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INTENSIFICATION OF INCLUSION BODY PROCESSING VIA SURFACE REFOLDING WITH CHEMICAL EXTRACTION

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Summary

Gloshedobin, a kind of thrombin-like enzyme (TLE), is recently isolated from the snake venom of *Gloydius shedaoensis*. The formation of inclusion body (IB) and truncated expression product however significantly complicated its production in *Escherichia coli*. This research aims to develop an efficient method to recover intact gloshedobin with biological activity via chemical extraction and molecular chaperone-mediated column refolding.

A novel folding-like-refolding strategy harnessing a bichaperone-based refolding cocktail comprising unpurified *E. coli* heat-shock proteins ClpB and DnaK/DnaJ/GrpE (DnaKJE) was first developed. Its efficacy was clearly demonstrated with efficient renaturation of a model protein, heat-denatured malate dehydrogenase (MDH), and further enhanced in the presence of polyethylene glycol (PEG). Prior to confirming the applicability of the proposed folding-like-refolding strategy to gloshedobin IBs, it was first shown that co-expression of ClpB rendered almost complete elimination of gloshedobin truncation products, allowing for the expression of intact gloshedobin (mostly in IB form though) without compromising the expression level. The following purification and refolding of gloshedobin IBs from the cell disruptates was performed based on bichaperone-mediated column refolding scheme using immobilized metal affinity chromatography (IMAC). The new refolding strategy taking advantage of ClpB/DnaKJE was shown to be superior to the

conventional refolding methods in either batch dilution or column refolding mode especially when refolding reaction was attempted at a higher protein concentration. The recovery process for gloshedobin IBs was further integrated through coupling of IMAC protein purification with chemical extraction to overcome the inefficiencies associated with traditional IB recovery method (e.g. requirement of additional unit operations such as mechanical cell disruptions and repeated centrifugations). Polyethyleneimine (PEI) as a new DNA precipitant during chemical extraction was studied. Compared to spermine-induced precipitation reported elsewhere (Choe and Middelberg, 2001b), PEI-mediated chemical extraction provided not only a higher DNA precipitation efficiency at a significantly lower cost but also the obviation of EDTA, which was reported to be essential for chemical extraction (Falconer et al., 1997; 1998). Since the residual PEI was effectively counteracted by addition of Mg^{2+}, the streamlined application of the extraction broth to IMAC protein purification was achieved. This offers the potential for further process intensification.

This study establishes new concepts for IB processing which include i) a folding-like-refolding strategy employing unpurified molecular chaperones to allow direct application of ClpB/DnaKJE bichaperone system, ii) reduction of truncation product through co-expression of molecular chaperone to provide a simple strategy to significantly improve the quality of protein expression, iii) bichaperone-mediated column refolding as an effective tool for refolding-recalcitrant proteins, and iv) PEI-mediated chemical extraction to achieve a more economically viable processing

route for the production of recombinant proteins whose expression is hampered by IB formation.

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Chapter 1
Introduction

1.1 Background

Proteins are fundamental components of all living cells and include many substances, such as enzymes, hormones and antibodies, which have wide applications in the medical, industrial and agricultural fields. The proteins which were previously available only in minute amounts from natural sources can now be produced in huge quantities in the host cells such as *Escherichia coli*. However, the over-expression of recombinant proteins in *E. coli* often leads to their intracellular accumulation as solid aggregates known as inclusion bodies (IBs) which show little (Garcia-Fruitos et al., 2005; 2007; Gonzalez-Montalban et al., 2006; Ventura and Villaverde, 2006) or none (Singh and Panda, 2005; Qoronfleh et al., 2007) biological activity. Nevertheless, the production of recombinant protein in IBs can also be advantageous, since i) a large amount of highly enriched target protein in IB form can be easily separated from other soluble proteins, ii) expressed protein trapped in IBs shows lower degree of degradation, and iii) the IB protein does not have toxic or lethal effects on the host cell (Vinogradov et al., 2003). Therefore, recombinant proteins expressed as IBs in *E. coli* have been most widely used for the commercial production of proteins (Singh and Panda, 2005), although a series of subsequent IB isolation and refolding strategies need to be incorporated in order to produce the soluble and correctly folded products.

The isolation of IB proteins is traditionally carried out by mechanical disruption techniques employing high-pressure homogenization and repeated centrifugation which are laborious and time-consuming (Falconer et al., 1997). A more efficient chemical extraction-based IB recovery method (Falconer direct extraction (FDE)) was developed (Falconer et al., 1997; 1998; 1999) which has the advantage to improve the economic of IB processing by integrating several primary extraction and recovery steps. A DNA precipitant, spermine, was further used to selectively precipitate DNA during FDE (Choe and Middelberg, 2001b) to reduce the high viscosity resulted from the concomitantly released DNA, enabling the direct coupling of following protein purification and refolding. The challenge is thus to convert these inactive, misfolded proteins into soluble and bioactive products (De Bernardez Clark, 2001; Middelberg, 2002).

Protein refolding involves intramolecular interaction which follows first order kinetics and protein aggregation, however, involves intermolecular interaction which is a kinetic process of second or higher order (Qoronfleh et al., 2007). Therefore, protein concentration during refolding must be carefully controlled at relatively low level in order to favor the productive refolding instead of the unproductive aggregation (Singh and Panda, 2005). Many novel high-throughput protein refolding methods have been developed so far for renaturation of IB proteins (Middelberg, 2002; Tsumoto et al., 2003a; Vallejo and Rinas, 2004a; Choe et al., 2006). The simplest refolding procedure is to dilute the concentrated protein-denaturant solution into refolding buffer that

allows the formation of native structure spontaneously. Other techniques for denaturant removal include transfer of the solubilized and unfolded protein to conditions allowing the recovery of enzymatic activity by dialysis or diafiltration systems (Maeda et al., 1995; Varnerin et al., 1998; West et al., 1998; Yoshii et al., 2000). Moreover, many different dilution or dialysis methods along with the use of refolding additives have been reported to further improve the protein refolding yield (Tsumoto et al., 2003a). Buffer exchange for denaturant removal can also be achieved by using chromatographic methods, such as protein refolding based on size exclusion chromatography (SEC) (Batas and Chaudhuri, 1999; Müller and Rinas, 1999), and matrix-assisted protein refolding (Zouhar et al., 1999; Li et al., 2002; Ueda et al., 2003). These processes essentially involve physical separation of the partially folded protein molecules during the buffer exchange, which helps in reducing the protein-protein interaction between these refolding intermediates thereby lowering aggregation and improving recovery of the bioactive product (Gu et al., 2001; Schlegl et al., 2003; Singh and Panda, 2005).

Besides those refolding techniques developed as above, molecular chaperones are extensively studied and have also been applied successfully to refold various proteins both *in vivo* and *in vitro*, marking the beginning of a new era in protein refolding. Molecular chaperones are a group of proteins conserved in all kingdoms, which play an essential role in preventing protein aggregation from various kinds of environmental stress, assisting folding/refolding and mediating degradation of

misfolded proteins (Hartl, 1996; Goloubinoff et al., 1999; Mogk et al., 2002). Among these chaperone systems, the ring-forming AAA+ chaperone Hsp100/ClpB which cooperates with an Hsp70 chaperone system (e.g. the bacterial DnaK/DnaJ/GrpE (DnaKJE) system) was demonstrated to efficiently solubilize and refold aggregated proteins (Mogk et al., 1999; Zolkiewski, 1999; Ziętkiewicz et al., 2004; 2006). The mechanism of the bichaperone system may rely on the extraction of individual polypeptide from the protein aggregate surface by translocation through the ClpB pore (possibly facilitated by DnaKJE), which initiates the unfolding of aggregated proteins. The extracted proteins are then captured and refolded by DnaKJE system to their native structure (Weibezahn et al., 2004b; Shorter and Lindquist, 2005; Haslberger et al., 2007).

1.2 Aims and scope of this project

This PhD work aims to develop an efficient recovery scheme for bacterial IB protein of gloshedobin, a recently isolated thrombin-like enzyme (TLE) from snake venom (Yang et al., 2002), whose expression in *E. coli* was impeded by the occurrence of truncated expression products besides the IB formation. The detailed characteristics of gloshedobin are discussed in the following section. The scope of this work includes: studying the feasibility of unpurified ClpB/DnaKJE-mediated protein refolding on a model protein, heat-denatured malate dehydrogenase (MDH); studying the synergistic coordination of refolding additives and ClpB/DnaKJE bichaperone system on heat-denatured MDH refolding; studying the possibility to reduce the truncated

expression products associated with the full-length gloshedobin; application of the molecular bichaperone system in the column-based refolding of gloshedobin IB proteins; and developing more efficient IB protein recovery strategy based on chemical extraction. The specific objectives of this thesis include:

1. To develop a refolding cocktail comprising unpurified ClpB/DnaKJE bichaperone system for IB protein renaturation. A systematic study on various components in the cocktail which may affect the refolding efficiency is first conducted on heat-denatured model protein, MDH. The synergistic coordination of commonly used refolding additives and ClpB/DnaKJE bichaperone system is investigated.

2. To develop efficient ways for the reduction of truncated expression products, which may complicate the purification and refolding of full-length gloshedobin.

3. To apply the developed refolding cocktail (unpurified ClpB/DnaKJE bichaperone system) to a column-based (IMAC) refolding strategy for the recovery of full-length gloshedobin IBs. Comparisons between the new column refolding strategy and traditionally used refolding methods (such as dilution refolding) are performed.

4. To further intensify the gloshedobin IB recovery strategy through coupling IMAC protein purification with chemical extraction. A more efficient DNA precipitant,

polyethyleneimine (PEI) is studied to reduce the high viscosity of the cell extracts by selectively precipitating the co-released high molecular weight DNA. This may further facilitate the direct coupling of chemical extraction with the subsequent protein purification and refolding steps in a more economically viable way, especially for large-scale protein production.

1.3 Model proteins used in this study

Two model proteins are used in this study, porcine heart MDH and recombinant gloshedobin expressed as IBs in *E. coli*.

1.3.1 MDH

Porcine heart MDH is commercially available from Sigma and commonly utilized elsewhere (Goloubinoff et al., 1999; Watanabe et al., 2002) in the testing of chaperoning activity of molecular chaperones. MDH is a homodimeric protein (molecular weight 35×2 kDa), containing 333 amino acids and an equivalent cofactor (NAD^+/NADH) binding site for each subunit (Sanyal et al., 2002). The subunits are associated in dimer by noncovalent bonds and dissociation of the subunits results in the loss of its activity (Birktoft et al., 1989).

1.3.2 Gloshedobin

Gloshedobin is a kind of TLE recently isolated from the snake venom of *Gloydius shedaoensis* (Yang et al., 2002). Snake venom TLEs are serine proteases affecting

hemostasis and thrombosis (Castro, 2004). More than 40 of them have been isolated and characterized since the coagulation studies on reptilase in 1957 (Blomback et al., 1957; Matsui et al., 2000; Castro, 2004). Unlike thrombins capable of converting fibrinogen into fibrin by splitting off Aα and Bβ chains of fibrinogen to finally release fibrinopeptide A and B, TLEs only cleave Aα chain and release fibrinopeptide A. As TLEs do not activate fibrin-stabilizing factor XIII (in contrast to thrombins), the TLE-induced uncross-linked clot is more susceptible to degradation by plasmin (Markland, 1998; Yuan et al., 2004). These enzymes can be potentially useful for the treatment of blood clotting disorders through their anti-coagulant action (Matsui et al., 2000). However, due to the difficulties faced in their separation and purification along with the limited supply of the natural snake venom, it is often difficult to obtain large quantity for studies and clinical applications (Yang et al., 2002). The production of these enzymes by genetic engineering is therefore the best alternative.

Due to the ease of handling and relatively high expression level, *E. coli* was initially selected for the expression of gloshedobin. The expression of unmodified gloshedobin (without fusion tag) was however unsuccessful (Yang et al., 2002), presumably due to the formation of stable secondary structure at the translation initiation region of its mRNA (Maeda et al., 1991; Yuan et al., 2004). The first successful expression of gloshedobin was reported in methylotrophic yeast *Pichia pastoris* and 10 mg/L of target protein was expressed in soluble form, exhibiting amidolytic activity of 31.2 U/mg (Yang et al., 2002). The low expression level associated with the yeast system,

however, requires further optimization for efficient gloshedobin expression. The plasmid pET-32a(+), designed for cloning and high-level expression of peptide sequences fused with the 109aa Trx•Tag™ thioredoxin protein and a 6×His-tags (LaVallie et al., 1993), was thus next used for gloshedobin expression in *E. coli* (Yang et al., 2003a). With this fusion construct (i.e. thioredoxin-6×His-tag-gloshedobin), gloshedobin was successfully overexpressed in *E. coli* BL21(DE3), but mostly as IBs largely contaminated with a major truncation product (Yang et al., 2003a; 2003b) probably arising from proteolytic degradation or secondary site translation initiation (Halling and Smith, 1985; Preibisch et al., 1988; Govind et al., 2001). Despite the presence of some proteins whose truncated forms were found to exhibit biological activities, truncated gloshedobin was inactive. Furthermore, the purification of intact gloshedobin by IMAC was hampered by the presence of significant amount of an unwanted product associated with the truncation (i.e. thioredoxin-6×His-tag containing N-terminal fraction of intact gloshedobin). Efficient strategies thus need to be developed for i) reduction of truncated expression product associated with the full-length gloshedobin expression, and ii) gloshedobin IB protein refolding.

Provided in the next chapter is the review of frequently used IB processing schemes. These techniques as well as the operating principles inspired our current research work.

Chapter 2
Literature review

Proteins are polymeric molecules composed of amino acid monomers joined together by peptide bonds. Examples of proteins include whole classes of important molecules, such as enzymes, hormones and antibodies, that are necessary for the proper functioning of an organism and have numerous applications in medical, industrial and agricultural fields. *Escherichia coli* have been most widely used for the production of recombinant proteins for commercial purposes (Baneyx, 1999; Swartz, 2001). However, high-level expression of recombinant proteins in *E. coli* often results in them accumulating *in vivo* as insoluble aggregates known as inclusion bodies (IBs) (Fahnert et al., 2004), thus requiring further solubilization, refolding and purification procedures to achieve functionally active products (Singh and Panda, 2005). In this chapter, several common IB recovery processes are reviewed.

2.1 Recombinant DNA and gene cloning

Recombinant DNA is DNA that has been created artificially through the combination or insertion of one or more DNA strands, allowing the creation of DNA sequences which would not normally occur (Berg et al., 2002). In 1973, two scientists, Herbert Boyer and Stanley Cohen, came together and laid the groundwork for recombinant DNA technology (Cohen et al., 1973), which initiated what is now the multibillion-dollar biotechnology industry. A circular piece of DNA called a plasmid

is first removed from a bacterial cell and then special proteins (restriction endonuclease or restriction enzyme) are used to cut the plasmid ring at specific sites. The host DNA that produces the wanted protein is inserted into the opened plasmid DNA ring and DNA ligase helps to connect the two fragments into a closed plasmid. The circular plasmid DNA that contains the host gene is inserted back into a bacteria cell in which it can multiply to make several copies of the wanted gene. Finally, the gene can be turned on in the cell to produce target proteins. Some of the basic techniques used such as Restriction Enzymes [Nobel Prize 1978], DNA Sequencing [Nobel Prize 1980], and Polymerase Chain Reaction (PCR) [Nobel Prize 1993] are milestones in the history of molecular biology.

2.2 Overview of IB processing schemes

2.2.1 IB formation

Transcription and translation are tightly coupled in the crowded milieu of the *E. coli* cytoplasm and it is reported that one protein chain is released from the ribosome every 35 seconds (Lorimer, 1996), resulting in an environment where macromolecule concentrations even reach up to 300-400 mg/mL. Correct protein folding and rapid production of recombinant proteins is thus an extraordinary challenge. The failure to rapidly reach a native conformation for a heterologous protein can lead to its partial or complete deposition into insoluble aggregates known as IBs (Betts and King, 1999). IBs are characterized as large, spherical particles which are clearly separated from the cytoplasm. The target protein typically accounts for 80-95% of the IB material and is

usually contaminated with several impurities such as host proteins (RNA polymerase, outer membrane proteins), ribosomal components and circular and nicked forms of plasmid DNA. In addition, IBs might contain the small heat-shock proteins (sHsps) IbpA and IbpB (Valax and Georgiou, 1993). Proteins trapped in IBs generally show little (Garcia-Fruitos et al., 2005; 2007; Gonzalez-Montalban et al., 2006; Ventura and Villaverde, 2006) or no (Singh and Panda, 2005; Qoronfleh et al., 2007) biological activity. Nevertheless, the production of recombinant protein in IBs can also be advantageous, since i) a large amount of highly enriched target protein in IB form can be easily separated from other soluble proteins, ii) expressed protein trapped in IBs shows lower degree of degradation, and iii) the IB protein does not have toxic or lethal effects on the host cell (Vinogradov et al., 2003). Therefore, recombinant proteins expressed as IBs in *E. coli* have been most widely used for the commercial production of proteins (Singh and Panda, 2005), although a series of IB isolation and refolding strategies need to be included to generate the biologically active products.

2.2.2 Traditional methods for IB recovery

The general strategy used to recover active protein from IBs involves four steps: Cell disruption and IB isolation; washing of IB proteins; solubilization of the aggregated proteins; and refolding of the solubilized proteins (De Bernardez Clark, 2001; Choe et al., 2006). Traditionally, cells containing IBs are disrupted by ultrasonication for small, French press for medium and high-pressure homogenization for large-scale protein production (Vallejo and Rinas, 2004a). The resulting suspension is treated by either

low-speed centrifugation or filtration to remove soluble proteins from the particulate containing the IBs. Methods used to solubilize prokaryotic membrane proteins can be adapted to wash IBs, especially to remove membrane-associated proteins released from cell envelope upon cell breakage, which is known to be the most difficult contaminants to eliminate in IB preparations (Rinas and Bailey, 1992). The commonly employed washing steps may utilize EDTA and low concentrations of denaturants with or without weak detergents, such as Triton X-100, deoxycholate and octylglucoside (Lilie et al., 1998; Middelberg, 2002).

After isolation, IB proteins are normally solubilized using high concentration of chaotropic agents such as guanidine hydrochloride (GdnHCl) and urea, renaturation is then accomplished by the removal of excess denaturants by either dilution or a buffer-exchange step, such as dialysis or diafiltration. Because of its simplicity, dilution of the solubilized proteins directly into renaturation buffer is the most commonly used method in small-scale refolding studies. Protein refolding involves intramolecular interaction which follows first order kinetics and protein aggregation, however, involves intermolecular interaction which is a kinetic process of second or higher order (Qoronfleh et al., 2007). Therefore, protein concentration during dilution refolding must be carefully controlled at relatively low level (usually 10-100 μg/mL in final concentration) in order to favor the productive refolding instead of the unproductive aggregation (Singh and Panda, 2005). This results in dilution refolding being time and cost inefficient due to the need for large refolding vessels and

additional concentration steps after protein renaturation (De Bernardez Clark, 2001). Dilution refolding can also be accomplished in multiple steps, known as 'pulse renaturation', in which aliquots of denatured protein are added to renaturation buffer at successive time intervals, or semi-continuously via fed-batch addition (Katoh and Katoh, 2000; Vallejo and Rinas, 2004a). By choosing the suitable protein concentration and time of successive additions of solubilzed proteins, relatively large quantities of proteins can be refolded in the same buffer tank, helping reduce the volume of buffer needed and improve the overall performance of the refolding process (Singh and Panda, 2005). In addition, buffer exchange to remove high concentration of denaturant can be utilized for protein refolding through diafiltration or dialysis with ultrafiltration membranes. However, renaturation yields using these membrane-based methods can be significantly affected by protein binding to the membranes (Maeda et al., 1995; Varnerin et al., 1998). Significant losses of unfolded proteins may occur via their transmission through the membrane fabricated with typical hydrophobic membrane material, such as polyether sulfone (West et al., 1998; Yoshii et al., 2000).

The composition of the refolding buffer is strongly protein-dependent and the choice of pH and redox reagents has the largest impact for a refolding process (Qoronfleh et al., 2007). Usually, the pH of a solution ranging from 4-9 is selected for refolding screens, which should also be more than 1-2 pH units away from the isoelectric point of target protein in order to minimize the aggregation formation. For proteins

containing disulfide bonds derived from the coupling of thiol groups, it is recommended that alkaline conditions (pH 7.5-10) are used for initial refolding, since the thiol reactivity will be lowered at pH below 7 (Gilbert et al., 1990). For non-disulfide or thiol-containing proteins, addition of reducing agents like dithiothreitol (DTT), β-mercaptoethanol or cysteine at a concentration of 1-5 mM in the refolding buffer helps to maintain cysteine residues in a reduced state and thus prevents non-native intra- or inter-disulfide bond formation during the refolding process (Fischer et al., 1993). For disulfide-containing proteins, a more elaborate refolding environment for the correct formation of disulfide bonds is required. A mixture of low molecular weight thiol and disulfide agents, such as reduced and oxidized glutathione (GSH/GSSG), cysteamine/cystamine, or cysteine/cystine are usually added to the refolding buffer to allow the correct disulfide bonds shuffling and formation (De Bernardez Clark, 2001; Vallejo and Rinas, 2004a). A total concentration of 5-15 mM with a molar ratio of reduced to oxidized compounds of 1:1 to 5:1 are usually tested for the initial protein refolding screens (Rudolph and Lilie, 1996; Vallejo and Rinas, 2004a).

During the process of protein refolding, the formation of incorrectly folded species, in particular aggregates, is usually the cause of decreased renaturation yield (De Bernardez Clark, 2001). The use of refolding additives to suppress aggregation is proved to be a very efficient strategy to inhibit the intermolecular interactions leading to aggregation and help in improving the yield of bioactive proteins (De Bernardez

Clark, 1998; 2001). Numerous kinds of additives, such as detergents, amino acids, salts, divalent cations, surfactants, polymers, polyols and sugars (Yasuda et al., 1998; De Bernardez Clark et al., 1999; Singh and Panda, 2005) have been tested and shown to be effective in the prevention of aggregation. These additives may influence both the solubility and stability of the unfolded protein, folding intermediate and the fully folded protein (De Bernardez Clark, 2001; Singh and Panda, 2005). Among them, L-arginine (usually 0.4-1 M) is the most commonly used additives and the positive effects have been demonstrated for the refolding of many kinds of proteins (Arora and Khanna, 1996; Arakawa and Tsumoto, 2003; Umetsu et al., 2003). Presumably by shielding hydrophobic regions of partially folded protein in the presence of L-arginine, the solubility of these refolding intermediates is enhanced and thus the formation of aggregates is impeded (De Bernardez Clark et al., 1999; Umetsu et al., 2003).

2.3 Principles of chemical extraction

As introduced in section 2.2, the recovery of recombinant IBs is traditionally achieved using mechanical cell disruption techniques (Falconer et al., 1997; De Bernardez Clark, 2001). The process is inefficient mainly due to the nature of the flowsheet, which is characterized by multiple unit operations operated repeatedly, and by the need to separate similarly sized cell debris and IBs by centrifugation (Choe et al., 2002). Falconer direct extraction (FDE) (Falconer et al., 1997; 1998; 1999), a chemical extraction method based on a combination of urea and EDTA, is a very attractive alternative way to the traditional IB recovery strategy. Additional chemicals

are not required in this extraction method and only the chemicals normally used for IB dissolution and protein refolding are present (Choe and Middelberg, 2001a).

The release of IB proteins (cytoplasmic Long-R3-IGF-I) by chemical extraction was first shown to be at an equivalent level to mechanical disruption at a lab scale (Falconer et al., 1998). Moreover, the extraction efficiency was not compromised by high density cell suspension of *E. coli* (up to OD_{600} = 160) and proved highly efficient (>90%) in extracting and solubilizing of His-tagged recombinant viral coat IB protein (Choe and Middelberg, 2001a). However, concomitant release of high molecular weight DNA during the extraction produced a highly viscous non-Newtonian post-extraction mixture (Choe and Middelberg, 2001b), posing a significant challenge to downstream operations (Fernández-Lahore et al., 1999). According to Choe and Middelberg (2001b), spermine was successfully used as a DNA precipitant to selectively precipitate the contaminant DNA associated with chemical extraction, allowing the direct coupling of chemical extraction with following primary capture methods. A new strategy for IB recovery which contains only 3 steps: chemical extraction, low-speed centrifugation and immobilized metal affinity chromatography (IMAC)-based expanded bed adsorption (EBA) was thus developed (Choe et al., 2002), leading to a very promising foreground for simple, efficient and cost-effective recovery of IB proteins.

2.4 Protein refolding by chromatographic methods

In recent years, many novel and high-throughput protein refolding methods based on chromatography have been developed for recovery of IB proteins. Among them, size exclusion chromatography (SEC) and matrix-assisted chromatography are the most widely studied tools for better protein recovery from solubilized IBs (Batas and Chaudhuri, 1996; Li et al., 2004). Since these processes essentially involve physical separation of partially folded protein molecules, the interactions between the refolding intermediates are prevented or at least minimized during the buffer exchange step. The refolding yield can thus be significantly increased especially when refolding was conducted at high protein concentration compared with traditionally used refolding procedures, such as dilution as mentioned above (Li et al., 2002; Lanckriet and Middelberg, 2003; Langenhof et al., 2005).

2.4.1 Size exclusion chromatography

SEC is a chromatographic method in which particles (e.g. proteins) of different sizes or hydrodynamic volumes elute through a stationary phase at different rates to realize the separation of each component. For a typical SEC refolding, solubilized IBs (in high concentration of denaturant) are first loaded into the SEC column which is pre-equilibrated with refolding buffer. Protein refolding and elution are then achieved by passing the column with the same buffer (Batas and Chaudhuri, 1996; 1999; Müller and Rinas, 1999; Fahey and Chaudhuri, 2000). During this refolding process, the formation of aggregates is greatly prevented since SEC restricts the available pore

volume for various protein forms in the gel matrix, thus facilitating the separation of correctly folded and aggregated species (Righetti and Verzola, 2001; Li et al., 2004). In addition, the delayed running front of the denaturant may re-solubilize the formed aggregates, giving the solubilized aggregates another opportunity to fold correctly (De Bernardez Clark et al., 1999). Therefore, the refolded protein in the eluate fraction has a significant higher concentration compared with normal dilution refolding process (Müller and Rinas, 1999). A SEC offers multiple advantages of buffer exchange, protein refolding and separation of monomer from aggregates, thus it will be an ideal method for the process intensification (De Bernardez Clark, 1999; Choe et al., 2006).

SEC refolding of bovine carbonic anhydrase B (CAB) was performed with special emphasis on media selection, sample application and residence time of the protein in column, showing that gels with higher resolution, using low protein concentrations in a larger application volume and high residence time are key factors to facilitate the refolding process to achieve highest activity recovery (Gu et al., 2003). The effects of column dimension on refolding yield, which is important for scaling up a process, were studied for b-lactamase refolding and the results demonstrated that refolding process was not very sensitive to changes in the column diameter, but a reduction in the column length gave rise to a poorer refolding performance (Harrowing and Chaudhuri, 2003). Besides the parameters that are mentioned above, it is also suspected that aggregation could occur immediately after injecting denatured protein sample to the refolding buffer (mobile phase). That is, during the passage of the

sample from injector to the column, aggregates could be possibly already formed irreversibly and could not properly refold later after entering the SEC column. Therefore, a 'chaperone solvent plug' strategy was developed by Liu and Chang (2003) to inhibit the formation of aggregate before the sample entered the column. This chaperone solvent plug was designed to escort the denatured protein (injected sample) from the injector into column under 'aggregation inhibition condition', thus providing the possibility to enhance protein refolding yield.

