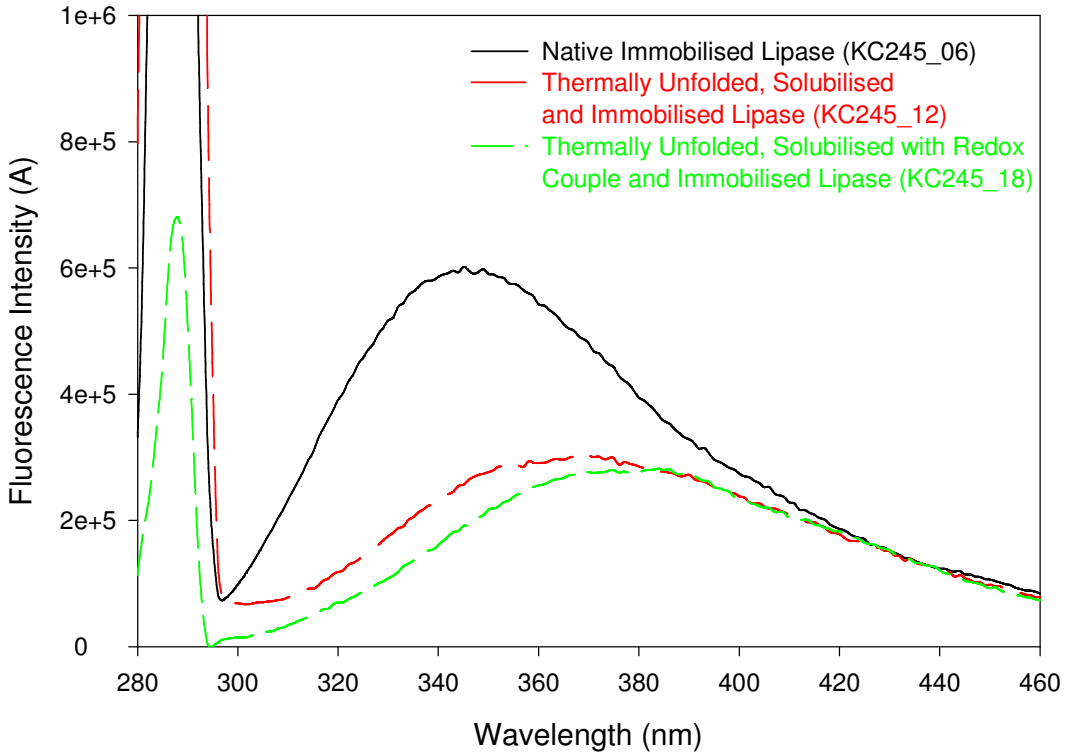


Figure 72: Fluorescence Spectra of Immobilised Lipase Samples

The fluorescence spectra of the reconstituted PCMC, show that although the solubility of the samples has improved somewhat, the protein immobilised upon the surface of the crystals is not in a folded state. The addition of the redox couple has no significant effect upon the state of the immobilised protein.

4.2.6 Immobilisation of Lipase from the Native and Thermally-Unfolded State with the Addition of a Catalytic Substrate

In an effort to trigger the refolding of thermally-unfolded lipase, a catalytic concentration (10 molar %) of enzymatic substrate was added to the solubilised protein in the incubation step. The incubation was carried out at both room temperature and at 37 ºC (the optimum temperature of the enzyme).

4.2.6.1 Immobilisation of Lipase from the Native State in the Presence of Catalytic Substrate at Room Temperature and 37 ºC - Results

PCMC samples of native lipase (both in the presence and absence of catalytic substrate) were analysed *via* the reconstitution of the immobilised protein preparation in 50 mM Tris/HCl, pH 7.8 with a view to subsequently determining the %MPL (Measured Protein Loading) and the specific activity.

Details of the PCMC sample preparation can be found in Appendix 21.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC253_01	Lipase on K_2SO_4, 1 % load in 2-PrOH	96.5	1.67	0.1787 (± 0.017)	95.2 (± 9.1)
KC253_02	Lipase on K_2SO_4, 5 % load in 2-PrOH	99.7	4.79	0.1699	90.5
KC253_03	Lipase on K_2SO_4, 1 % load in 2-PrOH at 37 ºC	90.7	1.34	0.1779	94.8
KC253_04	Lipase on K_2SO_4, 5 % load in 2-PrOH at 37 ºC	95.2	6.12	0.1729	92.1
KC253_05	Lipase + 10 molar % *p*-NPA on K_2SO_4, 1 % load in 2-PrOH	90.7	1.22	0.1775 (± 0.017)	94.6 (± 9.1)
KC253_06	Lipase + 10 molar % *p*-NPA on K_2SO_4, 5 % load in 2-PrOH	95.2	5.61	0.1691	90.1
KC253_07	Lipase + 10 molar % *p*-NPA on K_2SO_4, 1 % load in 2-PrOH at 37 ºC	96.2	1.66	0.1717	91.5
KC253_08	Lipase + 10 molar % *p*-NPA on K_2SO_4, 5 % load in 2-PrOH at 37 ºC	86.9	5.61	0.1693	90.2

Table 23: Immobilisation of Lipase from the Native State in the Presence of Catalytic Substrate - Results

Results for multiple measurements are indicated in the data by listing the mean value (± standard deviation). Standard errors for single measurements are anticipated to be of the same order of magnitude.

4.2.6.2 Immobilisation of Lipase from the Native State with the Addition of Catalytic Substrate - Discussion

The addition of a catalytic concentration of the substrate *p*-NPA appears to have a slight detrimental effect upon the measured solid recovery. The substrate is insoluble in water and therefore has to be dissolved in acetonitrile. The effect upon the solid recovery could perhaps be attributed to the excipient material being dissolved in the added in the coprecipitation.

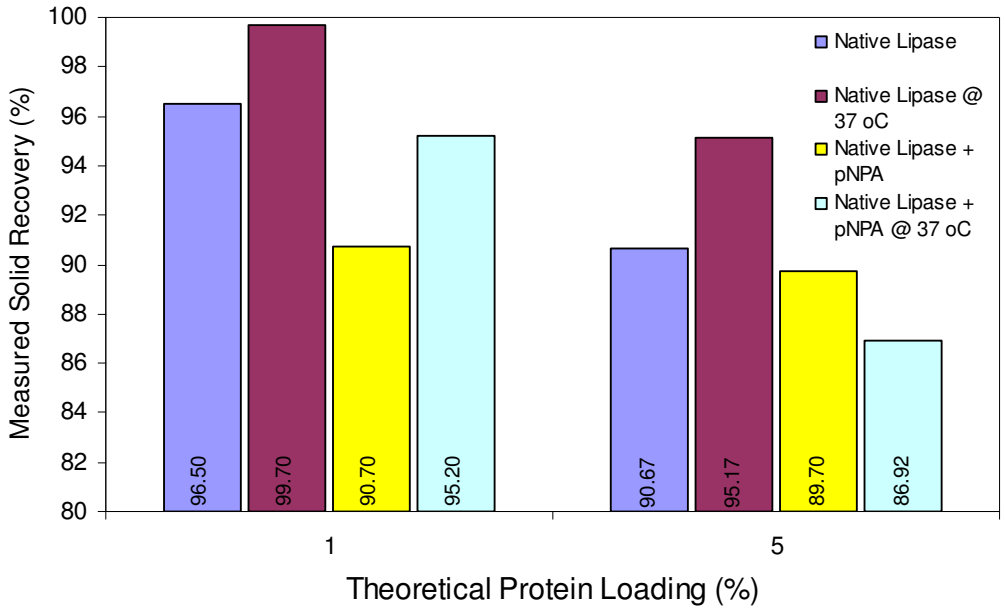


Figure 73: Immobilisation of Lipase from the Native State in the Presence of Catalytic Substrate at Room Temperature and 37 °C - Measured Solid Recovery (%)

It can be noted that the measured solids recovery of the PCMC which were prepared at 37 °C show a higher yield than their room temperature counterparts.

