Investigation of Lysozyme Refolding Using Ionic Liquids as Refolding Additives

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Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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INVESTIGATION OF LYSOZYME REFOLDING USING IONIC LIQUIDS AS REFOLDING ADDITIVES
(Thesis format: Integrated Article)

by

Harpreet Kaur

Graduate Program in Chemical and Biochemical Engineering

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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Abstract

A rapid and inexpensive method for the production of protein is crucial for the biopharmaceutical industry. The bottleneck in the case of genetically engineered systems lies in the recovery of active and properly refolded proteins from inclusion bodies. The correct refolding of a protein from its denatured state is dependent on several parameters including the concentration of the protein, the refolding buffers utilized, the approach employed and finally the type of refolding additives, if any, used in the process.

In this study, an emerging class of refolding additives, namely, ionic liquids were investigated for the refolding of denatured lysozyme protein. Lysozyme was used as a model system due to its rapid refolding kinetics and the fact that enzyme based assay could be carried out to measure the refolding yields. The ionic liquids selection was hypothesized based on the selection of cation and anion that constitute the ionic liquid. The efficacy of Imidazolium based ionic liquids as refolding additives was compared to conventional refolding approach that contains urea and redox-couple cysteine/cystine.

Using response surface methodology (RSM) and central composite design (CCD), an empirical model was developed and validated by experimental results. Different refolding strategies were also evaluated; dilution refolding, adsorptive on column packed bed refolding and adsorptive on column fluidized bed refolding.

Experimental results indicate that in comparison to the conventional refolding buffer, among different ionic liquids examined, ionic liquid 1-ethyl-3-methylimidazolium ([EMIM]$^+$ Cl) has a pronounced effect on the refolding yield and time required to refold the denatured lysozyme. The optimal conditions were identified in this study as 150 μg/ml of denatured lysozyme in presence of 75 mM [EMIM]$^+$ Cl in 50 mM HEPES buffer (pH 7.5). On-column refolding studies indicate that in comparison to the packed bed system, the fluidized bed ion-exchange system approach, with [EMIM]$^+$ Cl as the elution-refolding buffer could tolerate higher concentration of denatured lysozyme (up to 25 mg) with refolding yield ca. 90% and fractional mass recovery ca. 82%. This was due to potentially lower mass transfer limitations in the fluidized bed compared to the packed
bed. Thus, the versatility of ionic liquids coupled with on-column adsorptive refolding can possibly be the solution to the large scale downstream processing of proteins.

Keywords

Protein refolding, ionic liquids, on-column refolding, empirical model
Dedication

To my parents

Mr. Onkar Singh & Mrs. Kamaljeet Kaur

I love you.

P.S.: I’m coming home.
Every accomplishment starts with the decision to try.

It’s been 4 years and 3 months, since I first started work on this doctoral thesis. But the journey, in its truest sense, began about a decade ago. When my father asked me to write down my life goals for the next ten years and PhD was one of them. The journey, as they say, is as important as the destination, if not more. To say that this has been a rollercoaster ride would be an understatement. And there are many to whom I owe my most sincere gratitude for making this an enriching, memorable and fun experience.

My work was supervised by Prof. (Dr.) Amarjeet Bassi. To paraphrase Paulo Coelho, Dr. Bassi isn’t someone who teaches something, but someone who inspires the student to give his or her best in order to discover what he or she already knows. And to him, I owe my sincerest gratitude first and foremost for introducing me to the magical world of ionic liquids, for being a constant source of encouragement and support, for his understanding and mentoring of my sometimes-bumpy research and mostly for never giving up on me. I would also like to acknowledge and sincerely thank my advisory committee members, Dr. Lars Rehmann and Dr. Lars Konermann, whose irreplaceable advice and guidance helped overcome several obstacles and nurture the thesis to its fruition. The dissertation examination committee, Dr.Charles Trick, Dr.Keith Taylor, Dr. Hassan Gomaa and Dr. Anand Prakash; thank you for reviewing my thesis and providing me with your valuable feedback.

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“*What is the most resilient parasite? Bacteria? A virus? An intestinal worm? An idea. Resilient... highly contagious. Once an idea has taken hold of the brain it's almost impossible to eradicate. An idea that is fully formed - fully understood - that sticks; right in there somewhere*” – Inception (2010).

What started, almost ten years ago, as an idea has today materialized. And for this I have my parents to thank. Mama-Papa, I thank you for making me an independent and strong woman; for teaching me the valuable principles of life; for always providing me with the best of education and for never doubting my abilities but instead encouraging me to dream big. Above all else, I thank you for your unconditional love, patience and blessings, mostly, through these years that I’ve been away from home. I am, what I am, where I am today all because of you. One of my biggest critics and an even bigger fan has been, and continues to be, my brother Dr. Gurpreet Singh. He’s my friend, my confidante and the one person whom I respect and love with all my heart. And, today, I thank him for being the best friend and brother one could hope for, and also for being one of the strongest pillars of support in my life and for our family. I am very proud of your accomplishments and wish only the best for you, always.

“*Friendship is unnecessary, like philosophy, like art... It has no survival value; rather it is one of those things that gives value to survival.*” - C.S. Lewis

To my oldest and most cherished friends, Lavina Vaz, Gaurav Godambe, Jayendra Katti, Sudeep Desai, Gayatri Deshpande, Devanshi Mehta, Nikhil and Priyanka Churi, Samidha
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Chapter 1

1 Introduction

1.1 General Introduction

The biotechnology industry is estimated to be $93.0 billion industry with a current annual growth rate of 5.2% and projected rate of 8.9% in the coming five years (IBIS 2013). Proteins account for an important spectrum of products in this industry that have applications in medicine as biopharmaceuticals. The production, biochemical engineering and processing of these proteins, is thus an important consideration in biotechnology.

Industrial scale manufacturing of proteins can be categorized as a two-phase process – upstream, which is expression and production of recombinant proteins in host cells and downstream, which is the recovery and purification of the protein. Often, overexpression of proteins results in the production of aggregates or inclusion body proteins that are inactive. Recovery of the active protein from genetically expressed proteins is thus limited by several unit operations, starting from centrifugation and filtration of the inclusion body proteins to the renaturation of the solubilized

In the early 1980s, the ‘bottleneck’ in the bio-therapeutics industry centered on the large-scale production of proteins. Production titers have since skyrocketed from what was considered standard, 1g/L, to 10g/L and more recently to 27+g/L antibody titer by Crucell’s Per.C6® cell line (Scott, 2008). Production of intracellular inclusion bodies, even at high titre has shifted the aforementioned ‘bottleneck’ to downstream processing and specifically recovery of native proteins from solubilized inclusion body proteins. The rate-limiting step in the downstream processing of proteins is thus the renaturation of nascent polypeptides to form active, native proteins.

Chow et al (2006) published a database – REFOLD that summarized the literature published upto 2006 encompassing the different refolding strategies employed. Of the published articles, 44% of the articles discussed dilution refolding, 17% dialysis and 16% a combination of dilution and dialysis. Approximately 15% of them discussed the use of different on-column refolding
strategies such as immobilized metal affinity chromatography, size exclusion, ion exchange, and hydrophobic interaction etc.

Irrespective of the \textit{in vitro} methodology employed, protein refolding additives have a significant impact and critical to the successful downstream processing of proteins. Traditional refolding additives such as low concentrations of chaotrope (e.g. 2 M urea), amino acids and amino acid derivatives (L-arginine, glycine, arginamide), salts (e.g. ammonium sulfate), polymers (e.g. polyethylene glycol PEG, polyvinypyrrolidone PVP) have exhibited limited success. Since their first use as protein renaturation agents for denatured lysozyme by Summers and Flowers in 2000, the application of ionic liquids as protein refolding additives is being investigated by several groups worldwide.

1.2 Objectives

The overall objective of this study was to investigate the effect of a new class of protein refolding additives, specifically; the imidazolium based ionic liquids as refolding additives for on-column adsorptive refolding of denatured lysozyme. Lysozyme was chosen as the representative protein due to its relatively fast refolding kinetics and the fact that an enzyme-based assay can be applied to measure protein refolding yields.

Specific objectives:

The overall objective was further divided into the following specific sub objectives:

[1] First the conventional approaches currently applied for lysozyme protein refolding were compared. These included the traditional dilution refolding and comparison with an ion exchange based adsorptive refolding technique. Next ionic liquids were applied as refolding additives in a conventional dilution-refolding format and the various factors that affect refolding yield of lysozyme were investigated.

[2] The identification of the optimal conditions for refolding denatured lysozyme, with the aid of ionic liquid(s) was next attempted using response surface methodology and central composite design.
Finally, ionic liquids and in particular the ionic liquid [EMIM] Cl was investigated for both the elution and refolding of denatured lysozyme in two different ion exchange column configurations, i.e. packed bed and fluidized bed ion-exchange systems.

1.3 Thesis structure

The thesis is divided into the following chapters:

- Chapter 2: Literature review.

An exhaustive, up-to-date review of the published literature is presented in this chapter. Factors that affect protein refolding at a molecular level, in-vitro, are discussed. This is used to better understand the role of refolding additives, specifically ionic liquids. The potential for development of ionic liquids as refolding additives is evaluated and an unbiased, comprehensive review of the topic is presented.

- Chapter 3: Materials and Methods.

This chapter provides details of the materials and methods that are common to all the proceeding chapters. Details about the fluidized bed ion exchanger and its mode of operation are provided in this chapter. Materials and methods pertinent to individual chapters are presented accordingly in those chapters.

- Chapter 4: A comparative study of refolding lysozyme using conventional strategies.

This chapter begins by validating the requirement of refolding additives and low concentration of denatured protein for dilution refolding of denatured lysozyme. Next, it compares the refolding yield and fractional mass recovery, as a function of increase in protein concentration, for two different on-column modes of operation – packed bed and fluidized bed.

- Chapter 5: Investigation of dilution refolding of denatured lysozyme using imidazolium ionic liquids.

This chapter identifies the ionic liquid parameters, composition and concentrations of ionic liquid, that influence the refolding yield of lysozyme. Refolding yield is presented as function of
time, with change in concentration and composition of the ionic liquids tested. Influence of chaotropicity and kosmotropicity of the individual ions of the ionic liquid on the refolding yield is also discussed.

- Chapter 6: Application of response surface methodology and central composite design for the optimization of lysozyme refolding by [EMIM] Cl

A quick overview on the theory of response surface methodology and central composite design is provided. Experimental design and results thereof that aid and abet in the identification of optimal refolding conditions for denatured lysozyme by [EMIM] Cl are discussed in this chapter. A validated, empirical model based on the results is provided.

- Chapter 7: Adsorptive protein refolding with the aid of [EMIM] Cl in a packed bed/fluidized bed ion exchanger

The applicability and influence of [EMIM] Cl as an elution and refolding buffer for adsorptive refolding of denatured lysozyme is examined. Refolding yield and fractional mass recovery, due to incorporation of [EMIM] Cl in elution buffer, is presented as function of mode of operation and increase in concentration of protein load. Comparison on the efficacy of [EMIM] Cl as a refolding/elution enhancer to other ionic liquids is also reviewed by means of experimental results.

- Chapter 8: Concluding remarks and recommendations

Based on the experimental results acquired in this thesis, final concluding remarks and pertinent recommendations are provided.

1.4 Major contributions

The experimental results in this thesis can be summarized in the form of the major contributions, as follows:

[1] Experimental results obtained demonstrate the use of ionic liquids, specifically imidazolium based ionic liquids as refolding additives for denatured lysozyme without the support of other refolding agents such urea and cysteine/cystine.
For the first time, response surface methodology and central composite design were
implemented to identify the optimal conditions for refolding denatured lysozyme using
[EMIM] Cl.

Refolding of denatured lysozyme on on-column fluidized bed ion exchanger

i. A novel fluidized bed ion exchange system was proposed and investigated for the
adsorptive refolding of denatured lysozyme

ii. For the first time, ionic liquid was incorporated in the elution and refolding
studies of denatured lysozyme in a packed bed and fluidized bed ion exchanger

iii. With the aid of [EMIM] Cl in elution buffer, in comparison to packed bed,
fluidized bed mode of operation tolerates higher concentration of protein load (up
to 25 mg) without unfavorably affecting the refolding yield and mass recovery

1.5 Nomenclature

[EMIM] Cl : 1-ethyl-3-methylimidazolium chloride

1.6 References

IBISWorld US - Industry, Company and Business Research Reports and Information,
clients1.ibisworld.com.proxy1.lib.uwo.ca. IBIS World.
Chapter 2

2 Literature Review: Ionic liquids as refolding additives

An exhaustive, up-to-date review of the published literature is presented in this chapter. Factors that affect protein refolding at a molecular level, *in-vitro*, are discussed. This was used to better understand the role of refolding additives, specifically ionic liquids. The potential for development of ionic liquids as refolding additives is reviewed.

2.1 Abstract:

Globally, purification of proteins is steadily moving towards a single-unit-system that can handle higher concentrations of protein while maintaining the productivity and overall cost efficiency of the process. The bottleneck of protein production has shifted downstream, specifically, recovery of active proteins from inclusion body proteins. In principle, recovery is a two step process; solubilization of the inclusion body followed by refolding or renaturation of the unfolded, dissolved protein to its active, refolded state. Protein refolding, *in-vitro*, is a kinetically competitive process, which if not executed in the appropriate environment could result in formation of aggregates and/or misfolded proteins, thereby substantially reducing productivity. Renaturation can be achieved either by use of suitable additives that suppress aggregation and enhance structural stability of native protein, and/or chemicals that allow the formation of disulfide bridges. Protein refolding aids encompass a variety of chemicals – amino acids (L-arginine), chaotropic salts at non-denaturing concentrations (urea, guanidine hydrochloride), polyols, polymers, amino acid -esters and -amides, molecular chaperones (GroEL, GroES), etc. Ionic liquids, ‘green alternatives to organic solvents’, can be designed to serve variety of functions ranging from organo-catalysis to active pharmaceutical ingredient. In the past decade, reports of their ability to suppress protein aggregation and enhance structural stability against thermal denaturation have emerged that make them suitable targets as protein refolding additives. A comprehensive review of their mechanism of action has been provided by first understanding the fundamentals of protein refolding, need for refolding additives and the consequent effect of solutes as protein refolding additives.
2.2 Background Information:

Not more than a decade ago the “capacity bottleneck” in the bio-therapeutics industry centered on the large-scale-production of proteins. Production titers have since sky rocketed from what was considered standard, 1g/L, to 10g/L, and more recently to more than 27 g/L antibody titer by Crucell’s Per.C6® cell line (C. Scott, 2008). In this regard, bacterial expression such as in Escherichia coli (one of the most extensively studied and used prokaryotic organisms) offers several advantages including growth on inexpensive carbon sources, rapid biomass accumulation, amenability to high cell-density fermentations and simple process scale up (Baneyx & Mujacic, 2004; Jungbauer, Kaar, & Schlegl, 2004; Mergili, Eiberle, & Jungbauer, 2010). However, more often than not, over-expression leads to the accumulation of insoluble polypeptide aggregates, often termed as “inclusion body”. Inclusion body proteins are amorphous, electron refractile, devoid of biological activity, dense particles of aggregated proteins. They can be found in both the cytoplasm and the periplasm of the bacteria, depending on if they were engineered to secrete the proteins or not (Clark, Schwarz, & Rudolph, 1999; Singh & Panda, 2005; Vallejo & Rinas, 2004; Q. M. Zhang, Wang, Liu, & Wang, 2008). There are several advantages associated with expression of proteins as inclusion bodies (Burgess, 2009; Clark, 2001; Singh & Panda, 2005; Su, 2011; Vallejo & Rinas, 2004):

a) The expression levels are often very high, up to 30% of the total cell protein.

b) The proteins are largely protected from proteolytic degradation by host cell enzymes.

c) Inclusion body proteins usually have higher density (~1.3 mg/ml) than many cellular proteins in vivo. Easy isolation of inclusion bodies from cells due to differences in their size and density as compared with cellular contaminants

d) Homogeneity of protein of interest in inclusion bodies, which helps in reducing the number of purification, steps to recover the pure protein.

Nonetheless, the production of intracellular inclusion bodies (even at high titre) has shifted the aforementioned “bottleneck” to downstream processing, specifically recovery of native proteins. Protein refolding is a competitive process between folding and aggregation of proteins. As a
unimolecular process, protein folding is considered to be a first order reaction. While, on the other hand, aggregation is a second- or higher- order reaction, severely influenced by the concentration of the denatured protein.

However, the production of intracellular inclusion bodies even at high titre has shifted the aforementioned “bottleneck” to downstream processing and recovery of produced proteins. The pressure to develop better separation and purification protocols that combat the new process- and product-related impurities and higher titre levels without compromising on the safety, efficacy and quality of the product is continually increasing. The purification of proteins is steadily moving from pooling-elution-fractions-of-multiple-runs on a single column towards development of columns that can handle higher protein concentrations. In order to do so, it is pertinent to develop approaches that incorporate clarification, separation and purification.

In-vivo, the cell has developed safety mechanisms, in the form of molecular chaperones, to combat the issue of misfolding and aggregation that might occur due to premature termination of translation of proteins, environmental stress and/or failure for newly synthesized polypeptide to reach its native conformation. These molecular chaperones can be divided into three functional subclasses; folding chaperones (e.g. DnaK, GroEl), holding chaperones (e.g. IbpA/B) and aggregating chaperones (e.g. ClpB). Each of the subclasses serves a unique function. Folding chaperones, rely on ATP-driven conformational changes to mediate the net refolding/unfolding of substrates. Holding chaperones, hold the partially folded state of the proteins until folding chaperones are available to execute their activity. And lastly, aggregating chaperones promote the solubilization of aggregated proteins. (Hartl, 1996; Hartl, Bracher, & Hayer-Hartl, 2011; Kriegenburg, 2012; Obalinsky, 2006; Sinnige, Karagöz, & Rüdiger, 2001; Wittung-Stafshede, 2011) Some of these molecular chaperones, GroEl, GroEs, DnaK, have recently been examined and adopted for in-vitro refolding of denatured protein(Hartl & Hayer-Hartl, 2009). In-vitro, the focal point of research for the past several decades has been to develop better refolding additives that mimic the action of molecular chaperones in-vivo, while at the same time meet the economies of scale and production requirements.

Ionic liquids are organic salts whose melts are composed of discrete cations (Table 2.1), based on methylimidazolium, N-butylpyridinium, N-methyl-N-alkylpyrrolidinium, and anions (Table 2.2), such as hexafluorophosphate [PF6]−, tetrafluoroborate [BF4]−, alkylsulfates [R-SO4]−, halides
such as chloride [Cl$^-$], nitrate [NO$_3^-$] etc., Some noteworthy characteristics of ionic liquids include low to negligible vapor pressure, high thermal stability, non-ionizing, non-flammable properties, ability to dissolve wide range of organic, inorganic, organo-metallic compounds and polymers, electric conductivity, high electro elasticity, liquid crystal structures, potential to be reused and recycled, and immiscibility with either water or organic solvents (Hardacre, Holbrey, & Nieuwenhuyzen, 2007; Hough & Rogers, 2007; Turner, 2005; van Rantwijk & Sheldon, 2007). Also ionic liquids can be ‘tailored’ such that altering their structure affects its physico-chemical properties, which further enhances or impairs their functionality. Although, no thumb rule has been set to determine the physico-chemical properties of any novel ionic liquid, quantitative structure activity relationship (QSAR) is being investigated (Das & Roy, 2013; Jastorff et al., 2003). Many applications of ionic liquids have already been reported e.g. use as lubricants, additives, in organic and in-organic chemistry, separation technology, and biopharmaceuticals (Abbott & McKenzie, 2006; Freire et al., 2012; Greaves & Drummond, 2008; Han & Row, 2010; H. Lin, de Oliveira, & Veith, 2011; MacFarlane & Seddon, 2007; G. W. Meindersma, Maase, & De Haan, 2000; Rodrigues, Prosinecki, Marrucho, Rebelo, & Gomes, 2011; Zhu et al., 2006). In recent years, their use in refolding of denatured proteins has emerged. In order to assess their applicability, we must first understand the downstream processing of proteins and factors that influence protein refolding.

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<tr>
<th>Cation</th>
<th>Full name</th>
<th>Abbreviation</th>
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Table 2.1 List of commonly used cations of an ionic liquid
<table>
<thead>
<tr>
<th>Anion</th>
<th>Full name</th>
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<tbody>
<tr>
<td>[Cₙmim]^+</td>
<td>1-alkyl-3-methylimidazolium</td>
</tr>
<tr>
<td>[Cₙpyim]^+</td>
<td>1-alkyl-3-methylpyrolidinium</td>
</tr>
<tr>
<td>[Cₙpy]^+</td>
<td>N-alkylpyridinium</td>
</tr>
<tr>
<td>TRA</td>
<td>Tetraalkyl-ammonium</td>
</tr>
<tr>
<td>TRP</td>
<td>Tetraalkyl-phosphonium</td>
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Table 2.2 List of commonly used anions in ionic liquids
<table>
<thead>
<tr>
<th>Anion</th>
<th>Name</th>
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<tbody>
<tr>
<td>Cl⁻</td>
<td>Chloride</td>
</tr>
<tr>
<td>Br⁻</td>
<td>Bromide</td>
</tr>
<tr>
<td>BF₄⁻</td>
<td>Tetrafluoroborate</td>
</tr>
<tr>
<td>PF₆⁻</td>
<td>Hexafluorophosphate</td>
</tr>
<tr>
<td>RSO₃⁻</td>
<td>Alkylsulphate</td>
</tr>
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</table>

2.3 Downstream processing for protein recovery

Production of an active refolded protein from a mass of nascent polypeptide chains, often aggregated to form inclusion body proteins, involves several stages as elucidated in Figure 1. Irrespective of the cellular process used to express proteins, extraction of the protein is the first and foremost step. Cell lysis followed by separation using ultra-centrifugation and other such techniques are commonly employed to extract the inclusion body proteins. Filtration is ensued by solubilization of the inclusion body protein by employing high concentrations of chaotropic salts, such as urea and guanidine hydrochloride, detergents such as Triton X 100, deoxycholate and sodium-N-lauroyl sarcosine (SLS) (Burgess, 2009; Cabrita & Bottomley, 2004; Singh & Panda, 2005), and reducing agents such as dithiothrietol (DTT), β-mercaptoethanol that break the disulfide linkages of the protein and completely solubilize and denature the inclusion body proteins.
Recovery of folded proteins from denatured, solubilized inclusion bodies is thus dependent on multiple unit operations and involves a large number of parameters as shown in Figure 2.2.
Refolding of solubilized proteins involves approaches such as batch dilution, dialysis and pulse renaturation. These are traditionally employed methods that can be labor intensive, cost-ineffective, difficulty to automate and scale-up and often result in higher ratio of aggregates to active refolded proteins (Chen, Lin, Wu, & Liu, 2012; Mannall, Titchener-Hooker, Chase, & Dalby, 2006; Shiraki, 2010). (Jungbauer et al., 2004) review some of the noteworthy contributions in the field of on-column protein refolding strategies. It is evident a variety of preparatory modes of on-column refolding have been designed and tested for recovery and purification of refolded proteins from IBPs; expanded bed (EB), simulated moving bed (SMB), membrane chromatography etc. In comparison to packed bed and dilution refolding, EB & SMB offer significant advantages in terms of process efficiency, solvent and media utilization, reduced number of post-refolding processing steps such as separation, polishing, viral filtration and above all else flexibility to work with higher concentrations of protein titre. However, EB systems encounter fouling of
resin, protein/lipid build-up, and ligand loss and matrix disintegration rendering them a cost-ineffective option for industrial application. Although, SMB addresses some of the EB issues, it also suffers limitations owing to cost of construction, operation and maintenance (Chase & Draeger, 1992; Ferré et al., 2005; Freydell et al., 2010a; Gu et al., 2002; Lyddiatt, 2002; B.-J. Park, Lee, Mun, & Koo, 2006). High pressure refolding, although in its embryonic stages of development, is also being considered as potential refolding strategy at the industrial scale. Notable key advantages of the high pressure refolding method are: (1) performs solubilization and refolding/disaggregation of protein simultaneously, (2) requires little or no chaotropic agents, (3) is often independent of protein concentration, and (4) can disaggregate and refold proteins rapidly (Qoronfleh, Hesterberg, & Seefeldt, 2007). However, so far the published literature on the use of high-pressure protein refolding has been under extreme conditions; low protein concentration (0.65 mg/ml of growth hormone), high pressure 2000 bar, 65 °C for 24 hours, creating the element of doubt for scale up at industrial level (St John, 2001). Thus, there is a constant need to develop newer refolding strategies that are - economical, robust, cost- and process- efficient and generic.

2.4 Analysis of refolding efficiency

Irrespective of their final use, it is important that the produced-refolded protein meets the quality control and quality assurance standards. Besides high purity, activity of the compound, which is closely related to its native structure, is a critical quality control. Activity assays combined with circular dichroism (CD) spectroscopy and/or fluorescence spectroscopy are classified as adequate means to prove the identity of the product (Bekhouche, Blum, & Doumèche, 2012; Diego, Lozano, Gmouh, Vaultier, & Iborra, 2004; Helal & Melzig, 2013; Hennessey & Johnson, 1981; Kelly, Jess, & Price, 2005; Y. C. Lee & Yang, 2002). Typically, CD and fluorescence spectra of unfolded, native and refolded protein are compared and the latter ones have to show equal features to meet the activity criteria.

The correct formation of disulfide bonds can also be determined by chromatography techniques such as reverse phase chromatography, hydrophobic interaction chromatography and ion-exchange chromatography. Analytical size exclusion chromatography is often employed to detect and separate the aggregated and misfolded proteins from correctly folded proteins.
(Freydell, Van Der Wielen, Eppink, & Ottens, 2010b; Gu et al., 2002; Jungbauer et al., 2004). More recently, progressive proteomic techniques such as atomic force spectroscopy (Peterman & Wuite, 2011), light scattering (Gast, Damaschun, Misselwitz, & Zirwer, 1992; Peterman & Wuite, 2011), calorimetry (Baldwin & Rose, 2013; Farber, Darmawan, Sprules, & Mittermaier, 2010; C. Scott, 2008), ATR-FTIR spectroscopy (Baneyx & Mujacic, 2004; jungbauer, Kaar, & Schlegl, 2004; Mergili et al., 2010; Shivu et al., 2013; J. M. Walker, 2012) have been suitably adopted to study protein refolding kinetics, protein-protein interactions and oblige as a stronger validation protocol.

2.5 Role of refolding additives on protein refolding

Proteins are most soluble in aqueous solution when they are in their native, folded state. It is well established that protein refolding is a kinetically competitive process between the intermolecular hydrophobic interactions resulting in aggregates versus the intra-molecular interactions yielding native proteins. As a unimolecular process, protein folding is a first-order reaction with respect to protein concentration, while the aggregate formation is a second- or higher- order process caused by intermolecular interactions. Competition between the folding and aggregates determines the efficiency of the in-vitro refolding step.

Figure 2.3 depicts a simplified protein refolding kinetic model. In this model, the unfolded protein (U) undergoes the early phases of folding to form a transient intermediate (I) with native-like secondary structure and partial tertiary structure. This model can be further simplified to a parallel of reaction of the formations of the native state protein (N) and aggregates (A) from transient intermediate.
Figure 2.3 Kinetics of protein refolding. Key: U: unfolded and reduced protein; I: Intermediate stage; N: Native state

Aggregation results from non-specific interactions between hydrophobic regions or partially refolded structures of different polypeptides, it is usually irreversible, hence it’s critical that this step is suppressed so as to achieve high yields of native and active protein (Buswell & Middelberg, 2002; X.-Y. Dong, Huang, & Sun, 2004; Ferrone, 1999; Gast et al., 1992; Konermann, 2011; Nölting, 2006).