Improvement of the SEC protein refolding has also been achieved by harnessing a decreasing urea gradient with an increasing pH gradient on a Superdex 30 column for the refolding of recombinant single-chain antibody Fv fragments (scFvs) (Gu et al., 2002). One major advantage of using SEC for refolding, in this particular mode, is that the rate of denaturant removal and the change in pH can be carefully controlled by adjusting the gradient slope and the elution flow rate of the column. During the course of elution, the zone of denatured protein moved down the column at a speed approximately threefold higher than that of the denaturant. This means that the protein sample will gradually pass through areas of increasingly lower denaturant concentrations and higher pH, which promote the refolding of denatured protein into its native conformation (Gu et al., 2002).

As the batch-mode chromatography would be the limiting step in a column-based refolding downstream process, the application of SEC refolding in continuous

refolding system using pressurized continuous annular chromatography (P-CAC) was thus developed (Schlegl et al., 2003) for the refolding of bovine α-lactalbumin as well as a recombinant therapeutic protein (Schlegl et al., 2005a; 2005b). Another detailed study comparing batch dilution and SEC refolding of lysozyme using P-CAC placed special emphasis on the transferability of processes from batch columns to P-CAC and showed that the P-CAC elution profile was similar to that of a stationary column, making the scale-up and translation to P-CAC relatively simple (Lanckriet and Middelberg, 2004). More recently, simulated moving bed (SMB) chromatography using four SEC columns was also successfully developed for the renaturation of lysozyme as a model compound (Park et al., 2005; 2006), further broadening the application of SEC protein refolding in continuous mode.

2.4.2 Matrix-assisted chromatography

Adsorbing the solubilized and unfolded IB molecules of interest to a solid matrix prior to the changing from denaturing to native buffer conditions is another approach to avoid unwanted intermolecular interactions between these aggregation-prone folding intermediates (Singh and Panda, 2005). Simultaneous buffer exchange, refolding and purification of solubilized IB proteins are achieved by harnessing this kind of adsorption chromatography. Several combinations of the binding motives and matrices have been developed thus far for the binding of unfolded protein molecules to the solid support.

2.4.2.1 Ion exchange chromatography

Ion exchange chromatography (IEC) separates molecules on the basis of differences in their net surface charge. IEC takes advantage of the fact that the relationship between net surface charge and pH is unique for a specific protein (Creighton, 1986; Suttnar et al., 1994; Li et al., 2002; 2003). In an IEC separation, reversible interactions between charged molecules and oppositely charged IEC media are controlled in order to favor binding or elution of specific molecules and achieve separation. Since the first introduction of this adsorptive process through attaching the unfolded protein onto an ion exchange resin by Creighton (1986), many progresses have been achieved to improve the overall efficiency for protein refolding.

A two-buffer system used to improve activity yield and mass recovery was demonstrated by Li et al. (2002), in which a descending urea concentration gradient was introduced in parallel with an increasing ionic strength gradient. This allowed simultaneous structural rearrangement and elution of denatured target protein during its migration along the column. Refolding recovery at high protein concentration was further improved by another dual gradient IEC process wherein denatured hen egg-white lysozyme (a kind of disulfide-containing protein) bound to the IEC matrix was gently eluted with refolding buffer employing decreasing urea (from 6 to 1 M) and increasing pH (from 6.2 to 10) gradients (Gu et al., 2002). This is in line with the finding that pH of the refolding buffer exhibited critical effects on the formation of disulfide bonds (Gilbert et al., 1990). By avoiding high salt concentrations for elution,

and thus avoiding stronger hydrophobic interactions that might lead to aggregation of the folding intermediates, refolding yield was significantly improved.

Another important improvement to IEC refolding is the application of EBA technology, which is suitable for dealing with crude samples (Cho et al., 2001; Jin et al., 2005). An ion exchange matrix is used to adsorb a recombinant protein directly from cell homogenates, the following washing of cell debris and unbound components and exchanging the buffer to initiate refolding were all performed in expanded bed mode. Using this approach for the processing of non-clarified solutions, the target protein can be both purified and refolded on the same column, resulting in a substantial shortcut to the downstream steps of IB processing.

2.4.2.2 Immobilized metal affinity chromatography

IMAC has opened up new prospects for efficient simultaneous purification and refolding of recombinant proteins equipped with engineered N- or C-terminal polyhistidine tag (Poly-His-tag) (Ueda et al., 2003). The technique is based on the one-point immobilization of proteins onto a solid support through selective interaction between the electron donors groups on the proteins and on the transition-metal ions (Cu^{2+}, Ni^{2+}, Zn^{2+} or Co^{2+}) which are loaded on chelating ligands coupled to a solid support. Poly-His-tag form high-affinity complexes with immobilized divalent metal ions even in the presence of high concentration of chaotropic agents, thereby allowing the simultaneous isolation and refolding of tagged target proteins. A stepwise or

gradient decrease of denaturant concentration will induce the protein refolding and elution is often achieved by increasing the imidazole concentration or by using a decreasing pH-gradient (Ueda et al., 2003). The performance of IMAC to capture and release of proteins is a complex function of a large number of variables. The effects of the binding mechanisms can be evaluated by varying the pH and salt concentration of the adsorption solution and are also governed by a number of variables such as the nature of the chelating ligand, metal-ion, and surface amino acid composition (Sulkowski, 1985; Wong et al., 1991; Porath, 1992; Ueda et al., 2003).

IMAC-driven affinity capture followed by successful on-column refolding was reported for various kinds of proteins (Rogl et al., 1998; Glynou et al., 2003; Lemercier et al., 2003; Schauer et al., 2003; Jungbauer et al., 2004; Vincent et al., 2004). The aggregation of exopolyphosphatase during IMAC refolding was limited by including a suitable amount of detergent in the refolding buffer (Lemercier et al., 2003). Batch adsorption onto the solid support was performed for aequorin to avoid protein crowding at the top of the column, thus preventing aggregation due to molecule interactions (Glynou et al., 2003). A novel iterative column-based refolding process was recently developed for high concentration protein refolding (Hutchinson and Chase 2006a). Some reports suggested that only correctly folded protein molecules can be eluted after the adsorptive refolding, and these misfolded and aggregated species were still retained on the resin, accounting for the low refolding yield obtained (Hutchinson and Chase, 2006a; 2006b). In this iterative mode of

refolding strategy, the retained protein aggregates after elution of correctly folded proteins were first re-denatured in high concentration denaturant and then the refolding step was allowed to repeat. The final refolding yield was demonstrated to be greatly increased (Hutchinson and Chase 2006a).

IMAC coupled with EBA (Clemmitt and Chase, 2000) has further broadened the application of IMAC strategy in the area of protein purification and refolding. IBs obtained following cell disruption are dissolved by a denaturant and then directly fed to an EBA column to capture denatured protein on the solid-phase. The bound proteins are refolded following the gradual removal of denaturant and then eluted. In this way, several sequential steps of IB dissolution, cell debris removal, denaturant removal, oxidative refolding, and monomer fractionation could be integrated.

Although IMAC-based refolding has distinct advantages over standard refolding techniques (Ueda, 2003), it is necessary to take into account some disadvantages (Gutiérrez et al., 2007). i) IMAC refolding is mostly limited to proteins in which the inclusion of Poly-His-tag does not interfere with the formation of their native configuration (Jungbauer et al., 2004). Nevertheless, an octa-repeat sequence in the bovine prion protein refolding was successfully demonstrated to be a natural tag onto Ni-NTA using its metal-binding properties (Yin et al., 2003), achieving a production process of tag-less native protein, ii) If redox systems are required for proper refolding, they have to be adjusted very carefully in IMAC to avoid the reduction of

metal ions. If reducing agents are necessary for solubilization of IBs, a buffer exchange to remove the reducing agents has to be performed before adsorption onto the IMAC column (Hutchinson and Chase, 2006b; Gutiérrez et al., 2007). Alternative IMAC resins which may be more resistant to metal ion reduction would expand the range of operating conditions, and iii) There are unspecific bindings through ionic interactions. Higher salt concentration leads to an increase in hydrophobic forces favoring the aggregation of folding intermediates; therefore, binding, washing, and refolding conditions require distinct optimization.

2.4.2.3 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) has also been successfully used for protein refolding with concomitant removal of contaminating proteins during the renaturation process (Geng and Quan, 2002; Bai et al., 2003; Gong et al., 2004; Wang et al., 2004). HIC is a kind of chromatography technique in which reversed-phase packings are used to separate molecules by virtue of the interactions between their hydrophobic moieties and the hydrophobic sites on the solid support. High salt concentrations are used in the mobile phase and separations are effected by changing the salt concentration. The refolding of lysozyme, bovine serum albumin, α-amylase and recombinant γ-interferon were described by Geng and Chang (1992) by using a silica-based material crafted with polyethylene glycol (PEG) with a hydrophobic end group. A dual-gradient HIC strategy was further developed by Wang et al. (2006), consisting of decreasing GdnHCl concentration and increasing PEG concentration.

The refolding yield of denatured consensus interferon (C-IFN) was demonstrated to be greatly increased in the gradient HIC process with the presence of PEG. Mobile phase composition, gradient mode, and flow rate were found to be key factors in the mass and bioactivity recovery (Xiao et al., 2006; Wu et al., 2007).

Different from the other above-mentioned chromatographic methods, there is usually no requirement for the presence of typical refolding additives such as L-arginine during the HIC refolding (Bai et al., 2003). Moreover, refolding of the disulfide-containing protein proinsulin was even successfully conducted in the absence of a redox system in the mobile phase (Bai et al., 2003). It has been proposed that hydrophobic regions of the protein that adsorb to the HIC matrix form microdomains around which native structure elements can develop. During migration through the column, the protein will also pass through several steps of adsorption and desorption, controlled by the salt concentration and hydrophobicity of the intermediates, resulting finally in the formation of the native structure and better protein refolding yield (Geng and Quan, 2002; Vallejo and Rinas, 2004a).

2.5 Protein refolding by hydrostatic pressure

The use of pressurized tanks (150-200 MPa) as refolding reactors is suggested to facilitate protein refolding through promoting the folding mechanism while disfavoring aggregation (Gorovits and Horowitz, 1998; Foguel et al., 1999; Jungbauer and Kaar, 2007). For a typical hydrophobic pressure-mediated protein refolding

process, aggregates evolving from agitation, chaotrope-induced aggregates or bacterial IBs were first subjected to high-pressure treatment at non-denaturing GdnHCl concentration to unfold the protein aggregates (St. John et al., 1999; 2002). The gradually decreased pressure then prompted the unfolded proteins to recover their native structures. As high pressure disfavors interactions that lead to aggregation during protein refolding, aggregated proteins can be disaggregated and refolded at relatively high concentrations (up to 8.7 mg/mL) (St. John et al., 1999; 2001). Additional pressure treatment of IBs for a longer period of time led to significantly increased amount of active proteins. For disulfide-containing proteins, it is suggested that even though the use of high hydrostatic pressure by itself does not provide enough energy to break a covalent bond, correct formation of the protein's disulfide bonds can still occur because the high pressure process is compatible with standard redox reagents that allow shuffling of disulfide bonds (St. John et al., 2002; Seefeldt et al., 2004). Therefore, combining pressure-induced disaggregation and refolding with manipulation of GdnHCl concentrations and glutathione redox ratios would be a very efficient way for the refolding of protein from disulfide-linked aggregates. Other variables which may affect the refolding efficiency include: pH, ionic strength and temperature. By systematically changing formulation variables, optimal solution conditions for protein refolding can be determined (Phelps and Hesterberg, 2007). This method permits refolding reactions at higher concentration therefore reduces processing volumes and diminishes the need of chaotropes significantly.

2.6 Protein refolding by molecular chaperones

2.6.1 What are molecular chaperones?

Excessive protein misfolding and aggregation can be fatal. It may result from premature termination of translation, failure of a newly synthesized chain to reach a correct conformation or from loss of structure triggered by environmental stress. To deal with this situation, both prokaryotes and eukaryotes have evolved similar conserved molecular chaperone systems which can help in the proper *de novo* folding, refolding of partially folded proteins, dissolving aggregates and disposing irretrievably damaged proteins (Baneyx and Mujacic, 2004). Although these molecular chaperones are constitutively expressed under balanced growth conditions, many of them are upregulated upon heat-shock or other insults that increase cellular protein misfolding (including heterologous protein expression) and are therefore classified as stress or heat-shock proteins (Hsps), e.g. Hsp100 (ClpB, ClpA, ClpX), Hsp90 (HtpG), Hsp70 (DnaK), Hsp60 (GroEL), and small heat-shock proteins or sHsps (IbpA, IbpB) (*E. coli* chaperones in parenthesis) (Goloubinoff et al., 1999).

The investigation of chaperones has a long history (Ellis, 1996). The term 'molecular chaperone' was invented and appeared in 1978 from the discovering of the ability of nuclear protein called nucleoplasmin to prevent aggregation of folded histone proteins with DNA during the assembly of Nucleosomes (Laskey et al., 1978). With the finding of proteins that mediated the post-translational assembly of protein complex in 1987 (Ellis, 1987), the term was extended. In 1988, it was realized that similar

proteins mediated this process in both prokaryotes and eukaryotes (Hemmingsen et al., 1988). The details of this process were determined in 1989, when the ATP-dependent protein folding was demonstrated *in vitro* (Goloubinoff et al., 1989). With the continuous progresses, various kinds of molecular chaperones were discovered. Based on their different actions to aid protein folding, molecular chaperones can be divided into three functional subclasses (Figure 2.1) (Baneyx and Mujacic, 2004). 'Folding' chaperones (e.g. Trigger factor (TF), DnaK and GroEL) rely on ATP-driven conformational changes to mediate the net refolding/unfolding of their substrates (Hartl and Hayer-Hartl, 2002). 'Holding' chaperones (e.g. IbpB) maintain partially folded proteins on their surface to await availability of folding chaperones upon stress abatement (Veinger et al., 1998; Shearstone and Baneyx, 1999; Narberhaus, 2002). Finally, the 'disaggregating' chaperone of ClpB promotes the solubilization of proteins that have become aggregates as a result of stress (Zolkiewski, 1999; Diamant et al., 2000; Lee et al., 2003; Mogk et al., 2003; Schlieker et al., 2004).

As shown in Figure 2.1, TF, DnaK/DnaJ/GrpE (DnaKJE) and GroEL/GroES are three chaperone systems involved in the *de novo* folding in *E. coli* cytoplasm (Hartl, 1996). TF and DnaKJE have the overlapped substrate pool (Deuerling et al., 2003), except that TF is supposed to interact with short nascent chains or newly synthesized proteins while longer nascent chains may be captured by DnaK. DnaK is targeted to high-affinity sites by its co-chaperone DnaJ, which increases the ATP consumption or

hydrolysis and activates tight binding of DnaK to substrate. The release of substrate is controlled by GrpE who catalyzes ADP/ATP exchange. Once released, a newly synthesized protein may reach native conformation, undergo additional cycles of interactions with DnaK (and possibly TF) until it folds, or be transferred to the downstream GroEL/GroES system which handles about 10% of newly synthesized host proteins (Ewalt et al., 1997).

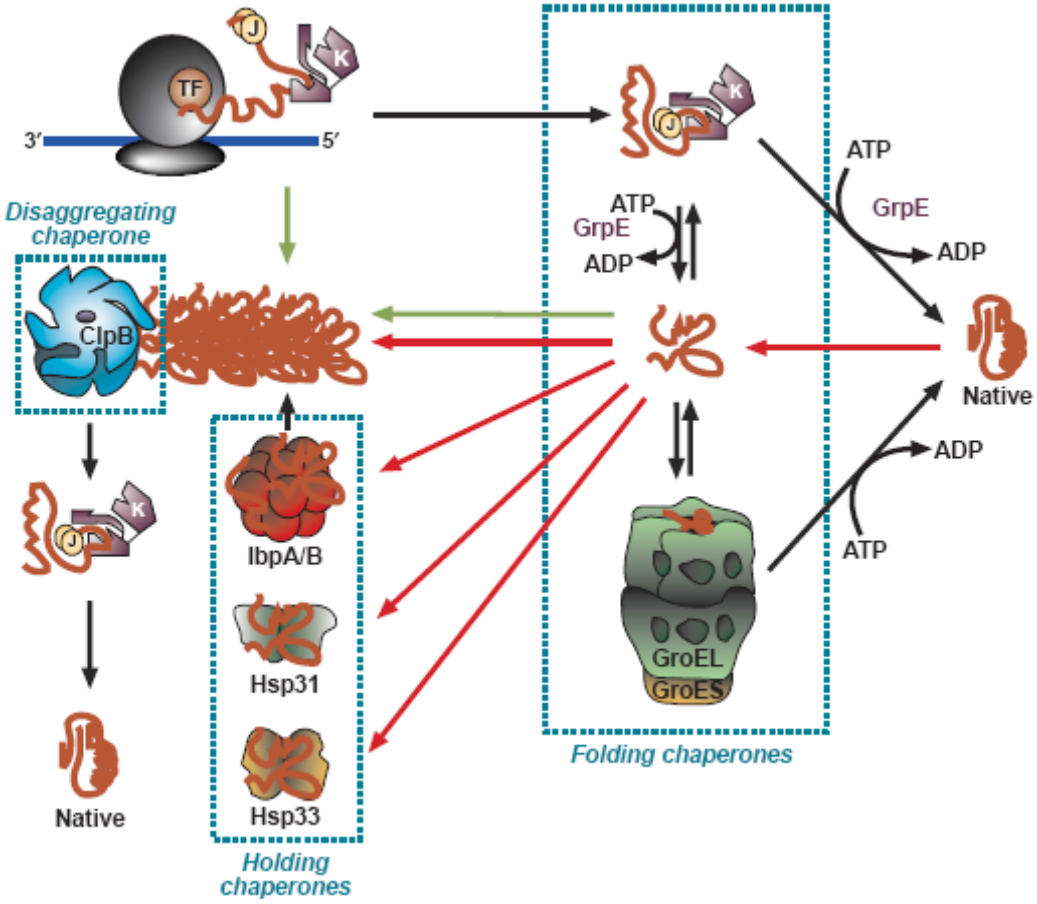


Figure 2.1 Chaperone-assisted protein folding in the cytoplasm of *E. coli* (Baneyx and Mujacic, 2004).

The bacterial IbpA and IbpB, which belong to sHsp family, are the most characterized holding chaperones (holdases). The increased expression of sHsp within cells may aid

mammalian and bacterial cells to resist heat, cold, and oxidant stresses (Mehlen et al., 1993; Van den Ijssel et al., 1994; Yei et al., 1997; Sato et al., 1999). Furthermore, it is demonstrated that the large oligomers formed by IbpB rely on temperature-driven exposure of structured hydrophobic domains to capture unfolded intermediates produced under heat-shock conditions, thereby leading to accumulation of misfolded intermediates which are protected from irreversible aggregation (Chang et al., 1996; Lee et al., 1997). This reservoir effect permits the misfolded proteins to stay in a folding-competent state for an extended period of time, and after the restoration of physiological condition, IbpB-bound species are engaged by DnaK system, and if necessary transferred to GroEL, for refolding (Ehrnsperger et al., 1997; Veinger et al., 1998).

When folding and holding chaperones fail to abrogate protein aggregation under severe or prolonged stress conditions, *E. coli* possesses a third line of defense to manage the deleterious effects associated with misfolding: active aggregate solubilization. Disaggregation is performed by ClpB, which belongs to the AAA+ (ATPase associated with various cellular activities) superfamily of proteins. AAA+ proteins self-assemble into barrel-shaped hexamer structures and use the energy derived from ATP hydrolysis to restructure their target substrates (Vale, 2000). Each ClpB protomer is composed of an N-terminal domain and two AAA+ domains separated by a coiled-coil middle domain (M-domain) which is essential for ClpB to realize hexamerization and chaperone function (Kedzierska et al., 2003; Lee et al.,

2003). Recent structural data of *Thermus thermophilus* ClpB (TClpB) showed that the hexamer of this protein forms a two tier-ring structure with a 16-Å hole in the top ring and six smaller openings on the lateral surface of the molecules (Lee et al., 2003). ClpB-mediated disaggregation is facilitated by intercalation of sHsps within the aggregates (Mogk et al., 2003) but renaturation requires the transfer of partially folded substrate from ClpB to DnaKJE chaperone system (Goloubinoff et al., 1999; Zolkiewski, 1999).

2.6.2 ClpB/DnaKJE, the most efficient bichaperone machine in protein disaggregation and renaturation

Protein aggregates formed under severe heat-shock conditions were not eliminated in the presence of ClpB within the cells (Laskowska et al., 1996; Mogk et al., 1999). Other *in vitro* studies showed that ClpB alone was not sufficient for disaggregation of proteins following their subjection to thermal stress. Instead, ClpB was reported to participate in multi-chaperone system which could efficiently inhibit and reverse protein aggregation (Glover and Lindquist, 1998; Goloubinoff et al., 1999; Zolkiewski, 1999). For instance, ClpB cooperates with DnaKJE chaperone system to efficiently solubilize protein aggregates and refold them into active proteins (Goloubinoff et al., 1999; Mogk et al., 1999; Zolkiewski, 1999; Ziętkiewicz et al., 2004; 2006). So far, the ClpB/DnaKJE is suggested to be the most efficient and promising bichaperone machine in protein disaggregation and renaturation.

The mechanism of ClpB/DnaKJE-mediated protein refolding is not fully understood yet and two models have been proposed (Figure 2.2) (Weibezahn et al., 2004a; Shorter and Lindquist, 2005). Firstly, ClpB possibly remodels the aggregated protein surface via crowbar activity to facilitate DnaKJE binding, which will in turn allow liberation of polypeptide by ClpB crowbar activity. The extracted polypeptide would then be refolded by DnaKJE. An alternative hypothesis is that aggregated proteins would be progressively fragmented into smaller pieces by ClpB via crowbar activity till individual polypeptides are released. The polypeptides are then refolded either spontaneously or with the aid of DnaKJE (Glover et al., 1998). The second model is proposed based on the translocation activity of ClpB, in which DnaKJE helps the extraction of individual polypeptides from the aggregate surface by translocation through the ClpB central pore. The extracted protein is then refolded and prevented from reaggregating by DnaKJE (Schlieker et al., 2004; Weibezahn et al., 2004b; 2005; Haslberger et al., 2007). It is also suggested that these two models need not be mutually exclusive since they may work sequentially or simultaneously in a protein-dependent manner (Shorter and Lindquist, 2005).

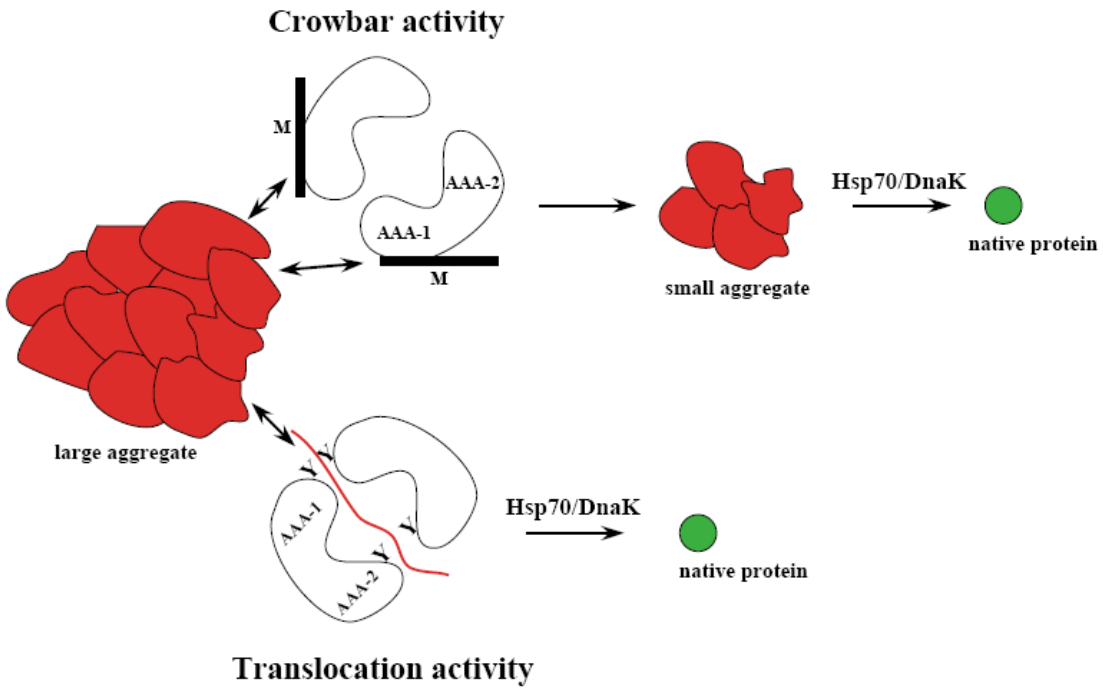


Figure 2.2 Potential mechanisms of protein disaggregation by ClpB/DnaKJE bichaperone system (Weibezahn et al., 2004a).

2.6.3 Application of artificial chaperones

Although natural chaperones have been applied successfully to refold various proteins *in vitro*, their routine application is still limited by their cost, the relatively high chaperone concentration required and the need for their removal after the refolding procedure (Buchner et al., 1992; Thomas et al., 1997). Therefore, a detergent-based micellar system mimicking the two-step mechanism of chaperone-assisted protein refolding is developed which may overcome these problems.

Rozema and Gellman (1996a) utilized a two-step method for the refolding of carbonic anhydrase B (CAB). In the first capturing step, chemically denatured CAB (by GdnHCl) was diluted in refolding buffer with a detergent which may form a complex with these non-native proteins, thereby preventing aggregation. The release of the

folding-component was induced by introducing cyclodextrin which strips the detergent away from the protein by forming tighter detergent-cyclodextrin complex. Cyclodextrin polymers of high molecular weight as stripping agent were reported to result in higher protein refolding yield compared with monomeric cyclodextrin (Machida et al., 2000). In addition, rapid application of cyclodextrin was found to be more favorable for the recovery of protein activity than slow addition (Rozema and Gellman, 1996a; 1996b). An important improvement was demonstrated by introducing a solid phase (polymeric β-cyclodextrin beads) as a detergent binding agent compared with previous method in which soluble phase was applied (Mannen et al., 2001). The utilization of these cyclodextrin polymer beads allows simple removal of the cyclodextrin-detergent complex by centrifugation and, moreover, these beads can be used in semicontinuous refolding processes using EBA (Mannen et al., 2001).

Aqueous solutions of hydrogel nanoparticles (nanogels) have also been used for the capturing step and a novel artificial chaperone system using cholesterol-bearing pullulan (CHP) nanogels as a host for a guest protein was developed (Figure 2.3) (Nomura et al., 2003). Hydrophobized polysaccharides such as CHP spontaneously form nanogels in water by inter-macromolecular self-association, which were able to form stronger protein-CHP complexes during refolding of GdnHCl denatured proteins. The complexed proteins were effectively released in their refolded native form upon dissociation of the nanogels in the presence of cyclodextrin (Figure 2.3). Furthermore, it is possible to design various functional nanogels such as surface-modified (cationic

as well as ionic) and stimulus-sensitive (heat, light) nanogels. Nanogels can also be immobilized on various surfaces including chromatographic matrices, which could be useful in column refolding or batch-wise renaturation. This may significantly simplify the protein recovery process and the nanogel system is thus considered to be promising as an efficient and versatile technique for protein refolding in the post-genome era.