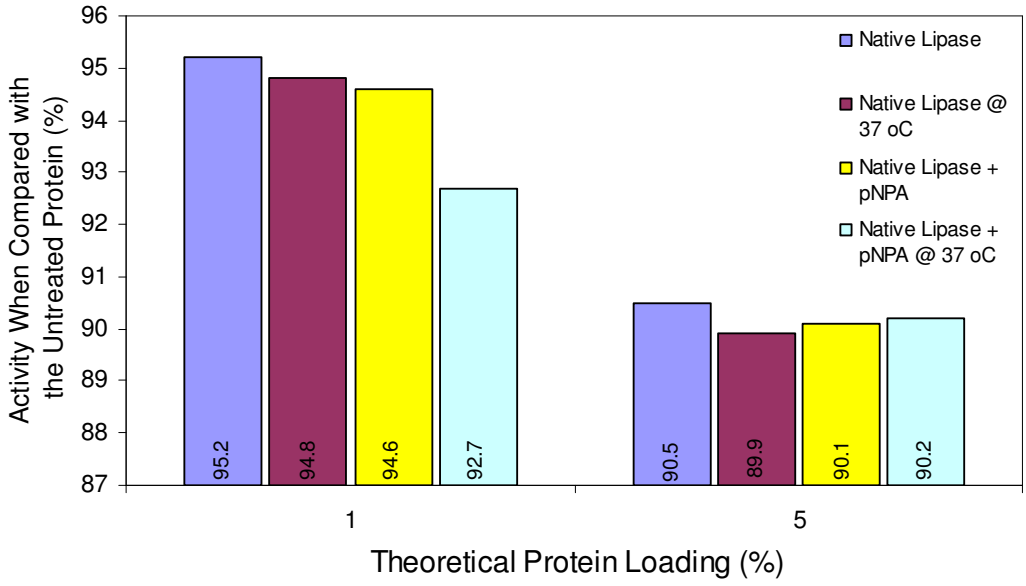


Figure 74: Immobilisation of Lipase from the Native State in the Presence of Catalytic Substrate at Room Temperature and 37 °C - Activity

Manufacturing PCMC at 37 °C showed no significant effect upon the retained activity of the immobilised native lipase samples. The presence of catalytic substrate also showed

little effect upon the activity retained during immobilisation when compared with the untreated protein.

4.2.6.3 Immobilisation of Lipase from the Thermally-Unfolded State with the Addition of Catalytic Substrate - Results

PCMC samples of thermally unfolded lipase (both in the presence and absence of catalytic substrate) were analysed *via* the reconstitution of the immobilised protein preparation in 50 mM Tris/HCl, pH 7.8 with a view to subsequently determining the %MPL (Measured Protein Loading) and the specific activity.

Details of the PCMC sample preparation can be found in Appendix 22.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC253_09	Lipase, heat unfolded, on K_2SO_4, 1 % load in 2-PrOH	95.0	**	**	**
KC253_10	Lipase, heat unfolded, on K_2SO_4, 5 % load in 2-PrOH	99.3	**	**	**
KC253_11	Lipase, heat unfolded, on K_2SO_4, 1 % load in 2-PrOH at 37 ºC	96.7	**	**	**
KC253_12	Lipase, heat unfolded, on K_2SO_4, 5 % load in 2-PrOH at 37 ºC	99.7	**	**	**
KC253_13	Lipase, heat unfolded + 10 molar % *p*-NPA on K_2SO_4, 1 % load in 2-PrOH	97.5	**	**	**
KC253_14	Lipase, heat unfolded + 10 molar % *p*-NPA on K_2SO_4, 5 % load in 2-PrOH	97.8	**	**	**
KC253_15	Lipase, heat unfolded + 10 molar % *p*-NPA on K_2SO_4, 1 % load in 2-PrOH at 37 ºC	94.3	**	**	**
KC253_16	Lipase, heat unfolded + 10 molar % *p*-NPA on K_2SO_4, 5 % load in 2-PrOH at 37 ºC	98.7	**	**	**

Table 24: Immobilisation of Thermally-Unfolded Lipase with the Addition of Catalytic Substrate - Results (**The sample was insoluble upon attempted reconstitution)

4.2.6.4 Immobilisation of Lipase from the Thermally-Unfolded State with the Addition of Catalytic Substrate - Discussion

The addition of *p*-NPA to the thermally-unfolded protein at the solubilisation stage appears to have no effect on the resultant PCMC when compared with samples prepared in the absence of substrate. The resultant crystals are found to remain insoluble in aqueous buffer and as such the %MPL and the specific activity cannot be determined.

The preparation of PCMC from lipase in the thermally-unfolded state at 37 °C results in no improvement in the observed solubility over the room temperature counterparts. The thermally-unfolded, immobilised samples prepared at room temperature and at 37 °C both

in the presence and absence of *p*-NPA show no evidence of refolding or increased solubility.

4.2.7 Immobilisation of Lipase from the Native and Thermally-Unfolded State Using a Syringe Pump

In order to determine if the gradual dilution of the denaturing salt from the environment of the thermally-unfolded, solubilised protein would help to regain activity from the protein, PCMC samples were prepared using a syringe pump. This equipment allows the gradual dilution of the GdnHCl from the environment before precipitation begins (therefore beginning the refolding process *via* dilution).

4.2.7.1 Immobilisation of Lipase from the Native and Thermally-Unfolded State Using a Syringe Pump - Results

PCMC were prepared of lipase (from both the native and thermally-unfolded, solubilised state) using a syringe pump to mix the excipient and protein together gradually (0.01 ml/min excipient into protein) followed by immediate precipitation of mixture into 2-PrOH.

These samples were analysed *via* the reconstitution of the immobilised protein in 50 mM Tris/HCl, pH 7.8 with a view to subsequently determining the %MPL (Measured Protein Loading) and the specific activity.

Details of the PCMC sample preparation can be found in Appendix 23.

Sample Identifier	Sample Description	Measured Solids Recovery (%)	Measured Protein Loading (%)	Specific Activity (A $min^{-1}mg^{-1}$)	% Activity of Native Protein
KC255_01	Lipase, on K_2SO_4, 5 % load in 2-PrOH	84.7	6.04	0.127	67.9
KC255_02	Lipase, heat unfolded, on K_2SO_4, 5 % load in 2-PrOH	78.9	**	**	**

Table 25: Immobilisation of Lipase from the Native and Unfolded State - PCMC Preparation using a Syringe Pump - Results (**The sample was insoluble upon attempted reconstitution)

4.2.7.2 Immobilisation of Lipase from the Native and Thermally-Unfolded State Using a Syringe Pump - Discussion

PCMC of thermally-unfolded, solubilised lipase prepared using syringe pumps were found to be insoluble in the reconstitution buffer, similarly to other thermally-unfolded samples. The gradual dilution of the denaturing salt from the environment of the lipase followed by subsequent precipitation therefore appeared to have no beneficial effect upon either the solubility of the crystals or the refolding of the protein.