Competition between folding and aggregation can be expressed by the following equations:

\[ \frac{dI}{dt} = -\kappa_N I - \kappa_A I^n \]  \hspace{1cm} \text{Equation 2.1}

When \( t = 0, I = U_0 \) and \( N = 0 \)

\[ \frac{dN}{dt} = \kappa_N I \]  \hspace{1cm} \text{Equation 2.2}

where, \( U_0 \) represents the initial unfolded protein concentration, \( t \) - the refolding time (min), \( \kappa_N \) and \( \kappa_A \) - folding and aggregation kinetic constants, respectively and \( n \) - reactor order of aggregate formation.

It is this competition between aggregates and/or misfolded proteins and native proteins that make recovery of active proteins from IBPs challenging and also the bottleneck step in downstream processing of proteins.
The refolding process is modulated, in vivo, by molecular chaperones (also known as heat shock proteins) that ensure the production of native, active protein. Absence or lack of molecular chaperones in humans gives rise to neurodegenerative diseases such as Alzheimer’s’ disease, Parkinson’s and various other prion diseases (Clark et al., 1999; Goedert, Clavaguera, & Tolnay, 2010; Jucker & Walker, 2011; Singh & Panda, 2005; T. Zhang & Ye, 2007). For a detailed understanding of the protein misfolding and its relationship with human diseases, the reader is directed to review by Chiti and Dobson (2003), in addition to other articles cited here (Burgess, 2009; Chiti & Dobson, 2006; Clark, 2001; F. Li, Dong, & Zhuang, 2009; Singh & Panda, 2005; Su, 2011; Vallejo, 2004)

*In vitro* the slightest deviation in up-stream process and/or product parameters such as pH, concentration of protein, temperature changes, mechanical stress such as shear strain, surface adsorption or foaming etc., can alter the structure of the protein and consequently promote ‘salting out’ and/or misfolding or aggregation (Hamada, Arakawa, & Shiraki, 2009; Ito et al., 2012; Karuppiah & Sharma, 1995; X. U. T. Lin, 2010; Mergili et al., 2010; Tsumoto, Ejima, Kumagai, & Arakawa, 2003; Yamaguchi, Yamamoto, Mannen, Nagamune, & Nagamune, 2012; L. Yang, Dong, & Yan, 2008). Often, production of proteins as inclusion body proteins (aggregates) is preferred owing to ease of separation, protection from proteolytic degradation and higher protein content. Once solubilized, inclusion bodies are open chain, polypeptides with hydrophobic core exposed to the solvent media. Gradual removal of denaturants (solubilization agents) generates an environment conducive to protein refolding.

Essentially during refolding proteins are not properly converted to the native state instead assume a partially folded state for a prolonged period of time (Biggar, Dawson, & Storey, 2012). Intermediates with hydrophobic regions that are exposed to the solvent play a crucial role in the partition between native and aggregated conformations. Folding intermediates are comprised of significant elements of the secondary structure but little of the native tertiary structure. Due to the expanded volume of these intermediates, the hydrophobic regions, which are usually buried in the native state, are exposed to the solvent. When so exposed, hydrophobic regions on separate polypeptide chains can be in contact with one another and can interact. This causes the intermediates to be diverted from the correct folding pathway, resulting in aggregate formation and loss of protein.
Thus, it is apparent that in order to achieve high refolding yield the protein structure must either be stabilized to minimize deviation and/or the structures that are not in the native state must be solubilized to avoid protein-protein interaction (aggregation/misfolding). This can be achieved by addition of solutes/refolding additives. In principle, solution/refolding additives can be classified into two categories a) denaturants – indirectly promote refolding by weakening the inter- and intra- hydrophobic bonding b) protein stabilizers- enhance protein refolding by modifying the water of hydration and thereby stabilizing the native structure.

Table 2.3 summarizes refolding aids typically utilized for production of proteins from nascent polypeptides and/or inclusion bodies. Chaotropic salts at non-denaturing concentrations (for urea below 2.5 M) anion- and non-ionic detergents, amino acids and its derivatives, osmolytes, amphiphilic polymers and varying salts have been used as refolding aids in the past.

Of these, the most popular refolding additives are urea (123 research articles since 2010) and arginine (48 research articles since 2010), as per number of research articles published and accessed via Scopus on July 23, 13. For detailed discussions on the mechanism of action of commonly used solution additives the reader is referred to what has already been covered by other authors (Hamada et al., 2009; Karuppiah & Sharma, 1995; Tsumoto, Ejima, Kita, & Arakawa, 2005; Yamaguchi et al., 2012; C. Yang, Yu, Dong, & Sun, 2012; Yasuda, Murakami, Sowa, Ogino, & Ishikawa, 1998)

<table>
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<tr>
<th>Sr. No.</th>
<th>Nature</th>
<th>Mechanism of action</th>
<th>Advantages &amp; Limitations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chaotropic Salt • Guanidine</td>
<td>• Solubilize hydrophobic moieties exposed to the solvent • Urea at concentrations below 3M promotes refolding</td>
<td></td>
<td>(Gu et al., 2002; Hamada et al., 2009;</td>
</tr>
</tbody>
</table>
Hydrochloride
- Urea

in folding intermediates or misfolded species.

Significantly enhances viscosity of the solution generating issues in chromatographic purification of the protein

Hardacre et al., 2007; Khademi and Mostafaei, 2010; Stumpe and Grubmüller, 2009

Detergent
- SDS (sodium dodecyl sulphate)
- Triton X-100
- Laroylsarcosin
- Tween -20
- CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate.)

SDS is an anionic detergent that imparts negative charge to entire protein.

Above critical micelle concentration, SDS completely denatures protein. Binds to the hydrophobic region of the polypeptide

SDS monomer can bind to the protein so tightly that dilution or dialysis does not completely dissociate SDS molecules.

Development of artificial chaperone protein refolding; solubilization of protein using SDS ensued by stripping off detergent by use of Cyclodextrin.

Toxicity of detergent imminent threat

Method is not versatile

(Daugherty et al., 1998; Gautam et al., 2012; Hendrick and Hartl, 1995; Rozema and Gellman, 1996; Yamaguchi et al., 2012; Yasuda et al., 1998)

Amino Acid and amino-acid derivatives
- Arginine
- Arginamide
- Arginine ethylester
- Proline
- Glycine

Increases solubility of aggregation prone molecules.

Also suppresses heat-induced aggregation of protein (exception arginine ethylester)

Expensive

Moderately toxic

Arginine ethylester tends to be hydrolyzed to alcohols and amino acids at high temperatures or alkaline pH

Expensive

Not – biodegradable

(Meng et al., 2001; Swartz, 2001; Yamaguchi et al., 2012; C. Yang et al., 2003; Hamada and Shiraki, 2007; C. R. R. Lange, 2009; Meng et al., 2001; Nakakido, 2009; Samuel et al., 2008; Tsumoto et al., 2005)

Table 2.3 cont’d

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<th>Sr. No</th>
<th>Nature</th>
<th>Mechanism of Action</th>
<th>Advantages &amp; Limitations</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Polymers</td>
<td>PEG binds to the first refolding intermediate to perturb self-association of aggregate-prone intermediates. Also decreases thermal</td>
<td>Expensive</td>
<td>(Meng et al., 2001; Swartz, 2001; Yamaguchi et al., 2012; C. Yang et al., 2003; Hamada and Shiraki, 2007; C. R. R. Lange, 2009; Meng et al., 2001; Nakakido, 2009; Samuel et al., 2008; Tsumoto et al., 2005)</td>
</tr>
<tr>
<td>glycol (PEG)</td>
<td></td>
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<tr>
<td>polyvinyl pyrrolidone (PVP)</td>
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<td>glycerol</td>
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<td>sorbitol</td>
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- PVP binds to early-molten globule like refolding intermediate, thus, prevents aggregation and enhances refolding.

5 SALTS
- Ammonium sulphate,
- Trimethyl ammonium oxide (TMAO)
- magnesium chloride/calcium chloride,
- sodium citrate/sulfate.

- Modify the interfacial tension between the protein surfaces and bulk solvent, leading to alteration of protein solubility
- Final effect dependant on protein concentration and salt concentration

2012; L. Yang et al., 2008)

An emerging class of refolding additives, the sixth category not included in the table above, is ‘Ionic liquids’. By definition, ionic liquids are ionic salts that are liquid at temperatures below 100 °C. Since the synthesis of ethylammonium nitrate (m.p. 12 °C) by Paul Walden in 1914, ionic liquids have evolved substantially and found application as lubricants, catalysts, in organic and in-organic chemistry, separation technology, and biopharmaceuticals etc. These have been reviewed in-depth by others and do not fall under the purview of our discussion. A brief overview on the history and nomenclature is provided in the section below.

2.6 History and Nomenclature of Ionic liquids
‘First-generation’ ionic liquids are mainly composed of N,N’-dialkylimidazolium and N-alkylpyridinium cations, and metal-halide anions. Due to hygroscopic nature of these anions, first-generation ILs have to be handled under inert gas, thus limiting their applications. ‘Second-generation’ ionic liquids, in comparison, are usually more stable in air and water and can be used on bench-top. This is widely attributed to the replacement of large- and oxygen-reactive anions by halides such as chloride, bromide, iodide or anions such as BF\textsubscript{4}+, PF\textsubscript{6}− and C\textsubscript{6}H\textsubscript{5}CO\textsubscript{2}. The activity, kinetic- and thermal- stability of different enzymes such as oxidases, lipases or cellulases in the presence of second-generation ILs has been successfully studied, and synthesis of various products has been carried out (W. G. W. Meindersma and De Haan, 2012; Moniruzzaman et al., 2010; Moon et al., 2006; Turner, 2005; van Rantwijk and Sheldon, 2007).

‘Third-generation’ of ionic liquids emerged in the early 2000s; are more task specific, chiral, and based on more hydrophobic-, biodegradable- anions and cations (Hough and Rogers, 2007; Hough et al., 2007; Turner, 2005). (Hough and Rogers, 2007) review the synthesis and application of some of these ILs such as ranitidine docusate (histamine H\textsubscript{2}-receptor antagonist, emollient) and didecyldimethylammonium ibuprofen (antibacterial anti-inflammatory).

Ionic liquids derived from 1-alkyl -3-methyl imidazolium and 1-alkylpyridinium cation are referred to as [C\textsubscript{n} mim]+, where the index ‘n’ stands for the number of carbons in one of the linear alkyl chains and ‘mim’ stands for N-methylimidazolium. 1-ethyl and 1-butyl derivatives are often referred to as [EMIM]+ and [BMIM]+ respectively. C-2 methylation of the imidazolium moiety leads to even more possibilities of different cation types. These cations are abbreviated [C\textsubscript{n}dmim]+ for 1-alkyl-2,3-dimethylimidazolium and [C\textsubscript{n}py]+ for 1-alkylpyridinium cation (Earle and Seddon, 2000; Plechkova and Seddon, 2008; Stark and Seddon, 2000). As per IUPAC nomenclature, a square bracket is drawn around polyatomic ions, and monoatomic anions are denoted without brackets. Thus [C\textsubscript{4} mim][Cl] is correct while [C\textsubscript{4} mim][Cl]- is not; [C\textsubscript{4} mim][NTf\textsubscript{2}] is correct, [C\textsubscript{4} mim]NTf\textsubscript{2} is not. The cation (if organic) must always be enclosed by square brackets.

While knowledge of ionic liquids that moderate protein refolding is crucial, it cannot be achieved without an understanding of the terms ‘water of hydration’, kosmotrope and chaotropes.

2.7 Water of hydration and structural stability of proteins
Proteins are linear macromolecules with a non-repetitive, specific covalent structure capable of forming a three-dimensional structure owing to amino-acid sequences that fold to generate compact domains. The nature and arrangement of the amino-acid side chains along with the protein backbone is responsible for the individual characteristics of the macromolecule (Chase & Draeger, 1992; Ferré et al., 2005; Freydell et al., 2010a; Gu et al., 2002; Lyddiatt, 2002; B.-J. Park et al., 2006; Vasantha, Attri, Venkatesu, & Devi, 2012).

In principle, when in solution, water in the vicinity of proteins can be classified into three categories (Attri, Venkatesu, & Kumar, 2012; Bekhouche et al., 2012; Collins, Neilson, & Enderby, 2007; Degreve et al., 2004; Rand, 2004; Zaccai, 2004):

(a) Internal water - buried in the bio-molecular structure,

(b) Water of hydration - at the surface of the biomolecule, and

(c) Bulk or free water - which, technically, no longer feels the influence of the dissolved biomolecule.

Of these, ‘water of hydration’ is of prime importance when studying protein refolding. During the course of any protein reaction the total surface area of the protein in contact with water changes. This is accompanied by a change in the number of water molecules with which it interacts, i.e., its extent of hydration. The water of hydration ‘$W_h$’ is the effective number of water molecules in a fluctuating cloud that interacts more or less favorably with the protein surface. Through Figure 2.4, (Timasheff, 2002), explained the ‘water of hydration’. It commences with an ideal situation, where free water molecules in pure water would be in equilibrium with the transient cluster (Figure 2.4.A). Next, upon introduction of a protein molecule (Figure 2.4.B), based on their associated free energies, the water molecules are either attracted to-, repelled from-, or indifferent to-, the surface of the protein. This translates in to the corresponding changes in the freedom of motion of the individual molecules, as depicted in Figure 2.4.C. For example, molecule ‘a’ (shaded grey) is repelled while molecule ‘b’ (shaded black) fluctuates between strong attraction and freedom. In Figure 2.4.D, each arrow on the water molecule represents the time average of its net non-random extent of motion in the same direction as that of the protein. Molecule ‘a’ being repelled from the surface is not transported.
Molecule ‘b’, however, due to its fluctuation between being attracted and repelled, migrates at a slow rate. The sum of the contributions of all the water molecules whose motion is momentarily (and non-randomly) oriented in the same direction as that of the protein molecule is the measured protein hydration, which divided by the molecular weight of the water results in the ‘number’ of water molecules, $W_h$, that interact with the protein molecules.
Figure 2.4 Illustration elucidating the concept of water of hydration (adapted from Timaseff, 2002).

Figure 2.4.A, ideal state where free water molecules are in equilibrium with transient cluster of water molecules. When a protein is introduced, Figure 2.4.B, water molecules interact with it with different free energies; represented by the vertical bars- downward pointing bars indicate attraction, upward indicate repulsion and absence of bars indicates indifference. In Figure 2.4.C, corresponding changes in the freedom of translational and rotational motions of the water molecules can be visualized. The consequences are seen in Figure 2.4.D. The large vector
(arrow) on the protein indicates its motion induced by the applied field (gravitational in sedimentation, electro kinetic in electrophoresis, chemical potential gradient in diffusion). Molecules with no vector are neutral thermodynamically and are indifferent to the transport of the protein molecule.

In the absence of hydrating water, proteins lack activity. The cloud of water molecules around proteins is affected up to at least a nanometer from its surface or 2 nm between neighboring proteins. Some of these water molecules interact with the protein surface, reorienting both themselves and the surface groups whereas other water molecules link these to the bulk in an ordered manner while continuing to remain in a dynamically active state. Thus, when in solution proteins possess a conformational flexibility, which encompasses a wide range of hydration states. Equilibrium between these states depends on the activity of the water within its microenvironment; that is, the degree of freedom that the water has to hydrate the protein.

It has been reported chemical solutes, including but not limited to ionic liquids, affect several characteristics of protein – stability, folding and aggregation - in a number of different ways (Ball, 2013; Record et al., 2013; van der Post et al., 2013). For many decades, several different studies have investigated these effects and with the evolution in scientific technology and analytical methods resulted in two different perspectives – the historical approach- Hofmeister series, and the more recent ‘new’ or evolved approach.

2.1.1 Effect of chemical solutes on proteins

The historical approach, which considers the concept ‘order makers’ and ‘order breakers’ as Hofmeister effects (chaotropicity, Kosmotropicity), is still considered by several scientists (Nostro & Ninham, 2012; Schwierz, Horinek, & Netz, 2013; Szalontai et al., 2013; Tietze et al., 2013) and a brief discussion is provided in the following section. A more recent revision indicates that this is an oversimplified approach lacking information at the sub-molecular level of interaction between hydrated state of individual solutes and proteins. Thereby implying that our traditional understanding of the concepts of chaotrope and kosmotrope could in fact be misleading.

Hofmeister (1888) developed the series bearing his name based on the concentrations of various salts (cations and anions) needed to ‘salt-in’ or ‘salt-out’ the proteins in solution (López-León, 2013; 2014).
Santander-Ortega, Ortega-Vinuesa, & Bastos-González, 2008; Peterman & Wuite, 2011). In principle, the ions in the series were arranged in the order of their ability to change water structure. Kosmotropes or “order makers” were those ions that exhibited strong interactions with water molecules and were therefore capable of breaking the water-hydrogen bonds (Gast et al., 1992; Peterman & Wuite, 2011; Tietze et al., 2013; H. Zhao, 2005). As per Hofmeister series, sulphate anion, for example, is a strong kosmotropic anion. Because of its strong interaction with water $\text{SO}_4^{2-}$ competes effectively for the water molecules originally associated with the protein molecule. This encourages the protein molecule to minimize its surface area exposed to the solvent, thereby favoring the acquirement of its native compact state due to the restored driving force of the hydrophobic effect. On the other hand, chaotropes are known as “order-breakers” because they exhibit strong interactions with protein vs. water. Chaotropic anion, such as cyanide for example, selectively partitions in water allowing the protein a degree of structural freedom that subsequently encourages protein extension and denaturation.

Recently, the ideas of the structure-maker/breaker concept have been revisited. The results indicated that the perturbation to water’s structure appeared to be confined to the first solvation shell of the ion (Baer & Mundy, 2013; Collins et al., 2007). Thus, the revised theories, move away from the effect of individual ions or solutes on the bulk-water phase and instead focus on the direct interaction of individual ions (cation/anion) with the protein. In general, they are thought to affect structural stability of proteins in three main ways (Ball, 2013; Nostro & Ninham, 2012; Y. Zhang & Cremer, 2010):

- Interaction with the protein backbone and side chains through ion-pairing
- Alteration of both surface tension and hydrogen-bonding network surrounding proteins
- The hydrophobic interaction of ions with water and proteins

Of special mention is the study by Collins et al (2007) since it addresses the issues with the micro-aqueous environment of the protein (Collins, 1997; Collins et al., 2007). They describe three different independent layers of hydration that occur at a protein surface; a) the solvation layer in close proximity to the protein surface, b) the transition layer, and c) the bulk layer that forms the solution. It is estimated that the solvation layer and bulk layer compete for hydrogen bond with the transition layer. The specific structure, charge and composition of
the protein determine the solvation layer, while the composition and properties of the bulk solution determine the behavior of the bulk layer. The first and third layers compete for hydrogen bonding with the second layer. (Nostro & Ninham, 2012). Thus, addition of ions or neutral solutes to the bulk layer affects the capability of the transition layer to solvate the protein surface. In particular, a kosmotrope in the bulk layer ‘deflects’ the transition layer molecules from participating in the hydration of the protein thereby minimizing the solvent exposed area. Whereas, the presence of a chaotropic solute in the bulk layer promotes the solvation of the protein by the transition layer; the solution becomes a better solvent for the protein, which unfolds, exposing more surface to the solvent. (Collins et al., 2007; Nostro & Ninham, 2012).

Thereafter, Pegram and Record (2008) proposed a solute partitioning model (SPM) that interprets chemical effects of nonelectrolyte solutes and Hofmeister salts on all aqueous processes (Pegram & Record, 2008). Molecular thermodynamic analysis of surface tension and hydrocarbon solubility data using SPM was employed to interpret the solute and salt effects on surface tension and on solubility of uncharged molecule quantitatively in terms of surface hydration, solute partition coefficient, the ratio of local to bulk solute concentration, and the extent to which the solute salt or ion accumulates at the surface. In general, it was observed that anions accumulated indifferently in proximity of the amide groups and increased solubility, while excluded solutes decreased solubility.

Similar studies by Mason et al (2009) investigated through molecular dynamic simulations the interaction between model peptide and tetrapropylammonium sulfate and guanidium chloride (Mason et al., 2009; Nostro & Ninham, 2012). Their results suggests that the Hofmeister series could be better understood by assessing the capability of ions to affect hydrogen bonding, salt bridges, and hydrophobic interactions in the protein and how these effects are altered by the counter- ion.

Thus, water of hydration of proteins, ion-specific interaction of solute molecules with protein surface and backbone have a cumulative effect on the refolding of proteins. A widely adopted and accepted policy to characterize these ion-specific effects is by the Jone-Dole viscosity B-coefficient of individual ions in solution.
2.1.2 Characterization of ions based on Jone-Dole viscosity B-Coefficients

Jone-Dole realized that some ions make water more viscous (kosmotrope) and some others make it more fluid (chaotropes) when compared to the viscosity of pure water at the same temperature (Jenkins & Marcus, 1995; Marcus, 1994; Nostro & Ninham, 2012). Please note hereafter for the purpose of this review article, kosmotrope is defined as the solute that increases the viscosity of solvent and chaotrope as the solute that reduces the viscosity of the solvent.

For a wide range of concentrations (between 0.005 M and 1 M) the specific viscosity of water solution can be fitted to the empirical equation, Equation 2.3, as expressed below.

\[
\frac{\eta}{\eta_0} = 1 + Ae^{1/2} + Bc + Dc^2 \ldots
\]

where \( \eta \) is the dynamic viscosity of the solution and \( \eta_0 \) dynamic viscosity of solvent, \( c \) is the molar concentration of the electrolyte, \( A \) reflects the viscous drag due to the ionic atmosphere, which delays the motion of the ion and makes the solution more viscous. \( B \)-coefficient depends on the ion-solvent interactions and is related to the volumes of the ions. The ion-specific \( B \)-coefficient can be negative (for chaotropes \(<\eta_0\)) or positive (for kosmotropes, \( \eta >\eta_0 \)). The term \( Dc^2 \) is needed only in very concentrated solutions (Jenkins & Marcus, 1995; Pusey, Paley, Turner, & Rogers, 2007; Z. Yang, 2009; H. Zhao, 2006).

Based on their viscosity \( B \)-coefficients in water Table 2, characterizes some of the commonly used ions in ionic liquids as either chaotrope or kosmotrope. This information could assist in the the selection and design of ionic liquids as protein refolding additives.

### Table 2.4 Characterization of ions commonly used in ionic liquids based on their viscosity \( B \)-coefficient

<table>
<thead>
<tr>
<th>Ion</th>
<th>Viscosity B-coefficient</th>
<th>Kosmotrope</th>
<th>Reference</th>
</tr>
</thead>
</table>


Using the Jones-Dole viscosity B-coefficients as scaling factors for the strength of water-water interactions, (Collins et al., 2007) demonstrated contact-ion pair formation is actually dominated by hydration-dehydration. This was reverberated by Zhao and Marcus as illustrated in Figure 2.4, (Marcus, 1994; H. Zhao, 2006). Hinckley noticed that the enzyme activity of laccase C from *Trametes* sp. decreased dramatically with the increase of concentration of 4-methyl-N-butylpyridinium tetrafluoroborate ([4-MBPy]BF₄⁻): 10% IL reduced half of the activity and 20%
IL reduced the activity more than 9-fold. It was postulated that the high chaotropicity of BF$_4^-$ ions might be an important factor causing the deactivation (B.-K. Liu, Wu, Xu, & Lin, 2007).

2.8 Role of ionic liquids as protein refolding additives

Addition of any solute to an aqueous protein solution, results in lowering of ‘water of hydration’ thereby allowing the water/solute molecules to interact with the protein, consequently affecting its stability and solubility (Kohno & Ohno, 2012). Ionic liquids, also known as ‘designer solvents’, in general consist of a salt where one or both the ions are large, and the cation has a low degree of symmetry which tends to reduce the lattice energy of crystalline form of the salt and thereby its melting point (Earle & Seddon, 2000). Ionic liquids are known to affect protein activity by either a) stripping off the internal water associated with the protein, b) penetrating into the micro-aqueous environment to interact with the protein by changing protein dynamics, conformational and/or protein’s active site, or c) by interacting with the substrates and products either by direct reaction with them or by altering the portioning between the aqueous and non-aqueous phases.

Recent molecular dynamics simulations of biomolecules in ionic liquid/water mixtures or ‘hydrated ionic liquids’ shed light on the interactions of ionic liquid in aqueous bio-molecular solutions (Attri et al., 2012; Haberler, Schröder, & Steinhauser, 2012; Hunt, 2006). One such simulation study of 1-ethyl-3-methylimidazolium trifluoromethanesulfonate/water mixture in the presence (and absence) of ubiquitin and zinc protein motif (Haberler, Schröder, & Steinhauser, 2011), demonstrated that the anion and cation of an ionic liquid have individual kosmotropic and chaotropic characteristics, respectively, encumbering the characterization of the whole ionic liquid as a kosmotropic or chaotropic salt.

The fact that ionic liquids dissolve and dissociate into individual ions rather than exist as an intact molecule is an obvious advantage over molecular organic solvent. Miscibility of the ionic liquid or its partition coefficient in water has a considerable effect on aqueous protein solutions. Upon dissociation, the individual component ions of ILs interact with water via ion-dipole interactions and their hydration state is then dependent on the type of ion and fraction of water present in the mixture. Consequently, this determines if the ion is behaving as a kosmotrope or
chaotrope. Each ion, cation and anion, in solution will interact with the protein in solution and either stabilize or destabilize the protein structure and conformation, as has been illustrated in Figure 2.7. So far, studies have shown that anions influence protein stability and activity via its hydrogen bonding forming capacity and nucleophilicity properties (Attri & Venkatesu, 2012a; Buchfink, Patil, Rudolph, Lange, & Tischer, 2010; Lange, Patil, & Rudolph, 2005; Vidya & Chadha, 2009; Weingärtner, Cabrele, & Herrmann, 2011). While changes in the alkyl chain length of the cations influence the viscosity, hydrophobicity and polarity of the ionic liquid, all of which have an effect on the solubility, stability and activity of targeted protein molecule (Bae, Chang, Koo, & Ha, 2012; Lange et al., 2005; Xiao et al., 2009; Yamamoto, Yamaguchi, & Nagamune, 2011).
Figure 2.5 Flow chart that illustrates the application of ionic liquids as protein refolding additives.

Ionic liquids when dissolved in aqueous protein solution dissociate into individual ions; either of which can behave as a kosmotrope or a chaotrope. For the purpose of enhancing protein-refolding yield in a downstream process, an ideal ionic liquid would be one that has a kosmotropic anion and a chaotropic cation.

2.8.1 Ionic liquid parameters that influence protein refolding

The first example of an enzyme in an IL was reported in 1984 when Magnuson and co-workers demonstrated the activity and stability of alkaline phosphatase in aqueous mixtures of ethyl ammonium nitrate (EAN). In 2000, Summers and Flowers reported the effective role of EAN as a protein aggregation suppressor which resulted in enhanced recovery and refolding of denatured lysozyme (Summers & Flowers, 2000). Since then, ionic liquids have been used to stabilize...
protein activity, to inhibit or reduce aggregation, and to improve the ‘in vitro’ refolding of denatured proteins (Attri et al., 2012; Attri & Venkatesu, 2011; 2012b; 2012a; Attri, Venkatesu, & Kumar, 2011a; Attri, Venkatesu, Kumar, & Byrne, 2011b; Bae et al., 2012; Byrne & Angell, 2009; Byrne, Belieres, & Angell, 2009; Lange et al., 2005; Yamamoto et al., 2011). There are several associated advantages of using proteins specifically enzymes in ionic liquid media; enhanced operational and thermal stability, increased solubility of sparingly soluble substances, ease of immobilization of enzyme in highly viscous ionic liquids, alteration of substrate specificity, ease of enzyme recovery by filtration or centrifugation, ease of substrate and product recovery simply by evaporation and/or mixing with non-polar organic solvents and/or supercritical carbon-dioxide (Moniruzzaman et al., 2010). For example, many ILs (based on BF$_4^-$, PF$_6^-$ and Tf$_2$N$^-$) in comparison to organic solvents have exhibited relatively less denaturation, higher enantioselectivity and higher catalytic activity of several enzymes (Naushad, ALOthman, Khan, & Ali, 2012; van Rantwijk & Sheldon, 2007). Alterations in the anion dramatically affect the chemical behavior and stability of the ionic liquid while the changes in cation have a profound effect on the physical properties, such as melting point, viscosity, and density (Matthews, 2001; Schwierz et al., 2013).