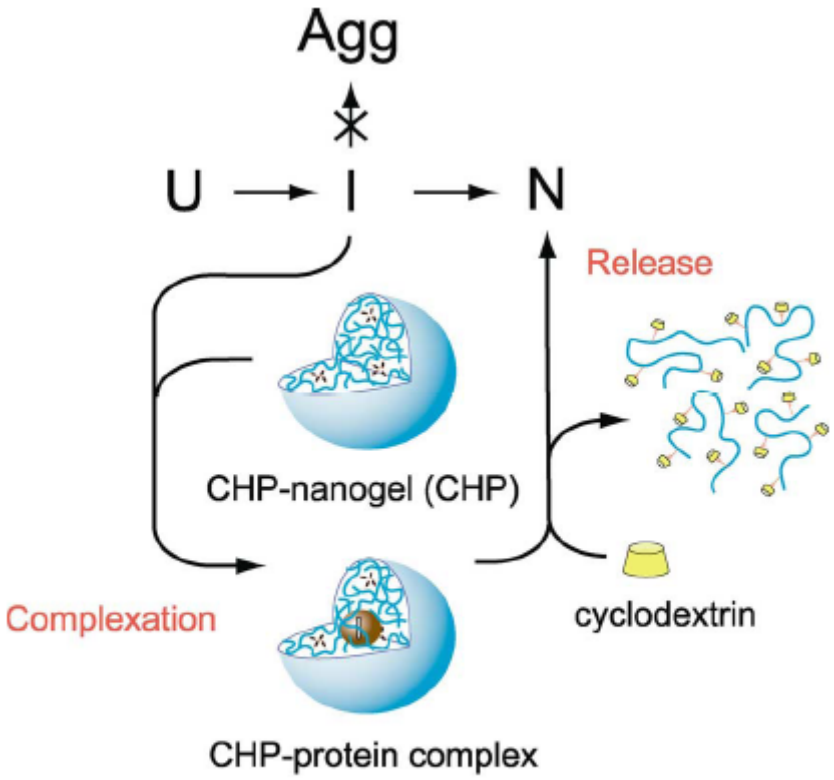


Figure 2.3 Schematic representations of artificial molecular chaperones (Nomura et al., 2003).

Chapter 3
Folding-like-refolding of heat-denatured MDH using unpurified ClpB and DnaKJE

Summary

The *Escherichia coli* heat-shock protein ClpB can efficiently solubilize protein aggregates and refold them into active form in cooperation with the DnaK/DnaJ/GrpE (DnaKJE) chaperone system. However, the application of this bichaperone system at a large-scale was restricted because of the difficulties and high cost to express and purify each of these molecular chaperones. The research in this chapter attempts to design an efficient alternative way for protein refolding through harnessing the unpurified molecular chaperones. We first constructed a plasmid encoding ClpB with a 6×His-tag at its C-terminus (His-ClpB) to facilitate its purification through immobilized metal affinity chromatography (IMAC). A different plasmid capable of expressing the DnaKJE was used to obtain a cell extract containing unpurified DnaKJE. The effect of purified His-ClpB and unpurified DnaKJE on the refolding of heat-denatured malate dehydrogenase (MDH) was investigated, and proved to be highly efficient for MDH refolding. Furthermore, the use of both unpurified His-ClpB and DnaKJE available in the cell extract enabled highly successful refolding of the heat-denatured MDH with efficacy comparable to the case where the purified His-ClpB was used. To the best of our knowledge, this is the first attempt to apply a refolding cocktail comprising unpurified bichaperone system to the refolding of a

heat-denatured protein, providing a practical and economically viable way to apply this bichaperone system in more protein refolding operations, especially for implementing a large-scale folding-like-refolding strategy.

3.1 Introduction

The *E. coli* chaperone ClpB, a heat-shock protein with a molecular weight of 100 kDa, belongs to the AAA+ (ATPase associated with various cellular activities) superfamily of proteins, which self-assemble into barrel-shaped hexamer structures and use the energy derived from ATP hydrolysis to restructure their target substrates (Vale, 2000). Each ClpB protomer is composed of an N-terminal domain and two AAA+ domains separated by a coiled-coil middle domain which is essential for ClpB to realize hexamerization and chaperone function (Kedzierska et al., 2003; Lee et al., 2003). Recent structural data of *Thermus thermophilus* ClpB (TClpB) showed that the hexamer of this protein forms a two tier-ring structure with a 16-Å hole in the top ring and six smaller openings on the lateral surface of the molecules (Lee et al., 2003). It has been suggested that large protein aggregates bind between the linker domains of adjacent ClpB molecules and ATP-induced conformational change of ClpB causes dissociation of the aggregates into smaller size aggregates. However, ClpB alone was not sufficient for disaggregation and renaturation of proteins following their subjection to thermal stress. Instead, ClpB was reported to participate in multi-chaperone system which could efficiently inhibit and reverse protein aggregation (Glover and Lindquist, 1998; Goloubinoff et al., 1999; Zolkiewski, 1999).

For instance, ClpB cooperated with DnaKJE chaperone system to efficiently solubilize protein aggregates and refold them into active proteins (Goloubinoff et al., 1999; Mogk et al., 1999; Ziętkiewicz et al., 2004; 2006).

However, the difficulties and the associated high cost in the purification of each molecular chaperone restrict the application of these molecular chaperones to *in vitro* refolding attempts, especially at a large-scale. In this chapter, we investigated the feasibility of using unpurified DnaKJE and ClpB for disaggregation of heat-denatured malate dehydrogenase (MDH) aggregates. First, we purified *E. coli* ClpB with a 6×His-tag in fusion (His-ClpB) and investigated the efficiency of the purified His-ClpB and unpurified DnaKJE system on the disaggregation and refolding of the MDH in the absence/presence of ATP regeneration system. Secondly, we assessed the efficacy of using a refolding cocktail comprising unpurified His-ClpB and DnaKJE on the refolding of the same model protein.

3.2 Materials and methods

3.2.1 Plasmids

The plasmid encoding ClpB (pClpB) was kindly provided by Dr Catherine Squires (Tufts University, Boston, MA, U.S.A.). This plasmid was used as a polymerase chain reaction (PCR) template and the DNA fragment encoding ClpB residues 1-857 (full length ClpB) was amplified, using two sets of oligonucleotides primers, 5’-GGAATTCCATATGCGTCTGGATCGTCTTAC-3’ and

5'-CCGCTCGAGTTACTGGACGGCGACAATCCGG-3', including *Nde*I and *Xho*I restriction sites. The amplified fragment was then digested by *Nde*I and *Xho*I and cloned into pET-32a(+) (Novagen) treated with the same restriction enzymes. The resultant plasmid was named as pET-ClpB which would produce ClpB fused with a 6×His-tag at its C-terminus (His-ClpB).

Chaperone Plasmid Set (3340, Takara Biotechnology) was used to express molecular chaperones. This set contains five types of plasmids (pG-KJE8, pGro7, pKJE7, pG-Tf2, pTF16) which were developed by HSP Research Institute, Inc. (Japan), each of which was designed to enable efficient expression of multiple molecular chaperones. pKJE7 was used in this study to express DnaKJE.

3.2.2 Proteins expression and purification

E. coli BL21(DE3) cells (69450-4, Novagen) were transformed with pET-ClpB to overexpress the His-ClpB. The transformed cells were grown at 37°C to $A_{600\ nm}$ ~ 0.6 in LB broth containing 0.1 mg/mL ampicillin. Protein expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h. The cell suspension, harvested at its stationary growth phase, was centrifuged at 5000*g* and 4°C for 20 min and resuspended in 50 mM Tris buffer (pH 7.5) containing 150 mM KCl and 20 mM $MgCl_2$. One Shot Cell Disrupter (Constant Cell Disruption System, UK) was used to disrupt the cells at 21.0 Kpsi. Following cell disruption, the cell lysates were centrifuged at 8000*g* and 4°C for 30 min and the supernatant was applied to a 2.5 cm

i.d.×15 cm column (Econo-Column Chromatography Column, 737-1517, Bio-Rad) packed with 20 mL of Ni^{2+}-charged iminodiacetic acid (IDA) resin following equilibration with a binding buffer containing 50 mM Tris, 150 mM KCl, 20 mM $MgCl_2$, 10mM imidazole, pH 7.5. The resin was washed with 10 column volume (CV) of washing buffer (50 mM Tris, 150 mM KCl, 20 mM $MgCl_2$, 20 mM imidazole, pH 7.5) to eliminate weakly bound proteins. To elute His-ClpB, the resin was passed with 5 CV of elution buffer (50 mM Tris, 150 mM KCl, 20 mM $MgCl_2$, 500 mM imidazole, pH 7.5).

Molecular chaperones, DnaKJE were expressed according to the standard protocol (Nishihara et al., 1998). Briefly, *E. coli* BL21(DE3) cells harboring plasmid pKJE7 (expressing multiple molecular chaperones of DnaK, DnaJ and GrpE) were grown to log phase in the presence of 34 μg/mL chloramphenicol at 37°C. To induce expression of chaperones, $_L$-arabinose was added to the culture medium to give a final concentration of 2.5 mg/mL. The cells were centrifuged at 5000*g* and 4°C for 20 min and the collected cell pellets were resuspended in buffer (50 mM Tris, 150 mM KCl, 20 mM $MgCl_2$, pH 7.5) and disrupted using the One Shot Cell Disruptor described as above. The soluble fractions following the centrifugal removal of cell debris (8000*g* and 4°C for 25 min) were kept at -20°C for further use as a DnaKJE stock solution (unpurified DnaKJE system).

MDH was from porcine heart muscle (M1567, Sigma). 2 μM of MDH was denatured

at 47°C for 40 min in 50 mM Tris, 150 mM KCl, 20 mM $MgCl_2$, 5 mM DL-Dithiothreitol (DTT), pH 7.5 (refolding buffer) as developed by Goloubinoff and coworkers (Goloubinoff et al., 1997). Protein refolding was initiated by diluting denatured MDH with 1.5 volumes of a refolding buffer containing chaperones and/or ATP at 25°C with or without ATP regeneration system (4 mM phosphoenol pyruvate (860077, Aldrich) and 20 ng/mL pyruvate kinase (P-9136, Sigma)) to give predetermined concentrations of chaperones and ATP in the refolding cocktail. MDH reactivation was measured according to published protocols (Schröder et al., 1993; Goloubinoff et al., 1999) in 3 mL reaction mixture. The final concentrations were 100 mM potassium phosphate buffer, pH 7.5, 0.13 mM ß-nicotinamide adenine dinucleotide in reduced form (NADH) (N8129, Sigma), 0.25 mM oxalacetic acid (OAA) (O4126, Sigma). The time-dependent oxidation of NADH catalyzed by MDH was monitored at 340 nm.

3.2.3 Analytical methods

Protein detection and quantification: Total protein concentration was measured using Coomassie Plus Protein Assay Kit (23236T, Pierce) based on the Bradford assay with bovine serum albumin (BSA) as the standard. Protein samples after expression and purification were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were diluted tenfold in sample loading buffer (4% (w/v) SDS, 5% (v/v) DTT, 50% (w/v) glycerol, 0.125 M Tris at pH 6.8, 0.002% (w/v) bromophenol blue) and boiled for 5 min. Following centrifugation at

16000*g* for 5 min, a 15 µL volume of sample was loaded into each well of a 12% polyacrylamide gel and electrophoresis was conducted using a Bio-Rad Mini-PROTEAN® 3 Cell system (165-3301 and 165-3302, Bio-Rad) with molecular weight marker (LC5925, Invitrogen). Protein bands were detected by Coomassie G-250 staining using GelCode® Blue Stain reagent (24590, Pierce). A Bio Imaging system (Gene Genius, Syngene, UK), equipped with a gel analysis software (GeneTools, Syngene, UK), was used to image and analyze the gels.

Circular dichroism (CD) spectroscopy: Far UV CD spectra of His-ClpB were measured with a Jasco J-720 (Jasco Corp., Japan) spectrometer using a 0.01-cm cylindrical cell. Purified His-ClpB was first dialyzed against 10 mM sodium phosphate, pH 7.5 and then the concentration of the dialyzed protein sample was adjusted to 0.1 mg/mL for CD measurement. Spectra were corrected by subtracting the buffer baseline and averaged 10 times. Mean residue ellipticity (θ), expressed in deg cm^2 $dmol^{-1}$, was calculated from the formula: $\theta = (MW\ \Theta)/(10\ cnl)$, where Θ is the ellipticity observed (mdeg), MW the molecular mass, c the protein concentration (mg/mL), l the path length of cuvette (cm) and n the number of amino acid residues. All experiments were conducted at room temperature.

His-ClpB ATPase activity determination: His-ClpB ATPase activity was determined according to Barnett and co-workers (Barnett et al., 2000). His-ClpB samples were incubated at 37°C in the reaction buffer (50 mM Tris, 20 mM $MgCl_2$ and 150 mM

KCl, 5 mM ATP, 1 mM EDTA, 1 mM DTT, pH 7.5). After incubation for 15 min, the inorganic phosphate concentration was determined using the Malachite Green Phosphate Assay Kit (POMG-05K, i-DNA Biotechnology Pte Ltd, Singapore). All ATPase assays were conducted at least in duplicate.

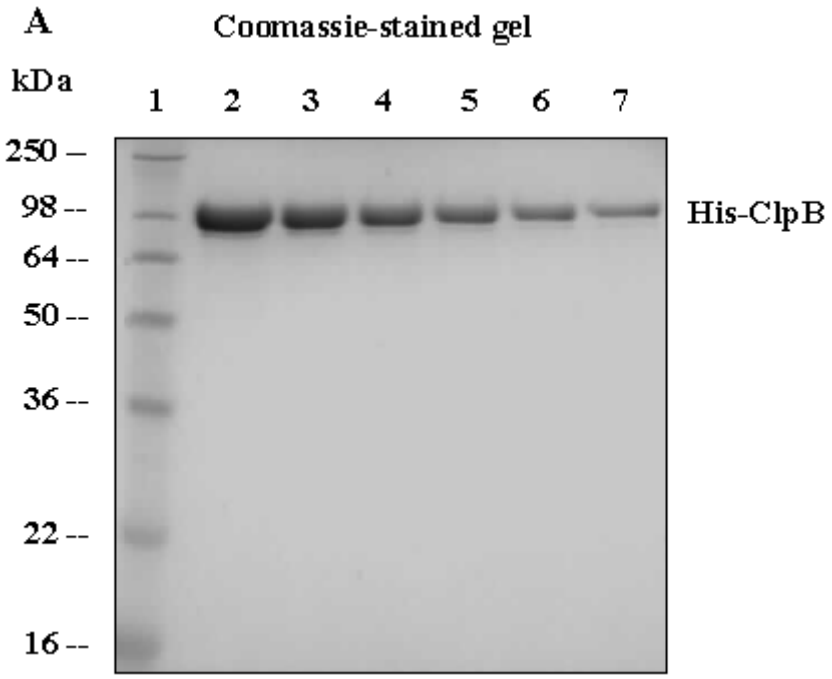
Tryptophan fluorescence spectra: Measurement of the intrinsic tryptophan fluorescence of His-ClpB was conducted with a PerkinElmer Life Science LS50B Luminescence spectrometer. Samples were measured in rectangular four sided quartz cuvettes at a pathlength of 1 cm. The emission spectra of tryptophan fluorescence of ClpB (0.5 μM) in the absence or the presence of 2 mM ATP/ADP were recorded in buffer A (50 mM Tris, 20 mM $MgCl_2$ and various concentrations of KCl, pH 7.5) between 300-400 nm at a fixed excitation wavelength of 290 nm. All experiments were conducted at room temperature.

3.3 Results and discussion

3.3.1 Purification and characterization of His-ClpB

In order to facilitate the production of purified ClpB, we constructed plasmid encoding ClpB with C-terminal extension containing a 6×His-tag (His-ClpB). The purity of His-ClpB following IMAC purification was over 98% as determined by densitometric quantification of proteins bands from Coomassie Blue stained SDS-PAGE gels (Figure 3.1A). The physical properties of His-ClpB were studied using MALDI TOF MS and circular dichroism (CD). The molecular weight of the

purified His-ClpB as measured by MALDI TOF MS was around 95 kDa (data not shown), which was consistent to those predicted from the amino acid sequence. CD study to determine the physical structure of His-ClpB revealed that the secondary structure of His-ClpB was predominantly α-helix as evidenced by the CD spectra showing a characteristic negative double band at 205-222 nm and a positive band at <200 nm (Figure 3.1B). This is in good agreement with the secondary structure of wild type ClpB (Barnett et al., 2000), indicating that the C-terminal fusion of a 6×His-tag did not affect the secondary structure of ClpB.



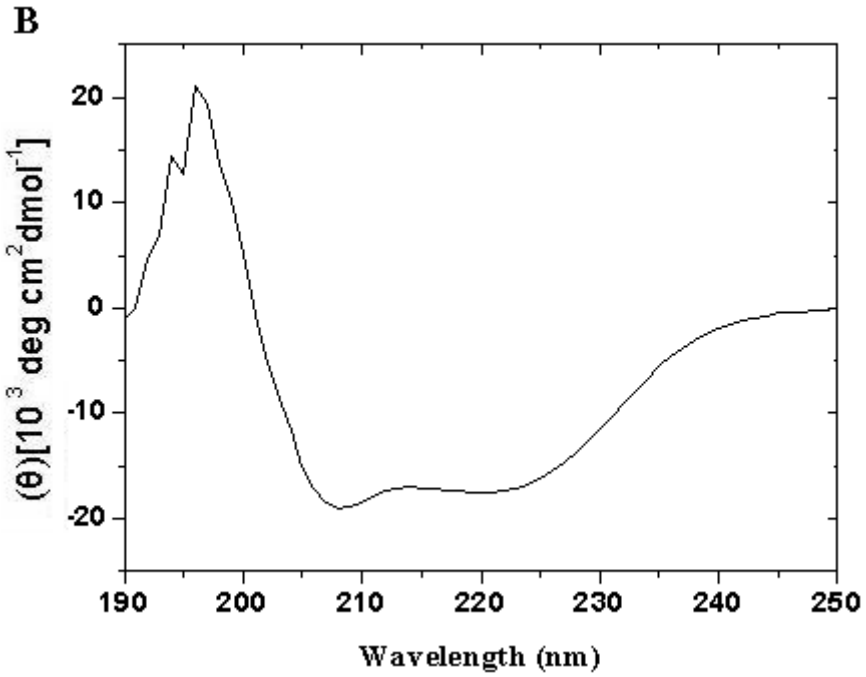


Figure 3.1 (A) SDS-PAGE for the analysis of His-ClpB protein expressed in *E. coli* BL21(DE3) harboring pET-ClpB. Molecular weight marker was loaded in lane 1. His-ClpB was purified by IMAC under native conditions. Lanes 2-7 represents series dilution of His-ClpB after purification. The purity of His-ClpB was quantified by gel analysis software, GeneTools from Syngene. (B) Secondary structure of His-ClpB. Far-UV circular dichroism spectra expressed as mean molar residue ellipticity (θ) [10^3 deg cm^2dmol^{-1}].

The ability of His-ClpB to hydrolyze ATP was also investigated and found to be similar to those of wild type ClpB reported previously (Zolkiewski, 1999; Barnett et al., 2000), i.e. dependent on the presence of active proteins and salt concentration. The ATPase activity increased approximately by 11 fold in the presence of α-casein, which confirmed that His-ClpB is a protein-activated ATPase. With the urea-denatured α-casein, His-ClpB showed only ~ 3 fold increase in the ATPase activity (Figure 3.2A). In addition, the salt concentration in the reaction buffer was found to significantly affect ATPase activity of His-ClpB as was evidenced by sharp decrease in its ATPase activity with the increment of the ionic strength in the buffer (Figure

3.2B). This phenomenon was also reported by Schirmer et al. (Schirmer et al., 1998) where high salt concentrations were found to inhibit the ATPase activity of Hsp104 (a homolog of ClpB in *E. coli*).

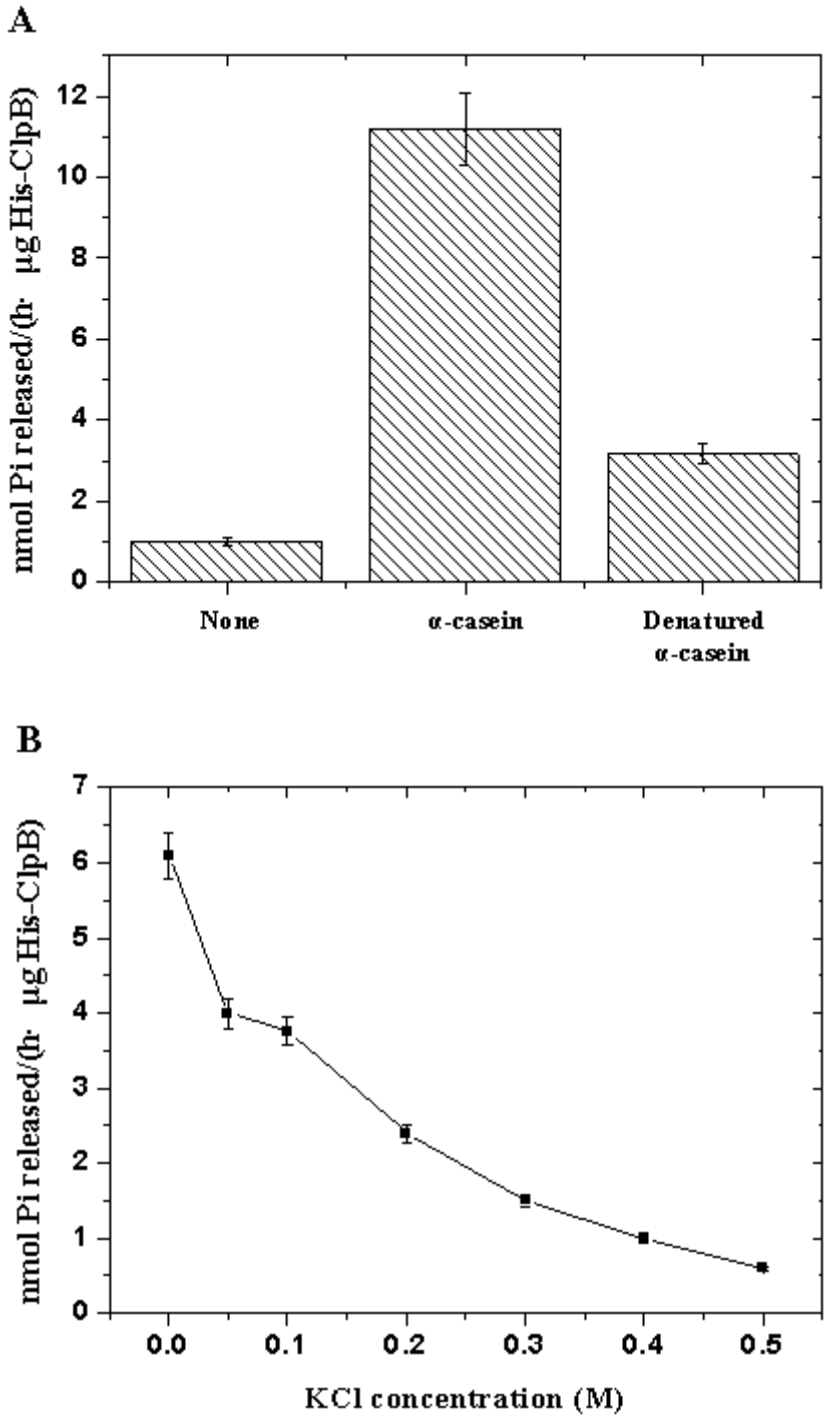


Figure 3.2 ATP hydrolysis by His-ClpB. (A) ATPase activity was measured by

incubating 0.5 μM of His-ClpB in reaction buffer (50 mM Tris, 20 mM $MgCl_2$ and 150 mM KCl, 5 mM ATP, 1 mM EDTA, 1 mM DTT, pH 7.5) at 37°C. The activity in the absence of any added proteins was expressed as 1 (column 1). ATPase activity in the presence of α-casein (column 2) and denatured α-casein (column 3) at 0.1 mg/mL were shown in the figure. (B) Effects of salts on His-ClpB ATPase activity. His-ClpB was incubated in buffer same as above except the concentration of KCl.

The changes in the ATPase activity in different concentration of salts may have arisen from the structural change in the protein. Hence, this possibility was further investigated by measuring intrinsic fluorescence of His-ClpB in solutions of different ionic strength (Figure 3.3). The fluorescence spectrum of His-ClpB exhibited a single emission maximum at 350 nm in the absence of nucleotide at a low ionic strength (Figure 3.3A). The addition of ATP rendered the fluorescence maximum shift to 347 nm, indicating structural change of His-ClpB with ATP binding. The ATP-dependent shift of fluorescence maximum was reverted at an increased ionic strength with the addition of 500 mM of KCl (Figure 3.3B), indicating that ATP binding to His-ClpB was hindered in a high salt condition. This provided direct explanation for the decreased ATPase activity of His-ClpB at a high ionic strength, and suggested that its ATPase activity is closely related to ATP-induced changes in His-ClpB structure, which in turn to be governed by ionic strength of a solution.