4.3 Discussion and Conclusions

In the refolding of wheat germ lipase, there are three stages at which the refolding of the enzyme can occur: during the mixing of the unfolded protein and excipient material (and associated dilution of denaturing agents); during the process of PCMC formation; and in the reconstitution of the material under aqueous conditions.

Activity measurements for lipase indicate that upon dilution (and subsequent removal) of the denaturing molecules from the protein environment, the enzyme has no catalytic activity.

Evidence obtained with lysozyme suggests that any possible folding of enzyme molecules occurs upon the formation of PCMC with the result that the protein is in a native conformation upon reconstitution under aqueous conditions.

Lipase from the chemically-unfolded state can be immobilised *via* the formation of PCMC with the regain of enzymatic activity. The immobilisation of lipase from the GdnHCl unfolded state upon carriers such as K_2SO_4 yielded crystals coated with the protein with the regain of up to 36.3 % of the activity of the untreated enzyme. Dialysis of GdnHCl unfolded lipase as a method for refolding regained 11.4 % of the activity of the native enzyme. The refolding of lipase *via* the formation of PCMC therefore compared favourably with a general protein folding method such as dialysis.

However, the immobilisation of lipase from the thermally-unfolded, solubilised state proved to be a difficult challenge for this methodology. Reducing enzyme concentration and protein loading appears to have a visible effect upon the level of aggregation, however, not to the extent required to complete further analysis. The introduction of redox couples and catalytic concentrations of enzymatic substrate seemed to have little or no effect upon the aggregation of the enzyme and as such PCMC prepared using lipase from these conditions gave rise to insoluble protein particles.

Dialysis of lipase from the thermally-unfolded, solubilised state fared no better than PCMC formation. Upon removal of the solubilising agent from the protein environment, the biomolecule was seen to form aggregates with no enzymatic activity. This was true for the introduction of a redox couple to the solubilised protein solution and also catalytic concentrations of substrate.

Chapter Five - Project Conclusions and Future Work

5 Project Conclusions and Further Work

At the commencement of this project, the aim was to develop a method for the one-step immobilisation and simultaneous refolding of proteins using PCMC formation. Two enzymes were unfolded utilising various methods and subsequently immobilised *via* the formation of PCMC. The purpose of this was to ascertain the effect of immobilisation upon enzyme structure and to determine if it is possible to refold proteins using this method. It was also an aim to determine when, if any, refolding took place; during the mixing of protein and crystal forming component; during coprecipitation or upon reconstitution of the powder back into the aqueous phase.

Lysozyme is known to be amenable to chemical unfolding and refolding experiments. Upon chemical unfolding and subsequent immobilisation of this enzyme by the formation of PCMC, the protein has been seen to have refolded to varying extents during co-precipitation. Solid State CD spectroscopy indicates that the refolding of lysozyme occurs upon the formation of PCMC and that the protein is in a native-like state on the surface of the coprecipitant material.

The refolding of thermally unfolded lysozyme proved to be a more difficult task for this immobilisation method. Upon reconstitution, the resultant immobilised protein coated crystals were insoluble and therefore further analysis was not possible.

Utilising the enzyme lipase for refolding studies also proved to be difficult. This enzyme is more reluctant to refold and this was evident in the work carried out here. Chemically unfolded lipase subjected to immobilisation lead to the regain of up to 36.3 % of the activity of the untreated enzyme. However, thermally-unfolded, solubilised lipase which was immobilised from a range of conditions, proved to be insoluble in the aqueous phase.

The scope for further work in this area is vast. There are a number of chemical modifications which remain to be investigated with this process. Parameters with scope for investigation include the choice of protein, solvent, crystal forming component, final water content of the solvent, rate of mixing of aqueous and organic phases and the addition of molecules known to promote protein folding such as arginine and detergents.

The determination of the chemical constituents of the formulated protein after precipitation and isolation by filtration is important. There are a number of different chemical components used in the manufacture of these species including buffers, solubilising agents,

solvents etcetera, and the identification of exactly what is remaining of these in the final product is important for the full understanding of their behaviour.

The greatest scope for further study of this methodology is the analytical methods which can be used to analyse the particles as a whole (protein + crystal forming component) or individually. Spectroscopic and imaging methods could provide a great deal of valuable information:

- The secondary structure of protein molecules can be investigated using methods such as FTIR (Fourier Transform Infra-Red) spectroscopy which would detect small changes in the structure of the backbone of the protein molecule or hydrogen bonding properties as a result of immobilisation (in the solid state) or upon reconstitution.

- NMR (Nuclear Magnetic Resonance) spectroscopy is a tool for the analysis of protein structure, detecting very small changes in protein conformation to great precision. This technique could be employed for the analysis of optimised formulations to determine the structural characteristics of the protein upon immobilisation and its conformational state.

- The PCMC could be subjected to powder XRD (X-Ray Diffraction) to determine the physical state of the core material and how this is affected upon coprecipitation with protein.

- SEM (Scanning Electron Microscopy) could be routinely used to ascertain the size, shape and general morphology of the precipitated material.

- AFM (Atomic Force Microscopy) would give information regarding the surface morphology of the precipitated material and the size and shape of the protein clusters present upon the excipient.

Scope for further work could also include measuring the stability of the formulations with respect to time under varying conditions of temperature and humidity stress. This would indicate the shelf-life of such refolded protein products.

The immobilisation of lysozyme from both the chemically and thermally-unfolded state is possible *via* the formation of PCMC. As a method for the refolding of chemically-

unfolded lysozyme or lipase, PCMC formation fares better than a generally applied method such as dialysis. However, further research is required to exhaust the possibility of using this immobilisation technique as a method for the refolding of protein molecules from the thermally-unfolded state.

Appendices

6 Appendices

6.1 Appendix 1

6.1.1 Immobilisation of Lysozyme from the Native State - Sample Preparation

Lysozyme (6 mg/ml in 50 mM Tris/HCl, pH 8.1) was prepared for immobilisation by mixing with an aqueous solution of excipient material (D,L-valine; 60 mg/ml). The protein and excipient solution were mixed in the appropriate ratio to achieve the desired protein loading before being added drop-wise to a stirring organic solvent (1000 rpm). The volume of solvent required was calculated so that the water content of the solvent after precipitation is between 4 and 5 %

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC158_01	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	1.3 ml D,L-valine solution	66 ml 2-PrOH	15.0
KC158-02	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.8 ml D,L-valine solution	56 ml 2-PrOH	20.0
KC158-03	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.6 ml D,L-valine solution	52 ml 2-PrOH	25.0

Table 26: Immobilisation of Lysozyme from the Native State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.1.2 Immobilisation of Lysozyme from the Guanidine Hydrochloride Unfolded State - Sample Preparation

Lysozyme (6 mg/ml in 50 mM Tris/HCl, pH 8.1) was subject to unfolding upon the addition of 6 M GdnHCl. The protein and excipient solution (D,L-valine; 60 mg/ml) were mixed in the appropriate ratio to achieve the desired protein loading and subsequently added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required was calculated so that the water content of the solvent after precipitation is between 4 and 5 %

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC162_01	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	1.3 ml D,L-valine solution	66 ml 2-PrOH	12.5
KC162_02	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	1.13 ml D,L-valine solution	63 ml 2-PrOH	15.0
KC162_03	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	0.93 ml D,L-valine solution	59 ml 2-PrOH	17.5
KC162_04	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	0.8 ml D,L-valine solution	56 ml 2-PrOH	20.0
KC162_05	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	0.7 ml D,L-valine solution	54 ml 2-PrOH	22.5
KC162_06	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	0.6 ml D,L-valine solution	52 ml 2-PrOH	25.0

Table 27: Immobilisation of Lysozyme from the Guanidine Hydrochloride Unfolded State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.2 Appendix 2

6.2.1 Immobilisation of Lysozyme from the Native State at Lower Protein Loadings - Sample Preparation

In order to determine if lysozyme could be immobilised and refolded successfully at lower protein loadings, samples were prepared of native and GdnHCl unfolded lysozyme (6 mg/ml) at protein loadings ranging from 0.6 - 20 %.