2.8.1.1 Effect of anion and cation

In comparison to anions of same charge density, cations are usually observed to show a less dominant effect, because anions are more polarizable and hydrate more strongly (Schwierz et al., 2013). Additionally, cations seem to present their impact indirectly via interaction with anions, and the kosmotropic behavior of anions could be lessened in the presence of kosmotropic cations. It was hypothesized that kosmotropic cations, as opposed to the chaotropic ones, have a higher tendency of ion-pairing with the kosmotropic anions, thus reducing the abundance of the free anions in solution to play their kosmotropic role. This was supported by the “law of matching water affinity” which states in aqueous salt solutions, the interactions of ions in the order of decreasing strength are as follows: kosmotrope–kosmotrope > kosmotrope–water > water– water>chaotrope–water>chaotrope–chaotrope (Degreve et al., 2004; Hess & van der Vegt, 2009; Schwierz et al., 2013; Vlachy et al., 2009; Z. Yang, 2009; Zaccai, 2004).
In principle, the law of matching water affinity suggests that oppositely charged ions tend to form contact ion-pairs in solution if they have equal affinities for water but will separate if their water affinities are very different. Thus, in an aqueous solution, a kosmotropic anion and a kosmotropic cation will bind together due to the strong interactions between them. A chaotropic anion and a chaotropic cation also tend to form an ion-pair because the relatively stronger water–water interactions will keep the two chaotropes together. Therefore, an optimal stabilization of enzymes or other biological macromolecules is usually achieved through the use of salts containing kosmotropic anions and chaotropic cations.

In general, it’s been observed that ILs with highly-fluorinated and charge-delocalized anions are highly hydrophobic and immiscible with water, while those with halides, phosphate or carboxylate anion are more miscible with water (Kohno & Ohno, 2012). A kosmotropic anion strongly interacts with water to stabilize the protein while a chaotropic anion exhibits strong interaction with the protein’s chaotropic moieties and results in destabilization. (Bae et al., 2012) investigated the refolding of lysozyme with imidazolium based ionic liquids by varying anion and alkyl chain length of the cation. They report highest refolding yield in the presence of chaotropic cation [EMIM]$^+$ or [BMIM]$^+$, and kosmotropic anion [MS]. Lange examined the effect of N-‘alkyl-N-methylimidazolium chlorides on the denaturation of lysozyme induced by heat and guanidinium chloride (GuHCl). All the tested ILs were found to reduce the melting temperature of the enzyme, suppress the protein aggregation, and lower the midpoint concentration for GuHCl-induced protein unfolding, with a higher tendency corresponding to a longer alkyl chain length. While the anion, more often than not, follows the Hofmeister series, there are a few exceptions and usually occur in the presence of komstropic cation, such as [BMIM]$^+$. The initial reaction rate of lipase-catalyzed enantioselective hydrolysis of d,l-phenylglycine methyl ester (Moon et al., 2006) in the IL-containing aqueous buffer varied in the order of [BMIm][BF$_4$]$^-$ > [BMIm]Cl$^-$ > [BMIm]Br$^-$ > [BMIm]NO$_3$$^-$ >[BMIm]HSO$_4$$^-$. The lower enzyme activity in the presence of a more kosmotropic IL anion in this situation could possibly be explained by the higher tendency of the kosmotropic cation to ion-pair with its kosmotropic counter-anion, thus reducing its abundance in the aqueous bulk solution to play its stabilizing/activating role, as per the law of matching water affinities.
Ionic liquids having long alkyl chains in their cation behave like surfactant in aqueous solutions and have a strong impact on the stability and activity of the protein (B.-K. Liu et al., 2007; Xiao et al., 2009). (Kohno & Ohno, 2012) reviewed the properties of ionic liquids/water mixtures and assert long alkyl chain lengths of cation dramatically reduces its miscibility with water. (Yamamoto et al., 2011) observed that increase in alkyl chain length of N-alkylpyridinium chloride and N-alkyl-N-methylpyrrolidinium chlorides destabilizes’ lysozyme. These results were corroborated with results reported by Lange et al., whose study indicated imidazolium cation with a longer hydrophobic alkyl chain has a higher preference of destabilizing the enzyme due to (1) its strong interaction with the kosmotropic moieties, such as the carboxylic groups, on the enzyme surface, and (2) its hydrophobic interaction with the inner hydrophobic moieties of the enzyme molecule, leading to the disruption of the enzyme’s native conformation.

Investigation of the influence of a series of N’-alkyl, and N’-(ω-hydroxyalkyl)-N-methylimidazolium chlorides on the behavior of hen egg white lysozyme (HEWL) and an antibody fragment ScFvOx indicated that in comparison to hydrophobic imidazolium cation carrying long alkyl chain, terminal hydroxylation of alkyl chain, made the salt more compatible with protein stability (Lange et al., 2005). (Constantinescu, Weingärtner, & Herrmann, 2007) used differential scanning calorimetry (DSC) to characterize the thermal denaturation of RNase A with addition of ILs holding Br⁻ and Cl⁻ as the common anions. Almost all ILs tested rendered the enzyme to have its transition temperature lowered, in the order of K⁺ > Na⁺ > [C₁,₁,₁,₁ N]⁺ > Li⁺ > [C₂,₂,₂,₂ N]⁺ ≈ [EMIM]⁺ > [BMIM]⁺ > [C₃,₃,₃,₃ N]⁺ > [HMIM]⁺ > [C₄,₄,₄,₄ N]⁺, which is satisfactorily consistent with the decreasing kosmotropicity order of the cations.

In summary, although it is safe to say a kosmotropic anion and chaotropic cation are the most desirable characteristics in an ionic liquid for protein refolding studies, these are not the sole determining factors. Other parameters such as viscosity of the ionic liquid that can be modified by addition of water or modulation of the temperature, also plays an instrumental role in designing the ionic liquid for biochemical processes.

2.8.1.2 Effect of viscosity of ionic liquids on protein refolding
The knowledge of the viscosity of ILs is of prime importance from an engineering point of view as it plays a major role in stirring, mixing and pumping operations; in addition, it also affects other transport properties such as diffusion. ILs are generally viscous liquids with viscosities ranging typically from 10 to 500 mPa s at ambient temperature (Y. Zhang & Cremer, 2006). This is comparable with the values obtained for oils, that is to say two or three orders of magnitude higher than viscosities of traditional organic solvents. Viscosity decreases with increase in temperature making ILs easier to apply at super ambient conditions. Using a vibrating densimeter and rheometer, Jacquemin et al (2006), studied the density and viscosity of six different ionic liquids $[\text{BMIM}]\text{PF}_6^-$, $[\text{BMIM}]\text{BF}_4^-$, $[\text{BMIM}]\text{NTf}_2^-$, $[\text{EMIM}]\text{NTf}_2^-$, $[\text{EMIM}]\text{EtSO}_4^{2-}$ and butyltrimethylammonium bis(trifluoromethylsulfonyl)imide as a function of temperature. For the ILs studied they observed that the imidazolium-based ionic liquids density decreases with increasing alkyl chain length in cation or anion, while the viscosity remains constant with increasing shear rates (from 0 to 200 s$^{-1}$)(Jacquemin, Husson, Padua, & Majer, 2006). This linear relationship between the shear stress and the shear rate corresponds to a Newtonian behavior. It is also noteworthy that the alkylammonium-based IL exhibited a higher viscosity than the imidazolium-based ILs with the same anion; furthermore, the viscosities of the latter ILs increased with the length of the alkyl chain on the imidazolium ring. Amongst the anions studied, the $\text{NTf}_2^-$ anion lowered the viscosity while the $\text{PF}_6^{2-}$ anion significantly increased the viscosity.

Simulations have shown that imidazolium ILs with side chains above butyl (and up to C12) exist in the pure liquid phases as micro-structured fluids, in which nonpolar domains are formed by the alkyl chains and, at the same time, the charged parts form ionic domains which tend to be continuous (channels) (Xiao et al., 2009). This segregation into domains was not observed for Emim$^+$ based ILs, since the non-polar part is too small, and in structural terms they just showed charge ordering like “simple molten salts”. The formation of the microstructures is likely to be responsible for the increase in viscosity and for the decrease in ion mobility (meaning a decrease in conductivity and in diffusion). Therefore, it is not a strong interaction between the non-polar parts that is giving rise to a more viscous fluid. In ILs with longer side-chains, the non-polar parts are being driven into domains, excluded by the strong electrostatic attraction between the anions and charged parts of the cations.
The addition of high viscosity glycerol and polyethylene glycol to the refolding buffer enhances refolding yield by inhibiting aggregation process and reducing the diffusivity of proteins in solution. In a similar manner, van Rantwijk and Sheldon explain that the high viscosity of ionic liquids slows down the conformational changes of proteins, allowing enzymes to maintain their native structure and activity. (Zeuner et al., 2011) observed that higher viscosity of [BMIM]PF$_6^{-}$ compared to [OH-MMIM]PF$_6^{-}$ is likely to cause lower reaction rates through mass transfer limitations in furuloyl esterase from Aspergillus niger. Zhao’s study of the CALB (Candida antarctica Lipase B) catalyzed trans-esterification of ethyl butyrate and 1-butanol in more than 20 ILs suggests that IL viscosity might affect the reaction rates in some cases.

**2.9 Ionic liquids as protein refolding additives - Examples**

Summers and Flowers paved the way for protein refolding using ionic liquids when they reported the successful use of ethyl ammonium nitrate to suppress aggregation of denatured hen-egg white lysozyme. Ever since, several groups worldwide have ventured into this emerging paradigm. While some have invested their time and effort towards decoding the mystery towards the mechanism of action of ionic liquids as refolding additives (Attri & Venkatesu, 2012a; Byrne et al., 2009; Esquembre et al., 2013; Fujita, MacFarlane, & Forsyth, 2005; Greaves & Drummond, 2008; Mangialardo, Gontrani, Leonelli, Caminiti, & Postorino, 2012; Mann, McCluskey, & Atkin, 2009; Stock et al., 2004; Weibels, Syguda, Herrmann, & Weingärtner, 2012), many others are exploring the vast variety of ionic liquids to find the ideal ‘novel designer liquid’ that holds the potential to solve the bottleneck of the downstream process (Buchfink et al., 2010; Fujita et al., 2005; Hough & Rogers, 2007; Mester, Wagner, & Rossmanith, 2012; Ohno & Fukumoto, 2007; Pusey et al., 2007; RAkita, n.d.; Ranke et al., 2004; Turner, 2005). Few of the most recent and relevant, successful refolding studies have been summarized in table 2.5. As can be seen from the table, a common trend has been to study the effect of the ionic liquid as a refolding additive by using it *in lieu* of urea or guanidine, in the presence of redox-couple, such as cysteine/cystine or GSSG/GSH and at very low final protein concentration (less than 0.5mg ml$^{-1}$) primarily attained by dilution or dialysis several fold into the refolding buffer. What is interesting to note is that irrespective of the ionic liquid studied, kosmotropicity of the
anions, alkyl chain length of the cation and thereby its hydrophobicity and finally molarity of the ionic liquid in the final solution play a critical role in determining the refolding yield or efficiency of the system. Thus far, barring a few exceptions (e.g. [HMIM]⁺), the studies indicate increasing the concentration (molarity) of the ionic liquid beyond 1M results in a decrease in the refolding yield.

Table 2.5 Summary of recent research findings of ionic liquids as refolding additives

<table>
<thead>
<tr>
<th>Ionic Liquid</th>
<th>Refolding protocol</th>
<th>Noteworthy findings</th>
<th>Ref</th>
</tr>
</thead>
</table>

Ethylammonium nitrate (EAN)

- 25 mg/ml of denatured lysozyme diluted with 143 mM Tris sulfate buffer, pH 8.5 containing 4 mM GSH and 4 mM GSSG varying concentration of EAN. Final concentration of lysozyme: 1.6 mg/ml. Duration of incubation: 24 h

- Addition of 5% EAN to lysozyme solution in buffer appears to stabilize enzyme against irreversible thermal denaturation

  **Proposed mechanism:**

  Ethyl group of EAN interaction with hydrophobic parts of protein and protects it from intermolecular association.

  Desalting with centriprep centrifugal filters

N’-alkyl and N’-(ω-hydroxy-alkyl)-N-methylimidazolium chlorides

- 1:60 dilution with refolding buffer (3 mM GSH, 0.3 mM GSSG, 1 mM EDTA, 0.1 M Tris/HCl (pH 8.2) containing Ionic liquids.

- Final concentration of lysozyme: 0.28 mg/ml

- Incubation: 18 h – 24 h

- Refolding Yield increased with increasing concentrations up to 1 M {Exception: 0.5 M HMIM Cl} (Lange et al., 2005)

Table 2.5 cont’d

<table>
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<td>Refolding Yield increased with increasing concentrations up to 1 M {Exception: 0.5 M HMIM Cl}</td>
<td>(Lange et al., 2005)</td>
</tr>
<tr>
<td>N’-substituted-3-methylimidazolium tetrafluoroborate</td>
<td>1:100 dilution with refolding buffer containing 0.1M Tris/HCl pH 8.1, 3mM GSH, 0.3mM GSSG and varying concentrations of ionic liquid</td>
<td>Chaotropicity of imidazolium-based cations decreased with increase in alkyl chain length. Kosmotropicity of anions: methylsulfate ~ sulfate &gt; Acetate &gt; CF₃COO⁻ &gt; Chloride &gt; tetraborofluorurate</td>
<td>(Bae et al., 2012)</td>
</tr>
<tr>
<td>N-alkyl-N-methylimidazolium cation based ionic liquids</td>
<td>Lysozyme: 1:60 dilution with refolding 3 mM GSH, 0.3 mM GSSG, 1 mM EDTA, 0.1 M Tris/HCl, pH 8.2 containing varying concentration ionic liquid. Incubate: 18 -24h. Final concentration of protein : 0.28mg ml⁻¹. rPA: 1:20 dilution with refolding buffer 7 mM GSH, 0.7 mM GSSG, 1 mM EDTA, 0.1 M Tris/HCl, pH 8.5) containing varying concentration of ionic liquid. Incubation : 8h.</td>
<td>Variation of the anion has a profound effect on the renaturation of proteins, lysozyme and rPA. Strongly destabilizing anions with higher alkyl substitutions were unable to promote refolding. The influence of the anion as refolding enhancers for rPA represented by the following series: Cl⁻ &gt; MDEGSO₄⁻ &gt; EtSO₄⁻ &gt; acetate &gt; tosylate &gt; Et₂PO₄⁻ ≈ HexSO₄⁻. Most effective refolding additive amongst those tested was : [EMIM] Cl⁻</td>
<td>(Buchfink et al., 2010)</td>
</tr>
</tbody>
</table>

### 2.10 Purity and Toxicity concerns of Ionic liquids

Purity of a raw material is of prime importance in any process. Downstream processing of proteins involves removal of product- and process- related contaminants. It is therefore essential that raw materials, in this case, ionic liquids being used be of the highest purity. The commonly
found contaminants in ionic liquids are halides, water, organic compounds or inorganic salts. (Scammells, Scott, & Singer, 2005) provide a comprehensive review of these impurities and state that they not only affect the physicochemical properties of the ILs but also pose a threat to the success of a reaction. For example, trace quantities of halide anions could deactivate the catalyst in transition metal catalyzed reactions (i.e. hydrogenations). An awareness of this issue is growing steadfast and methods to measure and remove such impurities from ILs are being developed (Austen Angell, Ansari, & Zhao, 2011; Hough & Rogers, 2007; Huddleston et al., 2001).

Primarily due to their non-volatile nature and low impact on human health, ionic liquids are increasingly being referred to as ‘green alternatives to organic solvents’. Studies on effect of ILs on environment have shown that the toxicity profile of ILs varies across trophic levels and organisms (Jastorff et al., 2003; Pham, Cho, & Yun, 2010; Ranke et al., 2004; Swatloski et al., 2004; Zhu et al., 2009). Literature findings indicate toxicity of ILs with eight-carbon-alkyl chains attached to imidazolium and pyridinium rings is higher than most organic solvents (acetone, acetonitrile, methanol etc.) (Pham et al., 2010; Stepnowski, Skladanowski, Ludwiczak, & Laczynska, 2004; Wood & Stephens, 2010). In contrast, study published by (Pernak, Sobaszkiewicz, & Mirska, 2002) advocate ILs with ten, eleven, twelve and fourteen carbon atoms in an alkoxy group of imidazolium cation exhibits very high antimicrobial property, while the anion has very little influence. A plausible explanation for this deviation could be increased hydrophobicity of cations, which accentuates permeation into cell membrane & cell death. Enhanced hydrophobicity of ionic liquids by virtue of changes in cation is being exploited for the synthesis of novel-target-specific third generation of pharmaceutically active ILs.

2.11 Economic feasibility

Meyer and Werbitzky state the global chemistry market is estimated at $2.92 billion and expected to grow to $3.24 billion by 2015 and to $4.012 billion by 2020, with industrial biotechnology and pharmaceutical biotechnology representing more than $50 billion and $70 billion, respectively (Meyer & Werbitzky, 2011). Approximately 6000 companies, globally, are active in pharmaceutical sector with an estimated compounded annual growth rate (CAGR) is
more than 20%. About 25% of the industrial biotechnology is fine chemicals, with the need

to develop ‘green chemicals’ or ‘green process’ being the prime focus. Meyer and Werbitzky
(2011) rightfully define ‘green chemistry’ as the guiding principle to encourage the development
of manufacturing processes and products with the lowest possible environmental impact or

footprint. As discussed earlier, research findings have demonstrated that ionic liquids are
effective substitutes for common organic solvents where control of volatile organic compound
emissions is critical (Hough & Rogers, 2007; Meyer & Werbitzky, 2011; RAkita, n.d.; Rogers,
Seddon, & American Chemical Society Meeting 224th : 2002 : Boston, 2003). It is this

negligible vapor pressure that permits the removal of products by distillation without further
contamination by the solvent, thereby facilitating recycling of ionic liquids and reduction in
operation costs of the process (Tavares, Rodríguez, & Macedo, 2013).

Due to their specific handling and poor handling production and application of first generation
ionic liquids has been expensive. In comparison, although second generation ionic liquids are
more stable, Gorke et al (2010) attribute their high cost to individual building blocks and
subsequent downstream processing (Gorke, Srieen, & Kazlauskas, 2010). Over the years,
changes in production strategy by means of ecological and economic optimization (ECO), has
resulted in the evolution of task-specific ionic liquids. The third-generation of ionic liquids, are
similar in cost to organic solvents, simple to prepare, biodegradable, and the purity of the starting
materials determines the purity of the final product while the choice of anion and cation
determines its toxicity profile (Gorke et al., 2010; Tavares et al., 2013). Reinhardt et al., (2008)
evaluated the greenness of [C₆MIM][BF₄], citric acid/N,N’-dimethyl urea, methanol,
cyclohexane, acetone and methanol/water for Diels-Adler reaction of cyclopentadiene and
methyl acrylate by means of ECO. In general, conventional solvents are easily available and
relatively cheap, and in order to be in the same range, Reinhardt estimate the price of ionic
liquids to be less than or equal to USD 30 kg⁻¹ (Reinhardt, Ilgen, Kralisch, König, & Kreisel,
2008).

At present, only a limited type and number of ionic liquids are produced in bulk quantities, most
of which are used in chemical synthesis. Table 5, lists comparative prices for ionic liquids in use
today. With increasing demand, and potential for application in biopharmaceutical industry,
BASF estimates the expects the prices to be below USD 40 kg⁻¹, for quantities ranging in metric
tons (BASF, 2013). Deep-eutectic solvents, like those manufactured by Scionix, are cheaper than imidazolium based ionic-liquids owing to the cost price of its principal component-choline chloride, which is manufactured in metric tonnes on a regular basis.
Table 2.6 Current market price of ionic liquids and other commonly used protein-refolding additives

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Ionic liquid</th>
<th>Price (USD per kg)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-ethyl-3-methylimidazolium chloride</td>
<td>110&lt;sup&gt;a&lt;/sup&gt;</td>
<td>BASF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>907&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Io-li-tec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>369&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>2</td>
<td>1-ethyl-3-methylimidazolium acetate</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>BASF</td>
</tr>
<tr>
<td></td>
<td></td>
<td>459&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Io-li-tec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1060&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>3</td>
<td>1-butyl-3-methylimidazolium chloride</td>
<td>294&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Io-li-tec</td>
</tr>
<tr>
<td></td>
<td></td>
<td>297&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>4</td>
<td>1-butyl-3-methylimidazolium acetate</td>
<td>964&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>5</td>
<td>1-hexyl-3-methylimidazolium tetrafluoroborate</td>
<td>1972&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Io-li-tec</td>
</tr>
<tr>
<td>6</td>
<td>1-methyl-3-propylimidazolium iodide</td>
<td>887&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Io-li-tec</td>
</tr>
<tr>
<td>7</td>
<td>Ethylammonium nitrate</td>
<td>899&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Io-li-tec</td>
</tr>
<tr>
<td>8</td>
<td>1-butyl-1-methylpyrrolidinium trifluoromethane sulfate</td>
<td>2320&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Io-li-tec</td>
</tr>
<tr>
<td>9</td>
<td>1-ethyl-3methylimidazolium thiocyanate</td>
<td>870&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Io-li-tec</td>
</tr>
<tr>
<td>10</td>
<td>1-ethyl-3-methylimidazolium tetrafluorosulfate</td>
<td>1435&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Io-li-tec</td>
</tr>
<tr>
<td>11</td>
<td>Glyceline (choline chloride: glycerol)</td>
<td>57&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Scionix, UK</td>
</tr>
<tr>
<td>12</td>
<td>Oxaline (oxalic acid: choline chloride)</td>
<td>57&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Scionix, UK</td>
</tr>
<tr>
<td>13</td>
<td>Ethaline (ethylene glycol : choline chloride)</td>
<td>57&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Scionix, UK</td>
</tr>
<tr>
<td>14</td>
<td>L-cystine</td>
<td>585&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>15</td>
<td>Cysteine</td>
<td>543&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>16</td>
<td>L-arginine</td>
<td>340&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>
Footnote Table 2.6.: Price: USD per kilogram. a: Calculated based on quotation received for 1000kg, as of September 2013. b: Calculated based on standardized price listed for 5kg in their in-house newsletter, Ionic liquids Today, dated December 2012. c: As per price listed on the Sigma-Aldrich website for 1kg of ionic liquid, as of September 2013. d: Standardized price listed on their website, based on order quantities greater than 50kg but less than 1 metric ton. Greater Britain Pound converted to USD as per foreign exchange on 20th September 2013.

When evaluating the economic and process feasibility of a chemical, it is important to note that there are lots of ways to look at cost-analysis and purchase price is only one part of the equation. Cost related to personal-protective equipment, emission control hardware and monitoring equipment, waste disposal and treatment, and the potential to recycle the chemical have a cumulative effect on the process feasibility and must not be ignored.

2.12 Conclusion

Production and purification of a therapeutic protein is a task that faces many challenges; upstream titre, aggregation of protein, membrane fouling, buffer contamination, efficient recovery of protein, purification of protein, and protein stability etc. However, the biggest challenge and bottleneck today is the cost-effective and process-efficient recovery of protein from inclusion body proteins. One or combination of several chromatographic or non-chromatographic methods can help achieve this. Irrespective of the methodology employed, protein refolding is complicated and dependent on several process- and product-related parameters. Hence, the use of a refolding additive is not only imperative but also critical to the success of the reaction. In theory, since protein refolding is a kinetically competitive process, it may be promoted by stabilizing the native state or suppressing the unspecific aggregation of unfolded peptide and/or intermediate-native structure formed during the process.

Since their inception into the scientific community in 1886 ionic liquids have carved a niche for themselves in the controversial field of ‘green chemistry’ (Byrne, Wang, Belieres, & Angell, 2007). While the characteristics that these ‘designer liquids’ are non-volatile and that they have low impact on human health classifies them as ‘green alternatives to organic solvent’, the fact that most of them are not completely biodegradable and highly toxic across several trophic levels tells a cautionary tale. The more recent third generation of ionic liquids developed are ‘target-specific’ chemical
agents that address the issues of biodegradability and toxicity, and some even serve as active pharmaceutical ingredients (Hough et al., 2007; Hough & Rogers, 2007).

The three dimensional structure of a protein is quintessential for its activity, which is maintained by disulfide bonds, hydrogen bonding and hydrophobic (Van der Waals contribution) interactions. In general, protein refolding additives function by either penetrating the micro-aqueous environment of the protein and stabilizing the protein (chaotropic cation) or by competing with the water associated the protein thereby limiting its exposure to the solvent and consequently driving the protein to its native state (kosmotropic anion). This dichotomy of preferential binding/exclusion originates from the Hofmeister series that is explicitly used to justify the effect of ionic liquids and other solute molecules on proteins. It is well documented that dependent on their physicochemical characteristics such as nucleophilicity, hydrophobicity and viscosity ionic liquids affect protein dynamics by their H-bonding and electrostatic and hydrophobic interactions with the protein.

The experimental data obtained thus far on the potential of ionic liquids as refolding additives is limited to very low protein concentrations. Of course, with the exception of the study by Byrne et al., who report long term stabilization against aggregation and hydrolysis of >200 mg ml\(^{-1}\) of lysozyme in trimethylammonium methane sulfonate (Byrne et al., 2007). As can be seen in Table 3, almost all studies have been based on the concept of ‘dilution refolding’, which albeit easy to adapt, is tedious, inefficient and difficult to scale up to industrial levels. Lange et al., by reporting the improved refolding yield by ionic liquids over traditional refolding additives such as L-arginine has demonstrated the potential of ionic liquids for industrial application (Lange et al., 2005). However, employing dilution refolding would be counter-productive at that scale. Hence a thorough investigation on the applicability of ionic liquids in chromatographic refolding strategies such as ion exchange, size exclusion, simulated moving bed and expanded bed chromatography is the need of the hour. In addition, the recovery efficiency and reusability of the ionic liquid (as a refolding additive) in the process, the bearing of ionic liquids on ‘clean-in-place’ protocols for chromatographic downstream processes, adaptability of the ionic liquid-assisted protein refolding process to in-house equipment
are all parameters that need to be examined and studied exhaustively to facilitate the smooth transition of ionic liquids from academic research to industrial application.

2.13 Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full form</th>
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<tbody>
<tr>
<td>IBPs</td>
<td>Inclusion body proteins</td>
</tr>
<tr>
<td>QSAR</td>
<td>Quantitative structure activity relationship</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>SLS</td>
<td>Sodium-N-lauroyl sarcosine</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>ATR-FTIR</td>
<td>Attenuated Total Reflectance- Fourier Transform InfraRed</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>4-MBPy][BF_4]</td>
<td>4-methyl-N-butylpyridinium tetrafluoroborate</td>
</tr>
<tr>
<td>EAN</td>
<td>Ethylammonium nitrate</td>
</tr>
<tr>
<td>Ils</td>
<td>ionic liquids</td>
</tr>
<tr>
<td>GuHCl</td>
<td>guanidinium chloride</td>
</tr>
<tr>
<td>ScFvOx</td>
<td>Small chain antibody fragment</td>
</tr>
<tr>
<td>HEWL</td>
<td>Hen egg white lysozyme</td>
</tr>
</tbody>
</table>
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biocatalysis. Journal of biotechnology, 144(1), 12–22.