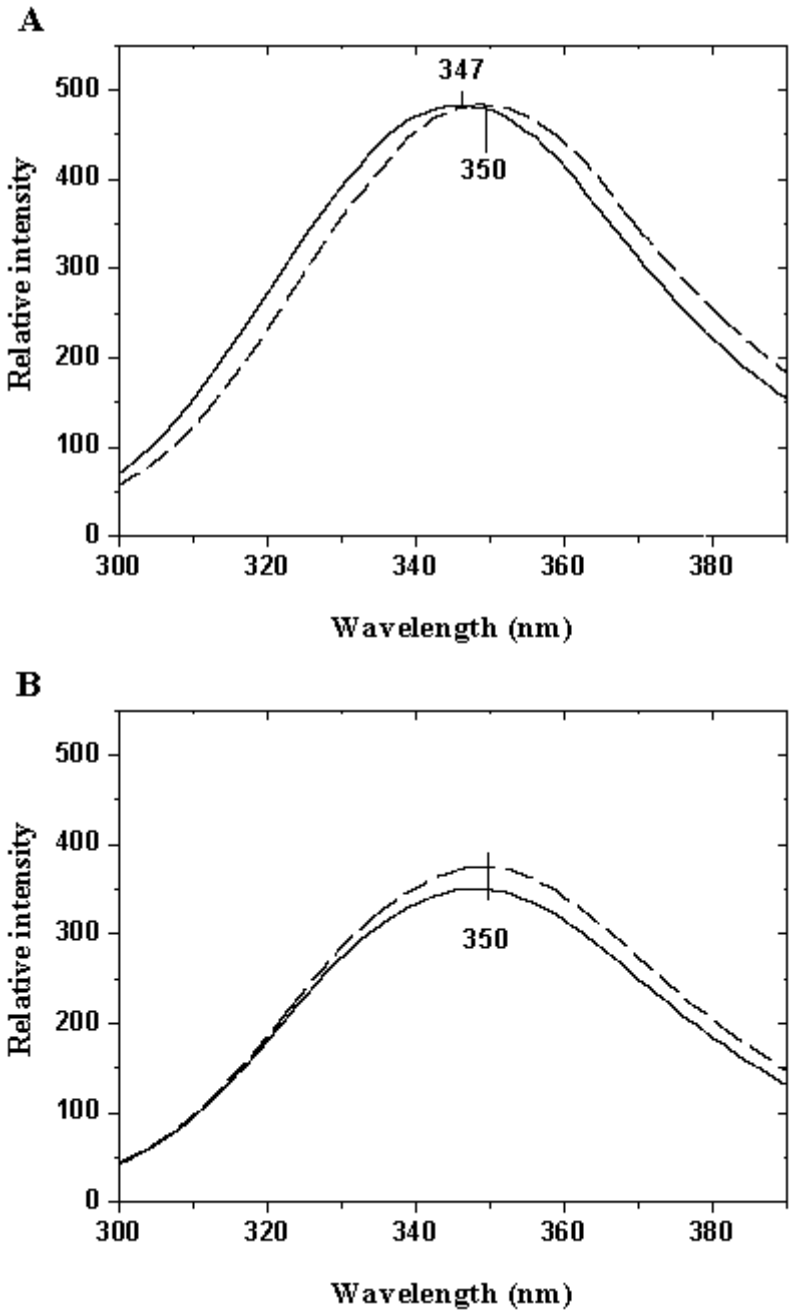
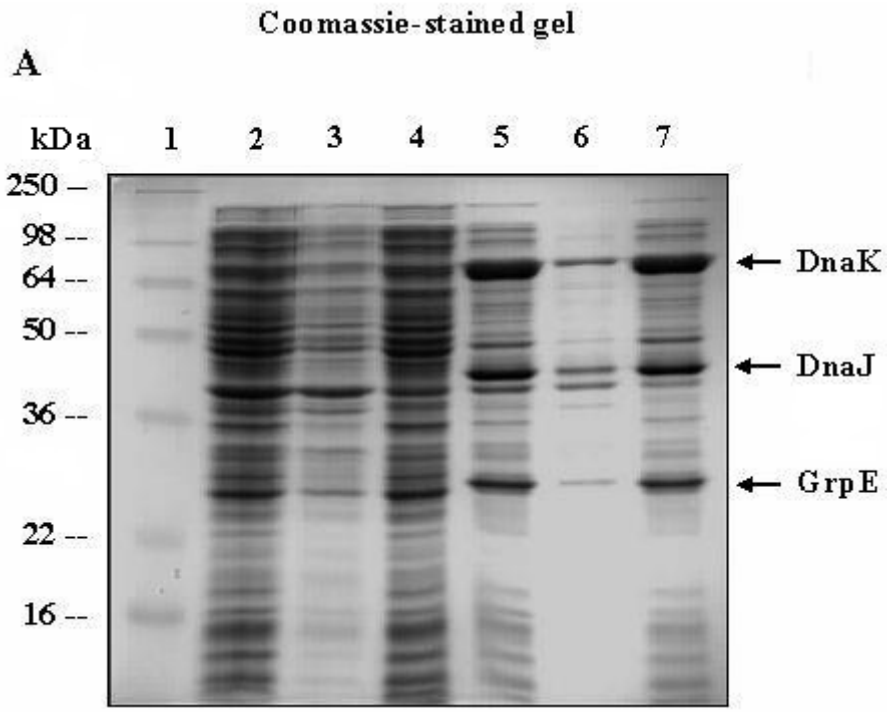


Figure 3.3 Intrinsic tryptophan fluorescence of His-ClpB was measured by incubating His-ClpB (0.5 μM) in reaction buffer (50 mM Tris, 20 mM $MgCl_2$ and different concentration of KCl, pH 7.5). (A) His-ClpB was incubated in reaction buffer at a moderate concentration of KCl (150 mM) in the presence (solid line) and absence (dashed line) of ATP. (B) His-ClpB was incubated in reaction buffer at a high KCl concentration (500 mM) in the presence (solid line) and absence (dashed line) of ATP.

3.3.2 Chaperoning activities of purified His-ClpB and unpurified DnaK/DnaJ/GrpE

Following the purification and characterization of His-ClpB, we investigated the chaperoning activity of purified His-ClpB with or without the assist of unpurified DnaKJE system (Figure 3.4A). DnaKJE, expressed from plasmid pKJE7 in *E. coli* strain of BL21(DE3), was analyzed by SDS-PAGE and the expression level of each chaperone estimated by densitometric quantification. The molar expression ratio of DnaK, DnaJ and GrpE was found to be approximately 3:4.2:5.7, respectively and the total concentration of DnaKJE accounted for more than 50% of the total proteins in the cell extracts (Figure 3.4B).



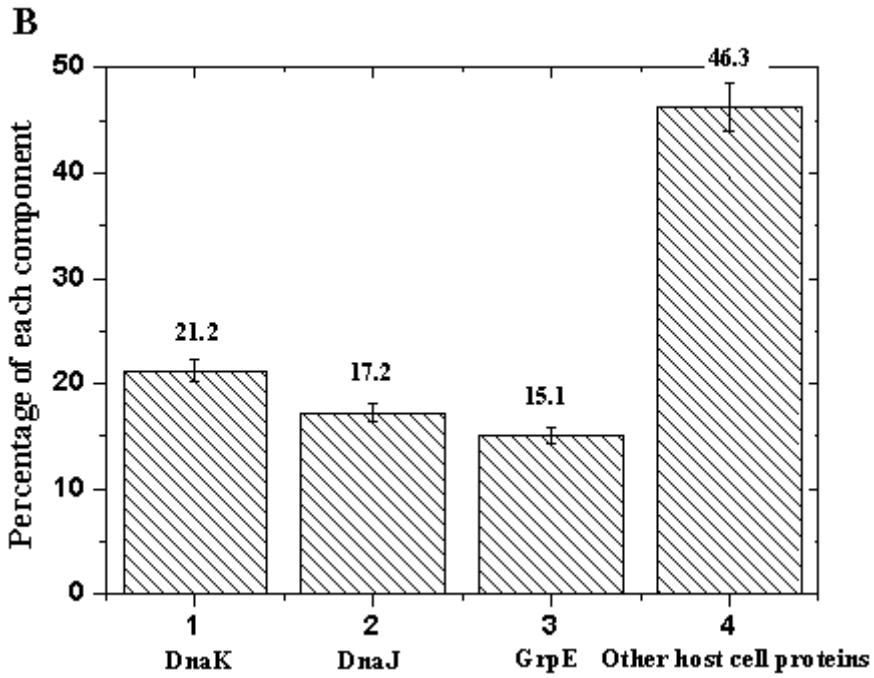
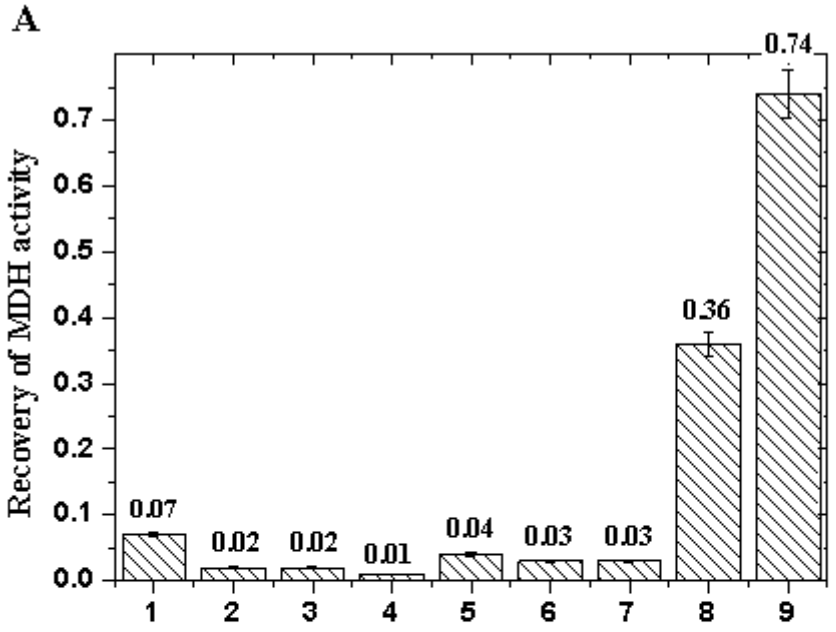


Figure 3.4 (A) SDS-PAGE for the analysis of DnaKJE expressed in *E. coli* BL21(DE3) harboring pKJE7. Molecular weight marker was loaded in lane 1. Lanes 2-4 are from the uninduced cells, representing the whole cell extracts after high-pressure cell disruption, the insoluble and soluble fraction in the cell extracts respectively. Lanes 5-7 are from the induced cells, representing the whole cell extracts after high-pressure cell disruption, the insoluble and soluble fraction in the cell extracts respectively (B) The expression level of DnaK, DnaJ and GrpE in the cell extracts. DnaKJE accounted for more than 50% of the total proteins in the cell extracts.

As a model protein, heat-denatured MDH, which was reported elsewhere (Goloubinoff et al., 1999; Watanabe et al., 2002) to test the chaperoning activity of ClpB, was used. In our study, it was confirmed that more than 99% of MDH activity was lost after heat treatment at 47°C with DTT for 40 min. The disaggregation and refolding of heat-denatured MDH was initiated by diluting 1 volume of denatured MDH solution with 1.5 volumes of a refolding buffer containing chaperones and ATP at 25°C with or without ATP regeneration system (4 mM phosphoenol pyruvate and 20 ng/mL pyruvate kinase in final concentration) to give predetermined concentrations of chaperones (final concentrations of 0.8 μM MDH, 5 μM His-ClpB,

0.2 mg/mL DnaKJE) and ATP (5 mM initial concentration) in the refolding cocktail. The refolding reaction was conducted for 3 h (after 3 h, no further increase in activity recovery was observed).



1 DnaKJE

2 His-ClpB

3 BSA

4 No molecular chaperones

5 *E. coli* cell lysate from uninduced cell harboring plasmid encoding His-ClpB

6 *E. coli* cell lysate from uninduced cell harboring plasmid encoding DnaKJE

7 Combination of cell lysates obtained from 5 and 6

8 DnaKJE and His-ClpB without ATP regeneration system

9 DnaKJE and His-ClpB with ATP regeneration system

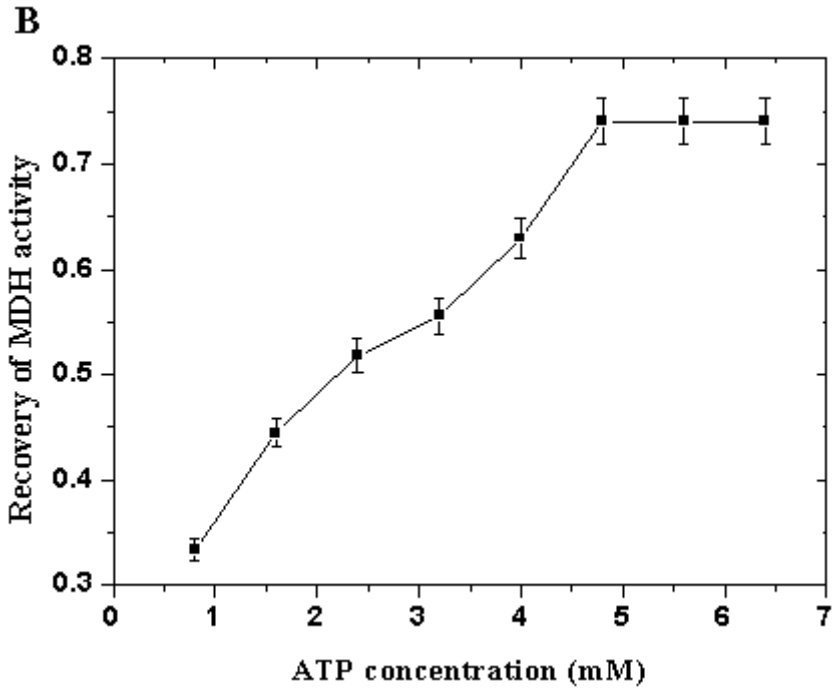


Figure 3.5 Refolding of heat-denatured MDH. (A) Investigation of individual or combinatorial chaperoning activity of purified His-ClpB and unpurified DnaKJE. Given yields correspond to recovered MDH activity after 3-hour incubation at 25°C. The concentrations of molecular chaperones supplemented to 0.8 μM of heat-denatured MDH were 5 μM of His-ClpB (expressed in protomers) and 0.2 mg/mL of DnaKJE mixture. For control experiments, a) 1 mg/mL of BSA was added instead of molecular chaperones; b) *E. coli* cell lysates from uninduced cells harboring plasmid encoding His-ClpB or DnaKJE or the combination of these two were added to the refolding cocktail to give a total protein concentration of 1 mg/mL. The initial ATP concentration was 5 mM and 4 mM of phosphoenol pyruvate and 20 ng/mL of pyruvate kinase were used for ATP regeneration system. (B) Effects of ATP concentration on the refolding yields in the presence of ATP regeneration system. The refolding condition remained the same except for the variation in ATP concentration.

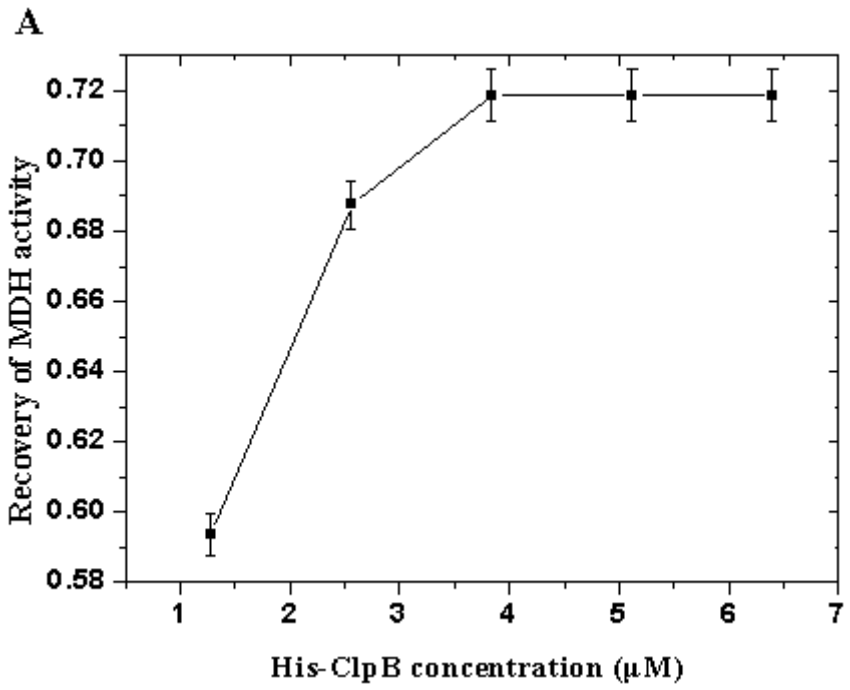
Figure 3.5A shows the reactivation of MDH with or without the assist of purified ClpB and/or unpurified DnaKJE. The recovery of MDH activity was negligible (less than 1%) when the refolding reaction proceeded without any chaperone added, well conforming to the previous study (Goloubinoff et al., 1999) where less than 3% recovery of MDH activity was reported in the absence of chaperones following 24 h of refolding reaction. It was found that the recovery of MDH activity strictly required

the presence of both His-ClpB and DnaKJE. With chaperoning of either purified His-ClpB and unpurified DnaKJE alone, the activity recovery from heat-denatured MDH was virtually absent (Figure 3.5A, columns 1 and 2). On the other hand, the denatured MDH regained 36% (without ATP regeneration system) and 74% (with ATP regeneration system) of its original activity when both His-ClpB and DnaKJE were present together in the refolding cocktail. Bovine serum albumin (BSA), added to the refolding cocktail as a control at an equivalent concentration of molecular chaperones (1 mg/mL), showed little effect on the refolding of denatured MDH. Furthermore, in order to investigate any potential contribution of endogenous chaperones in *E. coli* to MDH refolding, another control experiment was conducted using cell lysates containing neither DnaKJE nor His-ClpB (i.e. cell lysates from uninduced cells) at a final total protein concentration of 1 mg/mL. As shown in Figure 3.5A, MDH refolding in the cocktail lacking DnaKJE and/or His-ClpB was negligible (columns 5-7). This clearly demonstrates that His-ClpB and DnaKJE require each other for the collaborative and synergistic refolding of heat-denatured MDH and that the presence of other endogenous chaperones has little effect on the MDH refolding. Since the availability of ATP was found to exert a huge impact on the efficiency of refolding of denatured MDH (Figure 3.5A, columns 5 and 6), further investigation was made to determine the optimum ATP requirement in the presence of ATP regeneration system. As shown in Figure 3.5B, ATP concentration is crucial to the refolding yield and at least 5 mM of ATP was required to realize the optimum refolding yield in a given refolding cocktail comprising 0.8 μM MDH, 0.2 mg/mL DnaKJE and 5 μM

His-ClpB.

Next, the optimum concentration of His-ClpB or DnaKJE required to achieve a high efficiency refolding of MDH (0.8 μM) was explored in the presence of ATP regeneration system with an initial ATP concentration of 5 mM (Figure 3.6). It was found that the chaperoning efficiency of both His-ClpB and DnaKJE for MDH refolding was significantly affected by the concentration of each chaperone. For His-ClpB, refolding yield increased sharply with the increase of purified His-ClpB from 1 to 3 μM and then reached a plateau (Figure 3.6A). Under our experimental condition, the optimal concentration of His-ClpB was 4 μM, which corresponds to approximately 0.66 μM of $ClpB_6$ (expressed in hexamer). Unlike the His-ClpB, DnaKJE showed an optimum chaperoning activity around 0.15-0.25 mg/mL (with an optimal refolding yield of 0.73 at 0.15 mg/mL DnaKJE), and any further increase of DnaKJE in the refolding cocktail decreased the refolding yield (Figure 3.6B). It was however suspected that the presumable presence of increasing amount of various ATPases in the refolding cocktail due to the use of excess DnaKJE extracts above its optimum concentration (0.15-0.25 mg/mL) might possibly have caused the degradation of ATP (i.e. deprivation of available ATP required for chaperoning activity of His-ClpB), hence the lower refolding yields at increased DnaKJE concentrations. This led us to examine the effect of different starting concentrations of ATP on MDH refolding at excess DnaKJE concentrations (0.4-0.6 mg/mL). It was apparent from Figure 3.6C that the reduced MDH refolding yield at high DnaKJE

concentrations was not affected by the ATPase-driven hydrolysis of ATP, since MDH refolding yield remained virtually constant above 5 mM ATP (despite the increased initial supply of ATP) while exhibiting the same DnaKJE dependency (i.e. decreasing refolding yield with increasing DnaKJE). Hence, the MDH refolding yield appeared to be DnaKJE-dependent. This is in good agreement with the previous study where disaggregation and refolding activities of DnaK was found to depend on the chaperone to substrate ratio (Ben-Zvi et al., 2004).



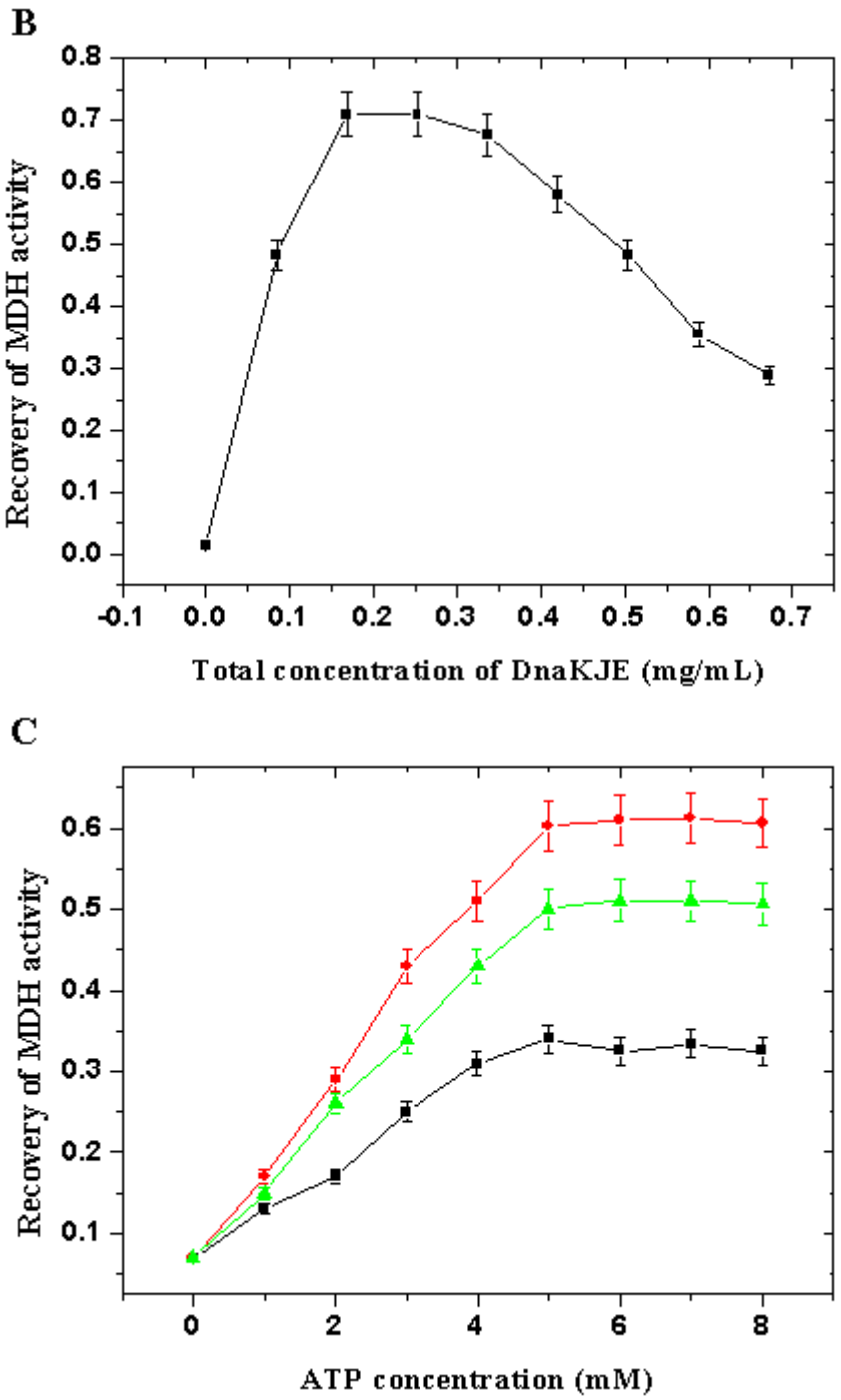


Figure 3.6 Refolding of MDH at varying purified His-ClpB and unpurified DnaKJE concentrations. (A) Effect of increasing concentrations of His-ClpB in the presence of constant amount of DnaKJE (0.2 mg/mL) and MDH (0.8 μM) on the refolding yield of MDH. (B) Effect of increasing concentrations of DnaKJE in the presence of constant amounts of His-ClpB (5 μM) and MDH (0.8 μM) on the refolding yield of MDH. (C) Effect of increasing initial concentrations of ATP on the refolding yield of MDH at varying DnaKJE concentrations (■, 0.6 mg/mL; ▲, 0.5 mg/mL; ●, 0.4 mg/mL) in the presence His-ClpB (5 μM) and MDH (0.8 μM).

In addition, the importance of DnaJ to DnaK ratio in determining the refolding yield was demonstrated (Ben-Zvi et al., 2004) in that the chaperoning activity of DnaK would decrease at a high DnaJ/DnaK ratio (larger than 0.175/1) probably by retarded release of DnaK. In our study, the molar expression ratio of DnaKJE mixture estimated by densitometry was about 3:4.2:5.7, which corresponded to the concentrations of 0.5 μM DnaK, 0.7 μM DnaJ and 0.95 μM GrpE. Since DnaK, DnaJ and GrpE were co-translated from a single plasmid (pKJE7), the ratio of these three chaperones were kept unchanged and the individual contribution of each chaperone for the refolding of heat-denatured MDH could be not be quantitatively assessed in the present study (an individual contribution of DnaKJE to the MDH refolding is currently under investigation).

Since the reaction rates and substrate conversion for biochemical reaction are strongly temperature dependent, MDH refolding in the optimum refolding cocktail composition was compared at different temperatures. As shown in Figure 3.7, MDH refolding was found to be optimum at 25°C and the refolding reaction was almost inhibited at 4°C where many previous refolding reactions have been attempted on the assumption that proteins should be most stable at this low temperature (Levine et al., 1998). Considering that most *E. coli* cells are cultured at 37°C, it is also interesting to note that the efficiency of MDH refolding at 37°C was lower than that at 25°C. This indicates that optimum temperature condition for cell growth may not necessarily be conducive to the folding of expressed proteins or *in vitro* refolding.

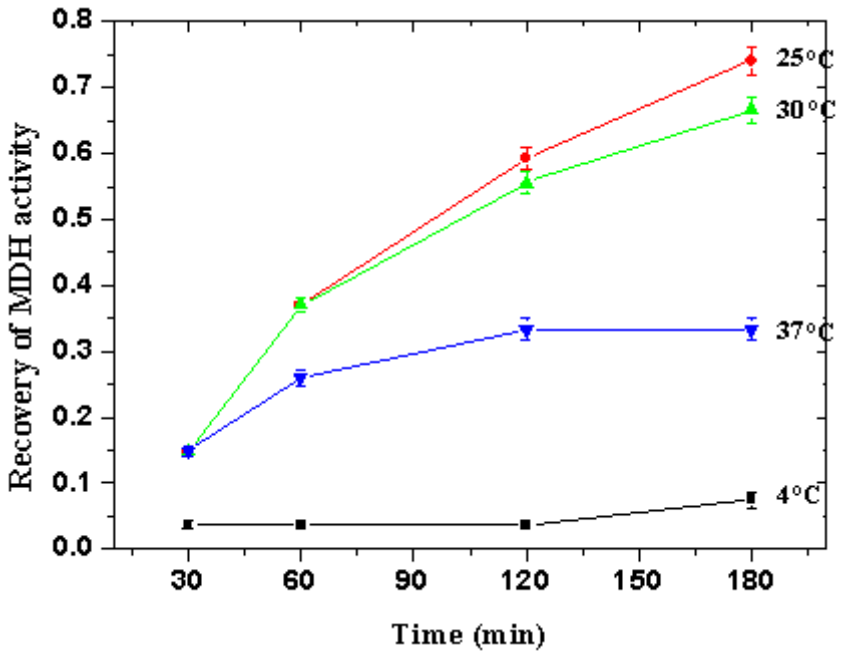


Figure 3.7 Time course of MDH refolding in the presence of His-ClpB (5 μM), DnaKJE (0.2 mg/mL) and ATP regeneration system with an initial ATP concentration of 5 mM at different temperatures.