Lysozyme (6 mg/ml in 50 mM Tris/HCl, pH 8.1) was prepared for immobilisation by mixing with an aqueous solution of excipient material (D,L-valine; 60 mg/ml) before being added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required was calculated so that the water content of the solvent after precipitation is between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC198_01	0.3 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	5.0 ml D,L-valine solution	125 ml 2-PrOH	0.6
KC198_02	6 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	1.9 ml D,L-valine solution	73 ml 2-PrOH	5.0
KC198_03	6 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.9 ml D,L-valine solution	48 ml 2-PrOH	10.0
KC198_04	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	1.12 ml D,L-valine solution	78 ml 2-PrOH	15.0
KC198_05	15 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	1.0 ml D,L-valine solution	87 ml 2-PrOH	20.0

Table 28: Immobilisation of Lysozyme from the Native state at Lower Protein Loadings - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.2.2 Immobilisation of Lysozyme from the Guanidine Hydrochloride Unfolded State at Lower Protein Loadings - Sample Preparation

Lysozyme (6 mg/ml in 50 mM Tris/HCl, pH 8.1) was subject to unfolding upon the addition of 6 M GdnHCl. The protein and excipient solution (D,L-valine; 60 mg/ml) were mixed and subsequently added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required was calculated so that the water content of the solvent after precipitation is between 4 and 5 %

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC187_05	0.3 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	5.0 ml D,L-valine solution	125 ml 2-PrOH	0.6
KC187_06	6 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	1.9 ml D,L-valine solution	73 ml 2-PrOH	5.0
KC187_07	6 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	0.9 ml D,L-valine solution	48 ml 2-PrOH	10.0
KC187_08	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	1.12 ml D,L-valine solution	78 ml 2-PrOH	15.0
KC187_09	15 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	1.0 ml D,L-valine solution	87 ml 2-PrOH	20.0

Table 29: Immobilisation of Lysozyme from the Guanidine Hydrochloride Unfolded State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.3 Appendix 3

6.3.1 Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump - Sample Preparation

In order to determine if more enzymatic activity could be regained by the gradual mixing of the protein solution and excipient; a syringe pump was employed. This allowed the excipient to be added to the protein containing solution at a slow rate (0.01 ml/min) to allow gradual dilution of the denaturant before the immobilisation step occurs.

Lysozyme (1 mg/ml in 50 mM Tris/HCl, pH 8.1) was chemically unfolded via incubation in 6 M GdnHCl for 2 hours. PCMC were prepared of both this GdnHCl unfolded protein and native lysozyme. The aqueous solution of excipient material (D,L-valine; 60 mg/ml) was then added to the unfolded protein at a rate of 0.01 ml min^{-1} in an attempt to determine if the gradual dilution of the denaturing salt from the protein environment can trigger refolding. When the gradual mixing is complete, the protein/excipient mixture was added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC258_16	3.0 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0
KC258_19	3.0 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 6 M GdnHCl	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0

Table 30: Immobilisation of Lysozyme from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.4 Appendix 4

6.4.1 Immobilisation of Urea Incubated and Urea Unfolded, Reduced Lysozyme - Sample Preparation

Lysozyme (12 mg/ml in 50 mM Tris/HCl, pH 8.1) was incubated in 8 M Urea to form urea incubated lysozyme. To prepare urea unfolded, reduced protein, lysozyme (12 mg/ml in 50 mM Tris/HCl, pH 8.1) was incubated in 8 M Urea with the addition of 32 mM DTT. The urea incubated and urea unfolded, reduced protein were prepared for immobilisation by mixing with the appropriate concentration of excipient material (D,L-valine; 60 mg/ml) before subsequent addition of the mixture drop-wise to stirring organic solvent (1000 rpm).

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC111_01	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	1.0 ml D,L-valine solution	30 ml EtOH	16.5
KC111_02	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	1.0 ml D,L-valine solution	30 ml 2-PrOH	16.5
KC111_03	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	1.0 ml D,L-valine solution	30 ml 1-PrOH	16.5
KC111_04	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Incubated in 8 M urea	1.0 ml D,L-valine solution	30 ml EtOH	16.5
KC111_05	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Incubated in 8 M urea	1.0 ml D,L-valine solution	30 ml 2-PrOH	16.5
KC111_06	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Incubated in 8 M urea	1.0 ml D,L-valine solution	30 ml 1-PrOH	16.5

Table 31: Immobilisation of Lysozyme from the Urea Incubated and Urea Unfolded, Reduced State Using Various Solvents - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.5 Appendix 5

6.5.1 Immobilisation of Lysozyme from the Urea Incubated and Urea Unfolded, Reduced State Using L-Glutamine as an Excipient - Sample Preparation

PCMC of urea incubated and urea unfolded, reduced lysozyme were manufactured utilising L-glutamine as an excipient. The urea incubated and urea unfolded, reduced protein were prepared for immobilisation by mixing with the appropriate concentration of excipient material (L-glutamine; 20 mg/ml) before subsequent addition of the mixture drop-wise to stirring organic solvent (1000 rpm).

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC111_07	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	1.0 ml L-glutamine solution	30 ml EtOH	37.5
KC111_08	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	1.0 ml L-glutamine solution	30 ml 2-PrOH	37.5
KC111_09	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	1.0 ml L-glutamine solution	30 ml 1-PrOH	37.5
KC111_10	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Incubated in 8 M urea	1.0 ml L-glutamine solution	30 ml EtOH	37.5
KC111_11	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Incubated in 8 M urea	1.0 ml L-glutamine solution	30 ml 2-PrOH	37.5
KC111_12	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Incubated in 8 M urea	1.0 ml L-glutamine solution	30 ml 1-PrOH	37.5

Table 32: Immobilisation of Lysozyme from the Urea Incubated and Urea Unfolded, Reduced State Using L-Glutamine as an Excipient - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.6 Appendix 6

6.6.1 Immobilisation of Lysozyme from the Native and Urea Unfolded, Reduced state at Lower Protein Loadings - Sample Preparation

Lysozyme (12 mg/ml in 50 mM Tris/HCl, pH 8.1) was incubated in 8 M Urea to form urea incubated lysozyme. To prepare urea unfolded, reduced protein, lysozyme (12 mg/ml in 50 mM Tris/HCl, pH 8.1) was incubated in 8 M Urea with the addition of 32 mM DTT.

The urea incubated and urea unfolded, reduced protein were prepared for immobilisation by mixing with the appropriate concentration of excipient material (D,L-valine; 60 mg/ml) before subsequent addition of the mixture drop-wise to stirring organic solvent (1000 rpm).