Chapter 3

3 Materials and Methods

This chapter describes all the common materials and methodology applied in this thesis. Commonly used analytical techniques are also presented. Subsequent chapters hereafter describe relevant materials and methods pertinent for that chapter only.

3.1 Materials

Lyophilized lysozyme from chicken-egg-white powder containing more than 90% protein (greater than or equal to 40,000 U/mg solid) was purchased from Sigma-Aldrich, (Oakville, Canada). *Micrococcus lysodeikiticus* (ATCC No. 4698), L-cysteine, cystine, ethylenediaminetetraacetic acid (EDTA), urea and sodium chloride were purchased from Sigma Aldrich (Oakville, Canada). Solutions of N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES) in distilled water (1M) and DL-dithiothrietol (DTT) (1M) solution (DTT) (1M) were also purchased from Sigma-Aldrich (Oakville, Canada).

3.2 Equipment

Grenier Bio-One 96 well, clear, flat-bottom, UV-Star® microplate was purchased from VWR International (Mississauga, Canada.) The detailed specification of which is given in Appendix A.

All packed-bed protein adsorption studies were performed on pre-packed HiTrap cation exchange columns connected to an ÄKTA FPLC equipped with the UNICORN software version 5.01 from GE Healthcare (Uppsala, Sweden). Specifications of the HiTrap column are provided in Appendix B.

Pre-packed HiTrap cation exchange columns (1 ml and 5 ml) were purchased from GE Life Sciences (Quebec, Canada). The HiTrap columns were pre-packed with Sepharose Fast Flow (FF) cation exchange resin media. The Sepharose FF media is made of particles with a macroporous gel structure and has neutral hydrophilicity based on chains of agarose that are arranged in bundles. Sepharose fast flow (FF) resin particles, with an
average particle diameter 90 μm, were used for all fluidized-bed ion-exchange lysozyme refolding studies.

The fluidized bed system used for adsorptive protein refolding was constructed in-house from Borosil® glass. A schematic diagram of the fluidized bed system is provided in Figure 3.1. The system consisted of a jacketed conical column connected to an air inlet. As illustrated in Figure 3.1, air was passed through the column (1); its flow was adjusted with a valve (2) and measured by an air flow meter (3). A sintered glass filter of pore size 40 to 60 μm (4) was at the bottom of the column, and placed 0.5 mm above the air-inlet pipe, ensured the resin remains fluidized throughout the duration of the operation. Sepharose FF resin beads were placed in the column and fluidized using a gentle airflow rate. Water was circulated in the fluidized bed by means of a peristaltic pump (10) for temperature control. Ports 6 and 7 served as water in-let and out-let ports for the water jacket surrounding the column. Port 5 was the discharge port for clean-in-place (CIP) protocols.
Figure 3.1 Schematic: Fluidized bed ion-exchanger for the refolding studies of denatured lysozyme.

Legend:
1. Air inlet
2. Ball valve with pressure guage
3. Air flowmeter
4. Sintered glass coarse filter (30-40 micron)
5. Discharge port for C-I-P
6. Water bath inlet
7. Water bath outlet
8. Peristatic pump inlet
9. Peristaltic pump outlet
10. Peristaltic pump
3.3 Analytical Methods

3.3.1 Protein quantification

The concentration of both native and denatured lysozyme were monitored in 96 well micro-plates using UV absorption spectrophotometer (Tecan 200pro, Männedorf Switzerland) at $A_{280nm}$. The protein concentration was determined using the Beer-Lambert law with molar extinction coefficients of 2.63 and 2.37 ml mg$^{-1}$ cm$^{-1}$ for native and denatured lysozyme, respectively.

The path-length (cm) of a well in a micro plate was experimentally calculated by the method suggested by Greiner Bio-one application note (GmbH, Germany) (Application Note, 2008). The sample path length was defined by Equation 3.1 below, and is the ratio of the difference in absorption of aqueous sample (path length unknown) measured at 977 nm and 900 nm to that of water (or reference buffer) with 1cm path-length measured at the same wavelengths.

$$ \text{Sample path length (cm)} = \frac{A_{977\text{(sample)}} - A_{900\text{(sample)}}}{A_{977\text{(reference)}} - A_{900\text{(reference)}}} \quad \text{Equation 3.1} $$

3.3.2 Lysozyme activity assay

Lysozyme enzymatic activity was measured using *Microcococcus lysodeiktius* (ATCC No. 4698) in a microwell plate using Tecan 200pro multimode spectrophotometer (Männedorf, Switzerland). A cell suspension of the bacteria was prepared by dispersing 9 mg of the bacteria in 30 ml of potassium phosphate buffer (0.06M at pH 6.2 units). The initial absorbance at 450nm was adjusted between 0.5 – 0.7 unit (Lee & Yang, 2002). An aliquot of cell suspension (190 μl) was accurately dispensed into each well of the microplate. To this, sample solution (10 μl) containing lysozyme (native, denatured or refolded) was added. Using Tecan microplate reader, with intermittent shaking, the initial rate of decrease in absorbance at 450 nm was measured every 5 seconds over a period of 2 min. The activity of lysozyme was calculated from the initial slope of the time course.
by linear regression of data points. The specific activity of the sample (units min\(^{-1}\) ml\(^{-1}\)) was computed by dividing the initial slope of the lysozyme activity by the concentration of lysozyme in reaction mixture. Equation 3.2 represents the specific activity of the solution.

\[
\text{Specific activity } (\text{units min}^{-1} \text{ ml}^{-1}) = \frac{A_{450\text{nm}}/\text{min}}{\text{concentration of lysozyme (mg)}} \quad \ldots \ldots \text{Equation 3.2}
\]

3.4 Performance indicators:

Definition of parameters used to determine refolding efficiency and process efficiency are given below as follows.

3.4.1.1 Refolding Yield

Refolding yield was defined as the percentage of specific activity (units min\(^{-1}\) ml\(^{-1}\)) of sample (or refolded protein) over the specific activity (units min\(^{-1}\) ml\(^{-1}\)) of the native lysozyme. It is computed using Equation 3.3

\[
\text{Refolding Yield(\%)} = \frac{\text{Specific activity}_{\text{sample}}}{\text{Specific Activity}_{\text{native}}} \times 100 \quad \ldots \ldots \text{Equation 3.3}
\]

3.4.1.2 Fractional mass recovery:

The ratio of total mass of protein (mg) recovered in elution to the total mass of protein (mg) loaded on to the ion-exchanger was defined as fractional mass recovery. It was calculated as per Equation 3.4 below.

\[
\text{Fractional mass recovery} = \frac{V_{\text{elute}}C_{\text{elute}}}{V_{\text{injected}}C_{\text{feed}}} \quad \ldots \ldots \text{Equation 3.4}
\]

where, \(V_{\text{elute}}\) and \(V_{\text{injected}}\) represent volume of protein (ml) eluted and injected, respectively. \(C_{\text{elute}}\) and \(C_{\text{feed}}\) indicate the concentration of protein (mg/ml) in the elute and feed, respectively.
3.5 Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full form</th>
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<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraaceteic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>SP FF</td>
<td>Sepharose Fast Flow</td>
</tr>
<tr>
<td>CIP</td>
<td>Clean-in-place</td>
</tr>
<tr>
<td>$V_{\text{elute}}$</td>
<td>Volume of elute (ml)</td>
</tr>
<tr>
<td>$V_{\text{injected}}$</td>
<td>Volume injected (ml)</td>
</tr>
<tr>
<td>$C_{\text{elute}}$</td>
<td>Concentration of protein in elute (mg/ml)</td>
</tr>
<tr>
<td>$C_{\text{feed}}$</td>
<td>Concentration of protein in feed (mg/ml)</td>
</tr>
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</table>

3.6 References


Chapter 4

A comparative study of refolding lysozyme using conventional strategies.

This chapter begins by validating the requirement of refolding additives and low concentration of denatured protein for dilution refolding of denatured lysozyme. Next, it compares the refolding yield and fractional mass recovery, as a function of increase in protein concentration, for two different on-column modes of operation – packed bed and fluidized bed.

4.1 Introduction:

Production of correctly folded and biologically active proteins in *Escherichia coli* can be a challenging process (Fang & Huang, 2001; Su, 2011). Often, proteins are recovered as insoluble inclusion bodies and need to be denatured and refolded into the correct structure. To address this, various process operations have been developed. Dilution refolding is the simplest and most-commonly used method, and involves refolding initiation by reducing the denaturant concentration. By carefully controlling the protein concentration at a low level, the formation of protein aggregates can be minimized. In large-scale protein refolding this leads to a high cost for reagents and buffers and an additional concentration step is often required for further processing after dilution (Gu et al., 2002; Leong & Middelberg, 2005.; Oganesyan, Kim, & Kim, 2004). Developing high efficiency and low cost protein refolding methods is a highlighted research focus in biotechnology. With speed being a driver for both structural biology and biopharmaceutical development, only a limited set of refolding strategies can be tested for any given protein. Technology for protein refolding should therefore be:

- Scale invariant – to ensure that results from screening can be translated to preparative systems and manufacture without significant changes in the technology used;
- Easily automated – to address the issue of speed and to enable high-throughput processing of samples;
• Generic for a broad range of similar proteins – so that the technology does not have to be reinvented for each new gene sequence;
• Economical – to ensure that resources are not being wasted if a large number of gene sequences or protein variants is being examined, and possibly to ensure streamlined transfer to manufacture.

These parameters are easily addressed with the use of on-column protein refolding processes and thus form the crux of research being conducted in the field of protein refolding and purification worldwide. On-column adsorptive refolding of denatured proteins is mainly achieved by size exclusion, ion exchange, or affinity processes. Ion exchange refolding of proteins permits the selective and controlled adsorption and elution of target protein and alleviates the aggregates formed in size-exclusion chromatography due to sharp decrease in denaturant concentration.

Considering the importance and need for the gradual removal of denaturants and gradual change in pH, an ion-exchange protein-refolding system was developed to improve the refolding recovery at high-protein concentration. This study aims to investigate the effectiveness of column refolding of denatured lysozyme under two different modes of operation – packed and fluidized bed.

4.2 Materials and methods:

All materials and analytical methods, and also equipment design and operation, pertinent to the study presented in this chapter, have been discussed and described in Chapter 3. The following section describes in detail the experimental methodology utilized.

4.2.1 Experimental methodology

4.2.1.1 Preparation of denatured lysozyme

Native lysozyme was dissolved in 50 mM HEPES, pH 7.0 to prepare a stock solution. Denaturation buffer contained 6M urea, 32 mM DTT, 1 mM EDTA in 50 mM HEPES buffer pH 8.1. Denatured lysozyme was prepared by diluting an appropriate volume of
native lysozyme in denaturation buffer and incubating in a water bath at 37° C for a period of 2 hours. The activity of the lysozyme was tested to ensure it was completely denatured.

4.2.1.2 Dilution refolding of denatured lysozyme

Three different buffers were investigated. Buffer A 50 mM HEPES, pH 8.1, Buffer B 2 M urea in 50 mM HEPES, pH 8.1 and Buffer C 2 M Urea, 0.03 mM cysteine, 0.3 mM cystine in 50 mM HEPES, pH 8.1 were prepared fresh on the day of the study. Denatured lysozyme stock solution was diluted with each of the refolding buffers to give a final enzyme concentration between 0.1 mg/ml and 2.0 mg/ml. The reaction mixtures were set aside, on bench-top shakers at 120 rpm, for a period of 3 hours. At time points, half hour, 1 hour and 2 hours appropriate volume of sample was aliquoted and analyzed for protein concentration and enzyme activity. The experimental results obtained are presented and discussed later.

4.2.1.3 Adsorptive refolding of denatured lysozyme in a packed bed ion exchange system

Figure 4.1 demonstrates the steps discussed below that were followed for the adsorptive refolding of denatured lysozyme in an ion-exchange system.

![Schematic representation of adsorptive on-column refolding of denatured lysozyme](image-url)
The pre-packed HiTrap SP FF column (5ml), connected to AKTA FPLC, was equilibrated with ten column volumes of equilibration buffer (50 mM HEPES, pH 8.7, 2M urea). The flow rate throughout the experiment was set at 0.4 ml/min. Denatured lysozyme solutions, in the range of 1 mg/ml to 10 mg/ml, were prepared. A protein loading of 2.5 ml of denatured lysozyme (at varying concentrations) was applied on to the column. After rinsing with five column volumes of equilibration buffer, the column was eluted by gradient elution. Elution was started by gradually increasing ratio of elution buffer/equilibration buffer from 0:100 to 100:0 at 0.4 ml/min within 12 column volumes. The composition of elution buffer was 50 mM HEPES, pH 8.1 containing 2 M urea, 0.03 mM cysteine, 0.3 mM cystine and 1M sodium chloride. Fractions were collected at every step and analyzed for protein concentration and lysozyme activity. The column was washed, cleaned-in-place (CIP) and regenerated for the next cycle.

4.2.1.4 Adsorptive refolding of denatured lysozyme in fluidized bed mode

A volume of exactly 5 ml of the Sepharose Fast Flow resin slurry, suspended in 50 mM HEPES (pH 7.0), was accurately aliquoted and dispensed in the fluidized bed column. The resin particles were equilibrated with 50 ml of equilibration buffer (2 M urea in 50 mM HEPES, pH 8.7) for a period of 30 minutes, by a steady-flow of air at 0.01 LPM flow rate. At the end of equilibration, fluidization was halted and the buffer was dispensed out of the column with the aid of peristaltic pump. Next, 10 ml of denatured lysozyme solution, at varying concentrations 1 mg/ml to 10 mg/ml, was loaded onto the re-fluidized resin particles. After 15 minutes of adsorption, the resin was allowed to settle and the buffer was dispensed out of the column with the aid of the peristaltic pump. Following this, the re-fluidized resin particles were washed with 25 ml of equilibration buffer for period of 10 minutes. After separating the washed-resin particles from the buffer, the resin particles were re-fluidized. Elution of bound lysozyme was initiated when the resin particles were allowed to interact with 100 ml of elution buffer (50 mM HEPES, pH 8.1 containing 2 M urea, 0.03 mM cysteine, 0.3 mM cystine and 1 M sodium chloride) for duration of 30 minutes. The elution pool was collected and analyzed for protein concentration and lysozyme activity. The resins were cleaned-in-place and
regenerated before use in the next cycle. Performance indicators, fractional mass recovery and refolding yield, were used to assess the efficiency of the process.

4.3 Results and Discussion

4.3.1 Refolding of denatured lysozyme by batch-dilution refolding

In principle, protein refolding was initiated by the gradual removal of denaturant, which initiated the collapse of the unfolded molecule and the formation of secondary or tertiary structure. This collapse exposed the hydrophobic core of the protein and therefore the molecule was susceptible to aggregation. Protein aggregation, often the rate-limiting step, is a second or higher order reaction and primarily dependent on the concentration of protein and the composition of redox environment. Chaotropic agents such as urea and guanidium chloride at non-denaturing concentrations solubilize protein aggregates and in effect enhance protein refolding. Addition of redox agents such as cysteine/cystine or GSH/GSSG, increase the rate of oxidation and break the non-native disulfide bonds. Thus refolding of a denatured protein to its native state is primarily dependent on, the type and concentration of refolding additives, the concentration of the protein and the duration of incubation. Figure 4.2 shows the experimental results obtained by variation of refolding buffer over a period of 2 hours at different concentrations of lysozyme.

Three different refolding buffers were investigated. Buffer A contained no refolding additive, Buffer B contained the chaotropic agent urea, and Buffer C contained both the chaotropic agent (urea) and redox couple (cysteine/cystine). Thus, a range of redox environments was investigated. The volume of the reaction mixture kept constant (10 ml) and the volume of denatured lysozyme was adjusted so appropriately to have varying concentrations of lysozyme, from 0.1 mg/ml to 2 mg/ml, in the final reaction mixture.

Refolding by dilution lowers refolding protein concentration to minimize the propensity of inter-molecular interactions of protein refolding intermediates (Basu, Li, & Leong, 2011). Figure 4.3, shows that increase in concentration of the lysozyme adversely affected the refolding yield, irrespective of the refolding buffer used. Also, despite the low protein concentration (0.1mg/ml and 0.2mg/ml), refolding buffer composition still
plays an important role in influencing protein-refolding kinetics (Figure 4.2). Highest refolding yield was obtained at the lowest concentration of lysozyme (0.1 mg/ml) in the presence of both a redox couple and chaotropic agent (Buffer C). This is substantially higher refolding yield, than that by Raman et al. (1996), who report ca. 75% renaturation yield in 5 h, upon refolding lysozyme at 0.05 mg/ml (Raman, Ramakrishna, & Rao, 1996).

Figure 4.2 Effect of refolding buffer composition, time (h) and concentration of protein (mg/ml) on protein refolding yield (%)

Three different buffer systems were investigated. Buffer A: 50 mM HEPES, pH 8.1; Buffer B: 2 M urea in 50 mM HEPES, pH 8.1; Buffer C: 2 M urea, 0.03 mM Cysteine, 0.3 mM Cystine in 50 mM HEPES, pH 8.1. Refolding yield obtained at different time points (0.5 h, 1 h and 2 h) was presented as a function of change in composition of buffer and change in concentration of lysozyme (0.1 mg/ml to 2 mg/ml). The error bars denote the standard error of mean between triplicates.

Diluting a denatured lysozyme by a solution containing 6 M urea and 32 mM DTT into refolding buffer containing 2 M urea has an effect on the residual concentration of the urea in the final reaction mixture. The concentration of residual urea determines which
protein-refolding intermediates proliferate. For example a 100-fold dilution of denatured lysozyme solution in refolding buffer A (no urea) would result in 0.01 M urea, while in buffer C (containing 2 M urea) would produce 2.04 M of urea in the final reaction mixture. Figure 4.3 shows the effect of residual urea (M) on the refolding yield of denatured lysozyme (0.2 mg/ml). Figure 4.3 shows that refolding yield of lysozyme is sensitive to the residual amount of urea.

At concentration above 2.25 M urea, irrespective of the duration of incubation, very little activity is regained. This can be explained by the increase in the chaotropic effect of urea. A chaotropic solute is one that disrupts the structure of macromolecules by interfering with the intramolecular interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces, and hydrophobic effects. Refolding additives such as urea, usually at concentrations greater than 2 M, disrupt the micro aqueous environment of the protein resulting in its denaturation, as has been demonstrated by previous research findings (Bian, Liang, Yang, & Liu, 2007; Duan et al., 2009; Hou, Hansen, Staby, & Cramer, 2010).

Figure 4.3 Changes in refolding yield due to urea (M) in the reaction mixture at two different time points, 0.5 h (△) and 2 h (□).
4.3.2 Adsorptive refolding of lysozyme in a packed bed ion exchange system

Figure 4.4 is the chromatograph generated by UNICORN when 16 mg of denatured lysozyme was passed through the HiTrap SP FF column. Upon injection of the load, as it passed through the column changes in UV absorption were recorded. The UV peak obtained between 0 and 20 on the x-axis in Figure 4.4 indicates some amount of the protein did not bind but instead flowed through. The second UV peak obtained between 40 and 80 on the x-axis in Figure 4.4 is the elution peak.

![Chromatogram generated by UNICORN upon loading 15 mg of denatured lysozyme on a pre packed HiTrap SP FF column (5ml).](image)

The blue line represents the change in UV absorption (mAU) at 280nm and the green line represents the change in concentration of elution buffer from 0:100 to 100:0.
Elution fractions C8 to C12 were pooled and analyzed for protein content and refolding yield. Analysis revealed 90% of the bound protein eluted and the refolding yield of the eluted peak was 69.34% with a fractional mass recovery of 0.53 units. Similar chromatograms were generated, upon varying the amount of load (mg) and the experimental results obtained are presented in the Table 4.1. It shows the experimental results obtained by loading varying concentrations of denatured lysozyme on 5 ml HiTrap Sepharose Fast Flow column and its corresponding effect on the refolding yield and fractional mass recovery of the protein. Table 4.1 also shows that increased in protein load resulted in decrease in refolding yield and fractional mass recovery. When the protein load (mg) onto the column was kept low (2.5 mg), there were enough adsorption sites for the denatured protein to bind and refold. At the same time, the denatured protein molecules because of their immobilization at different sites did not interact with each other. However, as the protein load increased (15 mg), the number of protein molecules exceeded the number of binding sites on the solid support, especially at the top of the column. Interaction between the denatured molecules, thus, was inevitable. This influenced the correct refolding and thereby decreased the fractional mass recovery and refolding yield of lysozyme.

Table 4.1 Effect of lysozyme load (mg) on the fractional mass recovery and refolding yield of lysozyme in a packed bed ion exchanger

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Load : Denatured lysozyme (mg)</th>
<th>Refolding Yield (%)</th>
<th>Fractional mass recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiTrap SP FF (5ml)</td>
<td>2.5</td>
<td>80.39</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>74.23</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>69.34</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Fractional mass recovery of protein is the ratio of concentration of eluted protein (mg) to the amount of the protein load (mg). In comparison to dilution refolding, adsorptive refolding of denatured lysozyme was successful at significantly higher protein load (0.2
mg in dilution vs 15 mg in packed bed system). This can be explained as follows. During an on-column ion-exchange refolding process, protein adsorb to the matrix through the interaction of its amino acid residues with the ion-exchange group on the solid support. This adsorption minimizes the intermolecular reaction between proteins, thereby decreasing the possibility of aggregation at higher concentrations of the protein. During gradient elution, as the concentration of the elution buffer increases (from 0:100 to 100:0), the concentration of the denaturants in the column reduces. This forces the partial-folded intermediate to move away from its original binding site and rearrange its structure to its correct native conformation.

In order to meet the economies of scale, and proactively debottleneck the downstream process, there is a strong demand in the industry for systems that can support high concentration of protein solutions. Packed bed refolding of lysozyme has already presented its limitation with regards to protein load (mg); whether this changes favorably or not, in a fluidized bed system was investigated in the following section.

4.3.3 Refolding of denatured lysozyme in a fluidized bed ion exchanger

In the fluidized mode of operation, resin particles are continuously “flowing” inside the column by an upward stream generated by gas, buffers or sample solutions. As result, the inter-particle porosity increases and mass-transfer between resin media and biological component is enhanced. This was supported by the experimental results of Trivedi (2006) who demonstrated the successful adaptation of a liquid-solid circulating fluidized bed for refolding denatured lysozyme (Trivedi, 2006). Although, 72% refolding yield was attained the mass recovery was less than 50%, therefore suggesting an improvement in the design of the fluidized bed column. The Glatt-Powder-Coater-Granulator (GPCG) is commonly used in the pharmaceutical industry for fluidized bed drying, particle coating, pelletizing and wet granulation. The fluidized bed system developed in-house for the purpose of this thesis, adopted the underlying principles of the GPCG while tackling the design issues raised by Trivedi (2006). Thus, a conical shaped fluidized bed was
designed, the construction and operation of which has already been described in Chapter 3.

In order to compare the efficiency of the fluidized bed ion-exchange refolding system to that of the packed bed, series of experiments were conducted (see Section 4.1.1.4). Samples from each step of the ion exchange process (adsorption, wash, elution) was collected and analyzed for both protein concentration and enzyme activity. The process performance indicators, discussed in analytical methods, were used to assess the efficiency of the system in comparison to the packed bed refolding system. Table 4.2 shows the experimental results obtained. Experimental results obtained by varying the amount of denatured lysozyme loaded onto the ion-exchange resin and its’ effect on the refolding yield and fractional mass recovery of the process is presented in Table 4.2. In comparison to results obtained in Table 4.1, the fractional mass recovery of the eluted protein and refolding yield was substantially higher, thereby indicating the superior efficacy of the fluidized bed system. Increasing the protein load to 15 mg of denatured lysozyme in the packed bed system reduced the fractional mass recovery to almost 50%. Whereas in the fluidized bed system with the same protein load (mg) the fractional mass recovery was almost 30% higher (Ferré et al., 2005; Hjorth, 1999).

Table 4.2 Effect of lysozyme load (mg) on the refolding yield of lysozyme in a fluidized bed ion-exchanger

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>Load : Denatured lysozyme (mg)</th>
<th>Fractional Mass Recovery</th>
<th>Refolding Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5</td>
<td>0.85</td>
<td>86.48</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0.81</td>
<td>79.98</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>0.80</td>
<td>77.21</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>0.78</td>
<td>75.43</td>
</tr>
</tbody>
</table>

In the more recent study by, Mazumder et al (2009), with the aid of mathematical modeling and simulation supported by experimental results, they showed that rate of
protein production and overall recovery increased with the increase in solid circulation rate (Mazumder, Zhu, Bassi, & Ray, 2009). Thereby, supporting our theory that increase in fluidization of resin particles enhances the mass-transfer and protein elution profile and thereby it’s recovery.

4.4 Conclusion

This study demonstrated the effectiveness of adsorption-based refolding technology to refold a model protein, lysozyme that contains 4 disulfide bonds, at refolding concentrations that are not attainable by dilution refolding (> 0.2 mg/ml). The influence of refolding additives, concentration of feed and mode of operation – packed bed vs fluidized bed, on lysozyme refolding yield was substantial. Increase in the amount of protein in the feed during packed bed mode of operation, in comparison to fluidized bed, resulted in decreased refolding yield and mass recovery. From this study, a potentially feasible process route based on on-column refolding for large-scale manufacture of proteins has been identified and demonstrated. The high refolding productivity achieved by column refolding compared to dilution refolding will translate into more rapid product delivery to market and a cheaper product, upon adequate optimization of the overall bioprocess. The ready availability of the chromatographic resin used, coupled with the simple and readily scalable on-column refolding process employed, indicates the widespread applicability of this technology for simultaneous refolding and purification of other protein molecules.

4.5 Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full form</th>
</tr>
</thead>
<tbody>
<tr>
<td>CIP</td>
<td>Clean-in-place</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td></td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>aci</td>
</tr>
<tr>
<td>SP FF</td>
<td>Sepharose Fast Flow</td>
</tr>
</tbody>
</table>
4.6 References


Chapter 5

Investigation of dilution refolding of lysozyme using imidazolium based ionic liquids

This chapter identifies the ionic liquid parameters, composition and concentrations of ionic liquid, that influence the refolding yield of lysozyme. Refolding yield is presented as function of time, with change in concentration and composition of the ionic liquids tested. Influence of chaotropicity and kosmotropicity of the individual ions of the ionic liquid on the refolding yield is also discussed.

5.1 Introduction

Proteins are most soluble in aqueous solution when they are in their native, folded state. It is well established that protein refolding is a kinetically competitive process between the intermolecular hydrophobic interactions resulting in aggregates versus the intramolecular interactions yielding native proteins. *In-vitro* the slightest deviation in upstream process and/or product parameters such as pH, concentration of protein, temperature changes, mechanical stress such as shear strain, surface adsorption or foaming etc., can alter the structure of the protein and consequently promote ‘salting out’ and/or misfolding or aggregation (Hamada, Arakawa, & Shiraki, 2009; Ito et al., 2012; Karuppiah & Sharma, 1995; Lin, de Oliveira, & Veith, 2011; Mergili, Eiberle, & Jungbauer, 2010; Tsumoto, Ejima, Kumagai, & Arakawa, 2003; Yamaguchi, Yamamoto, Mannen, Nagamune, & Nagamune, 2012; Yang, Dong, & Yan, 2008).