3.3.3 Chaperoning activity of unpurified His-ClpB and DnaKJE

To further broaden the application scope of bichaperone system comprising ClpB and DnaKJE, we tested the feasibility of harnessing refolding cocktail employing unpurified His-ClpB (Figure 3.8) and unpurified DnaKJE for the disaggregation and subsequent refolding of heat-denatured MDH. His-ClpB was expressed and the cell extracts containing unpurified His-ClpB obtained (His-ClpB extracts) as described in Materials and methods. In the cell extracts as shown in Figure 3.8, His-ClpB was found to account for approximately 40% of the total host cell proteins (15 mg/mL) according to densitometric quantification using GeneTools (data not shown). Separate cell extracts containing unpurified DnaKJE was prepared as stated in the previous section (DnaKJE extracts). His-ClpB/DnaKJE-mediated MDH refolding was initiated by diluting 1 volume of 2 μM heat-denatured MDH solution with 1.5 volumes of

refolding cocktail comprising His-ClpB extracts (0.2 volume), DnaKJE (0.1 volume), ATP stock solution (125 mM, 0.1 volume) with ATP regeneration system (0.1 volume) and refolding buffer (1 volume) to give a final concentration of a refolding cocktail at 0.8 μM MDH, 5 μM ClpB, 0.2 mg/mL DnaKJE with initial concentration of 5 mM ATP. The efficiency of MDH refolding by unpurified bichaperone system was almost indistinguishable from that achieved with the purified His-ClpB and unpurified DnaKJE (Figure 3.9), providing a significant potential to realize folding-like-refolding strategy in a simple, cost-effective way.

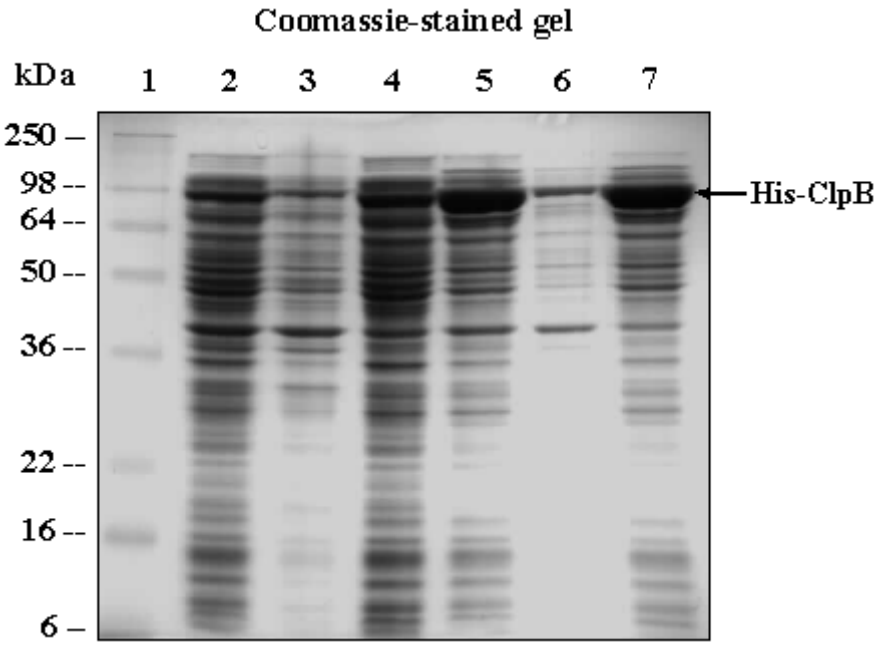


Figure 3.8 SDS-PAGE for the analysis of His-ClpB expressed in *E. coli* BL21(DE3) harboring pET-ClpB. Molecular weight marker was loaded in lane 1. Lanes 2-4 are from the uninduced cells, representing the whole cell extracts after high-pressure cell disruption, the insoluble and soluble fraction in the cell extracts respectively. Lanes 5-7 are from the induced cells, representing the whole cell extracts after high-pressure cell disruption, the insoluble and soluble fraction in the cell extracts respectively.

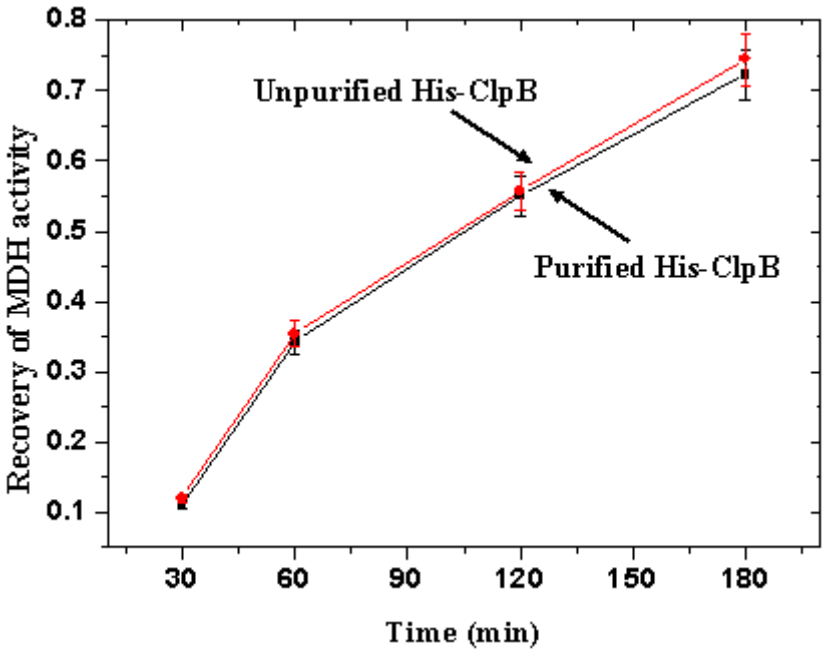


Figure 3.9 Time course of MDH refolding when unpurified His-ClpB or purified His-ClpB was added to the refolding cocktail containing 0.8 μM of heat-denatured MDH and 0.2 mg/mL of unpurified DnaKJE.

Our refolding strategy mimics *in vivo* folding mechanism which is typically assisted by protein quality control network harnessing chaperones and ATP (Baneyx and Mujacic, 2004). Most of the conventional refolding strategies relied on dilution-based refolding or column refolding with or without the use of purified and/or immobilized chaperones. The folding-like-refolding strategy employed in the present study departed from the traditional approaches and proved that refolding could be realized at a high efficiency in the crowded cell lysates by mimicking the folding mechanism. In a typical industrial-scale dilution refolding process, large volume of fluid handling is required. By using folding-like-refolding approach, it is envisioned that the process volume would be decreased, thus reducing cost while improving efficiency. The possible increase in viscosity as the process volume decreases, mainly due to the presence of high-molecular weight DNA, might lead to difficulties in fluid handling.

This however could be easily counteracted by the addition of DNA precipitants.

Overall, the demonstrated results herein indicate a potential to design a new refolding strategy of harnessing a refolding cocktail comprising overexpressed but unpurified molecular chaperones for the refolding of denatured protein aggregates typically encountered in the production of recombinant proteins in *E. coli*. Although the refolding efficiency of ClpB/DnaKJE chaperone system was illustrated using a model protein (MDH) herein, the refolding reaction was conducted in the presence of all the host contaminating proteins which were co-introduced with the addition of unpurified His-ClpB and/or DnaKJE to the refolding cocktail. Hence, the demonstrated refolding strategy has been proven to work at high efficiency in the complex milieu containing many undefined impurities, ensuring its applicability to the purification of actual target proteins. Moreover, in this study, the unpurified His-ClpB was demonstrated to be comparable to purified His-ClpB in terms of refolding efficiency. Hence, after the replacement of His-ClpB with ClpB (without His-tag) in the actual refolding process, IMAC can be easily incorporated to facilitate the isolation of his-tagged target proteins. The purification following the refolding reaction would not incur any extra difficulty compared to the conventional recombinant protein expression using *E. coli*. Lastly, the concept of refolding cocktail would be useful in the context of not only providing an economically viable platform technology for the refolding of IB route proteins at a large-scale but also in terms of decoupling maximum protein production stage and the subsequent refolding stage.

The further application of this bichaperone system is addressed in the following chapters (Chapters 4-6). Refolding additives which was reported to facilitate the protein refolding process is incorporated to this bichaperone system and the synergistic effect is evaluated first in the next chapter (Chapter 4). The refolding of a real bacterial IB protein (gloshedobin, a thrombin-like enzyme from snake venom) enhanced by the molecular bichaperone is then investigated in Chapters 5 and 6.

3.4 Conclusion

A refolding cocktail comprising unpurified His-ClpB and DnaKJE system was demonstrated to achieve high efficiency refolding of heat-denatured MDH in the presence of ATP regeneration system by mimicking the exquisite folding mechanism afforded by protein quality control network reported elsewhere (Schlieker et al., 2002; Baneyx and Mujacic, 2004). To our best knowledge, this is the first study to apply the unpurified bichaperone system to the folding-like-refolding of heat-denatured protein, providing a considerable scope to extend the application of ClpB/DnaKJE mediated protein refolding to large-scale refolding processes in an economically viable way.

Chapter 4
Synergistic coordination of polyethylene glycol with ClpB/DnaKJE bichaperone for refolding of heat-denatured MDH

Summary

In last chapter, the feasibility of using a refolding cocktail comprising the molecular bichaperone ClpB and DnaKJE was demonstrated to efficiently renature the heat-denatured malate dehydrogenase (MDH). In this chapter, the use of polyethylene glycol (PEG) as a refolding additive to the ClpB/DnaKJE bichaperone system was found to significantly enhance chaperone-mediated refolding of heat-denatured MDH. The critical factor affecting the refolding yield is the time point of introducing PEG to the refolding cocktail. The refolding efficiency reached approximately 90% only when PEG was added at the beginning of refolding reaction. The synergistic coordination of an inexpensive refolding additive PEG with the ClpB/DnaKJE bichaperone system may provide an economical route to further enhance the efficacy of ClpB/DnaKJE refolding cocktail approach, facilitating its implementation in large-scale refolding processes.

4.1 Introduction

During the process of protein refolding, the formation of incorrectly folded species (in particular protein aggregates) is the usual cause for decreased renaturation yield (De

Bernardez Clark, 2001). The use of refolding additives proved to be a very efficient strategy to inhibit the intermolecular interactions leading to aggregation and thus improves the yield of bioactive proteins (De Bernardez Clark, 1998; 2001). Numerous kinds of additives, such as detergents, amino acids, salts, divalent cations, surfactants, polymers, polyols and sugars, were shown to be effective in the prevention of protein aggregation (Yasuda et al., 1998; De Bernardez Clark et al., 1999; Singh and Panda, 2005) via alteration of both the solubility and stability of the unfolded, intermediate and native protein (De Bernardez Clark, 2001; Singh and Panda, 2005).

In last chapter (Chapter 3), a refolding cocktail comprising His-ClpB (ClpB with a 6×His-tag at its C-terminus) and unpurified DnaKJE system was developed and demonstrated to be efficient in the renaturation of heat-denatured malate dehydrogenase (MDH). This may broaden the application of this bichaperone system by providing a more practical and economically viable protein refolding strategy especially at large-scale basis. In this chapter, we further investigate the effects of various kinds of refolding additives, in particular PEG, on the ClpB/DnaKJE-mediated refolding of heat-denatured MDH.

4.2 Materials and methods

4.2.1 Plasmids

Plasmid pKJE7, designed to enable efficient expression of multiple molecular chaperones (DnaKJE), was bought from Takara Biotechnology, Japan (3340). Plasmid

pET-ClpB, capable of overexpressing ClpB with a 6×His-tag at its C-terminus, was constructed as described in Chapter 3.

4.2.2 Proteins

E. coli BL21(DE3) cells (69450-4, Novagen) were transformed with pET-ClpB to overexpress His-ClpB. Immobilized metal affinity chromatography (IMAC) purification of His-ClpB was conducted as in Chapter 3. DnaKJE were expressed according to the standard protocol (Nishihara et al., 1998) and the unpurified DnaKJE stock solution was prepared according to Chapter 3. MDH from porcine heart muscle is commercially available (M1567, Sigma). Total protein concentration was measured using Coomassie Plus Protein Assay Kit (23236T, Pierce) based on the Bradford assay with bovine serum albumin (BSA) as the standard.

4.2.3 MDH refolding

Heat-denatured MDH refolding was conducted as described in Chapter 3. 2 μM of MDH was heat-denatured at 47°C for 40 min in 50 mM Tris, 150 mM KCl, 20 mM $MgCl_2$, 5 mM DL-Dithiothreitol (DTT), pH 7.5 (refolding buffer) as developed by Goloubinoff and coworkers (1997). Protein refolding was initiated by diluting 1 volume of heat-denatured MDH with 1 volume of refolding buffer and another 0.5 volume of refolding buffer containing purified His-ClpB, unpurified DnaKJE and ATP (A2383, Sigma) with ATP regeneration system to give predetermined concentrations of chaperones and ATP in the refolding cocktail (0.8 μM

heat-denatured MDH, 5 µM His-ClpB, 0.2 mg/mL DnaKJE, 5 mM ATP, and ATP regeneration system (4 mM phosphoenol pyruvate (860077, Aldrich) and 20 ng/mL pyruvate kinase (P-9136, Sigma), given in final concentration unless stated otherwise). For the study of the effect of various additives on the refolding process, 1 volume of refolding buffer containing different kinds of refolding additives was added instead while maintaining the concentrations of all the other components in the refolding cocktail unchanged. Each additive was applied at the recommended final concentration as follows: 0.4 M glucose or sucrose (Lee and Timasheff, 1981), 0.5 M L-arginine hydrochloride (Tsumoto et al., 2004), 0.5 M glycine (Tsumoto et al., 2004), 20% (v/v) glycerol (Michaelis et al., 1995), 10 mM Triton X-100 (Wetlaufer and Xie, 1995), 10 mM Tween 20 (Yasuda et al., 1998), 20 mg/mL PEG (M_r = 8000) (Cleland et al., 1992a), 0.5 M KOAc or NaOAc, 0.5 M Na_2SO_4 (Michaelis et al., 1995), 0.5 M $MgCl_2$ or $MgSO_4$ (Singh and Panda, 2005), and 0.5 M $(NH_4)_2SO_4$. For all further studies to investigate the effect of PEG additive on MDH refolding, 20 mg/mL PEG was used unless otherwise stated.

MDH reactivation was measured according to published protocols (Schröder et al., 1993; Goloubinoff et al., 1999) in 3 mL reaction mixture. The final concentrations were 100 mM potassium phosphate buffer, pH 7.5, 0.13 mM ß-nicotinamide adenine dinucleotide in reduced form (NADH) (N8129, Sigma), 0.25 mM oxalacetic acid (OAA) (O4126, Sigma) and 0.8 µM MDH. The time-dependent oxidation of NADH catalyzed by MDH was monitored at 340 nm. Turbidity of protein aggregates was

measured in rectangular four sided quartz cuvettes at a pathlength of 1 cm and an excitation and emission wavelength of 550 nm (PerkinElmer Life Science LS50B Luminescence spectrometer) (Goloubinoff et al., 1999).

4.3 Results and discussion

As a model protein, MDH was previously used for the study of ClpB chaperoning activity (Goloubinoff et al., 1999; Watanabe et al., 2002). Herein, the potential effects of several refolding additives on ClpB/DnaKJE-mediated disaggregation and renaturation of heat-denatured MDH were studied. The refolding reaction was allowed to proceed for 3 h, afterwhich, no further recovery in activity was observed.

4.3.1 Effect of additives on the relative refolding yield of heat-denatured MDH

The synergistic effects of ClpB/DnaKJE bichaperone system and different kinds of refolding additives (without ATP regeneration system) were investigated (Figure 4.1). Among the 14 additives studied, only glycine, glycerol and PEG resulted in positive increment of the final refolding yield. The presence of Triton X-100, $MgCl_2$, $MgSO_4$, or $(NH_4)_2SO_4$ significantly inhibited the refolding. This inhibitive effect was lowered when glucose, L-arginine/HCl, or Tween 20 was used. Arginine, the most commonly used refolding additive, showed no improvement in the final refolding yield as MDH has no tryptophan residues (Scheich et al., 2004) to interact with the guanidino group of arginine to reduce protein aggregation (Arakawa and Tsumoto, 2003). Sucrose,

KOAc, NaOAc and Na_2SO_4 had little effect on the final refolding yield.

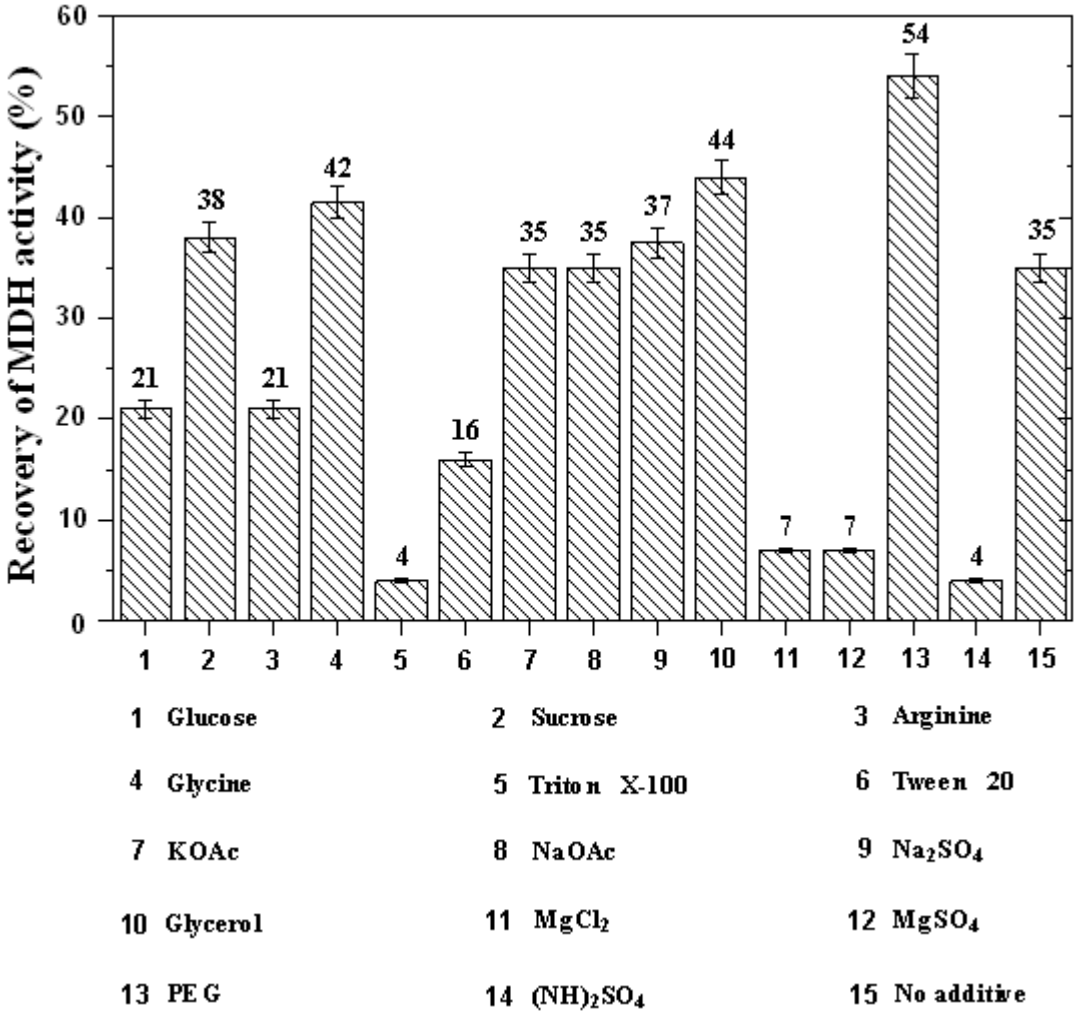


Figure 4.1 The effects of various additives on ClpB/DnaKJE-mediated refolding of heat-denatured MDH in the absence of ATP regeneration system.

Among the three additives which significantly increase the refolding yield, glycine possibly stabilizes individual protein structure through reducing attractive hydrophobic forces between refolding intermediates and thus facilitates protein refolding (Valente et al., 2005). Similarly, the stabilization of protein intermediate is likely to be the cause for improved refolding efficiency with glycerol (Gorovits et al., 1998). The efficiency of PEG (in the absence of molecular chaperones) on protein

refolding was extensively studied and proved to be highly protein-dependent. Although PEG showed no effect on the refolding of lysozyme (Goldberg et al., 1991; Yasuda et al., 1998), it was found that the addition of PEG was effective for improving the refolding yield of carbonic anhydrase B (Cleland et al., 1992a and 1992b; Cleland and Wang, 1992; Cleland and Randolph, 1992) and recombinant human tissue transglutaminase (Ambrus and Fésüs, 2001). According to Figure 4.1, PEG (column 13) gives the highest final refolding yield of 54% which is approximately 1.5-fold increment compared to the control experiment lacking PEG with a refolding yield of 35% (column 15). However, there are no reports thus far regarding the combinatorial effects of PEG and ClpB/DnaKJE bichaperone on protein refolding, necessitating further investigations in this aspect.

4.3.2 Effect of molecular chaperones on MDH refolding in the presence of PEG

Figure 4.2 shows the reactivation of MDH with purified ClpB, unpurified DnaKJE and/or PEG in the absence of ATP regeneration system. The recovery of MDH activity was previously shown in Chapter 3 to be negligible (less than 1%) without chaperone addition. Also, the presence of PEG alone exhibited low efficiency in the refolding of heat-denatured MDH (Figure 4.2, column 2). The recovery of MDH activity strictly required the presence of both ClpB and DnaKJE as demonstrated in Chapter 3. When either purified His-ClpB or unpurified DnaKJE was individually applied, the efficiency of MDH activity recovery was only 1.7% and 8.3%, respectively (Figure

4.2, columns 3 and 5). The addition of PEG with His-ClpB or DnaKJE alone showed no improvement on the refolding yield (Figure 4.2, columns 4 and 6). On the other hand, the denatured MDH regained 33% (without PEG) and 51% (with PEG) of its original activity when His-ClpB and DnaKJE co-existed in the refolding cocktail (Figure 4.2, columns 1 and 12). Bovine serum albumin (BSA), which was added to the refolding buffer (with or without PEG) as a control at a concentration equivalent to molecular chaperones used (1 mg/mL, as described in Chapter 3), showed negligible effect on the refolding of MDH (Figure 4.2, columns 7 and 8). This indicates that MDH refolding is specifically mediated by molecular chaperones and not just by the presence of other unspecific proteins (e.g. BSA). In addition, a second control experiment to investigate the potential contribution of endogenous chaperones in *E. coli* extract to MDH refolding was performed. The cell lysates containing neither His-ClpB nor DnaKJE (i.e. cell lysates from uninduced cells, Figure 4.2, columns 9-11) at a final total protein concentration of 1 mg/mL showed negligible effect on the refolding of heat-denatured MDH, indicating that endogenous chaperones in the cell lysates did not contribute to the refolding of MDH. The availability of ATP was however crucial for the ClpB/DnaKJE-mediated MDH refolding. The absence of which greatly reduced the activity recovery of MDH (Figure 4.2, columns 13-15) as ClpB and DnaKJE are ATP-dependent proteins (Baneyx and Mujacic, 2004).

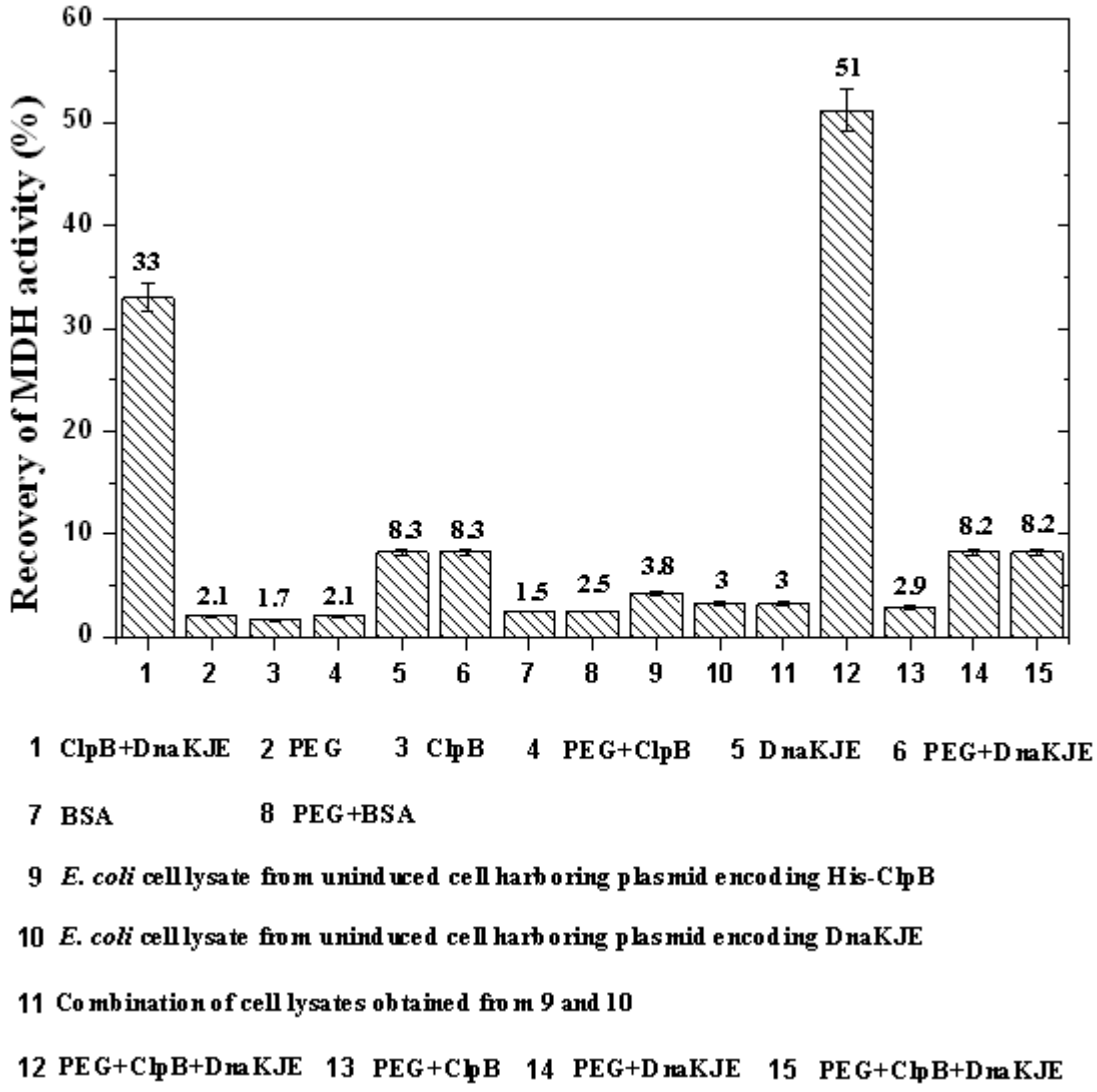
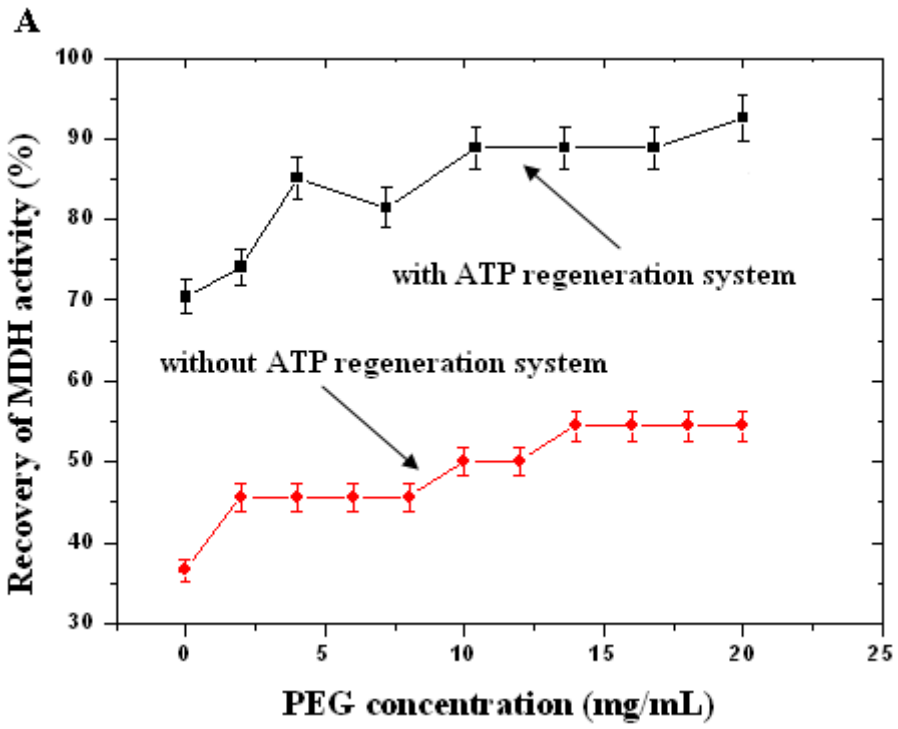


Figure 4.2 The individual or combinatorial chaperoning activity of purified His-ClpB and unpurified DnaKJE with or without the assistance of PEG (in the absence of ATP regeneration system). ATP was included in all experiments except for those to account for columns 13-15. For control experiments, a: 1 mg/mL of BSA with or without PEG was added instead of molecular chaperones (columns 7 and 8); b: *E. coli* cell lysates from uninduced cells harboring plasmid encoding His-ClpB or DnaKJE or the combination of these two lysates (columns 9-11) were added to the refolding cocktail at a total protein concentration of 1 mg/mL as a replacement for molecular chaperones.