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC119_01	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	2.0 ml D,L-valine solution	30 ml EtOH	9.0
KC119_02	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	2.0 ml D,L-valine solution	30 ml 1-PrOH	9.0
KC119_03	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	2.0 ml D,L-valine solution	30 ml 2-PrOH	9.0
KC119_07	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Incubated in 8 M urea	2.0 ml D,L-valine solution	30 ml EtOH	9.0
KC119_08	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Incubated in 8 M urea	2.0 ml D,L-valine solution	30 ml 1-PrOH	9.0
KC119_09	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Incubated in 8 M urea	2.0 ml D,L-valine solution	30 ml 2-PrOH	9.0

Table 33: Immobilisation of Lysozyme from the Native and Urea Unfolded, Reduced state at Lower Protein Loadings - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.7 Appendix 7

6.7.1 Immobilisation of Lysozyme from the Urea Unfolded, Reduced State at Lower Concentrations - Sample Preparation

The previously described PCMC were manufactured from various protein stocks at a concentration of 12 mg/ml lysozyme in 50 mM Tris/HCl, pH 8.1 containing 8 M Urea and 32 mM DTT. To determine if reducing the concentration of the protein stock from which samples were prepared could have any positive effect upon the solubility (and observed activity) of the PCMC, immobilised protein samples were prepared.

Lysozyme (6 mg/ml in 50 mM Tris/HCl, pH 8.1) was incubated in 8 M Urea to form urea incubated lysozyme. To prepare urea unfolded, reduced protein, lysozyme (6 mg/ml in 50 mM Tris/HCl, pH 8.1) was incubated in 8 M Urea with the addition of 32 mM DTT.

The urea incubated and urea unfolded, reduced protein were prepared for immobilisation by mixing with the appropriate concentration of excipient material (D,L-valine; 60 mg/ml) before subsequent addition of the mixture drop-wise to stirring organic solvent (1000 rpm).

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC77_01	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	2.0 ml D,L-valine solution	30 ml EtOH	9.0
KC77_02	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	2.0 ml D,L-valine solution	30 ml 2-PrOH	9.0
KC77_03	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	2.0 ml D,L-valine solution	30 ml EtOH	9.0
KC77_04	12 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	2.0 ml D,L-valine solution	30 ml 2-PrOH	9.0

Table 34: Immobilisation of Lysozyme from the Urea Unfolded, Reduced State at Lower Concentrations - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.8 Appendix 8

6.8.1 Immobilisation of Lysozyme from the Native and Urea Unfolded, Reduced State Using a Syringe Pump - Sample Preparation

In order to determine if more enzymatic activity could be regained by the gradual mixing of the protein solution and excipient; a syringe pump was employed. This allowed the excipient to be added to the protein containing solution at a slow rate (0.01 ml/ml) to allow gradual dilution of the denaturant before the immobilisation step occurs.

Urea unfolded, reduced lysozyme was prepared *via* incubation of 1 mg/ml protein in 50 mM Tris/HCl, pH 8.1 containing 8 M urea and 32 mM DTT. PCMC were prepared of both this urea unfolded, reduced protein and native lysozyme.

The aqueous solution of excipient material (D,L-valine; 60 mg/ml) was then added to the unfolded protein at a rate of 0.01 ml min^{-1} in an attempt to determine if the gradual dilution of the denaturing salt from the protein environment can trigger refolding. When the gradual mixing was complete, the protein/excipient mixture was added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC258_16	3.0 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0
KC258_20	3.0 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Unfolded with 8 M urea + 32 mM DTT	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0

Table 35: Immobilisation of Lysozyme from the Native and Urea Unfolded, Reduced State Using a Syringe Pump - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.9 Appendix 9

6.9.1 Immobilisation of Lysozyme from the Native and Thermally Unfolded, Guanidine Hydrochloride Solubilised State - Sample Preparation

Lysozyme (1 mg/ml in 50 mM Tris/HCl, pH 7.8) was thermally unfolded by heating to 100 °C and then allowed to cool to room temperature. The resultant protein aggregates were then solubilised by the addition of 6 M GdnHCl. This solution of thermally unfolded, solubilised protein was then used to prepare PCMC by mixing with a solution of excipient material (D,L-valine; 60 mg/ml) before precipitation in stirring organic solvent (1000 rpm). The volume of solvent required was calculated so that the water content of the solvent after precipitation is between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC258_01	0.6 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.99 ml D,L-valine solution	40 ml 2-PrOH	1.0
KC258_02	3 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0
KC258_03	0.6 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Thermally unfolded, solubilised with 6 M GdnHCl	0.99 ml D,L-valine solution	40 ml EtOH	1.0
KC258_04	3 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Thermally unfolded, solubilised with 6 M GdnHCl	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0

Table 36: Immobilisation of Lysozyme from the Native and Thermally Unfolded, Guanidine Hydrochloride Solubilised State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.10 Appendix 10

6.10.1 Immobilisation of Lysozyme from the Native and Thermally Unfolded, Urea Solubilised State - Sample Preparation

Lysozyme (1 mg/ml in 50 mM Tris/HCl, pH 8.1) was thermally unfolded by heating to 100 °C and then allowed to cool to room temperature. The resultant protein aggregates were then solubilised by the addition of 8 M Urea. This solution of thermally unfolded, solubilised protein was then used to prepare PCMC by mixing with a solution of excipient material (D,L-valine; 60 mg/ml) before precipitation in stirring organic solvent (1000 rpm). The volume of solvent required was calculated so that the water content of the solvent after precipitation is between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC258_01	0.6 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.99 ml D,L-valine solution	40 ml 2-PrOH	1.0
KC258_02	3 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0
KC258_05	0.6 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Thermally unfolded, solubilised with 8 M Urea	0.99 ml D,L-valine solution	40 ml EtOH	1.0
KC258_06	3 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Thermally unfolded, solubilised with 8 M Urea	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0

Table 37: Immobilisation of Lysozyme from the Native and Thermally Unfolded, Urea Solubilised State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.11 Appendix 11

6.11.1 Immobilisation of Lysozyme from the Native and Thermally Unfolded State Using a Syringe Pump – Sample Preparation

In order to determine if more enzymatic activity could be regained by the gradual mixing of the protein solution and excipient; a syringe pump was employed. This allowed the excipient to be added to the protein containing solution at a slow rate (0.01 ml/ml) to allow gradual dilution of the denaturant before the immobilisation step occurs.