In principle, during refolding, proteins are not promptly converted to the native state; instead assume a partially folded state for a prolonged period of time. Folding intermediates are comprised of significant elements of the secondary structure but little of the native tertiary structure. Due to the expanded volume of these intermediates, the hydrophobic regions, which are usually buried in the native state, are exposed to the solvent. When so exposed, hydrophobic regions on separate polypeptide chains can be in contact with one another and can interact. This causes the intermediates to be diverted from the correct folding pathway, resulting in aggregate formation and loss of protein.
Thus, it is apparent that in order to achieve high refolding yield the protein structure must either be stabilized to minimize deviation and/or the structures that are not in the native state must be solubilized to avoid protein-protein interaction (aggregation/misfolding). This can be achieved by addition of solutes/refolding additives. Solution- or refolding-additives can be classified into two categories a) denaturants – those that indirectly promote refolding by weakening the inter- and intra- hydrophobic bonding and b) protein stabilizers- those that enhance protein refolding by modifying the water of hydration and thereby stabilizing the native structure.

An emerging category of refolding additives is ‘Ionic liquids’, also known as designer liquids due to their tunable nature. By definition, they are ionic salts that are liquid at temperatures below 100 °Celsius. Usually, it is the cation in an ionic liquid that has a low degree of symmetry which tends to reduce the lattice energy of crystalline form of the salt and thereby it’s melting point (Earle & Seddon, 2000). Since the synthesis of ethylammonium nitrate (m.p. 12°C) by Paul Walden in 1914, ionic liquids have evolved substantially and found application as lubricants, catalysts, in organic and in-organic chemistry, separation technology, and biopharmaceuticals etc (Aparicio, Atilhan, & Karadas, 2010; Endres et al., 2010; Hough & Rogers, 2007; Ohno & Fukumoto, 2007; Pham, Cho, & Yun, 2010). So far, studies have shown that anions influence protein stability and activity via their hydrogen bonding forming capacity and nucleophilicity properties (Attri, Venkatesu, Kumar, & Byrne, 2011; Buchfink, Tischer, Patil, Rudolph, & Lange, 2010; Lange, Patil, & Rudolph, 2005; Vidya & Chadha, 2009; Weingärtner, Cabrele, & Herrmann, 2011). While changes in the alkyl chain length of the cations influences the viscosity, hydrophobicity and polarity of the ionic liquid, consequently affecting the solubility, stability and activity of targeted protein molecule (Bae, Chang, Koo, & Ha, 2012; Baer & Mundy, 2013; Lange et al., 2005; Xiao et al., 2009; Yamamoto, Yamaguchi, & Nagamune, 2011).

A common trend, in some of the recent studies, has been to study the effect of the ionic liquid as a refolding additive by using it in lieu of urea or guanidine, in the presence of redox-couple, such as cysteine/cystine or GSSG/GSH. This has primarily been achieved
by dilution or dialysis several fold into the refolding buffer, reducing the lysozyme concentration less than 0.10 mg/ml

In summary, refolding yield of a protein is dependent on several parameters:

- Composition of refolding buffer
- pH of the refolding buffer
- Type of refolding additive (chaotropic agent, redox couple, ionic liquids, detergents)
- Concentration of refolding additive (mM or %w/w)
- Concentration of denatured protein (mg/ml)
- Incubation time (hours)
- Methodology employed (dilution, dialysis, packed-bed, fluidized-bed, expanded-bed etc.)

It has already been established that at low concentrations of protein and in presence of redox couple, ionic liquids enhance refolding yield of denatured protein by suppressing aggregation of protein and stabilizing the native structure of the protein. However, to the best of our knowledge, published literature has not assessed the potential of ionic liquids as protein refolding enhancers without redox couple or in a short duration of time. Thus, with denatured lysozyme as the target protein, the aim of this study was to investigate the effect of imidazolium based ionic liquids on its refolding yield.

5.2 Materials and Methods:

5.2.1 Materials

In addition to the common raw-materials presented in Chapter 3, ionic liquids were purchased from purchased from Sigma-Aldrich (Oakville, Canada) and have been enlisted in Table 5.1, below. Also, all analytical methods used have been described and discussed in Chapter 3. The following section discusses the experimental methodology employed for the study presented in this chapter.
Table 5.1 List of ionic liquids used in this study

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Chemical</th>
<th>Abbreviation</th>
<th>Chemical Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-butyl-3-methylimidazolium acetate</td>
<td>[BMIM]Ac</td>
<td><img src="image1" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>2</td>
<td>1-butyl-3-methylimidazolium chloride</td>
<td>[BMIM]Cl</td>
<td><img src="image2" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>3</td>
<td>1-butyl-3-methylimidazolium methylsulfate</td>
<td>[BMIM]MS</td>
<td><img src="image3" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>4</td>
<td>1-ethyl-3-methylimidazolium acetate</td>
<td>[EMIM]Ac</td>
<td><img src="image4" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>5</td>
<td>1-ethyl-3-methylimidazolium chloride</td>
<td>[EMIM]Cl</td>
<td><img src="image5" alt="Chemical Structure" /></td>
</tr>
<tr>
<td>6</td>
<td>1-ethyl-3-methylimidazolium methylsulfate</td>
<td>[EMIM] MS</td>
<td><img src="image6" alt="Chemical Structure" /></td>
</tr>
</tbody>
</table>

5.2.2 Experimental Methods

5.2.2.1 Preparation of denatured lysozyme

Native lysozyme was dissolved in 50 mM HEPES, pH 7.0 to prepare a stock solution. Denaturation buffer contained 6 M urea, 32 mM DTT and 1 mM EDTA in 50 mM HEPES buffer pH 8.1. Denatured lysozyme was prepared by diluting appropriate volume of native lysozyme in denaturation buffer and incubating in water bath at 37 °C for period
of 2 hours. The activity of the lysozyme was tested examined after this period to ensure it is completely denatured.

### 5.2.2.2 Preparation of refolding buffer

Six different ionic liquids were investigated as refolding additives. Stock solution of each of the ionic liquids in 50 mM HEPES, pH 8.1 was prepared. This was further diluted with 50 mM HEPES, pH 8.1 to yield refolding buffers with varying concentration of each of the six ionic liquids. Controls, positive and negative, assure us that the experimental results obtained are valid, and hence are important in all research-based experiments. A positive control, gives the desired outcome of the experiment, provided that all the reagents and equipment are functioning properly. While a negative control, does not give the desired outcome. Based on the results presented in Chapter 4, buffer containing 2 M urea, 0.03 mM cysteine, 0.3 mM cystine in 50 mM HEPES, pH 8.1 demonstrated it’s efficacy in successfully refolding denatured lysozyme. Hence, this was used as positive control, and henceforth is referred to as ‘conventional buffer’. While, 50 mM HEPES buffer at pH 8.1 units, show negligible refolding yield (not desirable) and hence was used as the negative control.

### 5.2.2.3 Preparation of refolded lysozyme

Denatured lysozyme was diluted with equal volumes of appropriate refolding buffer (ionic liquid or conventional buffer), in micro-centrifuge tubes (1.5 ml). The tubes were incubated at room temperature for a period of 3 hours. Sample aliquots were withdrawn at 30 min and then at 1 h interval. These were analyzed for concentration of lysozyme and lysozyme activity as described earlier in Chapter 3.

### 5.2.2.4 Influence of ionic liquids on the protein quantification assay

A solution containing, a sample ionic liquid- [BMIM] acetate, at varying concentrations from 10 mM to 2.5 M, was prepared in 50 mM HEPES, pH 8.1. It’s absorbance without any protein content at 280 nm was recorded. Next, native lysozyme was dissolved in each of the ionic liquid containing buffers and the absorbance of the protein solution at 280 nm was again recorded. The absorbance of blank (without native lysozyme) was subtracted
from the absorbance of ionic liquid solutions containing lysozyme. The corrected (optical density) OD was used for calculation of concentration of lysozyme.

5.2.2.5 Influence of ionic liquids on the lysozyme activity assay

To examine whether ionic liquids have an effect on *Micrococcus lysodeikticus*, buffers containing 2 M [EMIM]Cl in 50 mM HEPES (pH 8.1) and 2 M [BMIM] Cl in 50 mM HEPES (pH 8.1), without any protein were treated as the sample solution in the lysozyme activity assay described earlier. Native lysozyme solution served as positive control. Change in absorbance at 450 nm representative of the change in turbidity of the cell suspension was recorded every 5 seconds for a period of 2 minutes. The experimental results are presented and discussed later.

5.2.2.6 Self-aggregation assay for lysozyme

The self-aggregation of lysozyme was assayed by measuring the light scattering intensity (Yamaguchi, Yamamoto, Tsukiji, & Nagamune, 2008). Three different refolding buffers were prepared. Buffer B was conventional buffer containing 2 M urea, 0.03 mM cysteine, 0.3 mM cystine in 50 mM HEPES buffer pH 8.1. This served as positive control. Buffer C was 100 mM [EMIM] Cl in 50 mM HEPES, pH 8.1 and Buffer A was 50 mM HEPES, pH 8.1, which served as negative control. The study was conducted in triplicate in microwell plates. Equal volumes of denatured lysozyme and each of the refolding buffers was mixed and monitored at 450 nm for a period of 2 hours. Absorbance was recorded every 10 min and the change in turbidity of the solution was noted.

5.3 Results & Discussion

5.3.1 Preparation of denatured lysozyme

Lysozyme is a single chain polypeptide of 129 amino acids cross-linked with four disulfide bridges. It hydrolyzes the β(1→4) linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in the peptidoglycans and between N-acetyl-D-glucosamine residues in chitodextrin (Gorin, Wang, & Papapavlou, 1971). Thus the natural substrate for lysozyme is the peptidoglycan of layer of bacterial cell wall. Of these, *Micrococcus lysodeikticus* is the most popular. An enzyme-substrate reaction is a
very target-specific and an accurate reaction. Any change in the enzyme structure would make it inaccessible to its specific substrate.

Lysozyme activity was measured by decrease in turbidity, represented by change in absorbance at 450 nm due to lysis of *Micrococcus lysodeikticus* cells. Decrease in absorbance indicated lysozyme activity and absence thereof implied either the denatured state of lysozyme or the presence of a blank (solution without protein).

Figure 5.1 illustrates the change in absorbance at 450 nm upon the addition of three different solutions, native lysozyme in 50 mM HEPES, pH 7.0, denatured lysozyme solution and blank, i.e., 50 mM HEPES, pH 7.0. Upon addition of native lysozyme the absorbance of cell suspension decreased linearly, and the initial slope was later used to compute the specific activity of lysozyme. Addition of blank (50 mM HEPES, pH7.0) and denatured lysozyme did not affect the micrococcus cell suspension and hence no change in absorbance was recorded.

![Figure 5.1](image)

**Figure 5.1** Graph illustrating the lysis of *micrococcus lysodeikticus* cell wall as a function of time (seconds) upon addition of different solutions; native (o) and denatured (Δ) and 50 mM HEPES pH 7.0 (✖️)**
5.3.2 Influence of ionic liquid on protein quantification

The influence of ionic liquid on the measurement of protein concentration was investigated. Solutions containing different concentration of native lysozyme (NL), 0.215 mg/ml, 0.43 mg/ml and 0.86 mg/ml, were prepared in buffers containing increasing concentration of [BMIM] acetate in 50 mM HEPES pH 8.1. Absorbance of these solutions at 280 nm was noted and a graphical representation of this data is presented in Figure 5.2. Increase in concentration of the native lysozyme and ionic liquid in buffer resulted in a linear increase in the absorbance at 280 nm, as noted in Figure 5.2. There’s a constant shift, corresponding to the increase in concentration of the protein. Hence, in order to compensate for ionic liquid, a baseline subtraction was done in all protein quantification assays.

**Figure 5.2** Change in $A_{280}$ with corresponding increase in concentration of native lysozyme (mg/ml) and [BMIM] Ac.

Absorption of radiation in the near UV region by proteins depends on the Tyrosine and Tryptophan content and to a very small extent on the amount of Phenylalanine and disulfide bonds. Recent studies have shown that imidazolium based ionic liquids have some absorption in the UV region (Katoh, 2007). It was therefore critical and essential when measuring the concentration of protein in buffers with ionic liquids that the absorbance of ionic liquid (at 280 nm) be subtracted from that of the solution containing...
the protein and ionic liquid. Figure 5.3 illustrates the increase in optical density of ionic liquids at 280 nm with corresponding increase in concentration (mol/l) of ionic liquid in 50 mM HEPES pH 7.0.

![Graph](image)

**Figure 5.3 Change in absorbance at 280 nm as function of change in concentration of ionic liquid (M).**

It was interesting to note, that different ionic liquids, at same concentration (M) had varying optical density at 280 nm.

### 5.3.3 Influence of ionic liquids on lysozyme activity assay

Figure 5.4 represents the change in the turbidity of micrococcus cell suspension over a period of 2 minutes upon the addition of three solutions. One of the solutions contains active, native lysozyme and hence decreases the optical density of the solution. The other two contain ionic liquids at high concentration (2 M). No change in absorbance is seen over period of time, which implies that the ionic liquids have no cell lysis ability of their own, and hence would not interfere with the assay of lysozyme activity or calculation of refolding yield.
Figure 5.4 Graph illustrating the lysis of *micrococcus lysodeikticus* cell wall as a function of time (seconds) upon addition of different solutions Native Lysozyme (◊), 2 M [EMIM]Cl in 50 mM HEPES pH 8.1 (Δ) and 2 M [BMIM] Cl in 50 mM HEPES, pH 8.1 (□).

5.3.4 Effect of ionic liquids as refolding additives for batch dilution refolding of denatured lysozyme

Miscibility of the ionic liquid or its partition in water has a considerable effect on aqueous protein solutions. Upon dissociation, the individual component ions of ILs interact with water via ion-dipole interactions and their hydration state is then dependent on the type of ion and fraction of water present in the mixture. Consequently, this determines if the ion is behaving as a kosmotrope or chaotrope. A kosmotropic anion strongly interacts with water to stabilize the protein while a chaotropic anion exhibits strong interaction with the protein’s chaotropic moieties and results in destabilization. The viscosity B-coefficient of Jones-Dole equation measures kosmotropicity of an ion. Table 5.2 represents the viscosity B-coefficients of certain anions and cations, as presented in literature.
Table 5.2 Characterization of ions based on their viscosity B-coefficient values

<table>
<thead>
<tr>
<th>Ion</th>
<th>Viscosity B-Coefficient</th>
<th>Kosmotrope/Chaotrope</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate (OAc-)</td>
<td>0.246</td>
<td>Kosmotrope</td>
<td>(Jenkins &amp; Marcus, 1995)</td>
</tr>
<tr>
<td>Chloride (Cl-)</td>
<td>-0.005</td>
<td>Chaotrope/kosmotrope</td>
<td>(Jenkins &amp; Marcus, 1995; Marcus, 1994)</td>
</tr>
<tr>
<td>Methylsulfate (MeSO₄⁻)</td>
<td>0.127</td>
<td>Chaotrope</td>
<td>(Jenkins &amp; Marcus, 1995; Marcus, 1994)</td>
</tr>
<tr>
<td>1-ethyl-3-methylimidazolium [Emim]</td>
<td>0.491</td>
<td>Chaotrope</td>
<td>(Zhao, 2006)</td>
</tr>
<tr>
<td>1-butyl-3-methylimidazolium [BMIM]</td>
<td>0.610</td>
<td>Kosmotrope</td>
<td>(Zhao, 2006)</td>
</tr>
</tbody>
</table>

For the purpose of this study, a selection of imidazolium based ionic liquids based on different combination of kosmotropic and chaotropic ions was made. Table 5.3 summarizes the chaotropicity and kosmotropicity of the cation and anion of the ionic liquid used for the purpose of this study. Thus, [EMIM] methylsulfate is a strong chaotropic ionic liquid, while [BMIM] acetate is a strong kosmotropic ionic liquid.
Table 5.3 Characterization of ionic liquids

<table>
<thead>
<tr>
<th>Anion</th>
<th>Acetate</th>
<th>Chloride</th>
<th>Methylsulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>[EMIM]⁺</td>
<td>C</td>
<td>C</td>
<td>C</td>
</tr>
<tr>
<td>[BMIM]⁺</td>
<td>K</td>
<td>K</td>
<td>K</td>
</tr>
</tbody>
</table>

Abbreviations: K- kosmotrope, C: chaotrope, K/C : Borderline kosmotrope/chaotrope

Efficiency of the ionic liquid as refolding aid was assessed on the basis of changes in refolding yield with respect to concentration of ionic liquid and duration of incubation. Conventional refolding buffer containing urea and cysteine-cystine as the refolding aids was used a positive control and 50 mM HEPES, pH 8.1 as the negative control. Refolding yield (mean ± SEM) of independent triplicates is plotted against the concentration of ionic liquid. The standard error of the mean (SEM) quantifies the precision of the mean and is a measure of how far the sample mean is likely to be from the true population mean. It is calculated by the sample standard deviation divided by the square root of the sample size.

Figures 5.5 and 5.6, compare the effect of different anions of 1-alkyl-3-methylimidazolium based ionic liquid on the refolding of denatured lysozyme. Figure 5.5 A, B and C, represent the change in refolding yield (%) of denatured lysozyme as function of time (hours) and concentration of [EMIM] based ionic liquid. The maximum refolding yield under the employed experimental conditions was 97 % in a period of an hour in the presence of 100 mM [EMIM] Cl. [EMIM] acetate proved to be moderately effective, with a renaturation yield of about 40% in 3 hours.
Figure 5.5 Change in refolding yield (%) of denatured lysozyme as a function of time and concentration of A) [EMIM] Cl, B) [EMIM] Acetate C) [EMIM] MS based ionic liquids.

Under similar experimental conditions, with a change in the ionic liquid composition to 1-butyl-3-methylimidazolium, a substantial change in refolding yield was observed. This has been illustrated in Figures 5.6A, B and C. In the presence of 1-butyl-3-methylimidazolium based ionic liquids, the maximum refolding yield obtained was in the
presence of 2.5 M [BMIM]Cl within the first half hour of incubation. Increase in incubation time resulted in the decrease of refolding yield. An anomaly was observed in the refolding experiment with 500 mM [BMIM] Cl.

Figure 5.6. A  
Figure 5.6B  
Figure 5.6 C

Figure 5.6 Change in refolding yield (%) of lysozyme as function of time and concentration of A) [BMIM] Cl, B) [BMIM] MS, C) [BMIM] Acetate
Ionic liquids are known to affect protein activity by either a) stripping off the internal water associated with the protein, b) penetrating into the micro-aqueous environment to interact with the protein by changing protein dynamics, conformational and/or protein’s active site, or c) by interacting with the substrates and products either by direct reaction with them or by altering the portioning between the aqueous and non-aqueous phases. The fact that ionic liquids dissolve and dissociate into individual ions rather than exist as an intact molecule is an obvious advantage over molecular organic solvents. Miscibility of the ionic liquid or its partition coefficient in water has a considerable effect on aqueous protein solutions. Upon dissociation, the individual component ions of ILs interact with water via ion-dipole interactions and their hydration state is then dependent on the type of ion and fraction of water present in the mixture. Consequently, this determines if the ion is behaving as a kosmotrope or chaotrope. Each ion, cation and anion, in solution then interacts with the protein in solution and either stabilizes or destabilizes the protein structure and conformation, thereby having an overall effect on the protein stability and refolding yield.

With respect to the influence of alkyl chain length of the cation, refolding yield was substantially less with BMIM based ionic liquids as opposed to EMIM based ionic liquids. In a similar set up, Lange et al. (2005), examined the effect of N-‘alkyl-N-methylimidazolium chlorides on the denaturation of lysozyme induced by heat and guanidinium chloride (GuHCl). They observed, at higher concentrations of [BMIM] Cl, HMIM Cl and OH-[HMIM] Cl, renaturation was effectively suppressed while for [EMIM] Cl refolding yield was maximized. They suggest imidazolium cation with a longer hydrophobic alkyl chain, such as [BMIM] Cl, has a higher preference of destabilizing the enzyme due to (1) its strong interaction with the kosmotropic moieties, such as the carboxylic groups, on the enzyme surface, and (2) its hydrophobic interaction with the inner hydrophobic moieties of the enzyme molecule, leading to the disruption of the enzyme’s native conformation.

In addition, literature review suggests, in comparison to anions of same charge density, cations are usually observed to show a less dominant effect, because anions are more polarizable and hydrate more strongly (Schwierz, Horinek, & Netz, 2013). Additionally,
Cations seem to present their impact indirectly via interaction with anions, and the kosmotropic behavior of anions could be lessened in the presence of kosmotropic cations. For example, [BMIM] is a kosmotropic cation and acetate a kosmotropic anion. The refolding yield observed with [BMIM] acetate is the least in comparison to the other five ionic liquids. It was hypothesized that kosmotropic cations (such as [BMIM]), as opposed to the chaotropic ones (such as EMIM), have a higher tendency of ion pairing with the kosmotropic anions (such as acetate), thus reducing the abundance of the free anions in solution to play their kosmotropic role. This is also supported by the “law of matching water affinity” which states in aqueous salt solutions, the interactions of ions in the order of decreasing strength are as follows: kosmotrope–kosmotrope > kosmotrope–water > water–chaotrope–water>chaotrope–chaotrope (Degreve et al., 2004; Hess & van der Vegt, 2009; Schwierz et al., 2013; Zaccai, 2004).

Figure 5.7 represents the refolding yield of denatured lysozyme in 1 hour as function of change in concentration of ionic liquid in refolding buffer. [EMIM] is a chaotropic cation and chloride a kosmotropic anion; increase in the concentration of the ionic liquid results in the increase of refolding yield. Whereas, BMIM a kosmotropic cation when paired with chloride, increase in its concentration results in decrease of refolding yield; as is substantiated by the law of matching water affinity.

![Figure 5.7](image-url)
The Jones-Dole empirical equation is commonly used to characterize ions based on their ion-specific effect on the viscosity of the solution. Kosmotropes are often classified as those that make the solution more viscous and Chaotropes as those that make the water more fluid. Equation 5.1 expresses the Jones-Dole empirical equation, where $\eta$ is the viscosity of the solution and $\eta_0$ is the viscosity of the solvent for most salts at low concentrations [(<0.5 M ) or (<0.1 M) for binary strong electrolytes)], the D or higher coefficients can be omitted, although they are necessary for higher concentrations.

$$\frac{\eta}{\eta_0} = 1 + Ac^{1/2} + Bc + Dc^2$$

Equation 5.1

A plot of refolding yield as a function of the viscosity B-coefficient of anion in 100 mM of EMIM and BMIM based ionic liquid is presented in Figure 5.8. It suggests that an increase in viscosity-B coefficient of the anion decreases the refolding yield of the denatured lysozyme. Increase in B-coefficient is usually associated with increase in kosmotropicity of the ion. However, how this pairs with the cation and affects the overall refolding yield of the denatured lysozyme, as can been explained the law of matching water affinity.
Figure 5.8 Graph illustrates the change in refolding yield (%) as a function of viscosity B-coefficient of anion with corresponding cations- [EMIM] (dark grey bar) and [BMIM] (light grey bar)

The refolding yield of lysozyme under the influence of six different ionic liquids is plotted. The X-axis represents the anion with its viscosity B-coefficient quoted in brackets. A positive value of B-coefficient for an ion represents the intensity of kosmotropic character for the ion. Thus, graphical results indicate effect of ionic liquid on refolding yield as a function of kosmotropicity with corresponding changes in cation.

Urea is a chaotropic salt that at low concentrations (less than 3M) solubilizes protein aggregates and enhances refolding of denatured protein. Results have already indicated that 100 mM [EMIM]Cl in 50 mM HEPES (pH 8.1) enhances refolding of denatured lysozyme.

A simple, semi quantitative, aggregation assay was conducted to compare the efficiency of [EMIM] Cl as an aggregation suppressor in comparison to the conventional refolding buffer containing urea and the redox couple, cysteine/cystine. The results of Figure 5.9 show that in the absence of any refolding additive (Buffer A), the optical density of
denatured lysozyme solution increases with time. Thereby implying the denatured proteins aggregate and come out of solution.

![Figure 5.9](image)

**Figure 5.9** Change in optical density of lysozyme solution over a period of time with change in composition of buffers. Buffer A (△), Buffer B (*) and Buffer C(ο).

Buffer A: 50 mM HEPES, pH 8.1 containing 2 M urea, 0.03 mM cysteine, 0.3 mM cystine.
Buffer B: 100 mM [EMIM] Cl in 50 mM HEPES, pH 8.1 Buffer C: 50 mM HEPES, pH 8.1

In the presence of Buffer B and Buffer C, each of which contains different refolding additives, the optical density of the denatured lysozyme solution was almost comparable. And in comparison to Buffer B, [EMIM] Cl in Buffer C had a higher impact on suppressing the aggregation of denatured protein. These results are corroborated by those presented by Yamaguchi *et al.* (2008) which revealed that increase in the hydrophobicity of N’-substituent of the cation augmented the suppression of aggregation but adversely affected the refolding yield. Hence, a fine balance between the hydrophobicity of the cation and the kosmotropicity of anion was desirable in an ionic liquid to function as an effective refolding additive(Yamaguchi *et al.*, 2008).

**5.4 Conclusion:**

It is evident that the refolding yield of denatured lysozyme in the presence of ionic liquids is influenced by the alkyl chain length of the cation, anion in the ionic liquid,
concentration of the ionic liquid as a whole and the duration of incubation. It now
remains to be examined if an optimum for maximized refolding yield can be identified.
To do so, a response surface methodology and central composite design was employed
and is presented in the next chapter.

5.5 Nomenclature

Greek letter

$\eta$ \hspace{1cm} viscosity of the solution
$\eta_0$ \hspace{1cm} viscosity of the solvent

Abbreviation

[BMIM]Ac \hspace{1cm} 1-butyl-3-methylimidazolium acetate
[BMIM]Cl \hspace{1cm} 1-butyl-3-methylimidazolium chloride
[BMIM]MS \hspace{1cm} 1-butyl-3-methylimidazolium methylsulfate
[EMIM] MS \hspace{1cm} 1-ethyl-3-methylimidazolium methylsulfate
[EMIM]Ac \hspace{1cm} 1-ethyl-3-methylimidazolium acetate
[EMIM]Cl \hspace{1cm} 1-ethyl-3-methylimidazolium chloride
OH-HMIM Cl \hspace{1cm} N-(6-Hydroxyhexyl)-N'-methylimidazolium chloride
C \hspace{1cm} Chaotrope
DTT \hspace{1cm} Dithiothreitol
EDTA \hspace{1cm} ethylene diamine tetraacetic acid
GuHCl \hspace{1cm} Guanidine hydrochloride
HEPES \hspace{1cm} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
K \hspace{1cm} Kosmotrope
K/C \hspace{1cm} borderline kosmotrope, chaotrope

5.6 References

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lysozyme with imidazolium-based room temperature ionic liquids: Effect of
hydrophobicity and sulfur residue. *SCIENCE CHINA Chemistry, 55*(8), 1657–1662.
Baer, M. D., & Mundy, C. J. (2013). An ab initio approach to understanding the specific
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Chapter 6

6 Application of response surface methodology and central composite design for the optimization of lysozyme refolding by the ionic liquid [EMIM]Cl

A quick overview on the theory of response surface methodology and central composite design is provided. Experimental design and results thereof that aid and abet in the identification of optimal refolding conditions for denatured lysozyme by [EMIM] Cl are discussed in this chapter. A validated, empirical model based on the results is provided.