Since the presence of PEG was found to exert a significant impact on the efficiency of denatured MDH refolding, further investigation was made to determine the optimum PEG requirement. As shown in Figure 4.3A, regardless of whether ATP regeneration

system is available, at least 15-20 mg/mL PEG in the refolding cocktail is required to achieve an optimal final refolding yield. At the optimum concentration of PEG and ATP regeneration system, the denatured MDH regained more than 90% of its native activity, highlighting the importance of balanced coordination of PEG with ATP-driven chaperoning of the ClpB/DnaKJE for MDH refolding. In contrast, the efficiency of MDH refolding for the equivalent condition except lacking ATP regeneration system was significantly lower, giving only 55% of activity recovery.



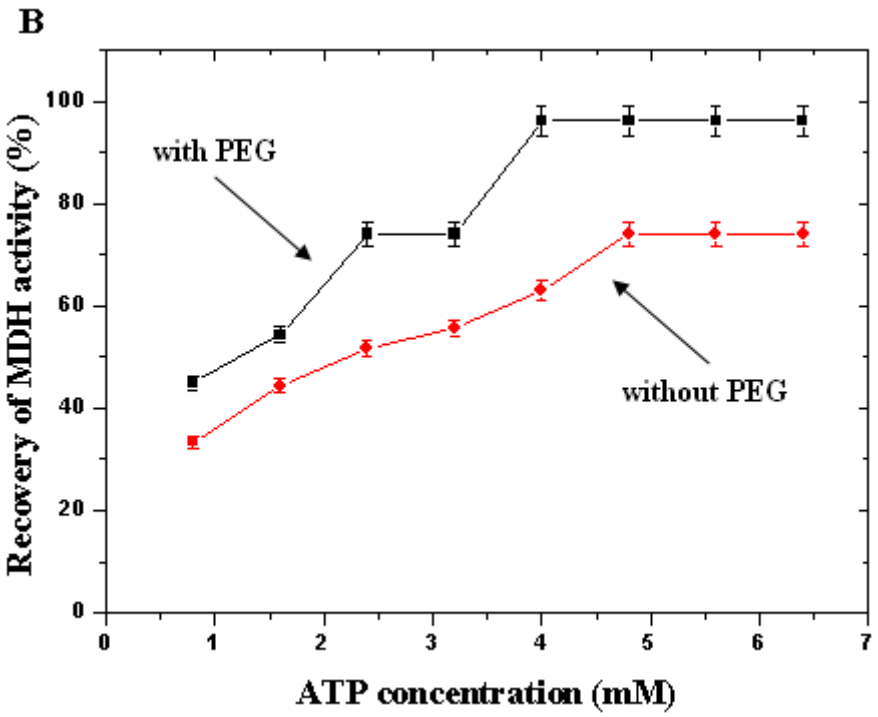


Figure 4.3 The effect of varying concentrations of PEG or ATP on ClpB/DnaKJE-mediated disaggregation and renaturation of heat-denatured MDH. (A) The effect of PEG concentration on the refolding yield of MDH with or without ATP regeneration system. (B) The effect of ATP concentration on the refolding yield of MDH (in the presence of ATP regeneration system) with or without the assistance of PEG.

The concentration of ATP (in the presence of ATP regeneration system) also significantly affected the final refolding yield. According to Figure 4.3B, 5 mM ATP is essential to obtain the maximum refolding yield with more than 90% of the MDH activity recovered in the presence of 20 mg/mL PEG while only 70% of its activity was regained without PEG. As described in Chapter 3, the chaperoning efficiencies of both His-ClpB and DnaKJE for MDH refolding were in addition affected by the concentration of each chaperone. Hence, in the presence of PEG, the effects of varying concentration of His-ClpB and/or DnaKJE were studied. With His-ClpB, the refolding yield increased with elevated concentration and an optimum refolding yield

was reached at concentrations above 5 μM (around 74% without PEG and 95% with PEG) (Figure 4.4A). Unlike His-ClpB, DnaKJE showed an optimum chaperoning activity around 0.15-0.25 mg/mL (with an optimal refolding yield of around 72% without PEG and 94% with PEG at 0.15 mg/mL DnaKJE), and further increase of DnaKJE in the refolding cocktail reduced the refolding yield (Figure 4.4B). It is suspected that the use of excess unpurified DnaKJE extracts may facilitate ATP hydrolysis in the refolding cocktail due to the concomitant introduction of increasing amount of various ATPases residing in the extracts. The resultant deprivation of available ATP required for chaperoning activity of His-ClpB could then account for the decreased refolding efficiency. However, the reduced MDH refolding yield at high DnaKJE concentrations is probably not due to ATPase-driven hydrolysis of ATP as even with additional supplement of ATP (at concentration higher than 5 mM), the MDH refolding yield remained virtually constant as stated in Chapter 3, showing that MDH refolding yield is DnaKJE-dependent.

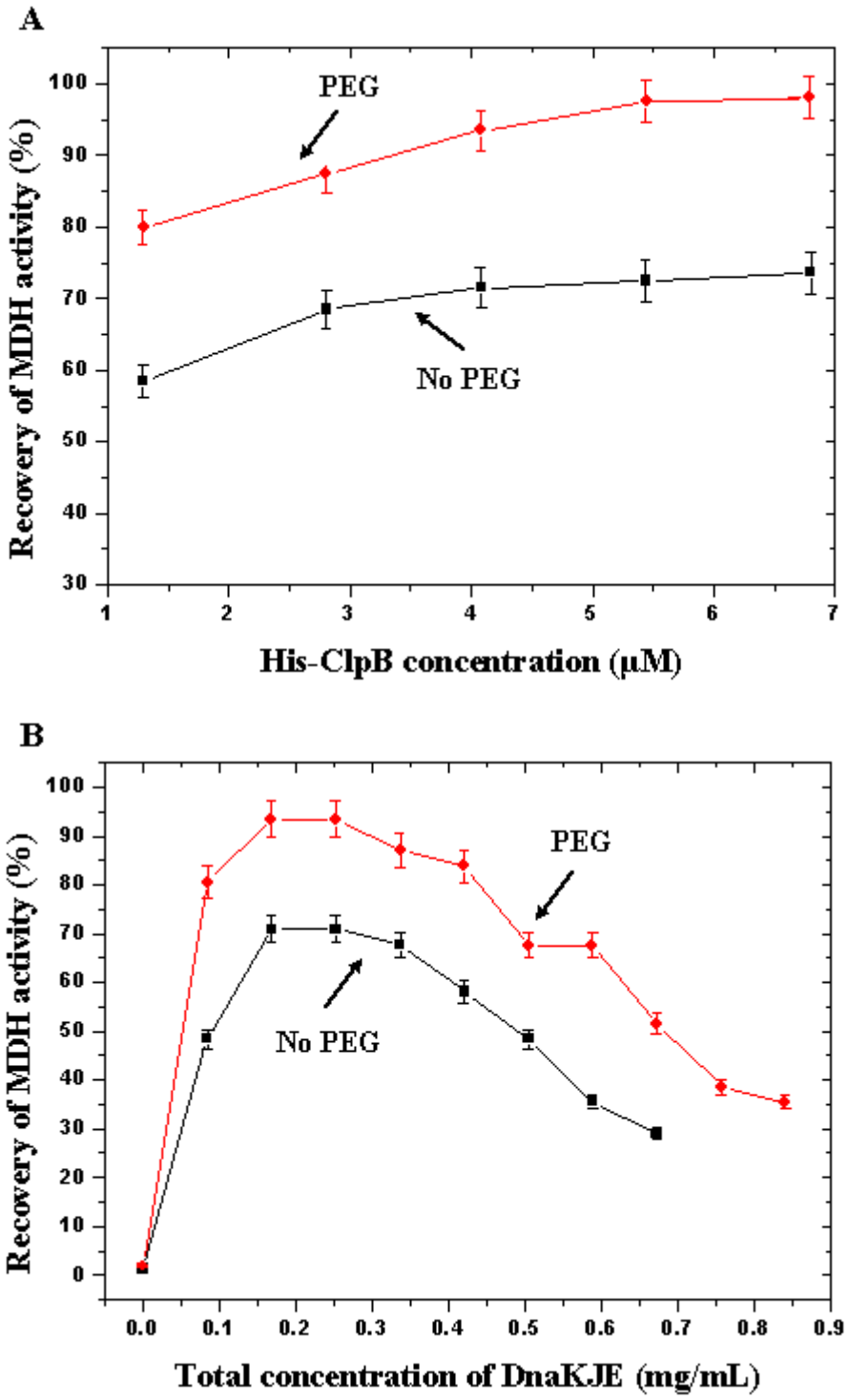


Figure 4.4 (A) Effect of increasing concentration of His-ClpB on the refolding yield of MDH. (B) Effect of increasing concentration of DnaKJE on the refolding yield of MDH. ATP regeneration system was included in all operations.

The time-dependent recovery of MDH activity was next investigated (Figure 4.5). ClpB/DnaKJE refolding cocktail containing both PEG and ATP regeneration system

gave the highest final refolding yield (condition a) when compared to condition b or c in which either PEG or ATP regeneration system was absent and condition d in which no PEG and ATP regeneration system were included. Figure 4.5B shows the apparent rates of MDH refolding corresponding to each condition in Figure 4.5A. Since the refolding yield displayed a non-linear increment, we divided the refolding process into two phases: Phase I is from 0 to 60 min and Phase II from 60 to 180 min. In the presence of ATP regeneration system (conditions a and b), PEG addition greatly increased the apparent refolding rates in Phase I (from 0.53 %/min in condition b to 0.92 %/min in condition a) as compared to Phase II, in which the apparent refolding rates become indifferent to the presence of PEG (around 0.33 %/min in both conditions a and b) (Figure 4.5B). A similar trend is also observed for MDH refolding devoid of ATP regeneration system (conditions c and d). Therefore, the efficacy of PEG on the ClpB/DnaKJE-mediated MDH refolding mainly occurred in the first 1 h (Phase I), during which the denatured MDH recovered around 60% of its enzymatic activity in the presence of PEG while the process lacking PEG only resulted in recovery of 35% of its activity.

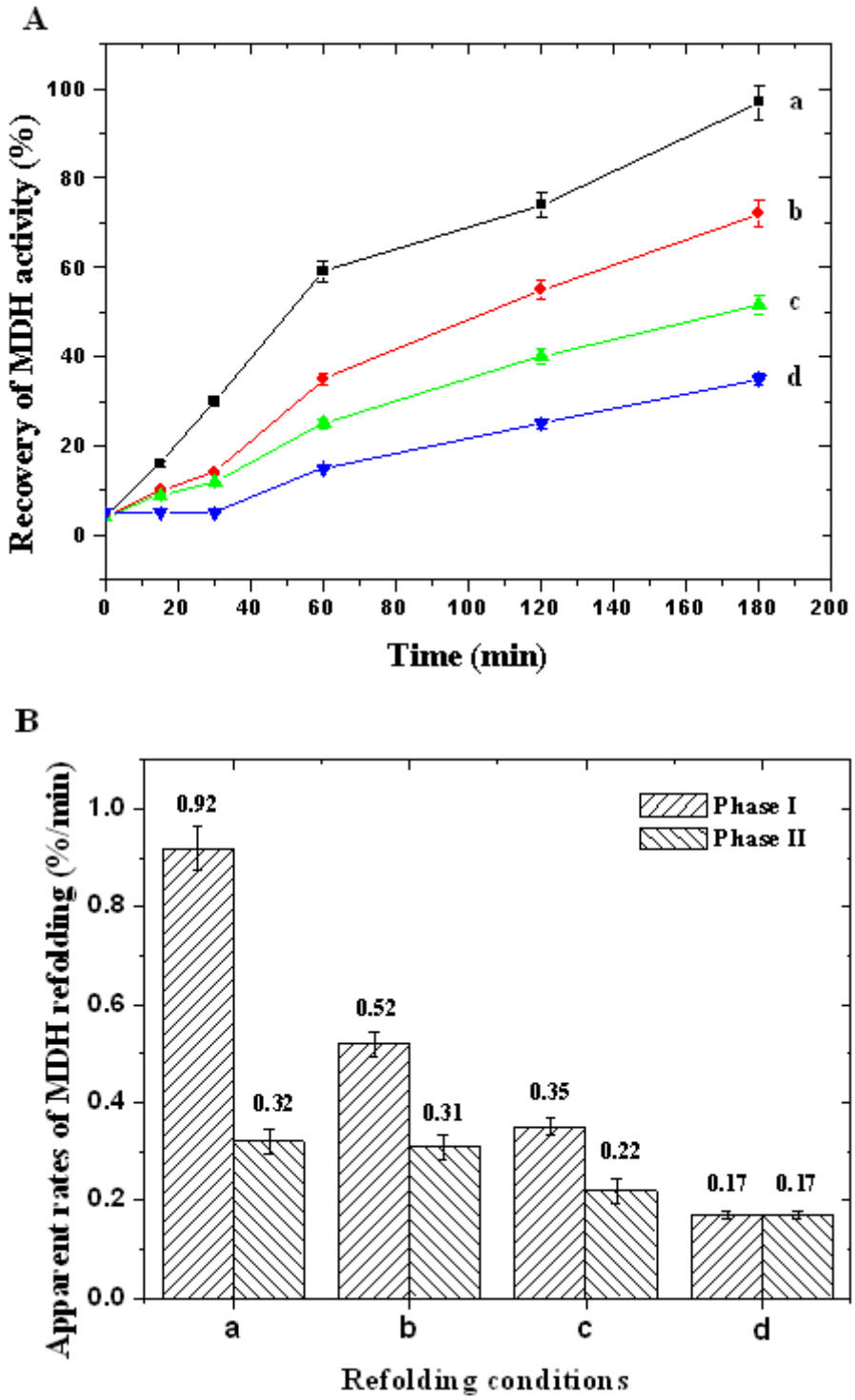


Figure 4.5 (A) Time-dependent MDH refolding in the presence or absence of PEG and ATP regeneration system. a, ClpB/DnaKJE refolding cocktail containing both PEG and ATP regeneration system; b, ClpB/DnaKJE refolding cocktail containing only ATP regeneration system; c, ClpB/DnaKJE refolding cocktail containing only PEG; d, ClpB/DnaKJE refolding cocktail without PEG and ATP regeneration system. (B) Apparent rates of MDH refolding under the various conditions in (A). The rates were expressed as percentage of reactivated MDH per minute.

4.3.3 Effect of PEG addition at different times

It was suggested that disaggregation and reactivation of heat-denatured MDH are two sequential reactions, in which the disaggregation starts immediately after the addition of molecular chaperones and completes around 45 min later while the refolding of solubilized MDH species only commences thereafter (Goloubinoff et al., 1999; Weibezahn et al., 2004). In our study, the turbidity of MDH solution rapidly decreased at the initiation of the disaggregation reaction and completed after around 1 h (Figure 4.6). In the presence of PEG, the decrease in the turbidity of MDH solution initiated by ClpB/DnaKJE addition was similar to that without PEG (Figure 4.6), suggesting that PEG may not stimulate the disaggregation of heat-denatured MDH. We further investigated the effect of PEG addition on the protein refolding by introducing PEG at various determined time points following the application of molecular chaperones to denatured MDH. Depending on the time point of PEG addition, the MDH substrates might exist primarily in the aggregated, intermediate or native state according to the extent of chaperone-mediated refolding reaction, consequently modulating the efficacy of PEG on MDH refolding. Figure 4.7A shows time course of MDH refolding upon addition of PEG at differential time points (i.e. 0, 15, 30 or 60 min) to the refolding cocktail containing denatured MDH and ClpB/DnaKJE chaperones. As a control experiment, equal volume of refolding buffer (without PEG) was also added at the various time points (Figure 4.7B).

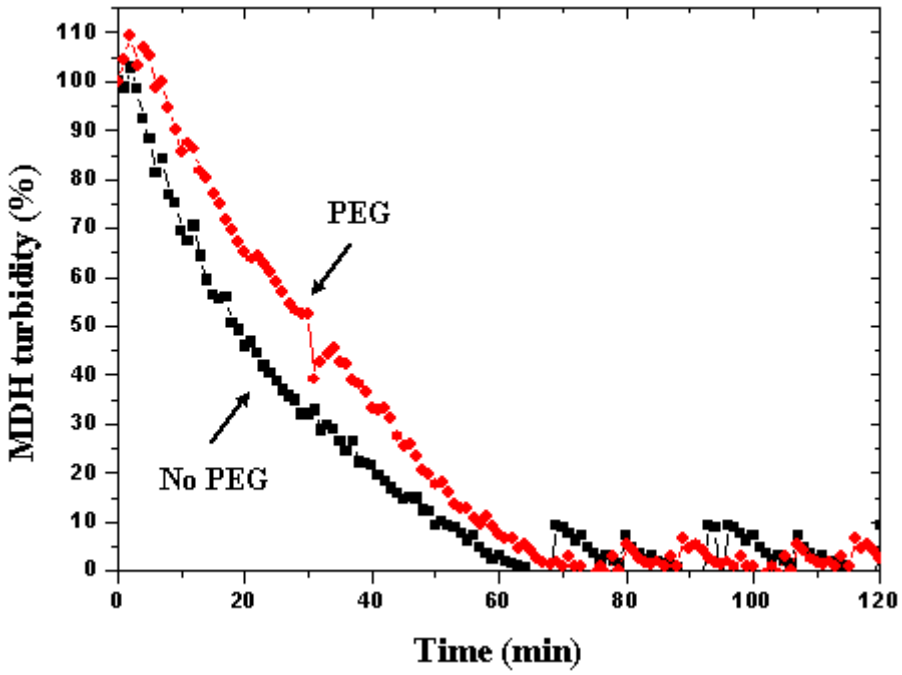
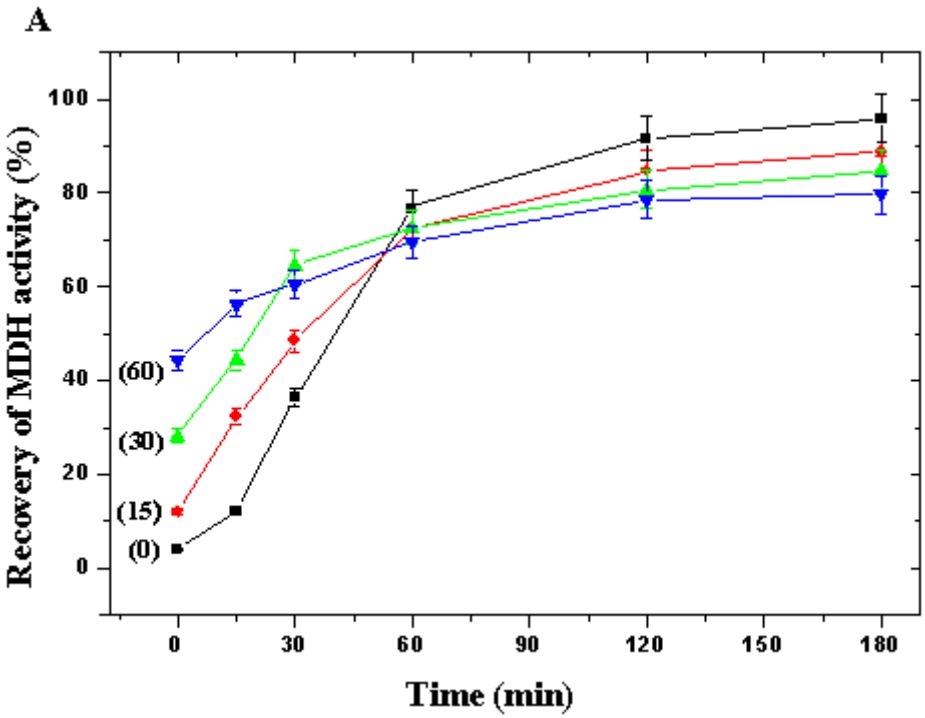


Figure 4.6 Time-dependent disaggregation of heat-denatured MDH by ClpB/DnaKJE bichaperone system (in the presence of ATP regeneration system).



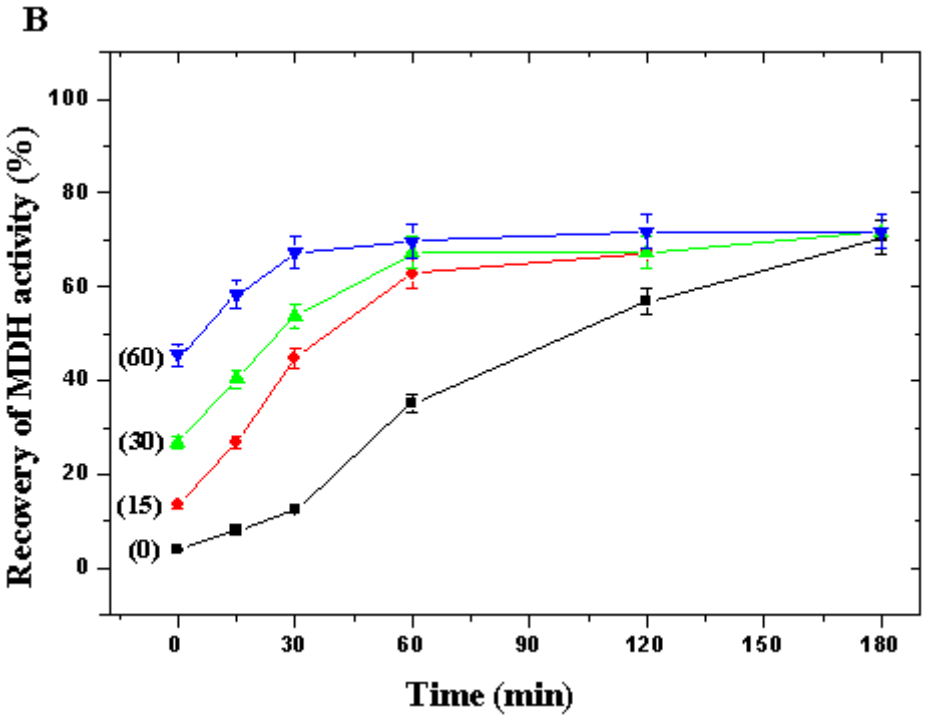


Figure 4.7 (A) Effects of PEG addition time on the MDH refolding. PEG was applied at various determined time (0, 15, 30 or 60 min) after the application of ClpB/DnaKJE molecular chaperones (with ATP regeneration system). (B) Equal volume of refolding buffer (without PEG) was added instead at 0, 15, 30 or 60 min as control experiments.

In the control experiment (Figure 4.7B), the final efficiencies of MDH activity recovery were all around 70% regardless of the pre-incubation period with molecular chaperones. In contrast, the time point of adding PEG to the refolding cocktail clearly affected final MDH activity recovery (Figure 4.7A). When PEG was added together with ClpB/DnaKJE bichaperone system without pre-incubation, the final refolding yield was around 95%. The MDH activity recovery gradually decreased (from 95% to 80%) along with the delay in PEG introduction (from 0 to 60 min). Moreover, the pre-incubation of PEG with heat-denatured MDH for different time periods showed no effect on the time-dependent MDH refolding compared to the condition when PEG was added together with ClpB/DnaKJE bichaperone system (data not shown). These

clearly demonstrated that the key point to realize the enhancement of ClpB/DnaKJE-mediated reactivation relied on the co-existence of PEG with molecular chaperones at the start of the disaggregation process of heat-denatured MDH.

The study of equilibrium unfolding and refolding of MDH showed that the transition processes are reversible and two equilibrium intermediate states are present (Sanyal et al., 2002). The first is a compact monomer formed immediately after subunit dissociation and the second an expanded monomer (less compact than the native monomer) exhibiting most of the characteristic features of a 'molten globule' state (Sanyal et al., 2002). Molten globule, a kind of collapsed intermediate state, has some native-like secondary structure, but generally lacking tertiary structure (Christensen and Pain, 1991; Dolgikh et al., 1984). PEG was reported to bind onto the molten globule intermediate of the bovine carbonic anhydrase B (CAB). Upon dilution of chemically denatured CAB (by guanidine hydrochloride) to non-denaturing chaotrope concentration, the unfolded protein rapidly forms 'molten globule' state intermediate. This intermediate subsequently either enters refolding/aggregation pathways or binds to PEG to form a non-associating complex (Cleland et al., 1992a; 1992b). The formation of this non-associating PEG-molten globule complex significantly inhibited aggregation, resulting in a reduction of self-association of this compact hydrophobic structure and hence the enhanced final refolding yields (Cleland and Randolph, 1992; Cleland et al., 1992a and 1992b; Cleland and Wang, 1992). In this 'dilution additive'

strategy, the transient interactions between the small molecule (e.g. PEG) and protein intermediate need to be sufficient to deter protein aggregation but not so strong as to prevent proper protein folding (Daugherty et al., 1998). The PEG enhanced protein refolding showed some analogies to GroEL/GroES chaperone system. It was observed that the naturally occurring chaperone, GroEL reversibly binds 'molten globule' folding intermediates of many proteins. However, this binding prevents both aggregation and refolding of the substrate protein, departing from the effect of PEG effect exhibited in CAB refolding. Release of the substrate and concomitant refolding are triggered by GroEL binding to GroES with ATP hydrolysis (Hartl, 1996; Rye et al., 1999; Houry, 2001). The two-step mechanism of GroEL/GroES system inspired an 'artificial chaperone' technique employing small molecules (artificial chaperones) to promote protein refolding from the chemically denatured state. A synthetic detergent (TOPPA) included in the refolding buffer was used first to capture non-native protein upon dilution of the denaturant to non-denaturing concentrations (Daugherty et al., 1998). Protein aggregation is greatly suppressed by the formation of protein-detergent complexes where the role of detergent is suggested to shield the hydrophobic regions of non-native protein molecules (Rozema and Gellman, 1995; 1996a; 1996b). Cyclodextrin is next added to initiate refolding reaction by stripping detergent from the protein-detergent complex (Daugherty et al., 1998). The stable interaction between non-native protein and the detergent in the artificial chaperone protocol is thus mechanistically distinct from their transient interaction in the 'dilution additive' strategy.