Lysozyme (1 mg/ml in 50 mM Tris/HCl, pH 7.8) was thermally unfolded by heating to 100 °C and then allowed to cool to room temperature. The resultant protein aggregates were then solubilised by the addition of either 6 M GdnHCl or 8 M Urea. An aqueous solution of excipient material (D,L-valine; 60 mg/ml) was then added to the unfolded protein at a rate of 0.01 ml min^{-1} in an attempt to determine if the gradual dilution of the denaturing salt from the protein environment can trigger refolding. When the gradual mixing was complete, the protein/excipient mixture was added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC258_16	3.0 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Native	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0
KC258_17	3.0 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Thermally unfolded, solubilised with 6 M GdnHCl	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0
KC258_18	3.0 mg lysozyme in 50 mM Tris/HCl, pH 8.1	Thermally unfolded, solubilised with 8 M Urea	0.95 ml D,L-valine solution	99 ml 2-PrOH	5.0

Table 38: Immobilisation of Lysozyme from the Native and Thermally Unfolded State Using a Syringe Pump - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.12 Appendix 12

PCMC of lipase from both the native and chemically unfolded state were prepared in order to ascertain the efficacy of this immobilisation method for the refolding of chemically unfolded lipase

6.12.1.1 Immobilisation of Lipase from the Native State - Sample Preparation

Lipase is prepared for immobilisation by dissolving in 200 µl of 50 mM Tris/HCl, pH 7.8 and subsequently mixed with an aqueous solution of excipient material (K_2SO_4; 100 mg/ml). The protein and excipient solution were mixed in the appropriate ratio to achieve the desired protein loading before being added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC214-01	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.6 ml K_2SO_4 solution	20 ml 2-PrOH	1.0
KC214-02	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.6 ml K_2SO_4 solution	20 ml 2-PrOH	5.0
KC214-03	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.54 ml K_2SO_4 solution	18.5 ml 2-PrOH	10.0
KC214-04	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.48 ml K_2SO_4 solution	17 ml 2-PrOH	15.0
KC214-05	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.48 ml K_2SO_4 solution	17 ml 2-PrOH	20.0
KC214-06	18.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.18 ml K_2SO_4 solution	10 ml 2-PrOH	50.0

Table 39: Immobilisation of Lipase from the Native State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.12.2 Immobilisation of Lipase from the Guanidine Hydrochloride Unfolded State - Sample Preparation

Lipase (6 mg/ml in 50 mM Tris/HCl, pH 7.8) was subjected to unfolding upon the addition of 6 M GdnHCl and incubated under these conditions for two hours. The unfolded protein was subsequently mixed with an aqueous solution of excipient material (K_2SO_4; 100 mg/ml) at an appropriate ratio so that the desired protein loading could be achieved. The protein/excipient mixture was then added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC214-07	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Unfolded with 6 M GdnHCl	0.6 ml K_2SO_4 solution	15 ml 2-PrOH	1.0
KC214-08	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Unfolded with 6 M GdnHCl	0.6 ml K_2SO_4 solution	22 ml 2-PrOH	5.0
KC214-09	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Unfolded with 6 M GdnHCl	0.54 ml K_2SO_4 solution	31.5 ml 2-PrOH	10.0
KC214-10	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Unfolded with 6 M GdnHCl	0.48 ml K_2SO_4 solution	50 ml 2-PrOH	15.0
KC214-11	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Unfolded with 6 M GdnHCl	0.48 ml K_2SO_4 solution	62 ml 2-PrOH	20.0
KC214-12	18.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Unfolded with 6 M GdnHCl	0.18 ml K_2SO_4 solution	80 ml 2-PrOH	50.0

Table 40: Immobilisation of Lipase from the Guanidine Hydrochloride Unfolded State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.13 Appendix 13

6.13.1 Immobilisation of Lipase from the Native and Guanidine Hydrochloride Unfolded State Using a Syringe Pump - Sample Preparation

In order to determine if more enzymatic activity could be regained by the gradual mixing of the protein and excipient solutions, a syringe pump was employed. This allowed the excipient to be added to the protein solution at a slow rate (0.01 ml/min) to allow gradual dilution of the denaturing salt before the immobilisation step occurs.

Lipase (1 mg/ml in 50 mM Tris/HCl, pH 7.8) was subjected to unfolding upon addition of 6 M GdnHCl and incubated under these conditions for 2 hours. The aqueous solution of excipient material (K_2SO_4; 100 mg/ml) was then added to the unfolded protein at a rate of 0.01 ml min^{-1}. When the gradual mixing is complete, the protein/excipient mixture was added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC255_01	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.57 ml K_2SO_4 solution	89 ml 2-PrOH	5.0
KC255_03	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Unfolded with 6 M GdnHCl	0.57 ml K_2SO_4 solution	89 ml 2-PrOH	5.0

Table 41: Immobilisation of Lipase from the Native and Chemically Unfolded State Using a Syringe Pump - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.14 Appendix 14

PCMC of lipase from both the native and thermally unfolded state were prepared in order to ascertain the efficacy of this immobilisation method for the refolding of thermally unfolded lipase.

6.14.1 Immobilisation of Lipase from the Native State with Various Excipients - Sample Preparation

Lipase was prepared for immobilisation by dissolving in 200 µl of 50 mM Tris/HCl, pH 7.8 and subsequently mixed with an aqueous solution of excipient material (K_2SO_4; 100 mg/ml or D,L-valine; 60 mg/ml). The protein and excipient solution were mixed in the appropriate ratio to achieve the desired protein loading before being added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC216_01	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.6 ml K_2SO_4 solution	20 ml 2-PrOH	1.0
KC216_02	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.6 ml K_2SO_4 solution	20 ml 2-PrOH	5.0
KC216_03	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.54 ml K_2SO_4 solution	18.5 ml 2-PrOH	10.0
KC216_04	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.48 ml K_2SO_4 solution	17 ml 2-PrOH	15.0
KC216_05	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.48 ml K_2SO_4 solution	17 ml 2-PrOH	20.0
KC216_06	18.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.18 ml K_2SO_4 solution	10 ml 2-PrOH	50.0
KC216_07	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	1.0 ml D,L-valine solution	30 ml 2-PrOH	1.0
KC216_08	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	1.0 ml D,L-valine solution	30 ml 2-PrOH	5.0
KC216_09	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.9 ml D,L-valine solution	27.5 ml 2-PrOH	10.0
KC216_10	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.8 ml D,L-valine solution	25 ml 2-PrOH	15.0
KC216_11	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.8 ml D,L-valine solution	25 ml 2-PrOH	20.0
KC216_12	18.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.3 ml D,L-valine solution	12.5 ml 2-PrOH	50.0

Table 42: Immobilisation of Lipase from the Native State with Various Excipients - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.14.2 Immobilisation of Lipase from the Thermally Unfolded, Solubilised State - Sample Preparation

Lipase (6 mg/ml in 50 mM Tris/HCl, pH 7.8) was thermally unfolded by heating to 100 °C and then allowed to cool to room temperature. The resultant protein aggregates were then solubilised by the addition of 6 M GdnHCl. This solution of thermally unfolded, solubilised protein was then used to prepare PCMC by mixing with a solution of excipient material (K_2SO_4; 100 mg/ml or D,L-valine; 60 mg/ml) before precipitation in stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC226_01	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.6 ml K_2SO_4 solution	20 ml 2-PrOH	1.0
KC226_02	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.6 ml K_2SO_4 solution	20 ml 2-PrOH	5.0
KC226_03	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.54 ml K_2SO_4 solution	18.5 ml 2-PrOH	10.0
KC226_04	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.48 ml K_2SO_4 solution	17 ml 2-PrOH	15.0
KC226_05	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.48 ml K_2SO_4 solution	17 ml 2-PrOH	20.0
KC226_06	18.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.18 ml K_2SO_4 solution	10 ml 2-PrOH	50.0
KC226_07	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	1.0 ml D,L-valine solution	30 ml 2-PrOH	1.0
KC226_08	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	1.0 ml D,L-valine solution	30 ml 2-PrOH	5.0
KC226_09	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.9 ml D,L-valine solution	27.5 ml 2-PrOH	10.0
KC226_10	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.8 ml D,L-valine solution	25 ml 2-PrOH	15.0
KC226_11	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.8 ml D,L-valine solution	25 ml 2-PrOH	20.0
KC226_12	18.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.3 ml D,L-valine solution	12.5 ml 2-PrOH	50.0

Table 43: Immobilisation of Lipase from the Thermally Unfolded, Solubilised State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.15 Appendix 15

In order to determine if the addition of a redox couple could assist in the potential refolding of thermally unfolded lipase, the solubilised lipase was incubated with the redox couple of cystine/cysteine (0.3 mM: 3 mM).