6.1 Introduction

Protein refolding, rate limiting step in the downstream processing of bio-therapeutics, is a kinetically competitive process between correct folding and unproductive side reactions such as aggregation and misfolding (Gautam, Mukherjee, Roy, & Gupta, 2012; Lange, Patil, & Rudolph, 2005). Aggregation, considered to be a second- or higher-order reaction, at high protein concentrations dominates over folding and leads to significant reduction in the refolding yield. Therefore a logical strategy to improve refolding yield is to prevent aggregation by adding low molecular weight compounds that interfere with unwanted protein–protein interactions (Dechavanne et al., 2011). In principle, protein refolding additives function by either penetrating the micro-aqueous environment of the protein and stabilizing the protein (chaotropic effect) or by competing with the water associated the protein thereby limiting its exposure to the solvent and consequently driving the protein to its native state (kosmotropic effect). Numerous additives such as chaotropic salts, detergent, polymers and amino acids etc., have been previously shown to impede aggregation and enhance refolding yield (Alibolandi & Mirzahoseini, 2011; Arakawa, Tsumoto, Kita, Chang, & Ejima, 2007; Hamada, Arakawa, & Shiraki, 2009; Meng, Park, & Zhou, 2001; Stumpe & Grubmüller, 2009; West, Guise, & Chaudhuri, 1997). An emerging class of additives is that of ionic liquids. Ionic liquids are known to affect protein activity by either a) stripping off the internal water associated with the
protein, b) penetrating into the micro-aqueous environment to interact with the protein by changing protein dynamics, conformational and/or protein’s active site, or c) by interacting with the substrates and products either by direct reaction with them or by altering the portioning between the aqueous and non-aqueous phases. This dichotomy of preferential binding/exclusion originates from the Hofmeister series that is explicitly used to justify the effect of ionic liquids as protein refolding additives.

Optimization refers to improving the performance of a system, a process of a product in order to obtain the maximum benefit from it (Bezerra, Santelli, Oliveira, Villar, & Escaleira, 2008). Traditionally, optimization has been carried out, by monitoring the influence of one-factor-at-a-time on an experimental response. The major disadvantage of the one-factor-at-a-time approach is that it ignores the interactions among the variables studied. As a consequence, this technique does not depict the complete effects of the parameters on the response. Another disadvantage of the one-factor optimization is the increase in the number of experiments necessary to conduct the research, which leads to an increase of time and expenses as well as an increase in the consumption of reagents and materials.

In order to overcome this problem optimization of procedures has been carried out by using multivariate statistic techniques. Among the most relevant multivariate techniques used is response surface methodology (RSM). Response surface methodology is a collection of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, which must describe the behavior of a data set with the objective of making statistical provisions (Aguirre & Bassi, 2013). It can be applied when a response or a set of responses of interest are influenced by several variables. The objective is to simultaneously optimize the levels of these variables to attain the best system performance.

Protein refolding yield, is affected by several factors. These factors are inherent to the system itself (chemical composition of refolding buffer, type of refolding additive, concentration of refolding buffer, pH, viscosity etc.), to the protein (molecular mass, charge, disulfide bonds) and interactions between the two (Rahimpour, Mamo, Feyzi,
Hence in this study, RSM design was developed to optimize the refolding yield of denatured lysozyme by ionic liquid, specifically \([\text{EMIM}]\) Cl.

The simplest model, which can be used in RSM, is based on a linear function. For its application, it is necessary that the responses obtained be well fitted to Equation 6.1 given below, where \(k\) is the number of variables, \(\beta_0\) is the constant term, \(\beta_i\) represents the coefficients of the linear parameters, \(x_i\) represents the variables, and \(\varepsilon\) is the residual associated to the experiments. The residual is the difference between the calculated and experimental result for a determined set of conditions. A good mathematical model fitted to experimental data must present low residuals values.

\[
y = \beta_0 \sum_{i=1}^{k} \beta_i x_i + \varepsilon \tag{6.1}
\]

Interactions among variables are depicted by curvature on the response surface. To evaluate curvature, a second-order model must be used. Two-level factorial designs are used in the estimation of first-order effects, but they fail when additional effects, such as second-order interaction effects, are significant. Among the more known second-order symmetrical designs are the three-level factorial design, Box–Behnken design, central composite design, and Doehlert design. These symmetrical designs differ from one another with respect to their selection of experimental points, number of levels for variables, and number of runs and blocks. Central composite design is the more popular and commonly used design for optimization of biological processes (Aguirre & Bassi, 2013; Akbari, Khajeh, Ghaemi, & Salemi, 2010; Branchu, Forbes, York, & Nyqvist, 1999; Steinberg & Bursztyn, 2010).

A central composite design consists of the following parts: (1) a full factorial or fractional factorial design; (2) an additional design, often a star design in which experimental points are at a distance \(\alpha\) from its center; and (3) a central point.

Figures 6.1 and 6.2 illustrate the full central composite design for the optimization of two and three variables.
Figure 6.1 Central composite design for the optimization of three variables (alpha = 1.68). Points of Factorial Design (■), Axial points (○) and Central points (□).

Figure 6.2 Central composite design for the optimization of two variables (alpha = 1.41). Points of Factorial Design (■), Axial points (○) and Central points (□).

A full uniformly rotatable central composite design is desired in circumstances where the experimenter doesn't know the location of the optimum point within the region of interest before the experiment is conducted, so it's desirable that all points a given distance (r)
from the center point in any direction have the same magnitude of prediction error. Such a central composite design presents the following characteristics:

(1) Requires an experiment number according to $N = k^2 + 2k + c_p$, where $k$ is the variable number and $(c_p)$ is the replicate number of the central point;

(2) $\alpha$ -values depend on the number of variables and can be calculated by $\alpha = 2^{(k-p)/4}$. For two, three, and four variables, they are, respectively, 1.41, 1.68, and 2.00;

(3) all factors are studied in five levels ($-\alpha$, $-1$, 0, +1, + $\alpha$).

6.1.1 Definition of key terms

It is pertinent to introduce and define key terms that are employed in the design of experiments.

*Factors or independent variables* are experimental variables that can be changed independently of each other. Typical independent variables comprise the pH, concentration of ionic liquid, concentration of protein, time and temperature, amongst others.

*Levels of a variable* are different values of a variable at which the experiments must be carried out. The variable pH, for example in this study, has been investigated at five levels: 6.6, 7, 7.5, 8, and 8.34 units.

*Responses or dependent variables* are the measured values of the results from experiments. Typical responses are the recovery of an analyte, resolution among chromatographic peaks and percentage refolding yield, among others.

There are several factors that affect the refolding of a protein; protein concentration, time, pH of the buffer, temperature, concentration of refolding additive and type of refolding additive. For the purpose of this study, two sets of experiments were conducted. First a three factor, central composite design was used to assess the significant factors that affect protein refolding. Next, two of the previously examined three factors that had significant
interaction, were used to optimize the refolding yield of lysozyme using EMIM Cl and derive an empirical model.

6.2 Materials and Methods

6.2.1 Materials

The ionic liquid of choice for this study, 1-ethyl-3-methylimidazolium chloride, [EMIM]Cl was purchased from Sigma-Aldrich (Oakville Canada).

UV-Star microplate used for protein quantification and lysozyme activity assay, was purchased from VWR International, (Mississauga, Canada). The specifications of plate are described in Appendix, Table 1. Micro centrifuge tubes (1.5ml) use for the purpose of this study was also purchased from VWR International, (Mississauga, Canada). Tecan multimode microplate readers, 200 pro and M1000, (Männedorf, Switzerland) were used for the purpose of the study. All other raw materials and analytical techniques used in this study have already been discussed in Chapter 3 and hence not reproduced. The follow section discusses the design of experiment and experimental methodology employed.

6.2.2 Design of experiments

6.2.2.1 Experimental design: RSM 1

Response surface methodology was employed to optimize the concentrations of denatured lysozyme, concentration of [EMIM]Cl and pH of the refolding buffer that produces the highest refolding yield. To fit the experimental data to a mathematical model, the central composite design (CCD) $2^3 + \text{star}$ was used with three factors and five levels. The CCD consisted of 17 experiments with three replicates for the central point (in parenthesis in Table 6.2) and $\alpha = 1.68$. Table 6.1 represents the range of experimental factors, which were investigated.
Table 6.1 Range of experimental factors considered for RSM-1

<table>
<thead>
<tr>
<th>Name</th>
<th>Units</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Amount of Lysozyme</td>
<td>mg</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>B: Ionic Liquid Concentration</td>
<td>mM</td>
<td>75.0</td>
<td>100.0</td>
</tr>
<tr>
<td>C: pH</td>
<td>Units</td>
<td>7.0</td>
<td>8.0</td>
</tr>
</tbody>
</table>

The data in table 6.2 represents the experimental conditions for the 17 independent runs. The experiments appear in the table in the order they were performed. Estimated response surface contour graphs were used to study to interactive effect of the variables on the refolding yield of lysozyme. The statistical analysis and optimum values for response variable was found based on mathematical models using Statgraphics Centurion XVI (StatPoint Technologies Inc., Warrenton, USA).
### Table 6.2 Experimental matrix for three factor central composite design

<table>
<thead>
<tr>
<th>Run</th>
<th>Denatured lysozyme (mg)</th>
<th>Concentration of [EMIM] Cl (mM)</th>
<th>pH (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.37</td>
<td>108.52</td>
<td>7.50</td>
</tr>
<tr>
<td>2</td>
<td>0.16</td>
<td>87.50</td>
<td>7.50</td>
</tr>
<tr>
<td>3 (Replicate 1)</td>
<td>0.37</td>
<td>87.50</td>
<td>7.50</td>
</tr>
<tr>
<td>4</td>
<td>0.37</td>
<td>87.50</td>
<td>6.66</td>
</tr>
<tr>
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<td>0.37</td>
<td>87.50</td>
<td>7.50</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td>75.00</td>
<td>7.00</td>
</tr>
<tr>
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<td>100.00</td>
<td>7.00</td>
</tr>
<tr>
<td>8</td>
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<td>75.00</td>
<td>8.00</td>
</tr>
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<td>9</td>
<td>0.59</td>
<td>87.50</td>
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<td>0.38</td>
<td>87.50</td>
<td>8.34</td>
</tr>
<tr>
<td>11</td>
<td>0.38</td>
<td>66.47</td>
<td>7.50</td>
</tr>
<tr>
<td>12 (Replicate 3)</td>
<td>0.37</td>
<td>87.50</td>
<td>7.50</td>
</tr>
<tr>
<td>13</td>
<td>0.50</td>
<td>100.00</td>
<td>8.00</td>
</tr>
<tr>
<td>14</td>
<td>0.50</td>
<td>75.00</td>
<td>7.00</td>
</tr>
<tr>
<td>15</td>
<td>0.25</td>
<td>100.00</td>
<td>8.00</td>
</tr>
<tr>
<td>16</td>
<td>0.25</td>
<td>75.00</td>
<td>8.00</td>
</tr>
<tr>
<td>17</td>
<td>0.25</td>
<td>100.00</td>
<td>7.00</td>
</tr>
</tbody>
</table>

### 6.2.2.2 Experimental Design (RSM -2)

Two significant parameters were identified in Experimental design 1. Response surface methodology was employed to optimize those factors, concentrations of denatured lysozyme and concentration of [EMIM] Cl, which produces the highest refolding yield. To fit the experimental data to a mathematical model, the central composite design (CCD) $2^2 +$ star was used with two factors and five levels. The CCD consisted of three blocks of 10 experiments and two central point replicates (in parenthesis in Table 6.4),
per block, and $\alpha = 1.14$. Table 6.3 represents the range of experimental factors, which
was examined.

**Table 6.3 Range of experimental factors in RSM 2**

<table>
<thead>
<tr>
<th>Name</th>
<th>Units</th>
<th>Low</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: Amount of lysozyme</td>
<td>mg</td>
<td>0.125</td>
<td>0.175</td>
</tr>
<tr>
<td>B: Ionic Liquid Concentration</td>
<td>mM</td>
<td>50.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Mathematical model describing the relationship between response variable, percentage
refolding yield, and manipulated variables, concentration of denatured lysozyme and
concentration of [EMIM] Cl, was developed by finding the coefficients of a second order
equation. All the calculations were done using the data obtained at time point 2 hours.
The accuracy of the model was calculated by the regression coefficients $R^2$ and adjusted
$R^2$. Significance of regression coefficients was determined with a confidence level of
95%. The statistical analysis and the optimum values for the response variable was found
based on the mathematical model using Statgraphics Centurion XVI (StatPoint
Technologies Inc., Warrenton, USA).
### Table 6.4 Experimental design for RSM 2

<table>
<thead>
<tr>
<th>Run</th>
<th>Denatured lysozyme (mg)</th>
<th>Concentration of EMIM Cl (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Replicate 1)</td>
<td>0.15</td>
<td>75.00</td>
</tr>
<tr>
<td>2</td>
<td>0.15</td>
<td>110.00</td>
</tr>
<tr>
<td>3</td>
<td>0.13</td>
<td>100.00</td>
</tr>
<tr>
<td>4</td>
<td>0.15</td>
<td>39.60</td>
</tr>
<tr>
<td>5</td>
<td>0.19</td>
<td>75.00</td>
</tr>
<tr>
<td>6</td>
<td>0.18</td>
<td>50.00</td>
</tr>
<tr>
<td>7</td>
<td>0.13</td>
<td>50.00</td>
</tr>
<tr>
<td>8</td>
<td>0.18</td>
<td>100.00</td>
</tr>
<tr>
<td>9</td>
<td>0.12</td>
<td>75.00</td>
</tr>
<tr>
<td>10 (Replicate 2)</td>
<td>0.15</td>
<td>75.00</td>
</tr>
<tr>
<td>11 (Replicate 3)</td>
<td>0.15</td>
<td>75.00</td>
</tr>
<tr>
<td>12</td>
<td>0.15</td>
<td>110.00</td>
</tr>
<tr>
<td>13</td>
<td>0.13</td>
<td>100.00</td>
</tr>
<tr>
<td>14</td>
<td>0.15</td>
<td>39.60</td>
</tr>
<tr>
<td>15</td>
<td>0.19</td>
<td>75.00</td>
</tr>
<tr>
<td>16</td>
<td>0.18</td>
<td>50.00</td>
</tr>
<tr>
<td>17</td>
<td>0.13</td>
<td>50.00</td>
</tr>
<tr>
<td>18</td>
<td>0.18</td>
<td>100.00</td>
</tr>
<tr>
<td>19</td>
<td>0.12</td>
<td>75.00</td>
</tr>
<tr>
<td>20 (Replicate 4)</td>
<td>0.15</td>
<td>75.00</td>
</tr>
<tr>
<td>21 (Replicate 5)</td>
<td>0.15</td>
<td>75.00</td>
</tr>
<tr>
<td>22</td>
<td>0.15</td>
<td>110.00</td>
</tr>
<tr>
<td>23</td>
<td>0.13</td>
<td>100.00</td>
</tr>
<tr>
<td>24</td>
<td>0.15</td>
<td>39.60</td>
</tr>
<tr>
<td>25</td>
<td>0.19</td>
<td>75.00</td>
</tr>
<tr>
<td>26</td>
<td>0.18</td>
<td>50.00</td>
</tr>
<tr>
<td>27</td>
<td>0.13</td>
<td>50.00</td>
</tr>
<tr>
<td>28</td>
<td>0.18</td>
<td>100.00</td>
</tr>
<tr>
<td>29</td>
<td>0.12</td>
<td>75.00</td>
</tr>
<tr>
<td>30 (Replicate 6)</td>
<td>0.15</td>
<td>75.00</td>
</tr>
</tbody>
</table>

#### 6.2.2.3 Validation of the empirical model

Amount of lysozyme in the final reaction mixture has a more profound effect on the final refolding yield than the concentration of ionic liquid. Hence, experimental validation of the model was done under conditions leading to optimal results, by varying the concentration of the denatured lysozyme (see Table 6.8). The predicted refolding yield
(%) was computed by an input of three different concentrations of denatured lysozyme, not previously tested in RSM- 2. Comparing to the observed refolding yield validated the accuracy of the model.

6.2.3 Experimental methodology

Preparation of native and denatured lysozyme:

Native lysozyme was prepared by dissolving lysozyme in 50 mM HEPES, pH 7.0. Composition of the denaturation buffer was 6 M urea, 32 mM DTT and 1 mM EDTA in 50 mM HEPES, pH 8.7. Denatured lysozyme was prepared by diluting appropriate volume of native lysozyme solution in denaturation buffer and incubating in hot-water bath at 37 °C for a period of 2 hours. Final concentration of the stock denatured lysozyme was 1 mg/ml.

Preparation of refolding buffers:

A concentration stock solution of [EMIM] Cl in 50mM HEPES was prepared. Refolding buffer, at its required concentration (mM), was prepared by diluting the stock solution of with 50 mM HEPES. The final pH (units) of the refolding buffer was adjusted per the design of experiments previously discussed. Either hydrochloric acid or sodium hydroxide was used to attain the desired pH value.

Refolding methodology:

Refolding of the denatured lysozyme was studied as per design of experiments (DOE), previously discussed. The experiments were conducted in micro centrifuge tubes (1.5ml) at ambient room temperature. The final volume of the reaction mixture was 1ml. Based on the requirement of DOE and calculations; denatured lysozyme solution (1 mg/ml) was diluted to a final volume of 1 ml with its corresponding refolding buffer (mM). Once the reaction mixture was prepared, the micro centrifuge tubes were placed on a shaker at 125 rpm for a period of 2 hours. Sample was carefully aliquoted and analyzed for protein concentration and refolding yield.
6.3 Results and Discussion

6.3.1 Refolding yield as a function of composition of refolding buffer and concentration of lysozyme

Results of the experimental set up, described in RSM-1, are presented in Table 6.5. It is evident that increase in the amount of denatured lysozyme (mg) adversely affected the refolding yield. Maximum refolding yield was obtained at the lowest concentration (0.165 mg) of denatured lysozyme, which was consistent with the results presented in Chapter 5.

Table 6.5 Experimental results of RSM -1

<table>
<thead>
<tr>
<th>Run</th>
<th>Denatured lysozyme (mg)</th>
<th>Concentration of [EMIM] Cl (mM)</th>
<th>pH (units)</th>
<th>Refolding Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.37</td>
<td>108.52</td>
<td>7.50</td>
<td>3.83</td>
</tr>
<tr>
<td>2</td>
<td>0.16</td>
<td>87.50</td>
<td>7.50</td>
<td>56.00</td>
</tr>
<tr>
<td>3 (Replicate 1)</td>
<td>0.37</td>
<td>87.50</td>
<td>7.50</td>
<td>33.50</td>
</tr>
<tr>
<td>4</td>
<td>0.37</td>
<td>87.50</td>
<td>6.66</td>
<td>22.70</td>
</tr>
<tr>
<td>5 (Replicate 2)</td>
<td>0.37</td>
<td>87.50</td>
<td>7.50</td>
<td>33.30</td>
</tr>
<tr>
<td>6</td>
<td>0.25</td>
<td>75.00</td>
<td>7.00</td>
<td>44.00</td>
</tr>
<tr>
<td>7</td>
<td>0.50</td>
<td>100.00</td>
<td>7.00</td>
<td>17.30</td>
</tr>
<tr>
<td>8</td>
<td>0.50</td>
<td>75.00</td>
<td>8.00</td>
<td>4.00</td>
</tr>
<tr>
<td>9</td>
<td>0.59</td>
<td>87.50</td>
<td>7.50</td>
<td>18.70</td>
</tr>
<tr>
<td>10</td>
<td>0.38</td>
<td>87.50</td>
<td>8.34</td>
<td>6.67</td>
</tr>
<tr>
<td>11</td>
<td>0.38</td>
<td>66.47</td>
<td>7.50</td>
<td>17.30</td>
</tr>
<tr>
<td>12 (Replicate 3)</td>
<td>0.37</td>
<td>87.50</td>
<td>7.50</td>
<td>33.20</td>
</tr>
<tr>
<td>13</td>
<td>0.50</td>
<td>100.00</td>
<td>8.00</td>
<td>10.70</td>
</tr>
<tr>
<td>14</td>
<td>0.50</td>
<td>75.00</td>
<td>7.00</td>
<td>12.00</td>
</tr>
<tr>
<td>15</td>
<td>0.25</td>
<td>100.00</td>
<td>8.00</td>
<td>10.40</td>
</tr>
<tr>
<td>16</td>
<td>0.25</td>
<td>75.00</td>
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<td>24.70</td>
</tr>
<tr>
<td>17</td>
<td>0.25</td>
<td>100.00</td>
<td>7.00</td>
<td>20.00</td>
</tr>
</tbody>
</table>

The visualization of the predicted model equation can be obtained by surface response plot. Based on the results obtained in Table 6.5, Figures 6.3, 6.4 and 6.5 illustrate the quadratic response surface plot obtained in this study.
Figure 6.3 Estimated response surface contour graph illustrating the refolding yield as a function denatured lysozyme (mg) and [EMIM] Cl (mM) at pH 7.5 units.

Figure 6.4 Estimated response surface contour graph of refolding yield as a function of concentration of [EMIM] Cl (mM) and change in pH (units) at 0.165 mg lysozyme.
Maximum refolding yield (56%) was obtained when the concentration of lysozyme was 0.165 mg using refolding buffer 87.5 mM [EMIM]Cl in 50 mM HEPES, pH 7.5. At constant pH, 7.5 units, increase in the concentration of denatured lysozyme results in a decrease in the refolding yield from 53% to 3%, Figure 6.3. In figure 6.4, change in refolding yield was presented as function of pH units and concentration of [EMIM] Cl (mM), at lysozyme concentration, 0.18 mg. A similar response surface contour graph was obtained when the lysozyme concentration was varied along with pH of the refolding buffer, but the concentration of [EMIM] Cl was kept constant at 75 mM.

6.3.2 Refolding yield as a function of lysozyme concentration (mg) and concentration of [EMIM] Cl (mM)

The response surface graphs obtained Figures 6.3, 6.4, and 6.5, show that the maximum (refolding yield) point was outside the experimental region and hence it was necessary to displace, if possible, the initial design to test it. Consequently, the RSM-2 design maintained the pH of the refolding buffer constant at pH 7.5 units but narrowed the range of denatured lysozyme and [EMIM] Cl. Table 6.6 presents the experimental results of the RSM-2 design described earlier. The repetition of central runs was carried out to provide
information on the variation of the responses about the average, the residual variance, and eventually estimate the pure experimental uncertainty.

Table 6.6 Experimental results of RSM-2

<table>
<thead>
<tr>
<th>Run</th>
<th>Denatured lysozyme (mg)</th>
<th>Concentration of EMIM Cl (mM)</th>
<th>Refolding Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.15</td>
<td>75</td>
<td>80.0</td>
</tr>
<tr>
<td>2</td>
<td>0.15</td>
<td>110</td>
<td>13.7</td>
</tr>
<tr>
<td>3</td>
<td>0.125</td>
<td>100</td>
<td>20.8</td>
</tr>
<tr>
<td>4</td>
<td>0.15</td>
<td>39.6</td>
<td>4.77</td>
</tr>
<tr>
<td>5</td>
<td>0.185</td>
<td>75</td>
<td>6.79</td>
</tr>
<tr>
<td>6</td>
<td>0.175</td>
<td>50</td>
<td>3.03</td>
</tr>
<tr>
<td>7</td>
<td>0.125</td>
<td>50</td>
<td>9.41</td>
</tr>
<tr>
<td>8</td>
<td>0.175</td>
<td>100</td>
<td>8.27</td>
</tr>
<tr>
<td>9</td>
<td>0.115</td>
<td>75</td>
<td>17.6</td>
</tr>
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<td>10</td>
<td>0.15</td>
<td>75</td>
<td>76.5</td>
</tr>
<tr>
<td>11</td>
<td>0.15</td>
<td>75</td>
<td>82.7</td>
</tr>
<tr>
<td>12</td>
<td>0.15</td>
<td>110</td>
<td>12.8</td>
</tr>
<tr>
<td>13</td>
<td>0.125</td>
<td>100</td>
<td>22.1</td>
</tr>
<tr>
<td>14</td>
<td>0.15</td>
<td>39.6</td>
<td>5.51</td>
</tr>
<tr>
<td>15</td>
<td>0.185</td>
<td>75</td>
<td>6.92</td>
</tr>
<tr>
<td>16</td>
<td>0.175</td>
<td>50</td>
<td>2.84</td>
</tr>
<tr>
<td>17</td>
<td>0.125</td>
<td>50</td>
<td>9.74</td>
</tr>
<tr>
<td>18</td>
<td>0.175</td>
<td>100</td>
<td>8.69</td>
</tr>
<tr>
<td>19</td>
<td>0.115</td>
<td>75</td>
<td>14.8</td>
</tr>
<tr>
<td>20</td>
<td>0.15</td>
<td>75</td>
<td>78.0</td>
</tr>
<tr>
<td>21</td>
<td>0.15</td>
<td>75</td>
<td>79.5</td>
</tr>
<tr>
<td>22</td>
<td>0.15</td>
<td>110</td>
<td>14.0</td>
</tr>
<tr>
<td>23</td>
<td>0.125</td>
<td>100</td>
<td>20.7</td>
</tr>
<tr>
<td>24</td>
<td>0.15</td>
<td>39.6</td>
<td>5.21</td>
</tr>
<tr>
<td>25</td>
<td>0.185</td>
<td>75</td>
<td>7.05</td>
</tr>
<tr>
<td>26</td>
<td>0.175</td>
<td>50</td>
<td>2.88</td>
</tr>
<tr>
<td>27</td>
<td>0.125</td>
<td>50</td>
<td>9.83</td>
</tr>
<tr>
<td>28</td>
<td>0.175</td>
<td>100</td>
<td>8.9</td>
</tr>
<tr>
<td>29</td>
<td>0.115</td>
<td>75</td>
<td>15.1</td>
</tr>
<tr>
<td>30</td>
<td>0.15</td>
<td>75</td>
<td>85.6</td>
</tr>
</tbody>
</table>
(Bezerra et al., 2008), state in order to find a critical point (maximum, minimum or saddle), it is necessary for the polynomial function to contain quadratic terms according to Equation 6.2, where $\beta_{ij}$ represents the coefficients of the quadratic parameter.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{1 \leq i < j \leq k} \beta_{ij} x_i^2 x_j + \varepsilon$$

Equation 6.2

Based on the results presented in Table 6.5, Equation 6.3 is the equation of the fitted model, where $Y =$ refolding yield (%), $X_1 =$ Amount of denatured lysozyme (mg), $X_2 =$ Concentration of [EMIM]Cl (mM), $X_1 X_2 =$ interaction effect of amount of lysozyme (mg) and [EMIM]Cl (mM).

$$Y = -1.51 \times 10^3 + (1.68 \times 10^4) X_1 + (9)X_2 + (5.58 \times 10^4) X_1^2 + (2.33)(X_1 X_2) - (0.0568)X_2^2$$

.....Equation 6.3

The quality of the fitted model was evaluated by the application of analysis of variance (ANOVA). The central idea of ANOVA is to compare the variation due to the treatment (change in the combination of variable levels) with the variation due to random errors inherent to the measurements of the generated responses (Bezerra et al., 2008; Hanrahan and Lu, 2006; Steinberg and Bursztyn, 2010).

The ANOVA table (Table 6.7) partitions the variability in refolding yield into separate pieces for each of the effects. As replicates of the central point were made, it was possible to estimate the pure error associated with repetitions. The statistical significance of each effect by comparing the mean square against an estimate of the experimental error was evaluated. In this study, 5 effects have P-values less than 0.05, indicating that they are significantly different from zero at the 95.0% confidence level. As per ANOVA, all parameters, including the interacting parameters $X_1 X_2$ are significant.
Table 6.7 Analysis of variance for the response surface quadratic model of refolding yield of lysozyme

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Degrees of freedom</th>
<th>Mean Square</th>
<th>F-value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_1$</td>
<td>384</td>
<td>1</td>
<td>384</td>
<td>85.08</td>
<td>0</td>
</tr>
<tr>
<td>$X_2$</td>
<td>269</td>
<td>1</td>
<td>269</td>
<td>59.56</td>
<td>0</td>
</tr>
<tr>
<td>$X_1^2$</td>
<td>16185</td>
<td>1</td>
<td>16185</td>
<td>3596.68</td>
<td>0</td>
</tr>
<tr>
<td>$X_1X_2$</td>
<td>25.3</td>
<td>1</td>
<td>25.3</td>
<td>5.62</td>
<td>0.0261</td>
</tr>
<tr>
<td>$X_2^2$</td>
<td>17102</td>
<td>1</td>
<td>17102</td>
<td>3800.51</td>
<td>0</td>
</tr>
<tr>
<td>Total error</td>
<td>108</td>
<td>24</td>
<td></td>
<td>4.51</td>
<td></td>
</tr>
<tr>
<td>Total (corr.)</td>
<td>2.43E+04</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R-squared = 99.6 %, R-squared (adjusted for d.f.) = 99.4 %, Standard Error of Est. = 2.12, Mean absolute error = 1.47, Durbin-Watson statistic = 2.36 (P=0.7300) and Lag 1 residual autocorrelation = -0.307.