For heat-denatured MDH, the aggregates are unable to spontaneously transform to 'molten globule' state intermediate to invite PEG binding, accounting for the low efficiency of heat-denatured MDH refolding when PEG alone (without molecular chaperones) was present. ClpB/DnaKJE bichaperone system was reported to trigger protein refolding by shearing protein aggregates to release individual polypeptides (Schlieker et al., 2004; Weibezahn et al., 2004). Upon solubilization, the unfolded polypeptides can either enter the chaperone network (Shorter and Lindquist, 2005; Weibezahn et al., 2005) or reach a 'molten globule' state to become substrate for PEG binding. We thus propose synergistic roles of PEG and ClpB/DnaKJE bichaperone system on the refolding of heat-denatured MDH where PEG may further stabilize 'molten globule' state intermediates triggered by bichaperoning activity of ClpB/DnaKJE during the initial stage of refolding process. The formation of PEG-intermediate complex possibly inhibits the reaggregation of molten globule intermediate and thus improves the final refolding yield.

4.4 Conclusion

PEG enhances the efficiency of ClpB/DnaKJE-mediated refolding of heat-denatured MDH only when PEG co-existed with ClpB/DnaKJE at the start of the refolding process. The binding of PEG onto the refolding intermediates initiated by chaperoning activity of ClpB/DnaKJE might inhibit the reaggregation of these intermediates. Since PEG does not display any disaggregation properties by itself, it is postulated that the presence of PEG at the start of the refolding process is necessary such that PEG can

bind onto the refolding intermediates before reaggregation occurs. The addition of this inexpensive refolding additive may further broaden the application of refolding cocktail approach using unpurified ClpB/DnaKJE bichaperone system, especially in large-scale refolding processes for commercial production of proteins.

Chapter 5
Effective reduction of truncated expression of gloshedobin in *Escherichia coli* using molecular chaperone ClpB

Summary

Snake venom thrombin-like enzymes (TLEs) have been widely studied for potential therapeutic applications as anti-coagulants in the treatment of blood clotting disorders. However, due to the cysteine-rich nature of these proteins, their expressions in *Escherichia coli* were often impeded by inclusion body (IB) formation. Moreover, the formation of a truncated expression product significantly complicated the production of gloshedobin, a recently isolated TLE from the snake venom of *Gloydius shedaoensis* (Yang et al., 2003a; 2003b). Therefore, prior to confirming the applicability of the proposed folding-like-refolding strategy developed in Chapter 3 to gloshedobin IBs refolding, we first investigated the possibilities to reduce the truncated expression product so as to facilitate the following purification and refolding work. In this chapter, it was first found that the expression of gloshedobin was strongly dependent on the expression host. The truncated expression was reduced by 25% when the protein was expressed in *E. coli* BL21(DE3)pLysS instead of BL21(DE3). It was also demonstrated that co-expression of ClpB (a molecular chaperone) in BL21(DE3) enabled the expression of gloshedobin mostly in intact form without compromising expression level, while almost completely eliminating its

truncation products. This suggests a new simple strategy to significantly improve the quality of protein expression in TLE production and may find its useful application for many other recombinant proteins whose expressions and/or purifications are hindered by the formation of truncation products.

5.1 Introduction

In general, *E. coli* offers a route for the rapid and economical production of recombinant proteins (Swartz, 2001), although the over-expression of recombinant proteins in *E. coli* often leads to their intracellular accumulation as solid aggregates known as inclusion bodies or IBs. These particles are classically perceived to contain misfolded proteins which lack biological activity (Carrió et al., 2000). However, this has been contested as some recent reports showed the presence of enzymatic activity associated with IBs, and thus reflecting that a significant fraction of embedded proteins occurs in a properly folded native-like form (Gonzalez-Montalban et al., 2005; Ventura and Villaverde, 2006; Garcia-Fruitos et al., 2007). However, gloshedobin expression (without the introduction of fusion tags or protein modification) in *E. coli* in either soluble or insoluble form has been largely unsuccessful. This difficulty of expression was also reported with several other thrombin-like enzymes (TLEs) (e.g. acutin and batroxobin) when their expressions were attempted in *E. coli* (Maeda et al., 1991).

It has been suspected that the formation of stable secondary structure at the translation

initiation region of mRNA might have prevented the protein expression (Maeda et al., 1991; Yuan et al., 2004). Hence, a gene encoding gloshedobin was cloned into pET-32a(+) to be positioned at the downstream of *trxA* (capable of encoding thioredoxin) (Yang et al., 2003a). With this fusion construct (i.e. thioredoxin-6×His-tag-gloshedobin), gloshedobin was overexpressed in *E. coli* BL21(DE3), but mostly as IBs largely contaminated with a major truncation product probably arising from proteolytic degradation or secondary site translation initiation (Halling and Smith, 1985; Preibisch et al., 1988; Govind et al., 2001). Despite the presence of some proteins whose truncated forms were found to exhibit biological activities, truncated gloshedobin was inactive. Furthermore, the purification of intact gloshedobin by immobilized metal affinity chromatography (IMAC) was hampered by the presence of significant amount of an unwanted product associated with the truncation (i.e. thioredoxin-6×His-tag containing N-terminal fraction of intact gloshedobin).

In this chapter, the focus is therefore to reduce the truncated expression of gloshedobin. By transforming the plasmid encoding gloshedobin into *E. coli* strain BL21(DE3)pLysS instead of BL21(DE3), the truncation product was reduced by 25%. We further constructed a plasmid containing genes encoding ClpB and gloshedobin (with Trx•Tag) which are separated by a stem loop and RBS to allow the separate expression of these two proteins. With ClpB co-expression, gloshedobin expression profile showed a huge transition: almost all expression products were in full-length form with no apparent truncation product. Following cell disruption and solubilization

of IBs, a single step metal affinity chromatographic purification was sufficient to recover full-length gloshedobin at a high purity (>99%).

5.2 Materials and methods

5.2.1 Plasmids

pET-32a(+)+TLE was kindly provided by Dr Qing Yang from Dalian University of Technology, Dalian, Liaoning, China. The plasmid encoding ClpB (pClpB) was kindly provided by Dr Catherine Squires (Tufts University, Boston, MA, U.S.A.). Plasmid encoding ClpB and gloshedobin was constructed as follows. Each of the following three sets of primers: F1, 5'-GGA ATT CCA TAT GCG TCT GGA TCG TCT TAC-3', R1: 5'-GCG CCA CGT TGT CGC AAA GAT TAA-3'; F2, 5'-TTT GCG ACA ACG TGG CGC AAA AAC GTG G-3', R2, 5'-GTC GGC CAC GGT GCC GCG AGA CTC AAG T-3'; F3, 5'-TCG CGG CAC CGT GGC CGA CAT CCT GAA AGC-3', R3, 5'-TCC TTC TTA AAG TTA ACT TTG TTA GCA GCC GGA TCC AAT TAC TGG ACG GCG ACA ATC CGG-3' was applied in a separate polymerase chain reaction (PCR) by using pClpB as template to give 300 bp, 100 bp and 2.3 kb DNA respectively, which together encoded full-length ClpB. pET-32a(+)+TLE was used in another PCR and a 400 bp DNA fragment encoding N-terminal of thioredoxin-6×His-tag-gloshedobin was obtained by using primers of F, 5'-TTA ACT TTA AGA AGG AGA TAT ATA TAT GAG CGA TAA AAT TAT TC-3', R, 5'-GAT GAT GAT GGT GCA TAT GGC CAG AA-3'. By using these four DNA fragments as templates and primers of F1 and R, a 3 kb DNA fragment encoding

full-length ClpB and part of gloshedobin was obtained. The amplified fragment was then digested by *Nde*I and cloned into plasmid of pET-32a(+)+TLE pre-treated with the same restriction enzyme. The resultant plasmid (pET-32a(+)+TLE+ClpB) was used to express ClpB and thioredoxin-6×His-tag-gloshedobin separately.

5.2.2 Protein expression

E. coli BL21(DE3) cells (Novagen) were transformed with pET-32a(+)+TLE+ClpB to enable the over-expression of thioredoxin-fused gloshedobin and ClpB separately. The transformed cells were grown at 37°C to OD_{600} ~ 0.8 in LB broth containing 0.1 mg/mL ampicillin. Protein expression was induced with 1 mM IPTG for 4 h during the exponential growth phase. The cell suspension, harvested at its stationary growth phase, was centrifuged at 5000*g* and 4°C for 20 min and resuspended in 50 mM Tris buffer (pH 7.4) containing 150 mM KCl and 20 mM $MgCl_2$. One Shot Cell Disrupter (Constant Cell Disruption System, UK) was used to disrupt the cells at 21.0 Kpsi. Following cell disruption, the cell lysates were centrifuged at 15000*g* and 4°C for 30 min, and the soluble and insoluble fractions (IBs of gloshedobin) were kept at -20°C for further use. Protein expression for *E. coli* strain BL21(DE3) or BL21(DE3)pLysS containing plasmid pET-32a(+)+TLE was conducted using the same protocol as above.

5.2.3 Protein purification

IBs (mainly gloshedobin with or without the truncated expression product) collected

as above were solubilized in Buffer A (50 mM Tris, 8 M urea, 150 mM KCl, 20 mM $MgCl_2$ and 20 mM imidazole, pH 7.4) by vigorous overnight stirring at room temperature. After centrifugation at 15000*g* and room temperature for 40 min, remaining insoluble particles were removed by filtration using 0.45 µm filter (704006, Munktell Filter). The filtrate was then applied at a flow rate of 1 mL/min to a 2.5 cm i.d.×15 cm column (Econo-Column Chromatography Column, 737-1517, Bio-Rad) packed with 5 mL of Ni^{2+}-charged iminodiacetic acid (IDA) resin after equilibration of the column with Buffer A. The resin was next washed with 20 column volume (CV) of Buffer B (50 mM Tris, 8 M urea, 150 mM KCl, 20 mM $MgCl_2$ and 40 mM imidazole, pH 7.4) to completely eliminate weakly bound proteins. The bound proteins were eluted by gradually replacing Buffer B with Buffer C (50 mM Tris, 8 M urea, 150 mM KCl, 20 mM $MgCl_2$, 500 mM imidazole, pH 7.4) over 60 min. The eluted fractions were analyzed by SDS-PAGE. The chromatography system used was BioLogic LP from Bio-Rad (731-8300 and 731-8301) and the obtained data was processed with LP Data View V1.03 software.

5.2.4 Analytical methods

Protein detection and quantification: Total protein concentration determination and SDS-PAGE were conducted as described in Chapter 3.

Western blotting: Protein bands on the SDS-PAGE gel were transferred to the Immun-Blot™ PVDF membrane (162-0177, Bio-Rad) using Mini Trans-Blot®

Electrophoretic Transfer Cell from Bio-Rad (170-3930 and 170-3935) at 100 V for 1 h. Following the transfer, the membrane was blocked overnight in a blocking solution (3% gelatin in TBS) with gentle agitation on an orbital shaker. After washing with TTBS buffer (350 μL Tween-20 in 700 mL TBS), 25 mL of primary antibody solution (1% gelatin in TTBS with 1:5000 dilution of primary antibody) was added. The primary antibody used was monoclonal anti-polyhistidine clone His-1 (H1029, Sigma). Subsequently, 30 mL of secondary antibody solution (1% gelatin in TTBS with 1:3000 dilution of secondary antibody) was added. The secondary antibody used was Goat Anti-mouse IgG (H+L) AP Conjugate from Bio-Rad (170-6520). The bound antibody was detected by adding color development reagent (Alkaline Phosphate Conjugate Substrate Kit, 170-6432, Bio-Rad).

5.3 Results and discussion

5.3.1 Expression and purification of gloshedobin produced from pET-32a(+)+TLE in *E. coli* strain BL21(DE3) or BL21(DE3)pLysS

Both BL21(DE3) and BL21(DE3)pLysS are *lon* and *ompT* protease-deficient host strains. They were selected as the host strains for gloshedobin expression on the presumption that the lack of *ompT* outer membrane protease might enhance the stability of recombinant proteins during the expression and/or purification (Grodberg and Dunn, 1988; Gupta et al., 1997). Since all the previous gloshedobin expressions were attempted using BL21(DE3), it was employed in the present study as a control.

The plasmid, pLysS, has been known to stabilize the target genes and thus reduces the basal expression of target proteins (Moffatt and Studier, 1987; Studier, 1991). Hence, we would like to investigate whether the truncated expression can be reduced by firstly preventing protein degradation (using protease-deficient host strains) and secondly reducing the unspecific basal-level expression with the use of pLysS.

E. coli cells (BL21(DE3) or BL21(DE3)pLysS) transformed with pET-32a(+)+TLE were induced with 1 mM IPTG at 37°C and the protein expression patterns were analyzed by Coomassie Blue staining of SDS-PAGE gels (Figure 5.1A). The presence of truncated gloshedobin (i.e. C-terminal region of gloshedobin with N-terminal truncation) was indirectly confirmed by detecting another truncation product (i.e. thioredoxin containing 6×His-tag and N-terminal region of gloshedobin) with anti-polyhistidine antibody in western blotting (Figure 5.1B). In Figure 5.1B, two protein bands which correspond to full-length gloshedobin (with an apparent molecular weight of approximately 50 kDa) and a truncation product containing a 6×His-tag (with an apparent molecular weight of approximately 28 kDa) were observed. The heterologous protein expression has often resulted in the generation of multiple gene products (Chapman et al., 1998; Tsubamoto et al., 1999). In particular, truncation products have lower molecular weight than full-length protein possibly due to 1) multiple initiation sites in mRNA, 2) premature translation termination and/or 3) specific or nonspecific proteolysis of the full-length polypeptides (Halling and Smith, 1985; Preibisch et al., 1988; Govind et al., 2001).

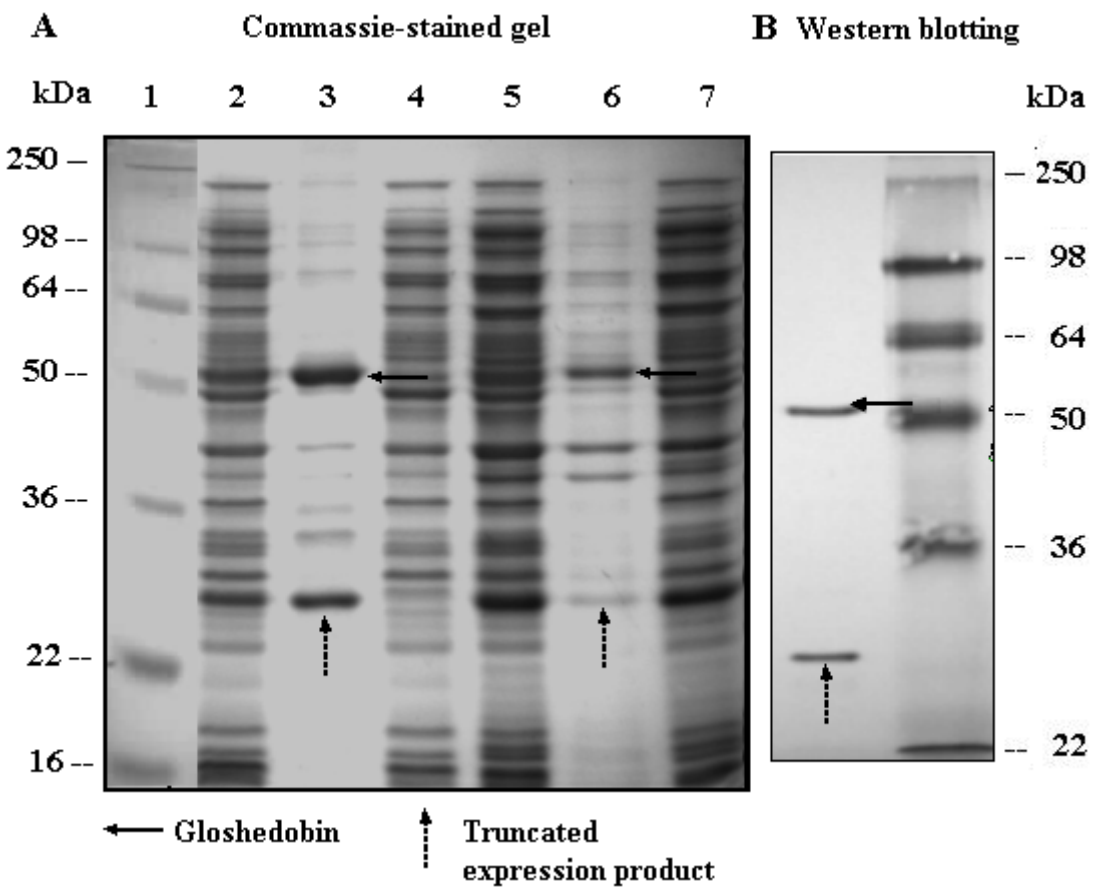


Figure 5.1 (A) SDS-PAGE for the analysis of gloshedobin expressed in *E. coli* BL21(DE3) or BL21(DE3)pLysS harboring pET-32a(+)+TLE. Molecular weight marker was loaded in lane 1. Lanes 2-4 are from *E. coli* BL21(DE3): the whole cell extracts (lane 2), the insoluble fraction (lane 3) and the soluble fraction (lane 4) in the cell extracts after high pressure cell disruption. Lanes 5-7 are from *E. coli* BL21(DE3)pLysS: the lane description is the same as in lanes 2-4. (B) Western blotting assay for gloshedobin expressed from *E. coli* BL21(DE3) harboring pET-32a(+)+TLE (corresponding to lane 3 in (A)). The lower molecular weight band (with apparent molecular weight of 27.5 kDa) represents thioredoxin containing 6×His-tag and N-terminal region of gloshedobin.

The presence of pLysS did not interfere with the transformation of pET-32a(+)+TLE and also showed negligible influence on the extent of growth of host cells. In contrast to our initial expectation, the basal level expression of gloshedobin was insignificant, hence the role of pLysS in this regard was not apparent (data not shown). It is noted, however, that the truncated expression in the presence of pLysS was significantly

reduced (Figure 5.1, lanes 3 and 6). However, this did not lead to the increase in intact gloshedobin since the expression of full-length gloshedobin was also reduced in the presence of pLysS. The effect of pLysS on the truncated expression was more clearly seen following the purification of gloshedobin using metal affinity chromatography: the purity of full-length gloshedobin from BL21(DE3), estimated by densitometry, was approximately 60% (Figure 5.2, lane 8) while that from BL21(DE3)pLysS was significantly higher (>85%) (Figure 5.3, lane 8). Although the exact role of pLysS in decreasing the truncated expression of gloshedobin requires further study, to the best of our knowledge, this is the first report on such efficacy and no previous study has reported reduced truncated expression of proteins including TLEs in the presence of pLysS. Despite the similar total protein production from both host strains (Table 5.1), the target protein (i.e. full-length gloshedobin) expression level in BL21(DE3)pLysS (ca 10%) was lower than that in BL21(DE3) (ca 13%), possibly due to the pLysS-modulated tighter regulation of protein expression (Studier, 1991). The lysozyme encoded by pLysS was reported to bind to T7 RNA polymerase, thereby inhibiting transcription and thus reducing the maximum expression level of target genes upon induction of T7 RNA polymerase (Zhang and Studier, 1997; Huang et al., 1999).

Table 5.1 Protein concentration in cell culture of *E. coli* BL21(DE3) or BL21(DE3)pLysS containing plasmid pET-32a(+)+TLE. The final OD of cell suspensions were adjusted to 80 before cell disruption.

OD=80	**Soluble protein concentration (mg/mL)**	**Insoluble protein concentration (mg/mL)**	**Total protein concentration (mg/mL)**
Protein expression in BL21(DE3)	8.9 ± 0.4	3.8 ± 0.2	12.7 ± 0.6
Protein expression in BL21(DE3)pLysS	10.8 ± 0.5	3.1 ± 0.1	13.9 ± 0.7

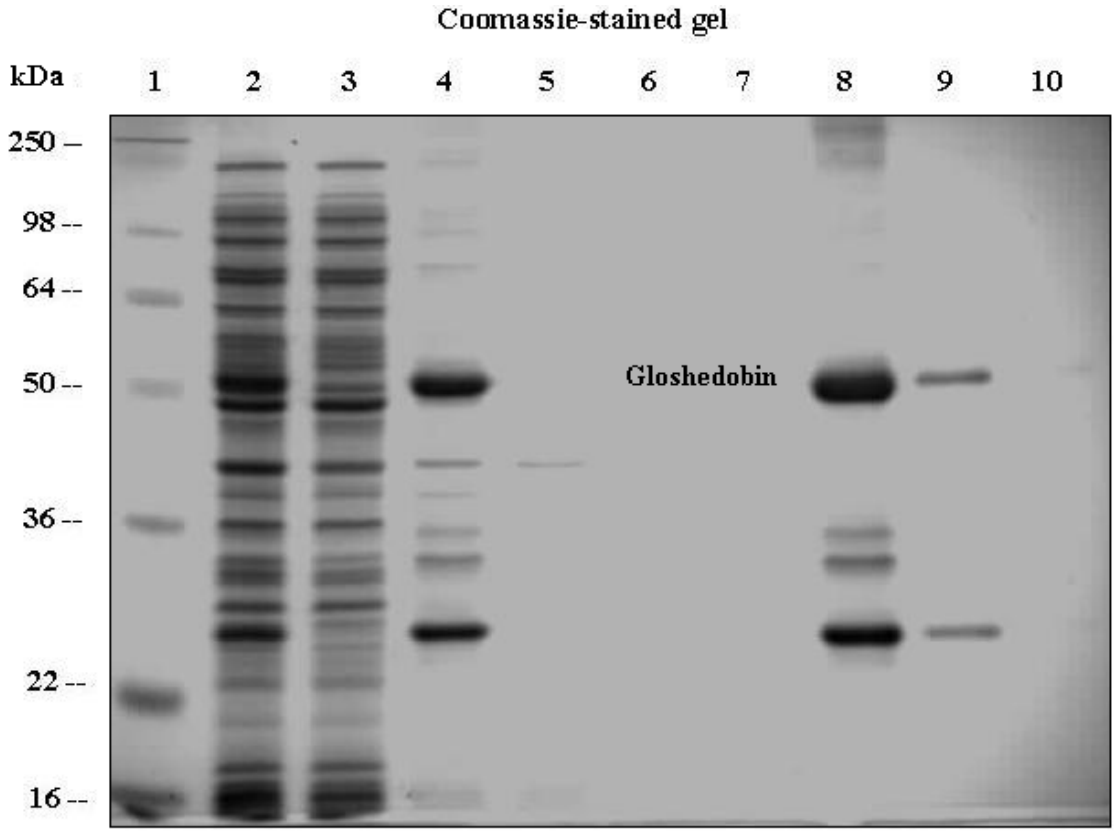


Figure 5.2 SDS-PAGE for the analysis of fractions collected during the purification of gloshedobin following its expression in *E. coli* BL21(DE3) harboring pET32-a(+)+TLE. Molecular weight marker was loaded in lane 1. Lanes 2-4 are from the BL21(DE3): the whole cell extracts (lane 2), the soluble fraction (lane 3) and the insoluble fraction (lane 4) in the cell extracts after high pressure cell disruption. Lane 5 is flow-through. Lanes 6 and 7 are wash fractions. Lanes 8-10 are selected fractions collected during the elution step.

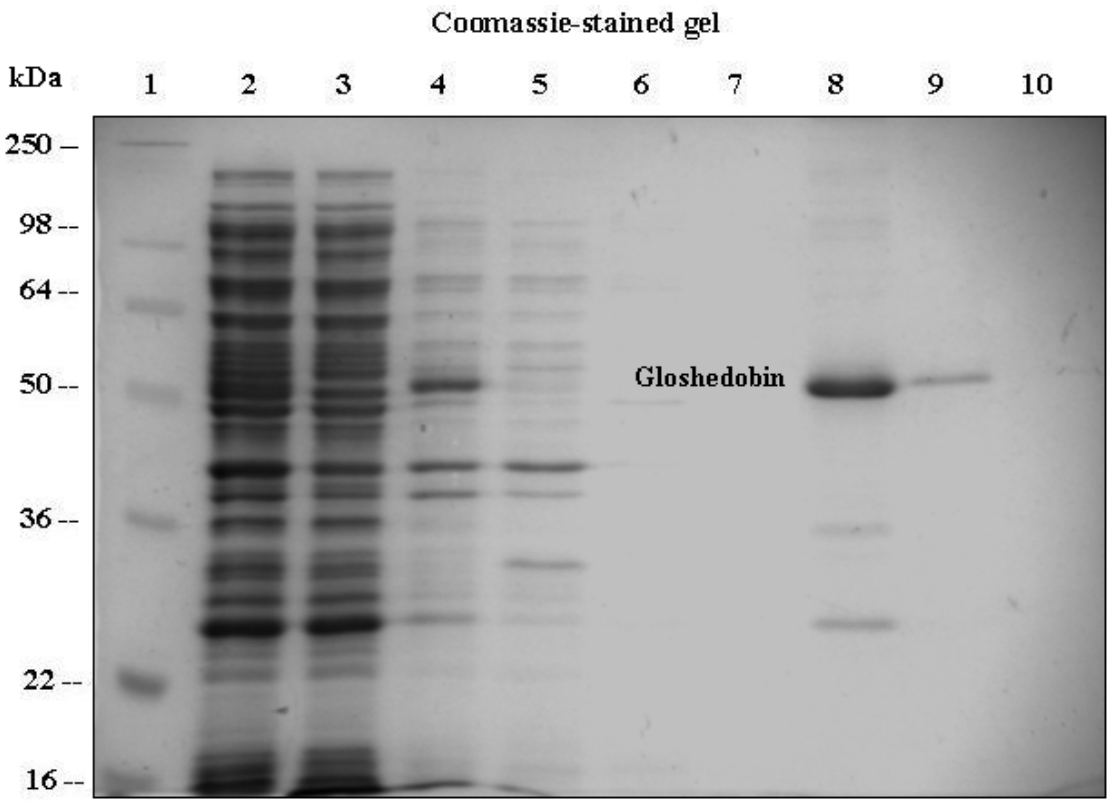


Figure 5.3 SDS-PAGE for the analysis of fractions collected during the purification of gloshedobin following its expression in *E. coli* BL21(DE3)pLysS harboring pET32-a(+)+TLE. The detailed lane descriptions are the same as in Figure 5.2.

5.3.2 Expression and purification of gloshedobin from *E. coli* strain BL21(DE3) harboring pET-32a(+)+TLE+ClpB

With the enhanced purity of intact gloshedobin, it is tempting to see improved solubility of the target protein. It was thus decided to co-express a molecular chaperone, ClpB (a homolog of Hsp100 in *E. coli*) whose function in disaggregating protein aggregates such as IBs has been extensively studied (Glover and Lindquist, 1998; Goloubinoff et al., 1999; Mogk et al., 1999; Zolkiewski, 1999) in the last decade. The major purpose of ClpB expression was to investigate if ClpB alone would be effective in diminishing the IB formation prior to its coordination with another versatile chaperone system known as DnaKJE (a homolog of Hsp70 in *E. coli*) for

subsequent refolding of disaggregated but misfolded polypeptides (De Marco et al., 2007). The transformation of *E. coli* BL21(DE3) with pET-32a(+)+TLE+ClpB, comprising gloshedobin coding region and *clpB* with an intervening stem loop (TTE, transcription termination element) and RBS, led to co-expression of 95 kDa ClpB in soluble form and 44 kDa gloshedobin (mostly in insoluble form) upon IPTG induction (Figure 5.4A). Note that the apparent molecular weight of gloshedobin as appeared on the SDS-PAGE gel was approximately 50 kDa, deviating by 13% from its molecular weight (44 kDa) determined by peptide sequence data or MALDI-TOF mass spectroscopy (data not shown). The size discrepancy of proteins often occurs when comparing molecular weight of proteins determined by SDS-PAGE gel versus peptide sequence data or MALDI-TOF (Squires et al., 1991). The cause for protein size deviation herein is probably insufficient amount of bound SDS per g protein and thus reduction in the negative charge of protein (Hjelmeland and Chrambach, 1981). However, in our experiment since the molecular weight of gloshedobin was reproducible as 50 kDa on the SDS-PAGE gels, the deviation from true molecular weight of the protein would not affect the use of SDS-PAGE in gloshedobin detection.