6.15.1 Immobilisation of Lipase from the Native State - Sample Preparation

Lipase was prepared for immobilisation by dissolving in 200 µl of 50 mM Tris/HCl, pH 7.8 and subsequently mixed with an aqueous solution of excipient material (K_2SO_4; 100 mg/ml and KCl; 300 mg/ml). The protein and excipient solution were mixed in the appropriate ratio to achieve the desired protein loading before being added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC240_01	3.0 mg lipase + 200 µl 50 mM Tris/HCl, pH 7.8	Native	0.57 ml K_2SO_4 solution	20 ml 2-PrOH	5.0
KC240_02	6.0 mg lipase + 200 µl 50 mM Tris/HCl, pH 7.8	Native	0.54 ml K_2SO_4 solution	19 ml 2-PrOH	10.0
KC240_03	9.0 mg lipase + 200 µl 50 mM Tris/HCl, pH 7.8	Native	0.51 ml K_2SO_4 solution	18 ml 2-PrOH	15.0
KC240_04	12.0 mg lipase + 200 µl 50 mM Tris/HCl, pH 7.8	Native	0.48 ml K_2SO_4 solution	17 ml 2-PrOH	20.0
KC240_05	3.0 mg lipase + 200 µl 50 mM Tris/HCl, pH 7.8	Native	0.19 ml KCl solution	10 ml 2-PrOH	5.0
KC240_06	6.0 mg lipase + 200 µl 50 mM Tris/HCl, pH 7.8	Native	0.18 ml KCl solution	10 ml 2-PrOH	10.0
KC240_07	9.0 mg lipase + 200 µl 50 mM Tris/HCl, pH 7.8	Native	0.17 ml KCl solution	9 ml 2-PrOH	15.0
KC240_08	12.0 mg lipase + 200 µl 50 mM Tris/HCl, pH 7.8	Native	0.16 ml KCl solution	9 ml 2-PrOH	20.0

Table 44: Immobilisation of Lipase from the Native State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.16 Appendix 16

6.16.1 Immobilisation of Lipase from the Thermally Unfolded, Solubilised State - Sample Preparation

Lipase (6 mg/ml in 50 mM Tris/HCl, pH 7.8) was thermally unfolded by heating to 100 °C and then allowed to cool to room temperature. The resultant protein aggregates were then solubilised by the addition of 6 M GdnHCl. This solution of thermally unfolded, solubilised protein was then used to prepare PCMC by mixing with a solution of excipient material (K_2SO_4; 100 mg/ml or D,L-valine; 60 mg/ml) before precipitation in stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC240_17	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.57 ml K_2SO_4 solution	27 ml 2-PrOH	5.0
KC240_18	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.54 ml K_2SO_4 solution	38 ml 2-PrOH	10.0
KC240_19	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.51 ml K_2SO_4 solution	50 ml 2-PrOH	15.0
KC240_20	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.48 ml K_2SO_4 solution	62 ml 2-PrOH	20.0
KC240_21	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.19 ml KCl solution	17 ml 2-PrOH	5.0
KC240_22	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.18 ml KCl solution	30 ml 2-PrOH	10.0
KC240_23	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.17 ml KCl solution	42 ml 2-PrOH	15.0
KC240_24	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.16 ml KCl solution	54 ml 2-PrOH	20.0

Table 45: Immobilisation of Lipase from the Thermally Unfolded, Solubilised State - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.17 Appendix 17

6.17.1 Immobilisation of Lipase from the Thermally Unfolded State with the Introduction of a Redox Couple - Sample Preparation

Lipase (6 mg/ml in 50 mM Tris/HCl, pH 7.8) was thermally unfolded by heating to 100 °C and then allowed to cool to room temperature. The resultant protein aggregates were then solubilised by the addition of 6 M GdnHCl and the redox couple of 0.3 mM cystine and 3 mM cysteine for disulfide rearrangement. This mixture was incubated at room temperature for two hours to equilibrate prior to being utilised in the formation of PCMC. This solution of thermally unfolded, solubilised protein was then used to prepare PCMC by mixing with a solution of excipient material (K_2SO_4; 100 mg/ml or D,L-valine; 60 mg/ml) before precipitation in stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC240_09	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.57 ml K_2SO_4 solution	27 ml 2-PrOH	5.0
KC240_10	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.54 ml K_2SO_4 solution	38 ml 2-PrOH	10.0
KC240_11	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.51 ml K_2SO_4 solution	50 ml 2-PrOH	15.0
KC240_12	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.48 ml K_2SO_4 solution	62 ml 2-PrOH	20.0
KC240_13	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.19 ml KCl solution	17 ml 2-PrOH	5.0
KC240_14	6.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.18 ml KCl solution	30 ml 2-PrOH	10.0
KC240_15	9.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.17 ml KCl solution	42 ml 2-PrOH	15.0
KC240_16	12.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.16 ml KCl solution	54 ml 2-PrOH	20.0

Table 46: Immobilisation of Lipase from the Thermally Unfolded State with the Introduction of a Redox Couple - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.18 Appendix 18

PCMC were prepared of lipase at low protein concentration and loadings to see the effect upon the immobilised native and thermally unfolded enzyme.

6.18.1 Immobilisation of Lipase from the Native State at Low Concentration and Loadings - Sample Preparation

Lipase (1 mg/ml in 50 mM Tris/HCl, pH 7.8) was prepared for immobilisation by mixing of the protein with an aqueous solution of excipient material (K_2SO_4; 100 mg/ml and KCl; 300 mg/ml). The protein and excipient solution were mixed in the appropriate ratio to achieve the desired protein loading before being added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC245_01	0.01 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	1.0 ml K_2SO_4 Solution	25 ml 2-PrOH	0.01
KC245_02	0.1 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.999 ml K_2SO_4 Solution	27.5 ml 2-PrOH	0.1
KC245_03	0.5 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.995 ml K_2SO_4 Solution	37.5 ml 2-PrOH	0.5
KC245_04	0.01 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.334 ml KCl Solution	8.5 ml 2-PrOH	0.01
KC245_05	0.1 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.333 ml KCl Solution	10.8 ml 2-PrOH	0.1
KC245_06	0.5 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.332 ml KCl Solution	20.8 ml 2-PrOH	0.5

Table 47: Immobilisation of Lipase from the Native State at Low Concentration and Loadings - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.19 Appendix 19

6.19.1 Immobilisation of Lipase from the Thermally Unfolded, Solubilised State at Low Concentration and Loadings - Sample Preparation

Lipase (1 mg/ml in 50 mM Tris/HCl, pH 7.8) was thermally unfolded by heating to 100 °C and then allowed to cool to room temperature. The resultant protein aggregates were then solubilised upon addition of 6 M GdnHCl. This solution of thermally unfolded, solubilised protein was then used to prepare PCMC by mixing with a solution of excipient material (K_2SO_4; 100 mg/ml and KCl; 300 mg/ml) before precipitation in stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC245_07	0.01 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	1.0 ml K_2SO_4 Solution	25 ml 2-PrOH	0.01
KC245_08	0.1 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.999 ml K_2SO_4 Solution	27.5 ml 2-PrOH	0.1
KC245_09	0.5 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.995 ml K_2SO_4 Solution	37.5 ml 2-PrOH	0.5
KC245_10	0.01 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.334 ml KCl Solution	8.5 ml 2-PrOH	0.01
KC245_11	0.1 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.333 ml KCl Solution	10.8 ml 2-PrOH	0.1
KC245_12	0.5 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.332 ml KCl Solution	20.8 ml 2-PrOH	0.5