The goodness of the model for yield of refolding can be checked by several standards. The regression coefficient, $R^2 = 99.6\%$, table 6.7, indicated that only 0.4% of the variability of the response was not explained by the model. The adjusted $R^2$, which was used to compared model, was 99.4%. These results indicated good accuracy of the model in the conditions investigated.

Further, Figure 6.6 represented the plot of experimental refolding yield versus the predicted, based on the model (Equation 6.3). The points cluster around the diagonal line indicating there was no significant deviation between the corresponding values.
Figure 6.6 shows the response surface and contour graph for refolding yield (%). Maximum refolding yield was obtained when the concentration of denatured lysozyme was 0.15mg and the concentration of EMIM Cl was 75 mM. It is also interesting to note that, when the amount of lysozyme is set at high value (0.19 mg), the refolding yield (Y) increases with increase in [EMIM]Cl concentration (from 35 mM to around 70 mM) and then decreases ([EMIM]Cl concentration from 80 mM to 110 mM).

Figure 6.7 Estimated RSM contour plot of refolding yield (%) as a function of concentration of [EMIM] Cl (ionic liquid) and lysozyme (mg)
The response surface model was validated with a random set of experiments to confirm the accuracy of the empirical model, equation 6.3. Experimental points, previously not tested, were examined. The predicted refolding yield was calculated by substituting the values of the experimental factors in Equation 6.3. Details of the experiment and obtained results along with predicted results are presented in Table 6.8.

**Table 6.8 Validation of empirical model. Experimental conditions, predicted response calculated from Eq. 6.6 and observed experimental results**

<table>
<thead>
<tr>
<th>Denatured lysozyme (mg)</th>
<th>EMIM Cl conc. (mM)</th>
<th>Predicted Refolding Yield (%)</th>
<th>Experimental Refolding Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.13</td>
<td>75</td>
<td>63.76</td>
<td>59.23</td>
</tr>
<tr>
<td>0.15</td>
<td>75</td>
<td>83.79</td>
<td>86.89</td>
</tr>
<tr>
<td>0.17</td>
<td>75</td>
<td>58.11</td>
<td>49.13</td>
</tr>
<tr>
<td>0.19</td>
<td>75</td>
<td>-10.84</td>
<td>0</td>
</tr>
</tbody>
</table>

The optimum concentration of [EMIM]Cl was kept constant and the refolding yield as function of change in amount of denatured lysozyme was observed, and also predicted using Equation 6.3. The calculated refolding yield was in close proximity of the predicted values; thus, validating the model.

Further, aggregation assay was used as means to evaluate the effect of [EMIM] Cl on the refolding of denatured lysozyme. The parameters were set as described in RSM-2. The experimental results obtained are presented in Table 6.9.
Table 6.9 Influence of [EMIM] Cl as protein aggregation suppressor

<table>
<thead>
<tr>
<th>Lysozyme (mg)</th>
<th>Concentration of [EMIM] Cl (mM)</th>
<th>% Refolding</th>
<th>% Turbidity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.13</td>
<td>100.00</td>
<td>12.79</td>
<td>28.65</td>
</tr>
<tr>
<td>0.15</td>
<td>110.00</td>
<td>13.68</td>
<td>29.81</td>
</tr>
<tr>
<td>0.12</td>
<td>75.00</td>
<td>17.62</td>
<td>32.69</td>
</tr>
<tr>
<td>0.18</td>
<td>100.00</td>
<td>28.27</td>
<td>32.31</td>
</tr>
<tr>
<td>0.17</td>
<td>87.50</td>
<td>42.00</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>75.00</td>
<td>88.27</td>
<td>21.15</td>
</tr>
<tr>
<td>0.19</td>
<td>75.00</td>
<td>36.79</td>
<td>40.00</td>
</tr>
<tr>
<td>0.25</td>
<td>75.00</td>
<td>34.00</td>
<td>49.38</td>
</tr>
<tr>
<td>0.38</td>
<td>87.50</td>
<td>15.72</td>
<td>11.89</td>
</tr>
</tbody>
</table>

Based on the results obtained in Table 6.9 and optimal conditions obtained earlier, Figure 6.8 is a graphical representation of the effect of 75 mM [EMIM] Cl in 50 mM HEPES as a refolding buffer for varying amount of denatured lysozyme (mg).

Figure 6.8 Graphical representation of the effect of 75 mM [EMIM] Cl in 50 mM HEPES, pH 7.5 on lysozyme refolding and aggregation.

Refolding yield (%) (black bar) and aggregation represented by turbidity (%) (grey bar) is presented a change in amount of lysozyme (mg) in final reaction mixture.

The results of Table 6.9 and Figure 6.8 support the empirical model and elucidate the effect of [EMIM] Cl on increasing concentration of lysozyme. It was, thus, evident that increase in the concentration of lysozyme increases the aggregation (%) hence poor refolding yield.
6.4 Conclusion

Refolding of denatured lysozyme, evidently, was grossly dependent on the amount of lysozyme in the final reaction mixture along with the concentration and type of refolding additive. Table 6.10 summarizes the published research findings of Lange et al (2005, 2010), Bae et al (2012), Buchfink et al (2010) and the results obtained in this thesis (chapter 4 and 6).

Table 6.10 Summary of experimental results obtained and data published by other scientists

<table>
<thead>
<tr>
<th>Protein</th>
<th>Refolding Buffer</th>
<th>Refolding duration (h)</th>
<th>Conc. of denatured protein (µg/ml)</th>
<th>Max. Refolding Yield (%)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>0.3 mM GSH, 0.03 mM GSSG, 1 mM EDTA, 0.1 M [EMIM] Cl, 0.1 M Tris/HCl (pH 8.2)</td>
<td>1:60 dilution for 24 hours</td>
<td>280</td>
<td>80</td>
<td>Lange et al (2005)</td>
</tr>
<tr>
<td>rPA (recombinant plasminogen activator)</td>
<td>0.3 mM GSH, 0.03 mM GSSG, 1 mM EDTA, 3 M [EMIM] Cl, 0.1 M Tris/HCl (pH 8.2)</td>
<td>1:60 dilution for 24 hours</td>
<td>280</td>
<td>16</td>
<td>Buchfink et al (2010)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.3 mM GSH, 0.03 mM GSSG, 1 mM EDTA, 1 M [EMIM] BF4, 0.1 M Tris/HCl (pH 8.2)</td>
<td>1:100 dilution for 24 hours</td>
<td>100</td>
<td>80</td>
<td>Bae et al (2012)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>2 M urea, 0.03 mM cysteine, 0.3 mM cystine 50 mM HEPES (pH 8.1)</td>
<td>1:100 dilution for 8 hours</td>
<td>100</td>
<td>90</td>
<td>Chapter 4, Kaur 2013</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>75 mM [EMIM] Cl 50 mM HEPES pH 7.5</td>
<td>1:6 dilution for 2 hours</td>
<td>180</td>
<td>84</td>
<td>Chapter 6, Kaur 2013</td>
</tr>
</tbody>
</table>
Thus far, only three research groups have published data on the refolding of denatured proteins using imidazolium based ionic liquids. The maximum refolding yield obtained in each of the studies is summarized in Table 6.10. None of the studies, by either research group, reports the exclusive use of ionic liquid as a refolding additive in lieu of cysteine/cystine. They however, do investigate the impact of ionic liquid as protein aggregation suppression agents. Also, the duration of the refolding process is almost always 24 hours, with results of the refolding yield at intermittent time intervals missing. Whether this yield was achieved within the initial few hours is not clear. The table also shows results obtained by the author in this thesis and serves as a comparison to published data.

As is evident from the Table 6.10, to the best of our knowledge, the results presented in this chapter, is the first report of using imidazolium based ionic liquids without any additional redox couple (cysteine/cystine) to refold any protein and in particular denatured lysozyme. This is also the first report of study using response surface methodology and central composite design to find the conditions that yield optimum refolding yield for denatured lysozyme using imidazolium based ionic liquids.

6.5 Nomenclature

**Greek symbol**

\( x_i \) Variables in a central composite design
\( \beta_{ij} \) Represents the coefficients of the quadratic parameter
\( c_p \) Replicate number of the central points
\( k \) Number of variables
\( \beta_0 \) Constant term
\( \beta_i \) Coefficients of linear parameters
\( \epsilon \) Residual associate to the experiments

**Abbreviation**  **Full Form**

[EMIM] Cl 1-ethyl-3-methyl imidazolium chloride
ANOVA Analysis of variance
CCD Central composite design
DTT Dithiothreitol
EDTA ethylenediaminetetraacetic acid
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
\( R^2 \) Regression coefficient
RSM Response surface methodology
\[
\begin{align*}
X_1 & \quad \text{Concentration of denatured lysozyme (mg)} \\
X_1X_2 & \quad \text{Interaction effect of concentrations of lysozyme (mg) and [EMIM] Cl (mM)} \\
X_2 & \quad \text{Concentration of [EMIM] Cl (mM)} \\
Y & \quad \text{Refolding yield (％)}
\end{align*}
\]

6.6 References


Chapter 7

7 Adsorptive protein refolding with the aid of [EMIM] Cl in a packed bed/fluidized bed ion-exchanger

The applicability and influence of [EMIM] Cl as an elution and refolding buffer for adsorptive refolding of denatured lysozyme is examined. Refolding yield and fractional mass recovery, due to incorporation of [EMIM] Cl in elution buffer, is presented as function of mode of operation and increase in concentration of protein load. Comparison on the efficacy of [EMIM] Cl as a refolding/elution enhancer to other ionic liquids is also reviewed by means of experimental results.

7.1 Introduction

The bottleneck in biotechnology, in the last decade, has shifted from upstream to downstream processing of biomolecules. The pressure to develop better separation and purification protocols that combat the new process- and product-related impurities and higher titre levels without compromising on the safety, efficacy and quality of the product is continually increasing. The purification of proteins is steadily moving from pooling-elution-fractions-of-multiple-runs on a single column towards development of columns that can handle higher protein concentrations. In order to do so, it is pertinent to develop integrated approaches that incorporate clarification, separation, recovery and purification. The recovery of active protein from inclusion bodies, to date, is the biggest challenge in downstream processing of proteins. Protein refolding is a kinetically competitive process. Aggregation caused by the hydrophobic interaction between folding intermediates competes with the on-pathway renaturation process, leading to low refolding yields. A potentially efficient strategy to minimize aggregation is to decrease this risk of intermolecular interaction by adsorbing the denatured protein molecules to a solid support, thus, effectively separating the individual protein molecules from each other during refolding.
Usually, adsorbent particles are packed in fixed beds which require pre-clarified feed, in order to avoid plugging particulate material in the packed bed, which can lead to increased pressure drop and operating failure (Thömmes, 1997). Fluidization of the protein adsorption media, increases the inter-particle distance of the resin particles, thereby allowing the feed to pass freely in the interstitial volume of the matrix. A significant advantage of this process is the integration of clarification, separation and recovery of the purified biomolecule in one unit operation.

Refolding additives, enhance refolding yield of a protein, by either suppressing aggregation or by stabilizing the native structure of the protein. Chaotropic salts at non-denaturing concentrations (for urea below 3 M), anion- and non-ionic detergents, amino acids and its derivatives, osmolytes, amphiphilic polymers and varying salts have been used as refolding aids in the past (Arakawa, Tsumoto, Kita, Chang, & Ejima, 2007; Daugherty, Rozema, Hanson, & Gellman, 1998; Hamada & Shiraki, 2007; Wang, Nema, & Teagarden, 2010; Yamaguchi, Yamamoto, Mannen, Nagamune, & Nagamune, 2012; Yasuda, Murakami, Sowa, Ogino, & Ishikawa, 1998). An evolving class of refolding additives is the “designer liquids” or ionic liquids. Ionic liquids, by definition are salts that are liquid at temperatures below 100 °C and consist entirely of ionic species. As they are made up of two components (the anion and cation), which can be varied, the solvents can be designed with a particular end use in mind, or to possess a particular set of properties. The effect of ionic liquids in biocatalysis has been previously reviewed by (van Rantwijk & Sheldon, 2007). In addition to experimental results presented and discussed in chapters 5 and 6 of this thesis, several other scientist have demonstrated the efficacy of ionic liquids as refolding additives via dilution refolding of denatured protein (Bae, Chang, Koo, & Ha, 2012; Buchfink, Tischer, Patil, Rudolph, & Lange, 2010; Lange, Patil, & Rudolph, 2005; Yamamoto, Yamaguchi, & Nagamune, 2011). The general consensus, so far, has been to use ionic liquids with a kosmotropic anion and chaotropic cation to achieve enhanced refolding yields.

The purpose of this work was to investigate the influence of one such imidazolium based ionic liquid [EMIM] Cl, on the refolding yield of denatured lysozyme in a packed bed/fluidized mode of operation. Process performance indicators such as refolding yield
and fractional mass recovery were used to compare and evaluate the efficiency of the different adsorptive refolding processes.

7.2 Materials and Methods

7.2.1 Materials

All materials and equipment used in this study have already been described in Chapter 3, with the exception of the ionic liquids 1-ethyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium chloride and methylimidazolium chloride, which were purchased from Sigma-Aldrich (Oakville, Canada). The following section discusses the experimental methodology employed. The reader is requested to refer Chapter 3 for discussion on the analytical techniques employed.

7.2.2 Experimental methodology

7.2.2.1 Preparation of denatured lysozyme

Native lysozyme was dissolved in 50 mM HEPES, pH 7.0 to prepare a stock solution. Denaturation buffer contained 6 M urea, 32 mM DTT, 1 mM EDTA in 50 mM HEPES buffer pH 8.7. Denatured lysozyme was prepared by diluting an appropriate volume of native lysozyme in denaturation buffer and incubating in a water bath at 37° C for a period of 2 hours. The activity of the lysozyme was tested to ensure it was completely denatured.

7.2.2.2 Investigation of adsorption of ionic liquid ([EMIM]Cl) on ion-exchange resin

A 5 ml pre-packed HiTrap SP FF column was connected to the AKTA FPLC system equipped with UV flow cell, conductivity cell and UNICORN version 5.01. The column was equilibrated with 10 column volumes of 50 mM HEPES, pH 8.7. Two column volumes of [EMIM] Cl in 50mM HEPES, pH 7.5 at varying concentrations, ranging from 10 mM to 150 mM, were applied to the column. The flow rate was kept constant at 0.4
ml/min. The load cycle was followed by wash cycle of 5 column volumes with equilibration buffer. Elution was started by gradually increasing the ratio of elution buffer/equilibration buffer from 0:100 to 100:00 within 12 column volumes. Composition of the elution buffer was 50 mM HEPES at pH 8.1. Changes in conductivity and absorbance were recorded to assess the adsorption-desorption of [EMIM] Cl on the ion-exchanger.

7.2.2.3 Investigation of the efficiency of [EMIM] Cl as an elution/refolding buffer

The pre-packed HiTrap SP FF column (5 ml), connected to AKTA FPLC, was equilibrated with ten column volumes of equilibration buffer (50 mM HEPES, pH 8.7, 2 M urea). A 1ml aliquot of denatured protein (2.5 mg/ml) was loaded on to the column. After rinsing with five column volumes of equilibration buffer above, the column was eluted by gradient elution. Elution was started by gradually increasing ratio of elution buffer/equilibration buffer from 0:100 to 100:0 at 0.4 ml/min within 12 column volumes. Four different elution buffers were examined. Composition of each elution buffer varied as follows;

Buffer A: 50 mM HEPES, pH 8.1 (negative control)

Buffer B: 50 mM HEPES, pH 8.1 containing 1M sodium chloride

Buffer C: 50 mM HEPES, pH 7.5 containing 75mM [EMIM] Cl,

Buffer D: 50 mM HEPES, pH 7.5 containing 75mM [EMIM] Cl 1M sodium chloride.

Buffer E: 50 mM HEPES, pH 8.1 containing 2M urea, 0.03mM cysteine, 0.3mM cystine and 1M sodium chloride

The entire study was conducted at a steady flow rate of 0.4 ml/min. Fractions were collected and analyzed for both protein concentration and lysozyme activity. The column was washed, cleaned-in-place (CIP) and regenerated for the next cycle. The study was conducted in triplicates and average results (mean ± SEM) are presented. The standard
The error of the mean (SEM) quantifies the precision of the mean. It is a measure of how far the sample mean is likely to be from the true population mean. SEM is computed by feeding the data to GraphPad Prism Software Inc. (LaJolla CA, USA). It is calculated by dividing the sample standard deviation by the square root of the sample size.

7.2.2.4 Adsorptive refolding of denatured lysozyme in a packed bed mode

The pre-packed HiTrap SP FF column (5 ml), connected to AKTA FPLC, was equilibrated with ten column volumes of equilibration buffer (50 mM HEPES, pH 8.7, 2 M urea). The flow rate throughout the experiment was set at 0.4 ml/min. Denatured lysozyme solutions, in the range of 1 mg/ml to 10 mg/ml, were prepared. A protein loading of 2.5 ml of denatured lysozyme (at varying concentrations) was applied on to the column. After rinsing with five column volumes of equilibration buffer, the column was eluted by gradient elution. Elution was started by gradually increasing ratio of elution buffer/equilibration buffer from 0:100 to 100:0 at 0.4ml/min within 12 column volumes. Composition of elution buffer was 50 mM HEPES, pH 7.5 containing 75 mM [EMIM] Cl and 1 M sodium chloride. Fractions were collected at every step and analyzed for protein concentration and lysozyme activity. The column was washed, cleaned-in-place (CIP) and regenerated for the next cycle.

7.2.2.5 Adsorptive refolding of denatured lysozyme in fluidized bed mode

A volume of exactly 5 ml of the Sepharose Fast Flow resin slurry, suspended in 50 mM HEPES (pH 7.0), was accurately aliquoted and dispensed in the fluidized bed column. The resin particles were equilibrated with 50 ml of equilibration buffer (2 M urea in 50 mM HEPES, pH 8.7) for a period of 30 minutes, by a steady-flow of air at 0.01 LPM flow rate. At the end of equilibration, fluidization was halted and the buffer was dispensed out of the column with the aid of peristaltic pump. Next, 10 ml of denatured lysozyme solution, at varying concentrations 1 mg/ml to 10 mg/ml, was loaded onto the re-fluidized resin particles. After 15 minutes of adsorption, the resin was allowed to settle.
and the buffer was dispensed out of the column with the aid of the peristaltic pump. Following this, the re-fluidized resin particles were washed with 25 ml of equilibration buffer for period of 10 minutes. After separating the washed-resin particles from the buffer, the resin particles were re-fluidized. Elution of bound lysozyme initiated when the resin particles were allowed to interact with 100 ml of elution buffer (50 mM HEPES, pH 7.5 containing 75 mM [EMIM]Cl and 1M sodium chloride) for duration of 30 minutes. The elution pool was collected and analyzed for protein concentration and lysozyme activity. The resins were cleaned-in-place and regenerated before use in the next cycle.

7.2.2.6 Investigation of [EMIM] Cl as a refolding additive in the elution buffer in a fluidized bed mode

A 5ml slurry of the Sepharose FF resin, re-suspended in 50 mM HEPES (pH 7.0) was accurately aliquoted and dispensed in the fluidized bed column. The resin particles were equilibrated with 50 ml of equilibration buffer (2 M urea in 50 mM HEPES, pH 8.7) for a period of 30 minutes, by a steady-flow at 0.01 LPM air flow rate. At the end of equilibration, fluidization was halted and the buffer was dispensed out of the column with the aid of peristaltic pump. Next, 10 ml of denatured lysozyme solution, at varying concentrations 1 mg/ml to 10 mg/ml respectively, was loaded onto the re-fluidized resin particles. After 15 minutes of adsorption, the resin was allowed to settle and the buffer was dispensed out of the column with the aid of the peristaltic pump. Following this, the re-fluidized resin particles were washed with 25 ml of equilibration buffer for period of 10 minutes. After separating the washed-resin particles from the buffer, the resin particles were re-fluidized using the same airflow rate. Elution of bound lysozyme commenced when the resin particles were allowed to interact with 100 ml of each of the elution buffers for duration of 30 minutes.

The composition of various elution/refolding buffers examined was as follows:

**Buffer D**: 50 mM HEPES, pH 7.5 containing 75 mM [EMIM] Cl and 1M sodium chloride
Buffer E: 50 mM HEPES, pH 8.1 containing 2 M urea, 0.03 mM cysteine, 0.3 mM cystine and 1M sodium chloride.

Buffer F: 50 mM HEPES, pH 7.5 containing 75 mM [MIM] Cl and 1M sodium chloride.

Buffer G: 50 mM HEPES, pH 7.5 containing 75 mM [BMIM] Cl and 1M sodium chloride.

The elution pool was collected and analyzed for both protein concentration and enzyme activity. The refolding efficiency and performance parameters were used to assess the efficiency of the process. The resins were cleaned-in-place and regenerated before next cycle.

7.3 Results and Discussion

The effect of ionic liquids via the on-column adsorptive refolding of denatured lysozyme is yet, to the best of our knowledge, unexplored. The purpose of this study was to evaluate investigate the application of ionic liquids, specifically [EMIM] Cl, as a refolding additive in the adsorptive refolding of denatured lysozyme in both a packed bed ion exchange system and fluidized bed ion exchange system.

7.3.1 Investigation of adsorption of [EMIM] Cl on ion-exchanger

It has already been established in chapters 5 & 6 that dilution refolding of denatured lysozyme with the ionic liquid, 1-ethyl-3-methylimidazolium chloride, ([EMIM] Cl) gave the high refolding yields (ca. 95%) in a short duration of time (1 hour). Imidazolium based ionic liquids are usually miscible in water. Upon dissolution they dissociate into individual ions. Therefore, it was essential to assess if [EMIM] Cl bound to the ion-exchange resin or not. Different concentrations of [EMIM] Cl were loaded onto the packed bed column as discussed previously.

Figure 7.1 is the chromatogram generated by UNICORN when 150mM of [EMIM] Cl was passed through the HiTrap SP FF column. Upon injection of the ionic liquid, as it flowed through the column, changes in UV (at 280 nm) and conductivity were recorded.
The UV peak observed between 0 and 5 on the x-axis in Figure 7.1 indicates that none of the ionic liquid bound to the column but instead flowed through. There is significant difference in the conductivity of the load ([EMIM]Cl in 50 mM HEPES pH 8.1) and elution buffer (50 mM HEPES, pH 8.1) and was evident in the Figure 7.1. It was thus noteworthy that [EMIM] Cl increases the conductivity of the buffer. This property was harnessed and utilized by incorporating [EMIM] Cl in the elution buffer.

**Figure 7.1 Chromatograph generated by UNICORN upon loading 150 mM [EMIM] Cl on to a Hi Trap SP FF column.**

The pink line at point 0ml indicates the injection of [EMIM]Cl. The blue line represents the change in UV absorption at 280 nm, the brown line represents the change in conductivity and the green line represents the change in concentration of elution buffer.

Ionic liquids tend to be viscous solutions. A real concern when working with packed bed systems is the column pressure that is generated due to use of viscous solutions or buffers. However, ionic liquids at concentration below 60 mM act as salts and thus do not
generate high back pressure in the system. It was therefore interesting to note that at high concentration of 150 mM [EMIM] Cl, no pressure issues were noted by UNICORN. This could be attributed to the low flow rate, of 0.4ml/min, at which all the steps of packed bed ion-exchange system were operated. Hence for all further experimental studies on packed bed system with ionic liquids, the flow rate was fixed at 0.4 ml/min for a 5 ml pre-packed column.

7.3.2 Examination of [EMIM] Cl as an elution/refolding buffer for lysozyme

Based on results from previous section (7.3.1), it would be safe to conclude that buffer containing [EMIM] Cl (up to 150 mM), when run through a 5 ml HiTrap SP FF (or Sepharose based) ion exchange system at 0.4 ml/min does not bind to the column but instead flowed through. Thus making it suitable for use with an ion exchange system. It was now important to investigate its efficacy as a refolding/elution buffer. Figure 7.2 represents the results of the study carried out as described in section 7.8.2. The concentration of [EMIM] Cl in the buffer was set at 75 mM. This was based on its efficacy in refolding denatured lysozyme via dilution refolding, as demonstrated earlier in Chapter 6. The efficiency of [EMIM] Cl as a refolding additive (in dilution refolding) has been reported in Chapters 5 and 6 of this thesis. The present study, evaluated its efficacy in packed bed refolding system.

Figure 7.3, shows that the percentage- elution and refolding yield was the least, 2.25% and 2.14% respectively, with Buffer A, which also served as the negative control in this experiment. Buffer E, (conventional buffer) containing 2M urea, 0.03mM cysteine, 0.3mM cystine and 1M sodium chloride in 50mM HEPES, pH 8.1 served as the positive control. In ion exchange based systems, increase in ionic strength of the buffer, the ions (usually sodium and chloride) compete with the bound protein for charges on the surface of the ion-exchange medium. This causes bound species to elute. Thus, addition of 1M sodium chloride to the Buffer A, increased the ionic strength (salt concentration) of the buffer and its subsequent effect on percentage elution (86.34% elution) was apparent in Figure 7.3. Sodium chloride is a salt that does not have any direct effect on the denatured protein.
protein. However, as Buffer B flowed through the column diluting the concentration of denaturant and simultaneously eluting the protein, it created an environment for some of the protein to refold. Hence, a slight increase in refolding yield (10.27%) in comparison to Buffer A was recorded. In comparison to Buffer B, Buffer C containing 75mM [EMIM]Cl, yielded substantially better refolding (50.22%), although it’s efficiency as elution buffer was not as high as in comparison to buffer B. Thus, a combination of Buffer B and C - Buffer D - was evaluated. A combination of the two salts (sodium chloride and [EMIM] Cl), considerably improved the elution and had much more pronounced effect on the refolding yield. Buffer D, containing 75mM [EMIM] Cl, enhanced the refolding yield of the denatured lysozyme by 62% in comparison to Buffer B and improved by 5% in comparison to the positive control (Buffer E). These results are substantiated by experimental results, as reported by Lange et al (2005).

![Figure 7.2 Effect of buffer composition on elution (%) and refolding yield (%) of adsorbed protein on HiTrap SP FF column.](image-url)
Five different buffers are presented. Buffer A (Negative control): 50 mM HEPES, pH 8.1
Buffer B: 50mM HEPES (pH 8.1) containing 1 M sodium chloride Buffer C: 50mM HEPES (pH 7.5) containing 75mM \([\text{EMIM}]\text{Cl}\), Buffer D: 50mM HEPES (pH 7.5) containing 75mM \([\text{EMIM}]\text{Cl}\) and 1 M sodium chloride. Buffer E (positive control): 50mM HEPES, pH 8.1 containing 2M urea, 0.03mM cysteine, 0.3mM cystine, 1M sodium chloride. Protein load : 2.5mg

Lange et al. (2005) examined a series of N-alkyl and N-hydroxyalkyl- N-methylimidazolium based ionic liquids as refolding additives for two different model proteins, lysozyme and ScFvOx. They compared the efficacy and efficiency of the ionic liquids as refolding additives to one of the more popular refolding additives, L-arginine hydrochloride. They found the results (refolding yield, stability of the protein and aggregation suppression) were comparable to, and in some instances better than, that obtained by L-arginine hydrochloride. The enhanced refolding yield was attributed to, suppression of aggregation coupled with stabilization of native structure of the protein, by ionic liquids. They also report, increase in the hydrophobicity of the cation and concentration of ionic liquid (M) adversely affected the refolding yield and aggregation of the target proteins (Lange et al., 2005).