Table 48: Immobilisation of Lipase from the Thermally Unfolded, Solubilised State at Low Concentration and Loadings - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.20 Appendix 20

6.20.1 Immobilisation of Lipase from the Thermally Unfolded, Solubilised State with the Addition of a Redox Couple - Sample Preparation

For the preparation of PCMC of lipase with the addition of a redox couple, the protein was thermally unfolded (1 mg/ml in 50 mM Tris/HCl, pH 7.8) by heating to 100 °C and subsequently allowed to cool to room temperature. The solubilised protein was subsequently mixed with an aqueous solution of excipient (K_2SO_4; 100 mg/ml and KCl; 300 mg/ml) prior to precipitation in stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC245_13	0.01 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	1.0 ml K_2SO_4 Solution	25 ml 2-PrOH	0.01
KC245_14	0.1 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.999 ml K_2SO_4 Solution	27.5 ml 2-PrOH	0.1
KC245_15	0.5 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.995 ml K_2SO_4 Solution	37.5 ml 2-PrOH	0.5
KC245_16	0.01 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.334 ml KCl Solution	8.5 ml 2-PrOH	0.01
KC245_17	0.1 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.333 ml KCl Solution	10.8 ml 2-PrOH	0.1
KC245_18	0.5 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl with redox couple	0.332 ml KCl Solution	20.8 ml 2-PrOH	0.5

Table 49: Immobilisation of Lipase from the Thermally Unfolded, Solubilised State at Low Concentration and Loading - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.21 Appendix 21

To determine if the addition of a catalytic concentration of enzymatic substrate would trigger refolding of thermally unfolded lipase and increase solubility of the immobilised samples, PCMC were prepared

6.21.1 Immobilisation of Lipase from the Native State with the Addition of Catalytic Substrate at Room Temperature and 37 ºC - Sample Preparation

Lipase (1 mg/ml in 50 mM Tris/HCl, pH 7.8) was prepared for immobilisation by mixing with 10 molar % *p*NPA before adding an aqueous solution of excipient material (K_2SO_4; 100 mg/ml) before subsequent precipitation in stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC253_01	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.6 ml K_2SO_4 Solution	30 ml 2-PrOH	1.0
KC253_02	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.57 ml K_2SO_4 Solution	89 ml 2-PrOH	5.0
KC253_03	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Native at 37 °C	0.6 ml K_2SO_4 Solution	30 ml 2-PrOH	1.0
KC253_04	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native at 37 °C	0.57 ml K_2SO_4 Solution	89 ml 2-PrOH	5.0
KC253_05	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Native in presence of *p*NPA	0.6 ml K_2SO_4 Solution	30 ml 2-PrOH	1.0
KC253_06	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native in presence of *p*NPA	0.57 ml K_2SO_4 Solution	89 ml 2-PrOH	5.0
KC253_07	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8 + *p*NPA	Native in presence of *p*NPA at 37 °C	0.6 ml K_2SO_4 Solution	30 ml 2-PrOH	1
KC253_08	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8 + *p*NPA @ 37 °C	Native in presence of *p*NPA at 37 °C	0.57 ml K_2SO_4 Solution	89 ml 2-PrOH	5

Table 50: Immobilisation of Lipase from the Native State with the Addition of *p*NPA at Room Temperature and 37 °C

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.22 Appendix 22

6.22.1 Immobilisation of Lipase from the Thermally Unfolded, Solubilised State with the Addition of Catalytic Substrate at Room Temperature and 37 ºC

Lipase (1 mg/ml in 50 mM Tris/HCl, pH 7.8) was thermally unfolded by heating to 100 °C before solubilisation upon addition of 6 M GdnHCl. A small concentration of enzymatic substrate (10 molar %) was added at this incubation step before the excipient material was added (K_2SO_4; 100 mg/ml). The protein/excipient mixture was then subjected to precipitation in stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC253_09	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.6 ml K_2SO_4 Solution	30 ml 2-PrOH	1.0
KC253_10	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl	0.57 ml K_2SO_4 Solution	89 ml 2-PrOH	5.0
KC253_11	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl at 37 °C	0.6 ml K_2SO_4 Solution	30 ml 2-PrOH	1.0
KC253_12	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Thermally unfolded, solubilised with 6 M GdnHCl at 37 °C	0.57 ml K_2SO_4 Solution	89 ml 2-PrOH	5.0
KC253_13	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8 + *p*NPA	Thermally unfolded, solubilised with 6 M GdnHCl in presence of *p*NPA	0.6 ml K_2SO_4 Solution	30 ml 2-PrOH	1.0
KC253_14	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8 + *p*NPA	Thermally unfolded, solubilised with 6 M GdnHCl in presence of *p*NPA	0.57 ml K_2SO_4 Solution	89 ml 2-PrOH	5.0
KC253_15	0.6 mg lipase in 50 mM Tris/HCl, pH 7.8 + *p*NPA @ 37 °C	Thermally unfolded, solubilised with 6 M GdnHCl in presence of *p*NPA at 37 °C	0.6 ml K_2SO_4 Solution	30 ml 2-PrOH	1.0
KC253_16	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8 + *p*NPA @ 37 °C	Thermally unfolded, solubilised with 6 M GdnHCl in presence of *p*NPA at 37 °C	0.57 ml K_2SO_4 Solution	89 ml 2-PrOH	5.0

Table 51: Immobilisation of Lipase from the Thermally Unfolded, Solubilised State with the Addition of pNPA at at Room Temperature and 37 °C

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

6.23 Appendix 23

6.23.1 Immobilisation of Lipase from the Native and Thermally Unfolded State Using a Syringe Pump – Sample Preparation

Lipase (1 mg/ml in 50 mM Tris/HCl, pH 7.8) was thermally unfolded by heating to 100 °C and solubilised upon addition of 6 M GdnHCl and incubated under these conditions for 2 hours. The aqueous solution of excipient material (K_2SO_4; 100 mg/ml) was then added to the unfolded protein at a rate of 0.01 ml min^{-1}. When the gradual mixing is complete, the protein/excipient mixture was added drop-wise to stirring organic solvent (1000 rpm). The volume of solvent required for precipitation was determined so as to keep the final water content of the solvent between 4 and 5 %.

Sample Identifier	Protein Solution	Protein Condition	Excipient Solution	Solvent	Theoretical Protein Loading (% TPL)
KC255_01	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Native	0.57 ml K_2SO_4 solution	89	5.0
KC255_03	3.0 mg lipase in 50 mM Tris/HCl, pH 7.8	Unfolded with 6 M GdnHCl	0.57 ml K_2SO_4 solution	89	5.0

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Table 52: Immobilisation of Lipase from the Native and Thermally Unfolded, Solubilised State Using a Syringe Pump - Sample Preparation

After precipitation, the PCMC samples were filtered and allowed to dry completely overnight in a pierced-parafilm covered petri-dish. The PCMC were subsequently scraped from the filter membranes and stored in glass vials on the bench top.

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