In summary, elution buffer D, containing 75 mM \([\text{EMIM}]\text{Cl}\) and 1 M sodium chloride in 50 mM HEPES, pH 7.5 assisted the elution and refolding of adsorbed denatured lysozyme from a strong cation exchange resin.

### 7.3.3 Packed Bed refolding of denatured lysozyme

Protein load is an important operational variable in the ion exchange refolding of denatured lysozyme because it directly affects the fractional mass recovery and volumetric productivity of the process. Varying concentrations of denatured lysozyme were loaded onto the pre-packed column, and fractions collected were analyzed to assess the influence of protein load. The experimental protocol has been discussed in materials and methods. Figure 7.3, elucidates a typical elution chromatogram of the cation-exchange protein refolding experiment when 5 mg of denatured lysozyme was loaded on to the column.
Figure 7.3 Chromatogram illustrating the elution of refolded lysozyme.

The blue line represents the change in UV absorption at 280nm, the brown line represents the change in conductivity and the green line represents the change in concentration of elution buffer. Load: 5mg of denatured lysozyme. The sharp blue peak represents the elution peak.

As discussed earlier (in Chapter 4), the nature of the protein load affected the adsorption, desorption and thereby refolding yield of the protein, and consequently efficiency of the process. Therefore, native and denatured lysozyme, at similar protein concentrations, was applied to the ion-exchange system. Experimental results from the series of experiments are represented in Figure 7.4 and Table 7.1. It was observed that, irrespective of the nature of the load, approximately 85 to 92% of the load, bound to the equilibrated ion-exchange resin.
Figure 7.4 Effect of protein load and feeding state (native or denatured) on the fractional mass recovery.

Different concentrations of native lysozyme (Δ) and denatured lysozyme (□) were loaded on to HiTrap SP FF 5ml column.

Figure 7.4 illustrates the effect of protein load (mg) and the feeding state (native or denatured) on the fractional mass recovery on a pre-packed HiTrap SP FF (5 ml) column. The results show that the natures of the protein load and the concentration both strongly affect the fractional mass recovery. As opposed to a native protein, the hydrophobic core of the denatured protein is exposed making it susceptible to protein-protein interactions and aggregations. Increase in protein concentration, thereby, increases the probability of aggregation affecting the fractional mass recovery adversely. Similar result is reported by Freydell et al., (2010). They examined the effect of feeding state (native or denatured or denatured-reduced) lysozyme on various cation exchange resins, including Sepharose fast flow beads (Freydell, Van Der Wielen, Eppink, & Ottens, 2010). A major drawback of their process and operation conditions was the inefficient elution of denatured lysozyme from Sepharose beads with sodium chloride. It is noteworthy that, the elution buffer they used did not contain any ionic liquid.
Table 7.1 represents the experimental results obtained by loading varying concentrations of denatured lysozyme on 5 ml HiTrap SP FF column and its corresponding effect on the refolding yield and fractional mass recovery of the protein.

**Table 7.1 Effect of load on refolding yield and fractional mass recovery of lysozyme in a packed bed ion exchanger**

<table>
<thead>
<tr>
<th>Ion Exchanger</th>
<th>Load : denatured lysozyme (mg)</th>
<th>% Refolding Yield</th>
<th>Fractional mass recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>HiTrap Sepharose Fast Flow (5ml)</td>
<td>2.5</td>
<td>84.34</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>85.39</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>84.29</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>54.29</td>
<td>0.53</td>
</tr>
</tbody>
</table>

Fractional mass recovery of the protein is the ratio of concentration of eluted protein (mg) to the concentration of protein load (mg). It is evident that increase in the protein load, up to 25 mg, had an adverse effect on the fractional mass recovery of the protein; however up to 10 mg of load the refolding yield was considerably high, 85%. A plausible justification for this could be loading under denatured state of protein. The probability of aggregation in a solution of denatured protein increases with increase in concentration of protein, by virtue of its exposed hydrophobic patches. This claim is further supported by similar trends reported by (Freydell et al., 2010; Langenhof, Leong, Pattenden, & Middelberg, 2005).

In order to meet the economies of scale there is a strong demand in the industry for systems that can support high concentration of protein solutions. Packed bed refolding of lysozyme has already presented its limitation with regards to protein concentration. Whether this changes favorably or not, in a fluidized bed system was investigated in the following section.
7.3.4 Protein refolding in a fluidized-bed ion-exchange chromatographic system

Previously optimized concentrations of refolding additives and buffer compositions were employed in the batch-refolding of denatured lysozyme in the fluidized bed ion-exchange system developed in-house and described earlier.

Similar to packed bed studies, series of experiments were conducted to assess the efficiency of the fluidized bed ion-exchange refolding system. Samples from each step of the ion exchange process (adsorption, wash, elution) was collected and analyzed for both protein concentration and enzyme activity. Process performance indicators, discussed in analytical methods, Chapter 3, were used to assess the efficiency of the system in comparison to the packed-bed refolding of denatured lysozyme and use of conventional refolding buffer containing a redox couple.

One of the main limitations in the use of a packed-bed system has consistently been its inability to handle higher protein concentration loads, and viscous solutions. Poor mass-transfer, resin fouling and saturation of the resin are commonly encountered issues. After a fixed period of cycles, the ion-exchange resin is often rendered unusable. On the other hand, in the fluidized mode of operation, resin particles are lifted inside the column by an upward liquid and/or air stream generated by buffers, sample solutions or air-inlet. As a result of increased inter-particle porosity, fluidized beads allow for treatment of solution with considerably higher viscosity without generation of any significant backpressure. It was therefore our hypothesis that increased mass transfer and inter-particle porosity would permit a denatured adsorbed protein to elute into an environment conducive for protein refolding. In order to test this hypothesis, experiments were executed at similar conditions as a packed bed, only in a fluidized bed system. It is shown here for the first time that ionic liquid aided adsorptive protein refolding is noticeably better in a fluidized bed with an efficiency ca. 90% versus 55% for packed-bed a system, for a protein load of 25mg.
Table 7.2 illustrates the experimental results obtained by varying the concentration of the denatured lysozyme loaded onto the ion-exchange resin. In comparison to the results presented in table 7.1, the fractional mass recovery of the eluted protein and the refolding yield was substantially higher, thereby indicating the superior efficiency of the fluidized bed.

It is interesting to note, that with increase in the protein load (mg), the refolding yield did not decrease substantially. Even at high protein load (25 mg), the refolding yield obtained was 35% higher than that obtained with a packed bed system. Also the fractional mass recovery was almost consistent through the range of protein load concentrations examined.

Table 7.2 Effect of load on the refolding yield and fractional mass recovery of lysozyme in a fluidized bed ion exchanger

<table>
<thead>
<tr>
<th>Ion Exchanger</th>
<th>Load: denatured lysozyme (mg)</th>
<th>Refolding Yield (%)</th>
<th>Fractional Mass Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepharose Fast Flow</td>
<td>2.5</td>
<td>97.71</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>93.38</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>92.04</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>89.54</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Comparing the protein elution (%), refolding yield (%) and fractional mass recovery (%) of two different adsorptive processes – packed bed and fluidized, Figure 7.5, elucidates the enhanced efficiency of ion-exchange system in a fluidized bed vis-à-vis a packed bed system. At lower protein concentration range, i.e., 2.5mg, percentage elution and percent mass recovery in packed bed and fluidized bed was almost similar; indicating the mass transfer limitations did not vary much. However, the refolding yield in fluidized bed was greater than packed bed by 14%. Increasing the protein load (mg) ten fold adversely affected the output in a packed bed system. The refolding yield in packed bed was only 54.29%, while in fluidized bed it was 89.54%. At both higher and lower concentrations of
protein load, fractional mass recovery in the fluidized bed was substantially higher than that in a packed bed system. Thereby, exemplifying the tolerance of a fluidized bed to higher concentrations of protein and its augmented efficiency in refolding denatured lysozyme.

Figure 7.5 Effect of mode of operation and protein load (mg) on the refolding yield, elution and fractional mass recovery of lysozyme.

Percentage elution (black bar), refolding yield (white bar) and fractional mass recovery (grey bar) are plotted against the load (mg) and mode of operation (packed bed, fluidized bed).

7.3.5 Comparing the efficiency of [EMIM]Cl as an elution/refolding buffer

Figure 7.7 describes the experimental results of similar refolding process in a fluidized bed ion-exchange system using different elution buffers at two different protein loads, 2.5
mg and 25 mg. It was evident from the previous study that [EMIM]Cl enhances refolding yield of denatured of lysozyme. How it compared with the conventional buffer containing the redox couple, cysteine/cystine was evaluated in this study.

Additional elution buffers, Buffer F and G were incorporated to test the efficiency of [EMIM]Cl in comparison to other imidazolium based ionic liquids, as a refolding additive. It was interesting to note that with regards to the fractional mass recovery, Buffer G containing BMIM Cl was almost similar (difference of less than 3%) to that of Buffer F. However, the refolding yield with the buffer containing the less hydrophobic ionic liquid, 1-methyl-3-methylimidazolium chloride (MIM Cl) was at least 25% higher than that with the buffer containing 1-butyl-3-methylimidazolium chloride (BMIM Cl); at either concentration of protein load. This could be explained by the increase in hydrophobicity and chaotropicity of the ionic liquid.

![Figure 7.6 Effect of load (mg) and buffer composition on the refolding yield and fractional mass recovery of lysozyme in a fluidized bed ion-exchanger](image)
Experimental results of four different buffers are graphically represented in the Figure 7.6. Composition of the buffers is as follows; Buffer D: 50mM HEPES, pH 7.5 containing 75mM [EMIM] Cl and 1M sodium chloride and buffer E: 50mM HEPES, pH 8.1 containing 2M urea, 0.03mM cysteine, 0.3mM cystine and 1M sodium chloride
Buffer F: 50mM HEPES, pH 7.5 containing 75mM [MIM] Cl and 1M sodium chloride,
Buffer G: 50mM HEPES, pH 7.5 containing 75mM [BMIM] Cl and 1M sodium chloride,

Studies have shown that a kosmotropic anion has low affinity for the amide group of the protein and instead competes for the water associated with the protein, thereby driving the protein to its native state. While, a chaotropic cation penetrates the micro aqueous environment of the protein and forms a protective, stabilizing layer around it (Bae et al., 2012; Lange et al., 2005a; Zhao, 2006). Thus, in order to achieve better refolding yields it is often advised that the ionic liquid be designed with a kosmotropic anion and chaotropic cation. In comparison to [MIM] and [EMIM], [BMIM] interacts poorly with water and has a destabilizing effect. It is therefore, no surprise that maximum refolding yield was obtained with Buffer D, containing [EMIM] Cl.

7.4 Conclusions

Proactive debottlenecking the downstream process of proteins is scrupulously dependent on the development of efficient, cost-effective refolding processes. In order to achieve this, it is important that the protein is provided with an environment, which promotes refolding and suppresses aggregation. This is commonly achieved by addition of refolding additives (L-arginine, 2 M urea, redox couples (cysteine/cystine) and GSH/GSSG etc) and employing strategies such as dilution, dialysis, or a combination of the two. Dilution and dialysis, albeit easy to adapt and execute, are plagued with limitations during scale up. The requirement of large volumes of refolding buffer, holding tanks, temperature and pH control, mixing and low concentration of denatured protein (less than 0.5mg/ml), make it inefficient and expensive. On-column adsorptive refolding of proteins, on the other hand, enhances refolding yield by reversibly binding the protein to the solid matrix, gradually removing the denaturants from the solution and providing the protein with sample space to refold to its natural state. The first report of on-column
protein refolding was by Creighton et al., in 1986 for horse cytochrome c and bovine pancreatic trypsin inhibitor. Since then, several methods have been developed to increase refolding yield of denatured-lysozyme for ion exchange refolding matrices, including but not limiting to, two and three buffer systems and dual gradient chromatography. Langenhoff et al., (2005) report a maximum refolding yield of 60% with bovine serum albumin (BSA) at protein load of 2 mg on an anion-exchange system using the redox couple GSH/GSSG (Langenhof et al., 2005). Li et al (2002), using redox couples GSH/GSSG and cation exchange system with lysozyme as target protein, demonstrated that avoiding high salt concentrations for elution, and thus avoiding stronger hydrophobic interactions, could lead to suppression of aggregation of the folding intermediates and thereby enhance refolding yield (Li & Su, 2002).

Ionic liquids, is an emerging class of refolding additives, that are gaining popularity due to their malleable nature and ‘green’ chemistry. Ionic liquids consist of organic cations such as N-alkyl-N-methylimidazolium, N-substituted pyridinium, tetraalkylated ammonium and tetraalkylated phosphonium, and either organic or inorganic anions. They represent a rather diverse class of organic solvents and co-solvents with a correspondingly assorted range of physico-chemical properties, which may be readily, engineered by changes in anion and cation substitution patterns (Austen Angell, Ansari, & Zhao, 2011). There are several publications that discuss the efficacy of ionic liquids as protein refolding additives in dilution refolding (Attri & Venkatesu, 2012; Lange et al., 2005; Summers & Flowers, 2000). However, to the best of our knowledge, none of the studies published so far, have incorporated ionic liquids as refolding additives for on-column adsorptive refolding of proteins.

The work presented in this study demonstrates, the efficacy of [EMIM] Cl as a refolding additive. When incorporated in the elution buffer, it increased the ionic strength of the buffer and assisted in the elution and subsequent refolding of the denatured protein. The presence of a chaotropic cation ([EMIM]) and kosmotropic anion (Cl) in the ionic liquid suppressed aggregation and enhanced refolding of denatured lysozyme. Further, two different modes of operation were compared, packed bed and fluidized bed. In comparison to a packed bed mode of operation, at denatured lysozyme concentration of
25 mg with the aid of 75mM [EMIM] Cl in the elution buffer, fluidized bed delivered refolding yield ca. 92%. This could be explained by the increase in the mass transfer and inter-particle porosity, offered by fluidized mode of operation, which directly translates into improved efficiency for the system. Where this improves at concentration higher than 25 mg of denatured lysozyme, is adaptable to different proteins and ionic liquids, remains to be examined. At present, these results pave the way for incorporation of ionic liquids as refolding additives in adsorptive refolding of denatured lysozyme, and for the development of economic, efficient refolding processes at an industrial scale.

7.5 Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full form</th>
</tr>
</thead>
<tbody>
<tr>
<td>[BMIM] Cl</td>
<td>1-butyl-3-methylimidazolium chloride</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothrietol</td>
</tr>
<tr>
<td>[EMIM] Cl</td>
<td>1-ethyl-3-methylimidazolium chloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
</tr>
<tr>
<td>[MIM]Cl</td>
<td>Methylimidazolium chloride</td>
</tr>
</tbody>
</table>

7.6 References


Chapter 8

Concluding remarks and recommendations

Based on the experimental results acquired in this thesis, final concluding remarks and recommendations are provided.

8.1 Summary

Protein refolding is one of the most severe rate limiting steps in the downstream processing of proteins. The efficient conditions required for refolding proteins generally have to meet the following requirements; a) concentration of denaturants should be reduced to a level where intramolecular non-covalent interactions are retained, b) the refolding process should be performed under an appropriate environment to form the disulfide bridges and c) the concentration of the denatured protein should be maintained at a low level (usually less than 100μg/ml) to avoid intermolecular aggregation.

Low protein concentration, is traditionally accomplished by diluting the solubilized protein in the refolding buffer. This also results in dilution of the denaturants. A rapid change in concentration of denaturants, however, may result in the formation of misfolded protein or aggregates. Due to the ease of automation and ability to handle high protein concentrations with integrated purification steps, the use of on-column refolding has gained momentum. However, irrespective of the methodology employed, dilution or on-column refolding, refolding additives play a significant role in the overall development of the process. These can be broadly classified into categories based on their mechanism of action, aggregation inhibitors or stabilizers of native protein structure.

Ionic liquids, are gaining popularity in the biotechnology and pharmaceutical industry, primarily due to their characteristic low vapor pressure that renders them valuable as ‘green solvents’ in comparison to the other molecular solvents. They have been successfully applied in biocatalysis, synthesis of active pharmaceutical ingredients and to enhance protein stability (Austen Angell, Ansari, & Zhao, 2011; Hough & Rogers, 2007; van Rantwijk & Sheldon, 2007). The effect of ionic liquids on protein structure, at a
molecular level has been examined, which exposed their role as protein aggregation suppressors and native structure stabilizers; both ideal characteristics required of a refolding additive (Endres et al., 2010; Kohno & Ohno, 2012; Mann, McLuskey, & Atkin, 2009; H. Zhao, 2010).

A thorough, yet comprehensive, review of research findings (with respect to ionic liquids) has been provided in the literature review section of this thesis. It is apparent that, although there are numerous papers that discuss the role of ionic liquids as refolding additives, none so far have examined their worth in adsorptive, on-column refolding of denatured proteins. Hence the main objective of this thesis was to identify the potential of ionic liquids as refolding additives for on-column refolding of proteins, specifically denatured lysozyme. Each ion (cation and anion) of the ionic liquid has a significant impact on the protein. Dependent on their behavior, they are often classified as kosmotrope – order makers - or as chaotropes – order breakers. Ideally, for an ionic liquid to be effective protein refolding additive, the most desirable combination is that of a kosmotropic anion and a chaotropic cation. We reviewed this theory by using six different ionic liquids, encompassing a range of kosmotropic and chaotropic ions, for the refolding studies of denatured lysozyme. The ionic liquids that were investigated as refolding additives were all imidazolium based ionic liquids with varying alkyl chain lengths of the cation and varying anions.

It is an established fact that protein-refolding yield is dependent on the type- and concentration -of- protein and refolding additive, duration of incubation, pH and refolding strategy employed. Successful dilution refolding demands low concentration of protein. Hence, by maintaining the concentration of denatured lysozyme, the refolding yield was assessed as a function of change in concentration of ionic liquid and incubation duration for each of the chosen six ionic liquids. The results showed maximum refolding yield close to 90% (within an hour of incubation) in the presence of [EMIM] Cl. Next, by using response surface methodology and central composite design optimized refolding conditions, for [EMIM] Cl and denatured lysozyme were identified and an empirical model was developed. The conditions for refolding denatured lysozyme that yield
optimal results are; 75 mM [EMIM]Cl in 50 mM HEPES, pH 7.5 at denatured lysozyme concentration 0.18 mg/ml. The model was validated by experimental results.

Ionic liquids dissociate into individual anion and cation when dissolved in water. It was therefore critical and pertinent to the development of on-column refolding process to test if [EMIM] Cl in buffer adsorbed to the cation exchange resin, Sepharose Fast Flow. At the tested concentration of [EMIM] Cl (up to 150 mM in 50 mM HEPES, pH 7.5), there was no evidence of binding to the resin. Therefore, it was safe to use the ionic liquid in the elution buffer for the on-column refolding of denatured lysozyme.

Protein load has a significant impact on the fractional mass recovery and refolding yield. Hence, by varying the concentration of denatured lysozyme load (mg), on the packed bed and fluidized bed resin, the efficiency of [EMIM] Cl in the elution buffer as a refolding additive was assessed. Elution buffer containing 75 mM [EMIM] Cl, 1 M NaCl in 50 mM HEPES, pH 7.5 not only enhanced the elution of the adsorbed protein but also increased the refolding yield in comparison to conventional refolding buffer tested in Chapter 4. This was found to be independent of the mode of operation, packed bed vis-a-vis fluidized bed.

Further, the efficiency of on-column adsorptive refolding of denatured lysozyme in packed bed was compared to that of that in a fluidized bed. At low protein load (2.5 mg) the refolding yield and recovery was comparable in both modes of operation. However, as the protein load increased, the enhanced mass transfer and inter-particle porosity in the fluidized-bed mode of operation enhanced the elution protein and refolding yield of the protein. In comparison to fluidized bed, the reduction in the number of available sites for protein adsorption, especially at the top of the column in the packed bed system resulted in poor recovery and refolding yield.

In summary, based solely on the experimental results presented in this thesis, it is safe to that ionic liquids, particularly [EMIM] Cl is an effective refolding additives for refolding denatured lysozyme. On-column adsorptive refolding of denatured lysozyme is enhanced in the fluidized bed and by incorporation of [EMIM] Cl in the elution-refolding buffer.
8.2 Recommendations

Purity, toxicity and economic feasibility of the application of ionic liquids at industrial scale need to be rigorously evaluated. The reusability of the ionic liquids by suitably modifying the fluidized bed to a circulating fluidized bed is a proposed plan to combat the economical and ecological aspects of ionic liquids. The efficacy of the ionic liquid as a refolding additive in on-column refolding process was demonstrated by inclusion in the elution buffer. However, the separation of the refolded protein from the ionic liquid elution pool, remains to be evaluated. Summers and Flowers (2000) did successfully separate refolded lysozyme from ethylammonium nitrate [EAN] by use of advanced centrifugal filters (Summers and Flowers, 2000). Whether this is applicable to other ionic liquids, based on imidazolium, pyrrolidinium or pyridinium etc., is yet to the examined. Also, how this would translate into economies of scale at the industrial level.

Thus, in summary, while the use of fluidized bed ion exchange refolding system is an effective means to refold high concentrations of denatured lysozyme, an organized, systematic assessment of its applicability to other proteins, in the presence of other refolding additives including different types of ionic liquids, could in the long run help over come the biggest obstacle in downstream processing today – protein refolding.

8.3 Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full form</th>
</tr>
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<tbody>
<tr>
<td>[EAN]</td>
<td>Ethyl ammonium nitrate</td>
</tr>
<tr>
<td>[EMIM] Cl</td>
<td>1-ethyl-3-methylimidazolium chloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
</tbody>
</table>

8.4 References


Appendices

Appendix  A Specification of the UV-Star 96 well microplate

<table>
<thead>
<tr>
<th></th>
<th>Description/Specification</th>
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</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Description</td>
</tr>
<tr>
<td>1.2</td>
<td>Plate Length</td>
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<tr>
<td>1.3</td>
<td>Volume</td>
</tr>
<tr>
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<td>Material/ Resin</td>
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<td>Color</td>
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<td>Sterilisation</td>
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<tr>
<td>1.7</td>
<td>Quality Control</td>
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<td>1.8</td>
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<tr>
<td>2.1</td>
<td>Basic Features</td>
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<td>Centrifugation, max. RCF</td>
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<td>Chemical Resistance</td>
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<td>2.6</td>
<td>Shelf Life</td>
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</table>
## Appendix B Specifications of HiTrap Sepharose Fast Flow columns (1ml and 5ml)

<table>
<thead>
<tr>
<th></th>
<th>5ml Column</th>
<th>1ml Column</th>
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<tbody>
<tr>
<td><strong>HiTrap SP FF</strong></td>
<td><strong>5ml Column</strong></td>
<td><strong>1ml column</strong></td>
</tr>
<tr>
<td><strong>Bed Dimensions</strong></td>
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<td>7 × 25 mm</td>
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<td><strong>Bed Volume</strong></td>
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<td>1 ml</td>
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<tr>
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<td>&lt;4 ml/min1)</td>
</tr>
<tr>
<td><strong>Flow Rate</strong></td>
<td>&lt; 20 ml/min2)</td>
<td>&lt; 4 ml/min2)</td>
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<td><strong>Pressure Max. [Over the Packed Bed During Operation]</strong></td>
<td>3 bar [0.3 MPa] (42 psi)</td>
<td>3 bar [0.3 MPa] (42 psi)</td>
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<tr>
<td><strong>Storage Conditions</strong></td>
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<td>4 to 30°C, 0.2 M Sodium Acetate in 20% Ethanol</td>
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<tr>
<td><strong>Media</strong></td>
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</tr>
<tr>
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</tr>
<tr>
<td>Ligand</td>
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<td>Sulphopropyl</td>
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<tr>
<td>Average Particle Size</td>
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<tr>
<td>Ion Exchanger Type</td>
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<td>Strong cation exchanger</td>
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<tr>
<td>Matrix</td>
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<td>6% cross-linked agarose</td>
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<tr>
<td>Particle Size</td>
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<td>Storage Conditions</td>
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<td>4 to 30°C, 20% Ethanol + 0.2 M Sodium Acetate</td>
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<tr>
<td>Chemical Stability</td>
<td>Stable to all commonly used aqueous buffers:1 M NaOH, 8 M urea, 8 M guanidine hydrochloride, 70% ethanol1)</td>
<td>Stable to all commonly used aqueous buffers:1 M NaOH, 8 M urea, 8 M guanidine hydrochloride, 70% ethanol1)</td>
</tr>
</tbody>
</table>
Appendix C. Clean-in-place protocol for Sepharose Fast Flow resin

Cleaning-in-place (CIP) is a cleaning procedure that removes contaminants such as lipids, precipitates, or denatured proteins that may remain in the packed column after regeneration. Such contamination is especially likely when working with crude materials. Regular CIP also prevents the build-up of these contaminants in the media bed and helps to maintain the capacity, flow properties and general performance of the media.

Standard CIP protocol followed:

Step 1. Wash with 0.5 column volume of 1 M sodium hydroxide solution, contact time 15 minutes.

Step 2. Wash with 4 column volumes of Milli Q water, contact time 30 minutes.

Step 3. Re-equilibrate Resin : Wash with 5 column volumes of equilibration buffer or until the base line is stabilized.
Appendix D. Sample calculation - refolding yield of lysozyme

Preparation of denatured lysozyme solution:

Native Lysozyme solution (10 mg/ml) in 50 mM HEPES, pH 7.0 was prepared by dissolving 100 mg of lyophilized lysozyme in 10 ml of 50 mM HEPES buffer (pH 7.0). Next, native lysozyme solution, 1 ml aliquot, is diluted with 9 ml of denaturation buffer (6 M urea, 32 mM dithiothreitol, 1 mM EDTA in 50 mM HEPES, pH 8.7) and placed in water bath for 2 hours at 37 °C. At the end of 2 hours, denaturation is confirmed by lysozyme activity assay.

Concentration of denatured lysozyme: 1 mg/ml

Experimental methodology

Refolding buffer: 75 mM [EMIM] Cl in 50 mM HEPES, pH 7.5

Technique: Fluidized bed refolding.

Load: 2.5 mg of denatured lysozyme (i.e. 2.5 ml)

Raw Data: Lysozyme activity assay

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
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<td>0.616</td>
<td>0.558</td>
<td>0.516</td>
<td>0.469</td>
<td>0.469</td>
<td>0.405</td>
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<tr>
<td>Elute</td>
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<td>0.555</td>
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<td>0.453</td>
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<tr>
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<td>0.395</td>
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<td>0.325</td>
<td>0.304</td>
<td>0.297</td>
<td>0.294</td>
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</tbody>
</table>

Average Slope (GraphPad Prism, linear regression) -0.002614 ± 0.0001046

Using Equation 3.2, specific activity of elute = -0.105 units mg⁻¹ ml⁻¹

Specific activity of native lysozyme: -0.114 units mg⁻¹ ml⁻¹

Therefore, substitute the values in Equation 3.3, Refolding yield = 92.13 %
Curriculum Vitae

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Institute of Chemical Technology, Mumbai, Maharashtra, India

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2009-2013 Ph.D. Biochemical Engineering

Related Work Experience

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Research Associate – R&D Novel Drug Delivery Development
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Graduate Research Associate – Monoclonal Antibody Division
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Publications:


Conferences