The expression level of full-length gloshedobin was about 11% which is slightly lower than the expression level (13%) obtained from BL21(DE3) harboring pET-32a(+)+TLE. However, the co-expression of ClpB did not compromise the volumetric productivity of gloshedobin since total protein concentration was around 1.3 times higher in the presence of ClpB compared to the condition lacking ClpB. It

was also found that ClpB alone is necessary but not sufficient for the disaggregation of IBs since no solubility increase in the expressed gloshedobin was seen (Figure 5.4A, lane 4). This is consistent with previous studies where reactivation of aggregated proteins following thermal stress required coordinated chaperoning of ClpB with *E. coli* Hsp70 (DnaK) and its co-chaperones (DnaJ, GrpE) (Mogk et al., 1999; Ziętkiewicz et al., 2004; 2006). Interestingly, co-expression of ClpB was very effective in reducing the truncation of gloshedobin at the N-terminus. Hence, no truncation product was detected in the insoluble fraction of cell disruptate by SDS-PAGE analysis (Figure 5.4A, lane 3). As discussed earlier, truncation product may arise from proteolysis of the full-length polypeptides or the presence of internal pseudo RBS sequence in mRNA or premature translation termination (Halling and Smith, 1985; Preibisch et al., 1988; Govind et al., 2001).

It is hard to envisage that *clpB* fusion to the downstream of gloshedobin encoding gene would affect the internal translation initiation or premature translation termination. Thus it is postulated that the truncation product is more likely to arise from the proteolytic degradation of full-length gloshedobin by various proteases populated in the cell. The Clp family comprises several closely related protein-activated ATPases that associate with peptidase subunits to form ATP-dependent protease complexes (Zolkiewski, 1999). Among the Clp family members, ClpP peptidase requires interactions with ClpA and ClpX for its stimulation, whereas ClpQ (a different peptidase) is activated via interactions with ClpY

(Gottesman et al., 1997). ClpB, despite a 42% sequence identity and 64% sequence similarity with ClpA (Gottesman et al., 1997), does not support protein degradation by Clp peptidases (Woo et al., 1992). Thus, the co-expression of ClpB itself is unlikely to affect proteolytic digestion of gloshedobin as evidenced by the presence of no other smaller His-tagged truncation products following purification with metal affinity chromatography. Taken together, the reduced truncated expression of gloshedobin seemed to be associated with an unknown function of ClpB: a possible protection of full-length gloshedobin against proteolysis. ClpB was reported to form hexamers in the presence of ATP and the hexamers were able to then bind onto large protein aggregates (Lee et al., 2003; Kedzierska et al., 2003). It was thus postulated that the formation of a large hexameric structure of co-expressed ClpB (molecular weight of hexamers >600 kDa) and its subsequent binding onto the protein aggregates might restrict the access of other proteases, thereby protecting protein aggregates against proteolytic degradation. As a result, this protease inhibitor-like effect of ClpB may have minimized the formation of truncation products.

A logical next expectation should be to see the synergistic effect of pLysS and ClpB co-expression on the reduction of truncation products. Hence, BL21(DE3)pLysS was transformed with pET-32a(+)+TLE+ClpB. However, gloshedobin expression (in either full-length or truncated form) was significantly hampered (expression level of full-length gloshedobin was only about 1.5%) and it was impossible to see the expected synergistic effect (Figure 5.4B).

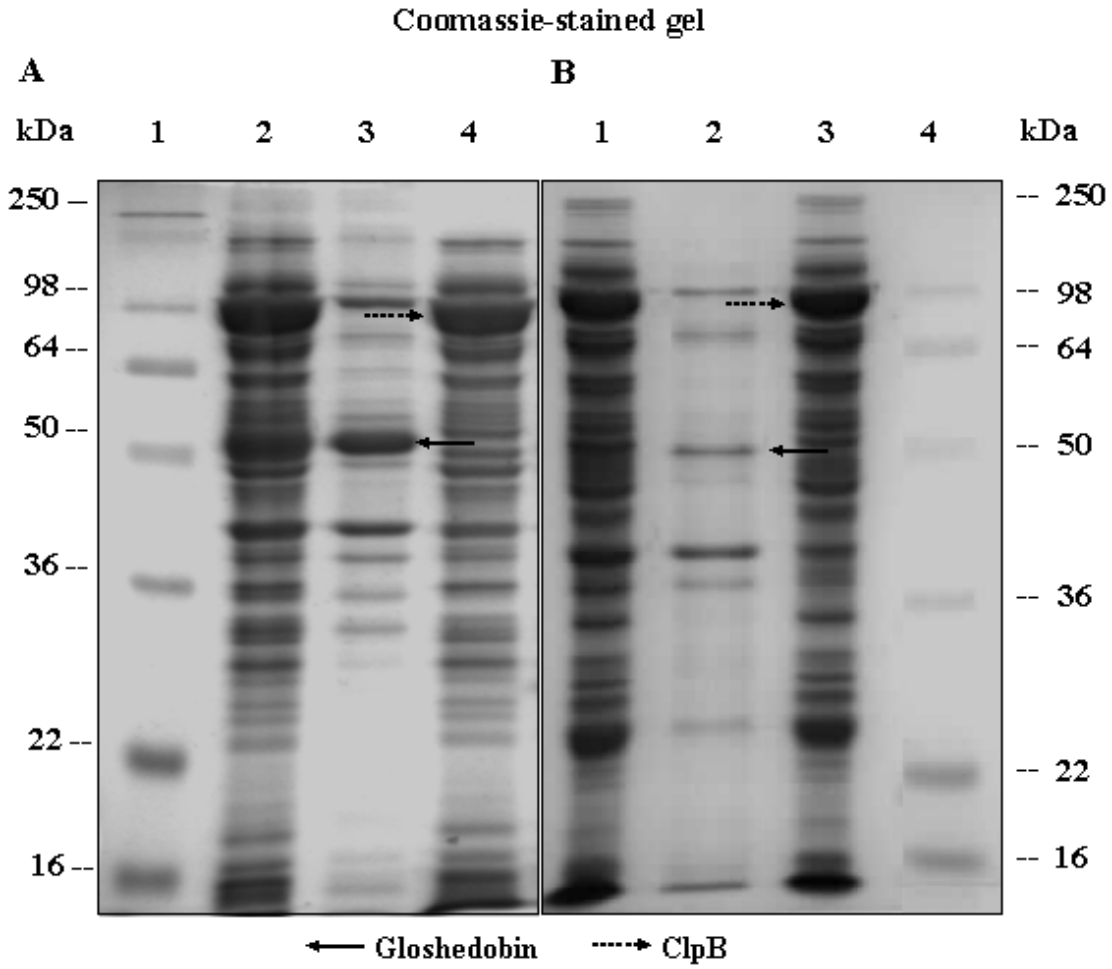


Figure 5.4 (A) SDS-PAGE for the analysis of gloshedobin following its expression in *E. coli* BL21(DE3) harboring pET-32a(+)+TLE+ClpB. Molecular weight marker was loaded in lane 1. Lanes 2-4 represent the whole cell extracts (lane 2), the insoluble fraction (lane 3) and the soluble fraction (lane 4) in the cell extracts after high pressure cell disruption. (B) SDS-PAGE for the analysis of gloshedobin expressed in *E. coli* BL21(DE3)pLysS harboring pET-32a(+)+TLE+ClpB. Lanes 1-3 represent the whole cell extracts (lane 1), the insoluble fraction (lane 2) and the soluble fraction (lane 3) in the cell extracts after high pressure cell disruption. Molecular weight marker was loaded in lane 4.

Following the enhanced expression of intact gloshedobin in BL21(DE3) harboring pET-32a(+)+TLE+ClpB, the cell pellets were disrupted and IB fraction was collected as described in Materials and methods section. Solubilized IBs (mainly containing full-length gloshedobin) were purified with metal affinity chromatography using Ni^{2+}-IDA. With the presence of negligible truncation product, the purification was

greatly facilitated. Highly purified full-length gloshedobin (>99%) shown in Figure 5.5 emphasized the benefits of reduced truncated expression in terms of both increased recovery yield and purity of functional target product. After obtaining the purified full-length gloshedobin, the challenge now is to convert the denatured proteins to native form with biological activity. A column-based refolding strategy with the assist of unpurified ClpB/DnaKJE bichaperone system developed in Chapter 3 is investigated in the following chapter. Dilution refolding, the commonly used refolding technique, is also conducted as a comparison.

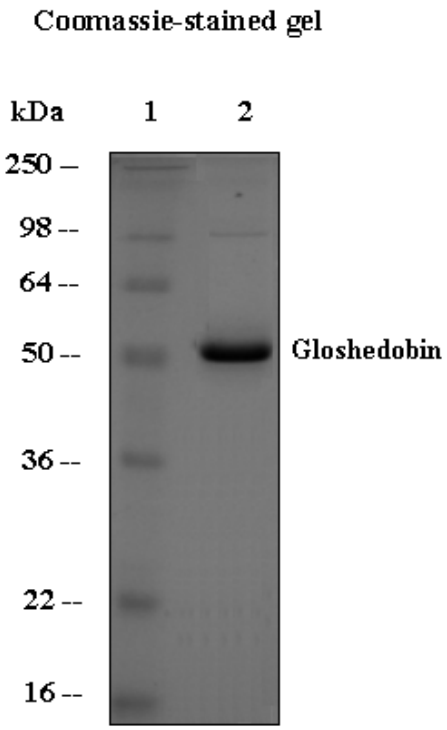


Figure 5.5 Purified gloshedobin by IMAC under denaturing condition following its expression in *E. coli* BL21(DE3) harboring pET-32a(+)+TLE+ClpB. The apparent molecular weight of purified gloshedobin as appeared on the SDS-PAGE gel was around 50 kDa.

5.4 Conclusion

Heterologous protein expression in *E. coli* occasionally leads to both full-length target protein and truncation products. The presence of truncated expression products is likely to affect the purification efficiency severely, thus compromising final target protein purity and recovery yield. The present study demonstrated that the truncated expression of gloshedobin (one of the promising TLEs) could be effectively reduced by simply switching the expression host from BL21(DE3) to BL21(DE3)pLysS. Furthermore, co-expression of a molecular chaperone, ClpB, in BL21(DE3) almost completely eliminated truncated expression of gloshedobin, facilitating the high purity (>99%) purification of the target protein by metal affinity chromatography.

Chapter 6
Chaperone-assisted column refolding of gloshedobin with the use of refolding cocktail

Summary

Gloshedobin, a recently isolated thrombin-like enzyme (TLE) from the snake venom of *Gloydius shedaoensis*, is expressed mainly in the form of inclusion bodies (IBs) in *Escherichia coli* due to its cysteine-rich nature. Moreover, truncated expression products significantly contaminated the purification of full-length gloshedobin through immobilized metal affinity chromatography (IMAC). In Chapter 5, it was shown that co-expression of ClpB rendered almost complete elimination of gloshedobin truncation products, allowing for the expression of intact gloshedobin (mostly in IB form though) without compromising the expression level. Following extraction and solubilization of the IBs, one-step immobilized metal affinity chromatography purification produces highly purified (>99%) denatured-solubilized gloshedobin ready to enter the subsequent refolding process. However, the traditional dilution or column refolding strategy, based on gradual denaturant removal, was found to be inefficient for the recovery of protein activity. In this chapter, a new refolding strategy harnessing the ClpB and DnaK/DnaJ/GrpE bichaperone system developed in Chapter 3 is demonstrated to be superior to the conventional refolding methods in either batch dilution or column refolding mode. It is noted that the

efficacy of bichaperone-mediated column refolding strategy is further highlighted especially when refolding reaction is attempted at a higher protein concentration with the recirculation of the refolding cocktail containing the bichaperone system. This is evidenced by an uncompromised refolding efficiency (c.a. 21.4%) achieved at 2000 μg/mL of initial protein concentration, which is comparable to the refolding efficiency (c.a. 22.5%) obtained at 20 times lower protein concentration (i.e. 100 μg/mL) in the conventional batch dilution refolding technique. The demonstrated chaperone-assisted column refolding strategy thus provides an effective tool for refolding-recalcitrant proteins whose reactivation is otherwise difficult to achieve.

6.1 Introduction

It was shown in Chapter 5 that with the co-expression of a molecular chaperone ClpB, the truncated expression associated with the full-length gloshedobin expression was almost completely eliminated, facilitating the subsequent purification by immobilized metal affinity chromatography (IMAC). The current challenge is thus to convert these inactive, misfolded protein aggregates into soluble and bioactive products (De Bernardez Clark, 2001; Middelberg, 2002). The various IB processing technologies developed for this purpose are well reviewed elsewhere (Choe et al., 2006).

The immobilization of protein onto an adsorbent matrix during column refolding significantly limits intermolecular interactions between partially folded proteins, hence aggregate formation can be minimized (Li et al., 2004; Langenhof et al., 2005).

The use of affinity tags (e.g. His-tag) engineered on the proteins to facilitate their binding to IMAC column provides an additional advantage to refolding processes by eliminating host-derived contaminating proteins while allowing a large section of protein to refold unconstrained (Stempfer et al., 1996 ; Langenhof et al., 2005). In this Chapter, the focus was to assess refolding efficiency of IMAC-purified denatured gloshedobin in either batch dilution or column refolding mode in the presence or absence of the molecular bichaperone system comprising ClpB and DnaK/DnaJ/GrpE (ClpB/DnaKJE) as developed in Chapter 3 in the refolding milieu.

6.2 Materials and methods

6.2.1 Plasmids

Plasmid pET-32a(+)+TLE for the expression of 6×His-tagged recombinant gloshedobin was kindly provided by Dr Qing Yang at Dalian University of Technology (Dalian, Liaoning, China). Plasmid pET-32a(+)+TLE+ClpB capable of co-expressing ClpB and gloshedobin separately was constructed as described in Chapter 5. Plasmid encoding ClpB (pClpB) was kindly provided by Dr Catherine Squires at Tufts University (Boston, MA, USA). Plasmid pKJE7 (3340) designed to express DnaKJE was purchased from Takara Biotechnology, Japan.

6.2.2 Protein expression

Protein expression was achieved in antibiotics-supplemented LB broth using *E. coli* BL21(DE3) (Novagen) as an expression host. Cells harboring pET-32a(+)+TLE or

pET-32a(+)+TLE+ClpB alone or together with pKJE7 were grown to log phase ($OD_{600\ nm}$ ~ 0.8) in the presence of 100 μg/mL of ampicillin (additional 34 μg/mL chloramphenicol was added when pKJE7 was present) at 37°C. Gloshedobin expression was induced with 1 mM IPTG for 4 h. For cells co-transformed with pKJE7, $_{L}$-arabinose (10845, Fluka) was first added to a final concentration of 2.5 mg/mL to induce the expression of DnaKJE for 30 min. Gloshedobin expression was then initiated as described above. The cell suspension was centrifuged at 5000*g*, 4°C for 20 min and the cell pellets resuspended in binding buffer (50 mM Tris, 300 mM KCl, 20 mM $MgCl_2$ and 40 mM imidazole at pH 8). One Shot Cell Disrupter (Constant Cell Disruption System, UK) was used to disrupt the cells at 21.0 Kpsi. The cell disruptates were then centrifuged at 15000*g*, 4°C for 30 min. The soluble and insoluble fractions were immediately used in the following purification and refolding experiments.

Molecular chaperone ClpB was expressed according to Woo et al. (1992). DnaKJE were expressed according to Nishihara et al. (1998). Following expression, the culture broth was centrifuged at 5000*g*, 4°C for 20 min, and the cell pellets collected and resuspended in Buffer A (50 mM Tris, 150 mM KCl and 20 mM $MgCl_2$ at pH 8) and disrupted as above. The supernatant containing unpurified ClpB or DnaKJE was stored at -20°C as a stock solution until further use as described in Chapter 3.

6.2.3 Protein purification and refolding

6.2.3.1 Protein purification under native condition

The soluble fraction recovered following the co-expression of gloshedobin with DnaKJE was filtered using a 0.45 μm filter (704006, Munktell Filter) and loaded at 0.5 mL/min to a 2.5 cm i.d.×15 cm column (Econo-Column Chromatography Column, 737-1517, Bio-Rad) packed with 15 mL of Ni^{2+}-charged iminodiacetic acid (IDA) resin (Chelating Sepharose™ Fast Flow, 17-0575-02, GE Healthcare) following pre-equilibration with binding buffer. The resin was next washed at 1 mL/min with 10 column volume of washing buffer (50 mM Tris, 300 mM KCl, 20 mM $MgCl_2$, 60 mM imidazole, pH 8). The target protein was then eluted at 1 mL/min using a linear gradient from the washing buffer to elution buffer (50 mM Tris, 300 mM KCl, 20 mM $MgCl_2$, 500 mM imidazole, pH 8) over 30 min. The eluates were collected in 1 mL fractions and the purity of gloshedobin was analyzed by SDS-PAGE. The chromatography system used was BioLogic LP (731-8300 and 731-8301, Bio-Rad). The fractions containing gloshedobin were pooled and filtered through a 0.22 μm filter (SLGP033RS, Millipore) and then subjected to gel filtration chromatography at 4°C using a HiLoad™ 16/60 Superdex™ 200 pg column (17-1069-01, GE Healthcare) on ÄKTA purifier from Amersham Biosciences. 1 mL sample was loaded each time at 0.2 mL/min. Buffer A was used for column equilibration and protein elution. The eluents were collected in 1 mL fractions and the purity of target protein was analyzed by SDS-PAGE. The enzymatic activity of the purified gloshedobin was analyzed immediately as described in the analytical methods section.

6.2.3.2 Protein purification under denaturing condition

The insoluble IBs containing gloshedobin were solubilized in denaturing binding buffer (50 mM Tris, 8 M urea, 300 mM KCl, 20 mM $MgCl_2$, 40 mM imidazole, pH 8) by vigorous magnetic stirring for 6 h. After centrifugation at 15000*g* for 30 min, the insoluble particles were removed with a 0.45 μm filter. The purification was conducted as in the native condition except that 8 M urea was added to all the buffers used. The fractions containing gloshedobin were pooled and desalted against buffer (50 mM Tris, 8 M urea, 300 mM KCl and 20 mM $MgCl_2$ at pH 8) to remove imidazole using a HiPrep^{TM} 26/10 Desalting column (17-5087-01, GE Healthcare) on ÄKTA purifier. The urea-denatured gloshedobin was further concentrated by ultrafiltration using Vivaspin Sample Concentrators (28-9322-47, GE Healthcare) with a molecular weight cut-off (MWCO) of 10 kDa. To counteract any potential air-oxidation of denatured gloshedobin during the purification process, DTT (D0632, Sigma) was added to the urea-denatured gloshedobin (after desalting) to a final concentration of 50 mM, and incubated for 3 h in order to ensure complete reduction of intra- or inter-molecular disulfide bonds. Removal of DTT was effected by an additional buffer exchange step immediately prior to refolding experiments (Lanckriet and Middelberg, 2003; Hutchinson and Chase, 2006b; Leong and Middelberg, 2006).

6.2.3.3 Protein refolding by dilution

Following DTT removal, the protein concentration was adjusted to fall between 1 to 40 mg/mL using the desalting buffer (Hutchinson and Chase, 2006b). A 1 mL aliquot

of the purified and denatured gloshedobin was rapidly diluted 20-fold under vigorous mixing with refolding buffer (50 mM Tris, 45 mM KCl, 20 mM $MgCl_2$, 3 mM glutathione (GSH), 3 mM glutathione disulphide (GSSG), pH 8) to a final concentration ranging from 100 to 2000 μg/mL at an urea concentration of 0.4 M. The refolding mixture was incubated for 20 h and 500 μL aliquots of samples were collected at regular time intervals for enzymatic activity analysis.

For chaperone-assisted dilution refolding, 1 mL protein sample was diluted 20-fold into the refolding buffer supplemented with a bichaperone refolding cocktail as developed in Chapter 3 with some modifications. Wild type ClpB, instead of His-tagged ClpB, was used to avoid chaperone binding to IMAC matrix during the refolding process. ClpB extracts, DnaKJE extracts, ATP stock solution and ATP regeneration system were mixed with the refolding buffer to give a final concentration of 5 μM ClpB, 0.2 mg/mL DnaKJE, 5 mM ATP and an ATP regeneration system comprising 4 mM phosphoenol pyruvate and 20 ng/mL pyruvate kinase.

6.2.3.4 Protein refolding by IMAC

Column refolding was conducted on a 2.5 cm i.d.×15 cm column (the same as that used in the protein purification step) which was packed with 2.3 mL of Ni^{2+}-charged IDA resin. Following equilibration of the resin with denaturing binding buffer, a denatured gloshedobin sample prepared at varying concentrations (100-2000 μg/mL) was loaded at 0.25 mL/min. The refolding was initiated by applying a decreasing

linear urea gradient (from 8.0 M to 0.4 M) to the bound proteins over 120 min at a flow rate of 1 mL/min, starting with the binding buffer and ending with refolding buffer (50 mM Tris, 0.4 M urea, 300 mM KCl, 20 mM $MgCl_2$, 3 mM GSH, 3 mM GSSG, pH 8). The column was then further washed with the same refolding buffer for 30 min.

For chaperone-assisted column refolding, a refolding cocktail containing ClpB and DnaKJE was prepared as described in the dilution refolding. Following the completion of downhill urea-gradient induced refolding reaction as above, the column was continuously washed by recycling 20 ml of refolding cocktail (50 mM Tris, 0.4 M urea, 150 mM KCl, 20 mM $MgCl_2$, 3 mM GSH, 3 mM GSSG, 5 μM ClpB, 0.2 mg/mL DnaKJE and 5 mM ATP with ATP regeneration system, pH 8) for 0.5-4 h at 0.25 mL/min. The column was next extensively washed with the refolding buffer containing no molecular chaperone and the elution was effected by applying an increasing linear imidazole gradient (0 to 500 mM imidazole in refolding buffer) at 1 mL/min over 30 min. The eluates were collected in 1 mL fractions and analyzed for protein concentration and enzymatic activity.

Chaperone-assisted column refolding was next conducted using an alternative strategy. Instead of applying the refolding cocktail after the gradual removal of urea, refolding reaction was directly initiated by applying a linear gradient from denaturing washing buffer to refolding cocktail over 120 min at a flow rate of 1 mL/min. An additional 20

mL of refolding cocktail was continuously recycled for different time periods (0.5-3 h) at a flow rate of 0.25 mL/min to obtain the optimized refolding yield. The column was then washed, and the target protein eluted and characterized.

6.2.3.5 Purification of refolded gloshedobin using gel filtration chromatography

Refolded gloshedobin from chaperone-assisted column refolding (with the optimum refolding yield) was further purified by gel filtration chromatography at 4°C on ÄKTA purifier. 3.5 mL of protein sample (concentrated to around 1.5 mg/mL) was loaded to the column at 0.2 mL/min. Buffer A was used for column equilibration and protein elution. The eluents were collected in 2 mL fractions. The protein standard (Bio-Rad, 151-1901) was used to estimate the molecular weight of the eluted proteins.

6.2.4 Analytical methods

Protein detection and quantification: Total protein concentration determination and SDS-PAGE were conducted as described in Chapter 3.

Fibrinogenolytic activity determination: Fibrinogenolytic activity was analyzed according to Shimokawa and Takahashi (1995). Bovine fibrinogen (46312, Fluka) was dissolved in buffer containing 50 mM Tris, 6 mM $CaCl_2$ at pH 8 to give a final concentration of 0.4 mg/mL. 800 μL of fibrinogen solution was incubated with 200 μL gloshedobin solution (0.5 mg/mL). The mixture was incubated at 37°C for 12 h

and then assayed by SDS-PAGE.

Amidolytic activity assay: Amidolytic activity was measured according to published protocols (Lottenberg et al., 1981; Cho et al., 2001; Yang et al., 2002) using chromogenic protease N-(*p*-Tosyl)-Gly-Pro-Arg *p*-nitroanilide (T1637, Sigma) as the substrate. Activity was assessed by mixing 100 μL of gloshedobin solution with 900 μL of the 0.5 mM substrate in 50 mM Tris at pH 8. The amount of *p*-nitroanilide released was determined by recording the increase in absorbance at 405 nm for 10 min. One unit of amidolytic activity was defined as the amount of enzyme necessary to hydrolyze 1 μM substrate per min. Ancrod (A5042, Sigma), a kind of TLE from *Calloselasma rhodostoma*, was used as the standard (Yang et al., 2002).

RP-HPLC analysis: RP-HPLC was performed using Zorbax 300 SB-C18 column (5 μm, 4.6×250 mm, 880995-902, Agilent Technologies) on Shimadzu LC-10AVP HPLC system. All the HPLC-grade buffers used were supplemented with 0.1% v/v formic acid. 100 μL of protein sample was injected for each analysis and then eluted from the column using an acetonitrile-water gradient (10-90% v/v acetonitrile, over 60 min) at a flow rate of 0.75 mL/min. Between each run, the column was re-equilibrated with 10% v/v acetonitrile for 20 min. The protein absorbance was measured at 280 nm. All samples were centrifuged for 5 min at 8000*g*, 4^{o}C and then filtered using a 0.22 μm filter prior to RP-HPLC analysis. Data acquisition and processing were conducted using the Class-VP 7.2.1 software (Shimadzu, Australia).

Circular dichroism (CD) spectroscopy: Far UV CD spectra for denatured, native, and refolded gloshedobin were obtained with a Jasco J-720 (Jasco Corp., Japan) spectrometer using a 0.01-cm cylindrical cell. Native and refolded gloshedobin (purified after gel filtration) was first dialyzed against 10 mM sodium phosphate at pH 8 to remove salt interferences in CD analysis. Denatured gloshedobin was prepared as stated in section ***6.2.3.2 Protein purification under denaturing condition***. DTT was removed before CD measurement as DTT absorbs far UV in the analytical region. Protein concentration was adjusted to 0.2 mg/mL for all CD measurements. Spectra, corrected by subtracting the buffer baseline, were averaged over 10 readings. Mean residue ellipticity (θ), expressed in deg cm^2 $dmol^{-1}$, was calculated as described in Chapter 3.

6.3 Results and discussion

His-tagged recombinant gloshedobin was expressed and purified using IMAC under either native or denaturing condition (Figure 6.1). The refolding of denatured gloshedobin was achieved by dilution or on-column approach (with/without molecular chaperones) (Figure 6.1). As demonstrated by Yang et al. (2005), the presence of thioredoxin did not inhibit the enzymatic activity of gloshedobin, hence the removal of this fusion partner was not conducted. The enzymatic activity of the refolded gloshedobin was compared to that of native purified gloshedobin (Table 6.1).

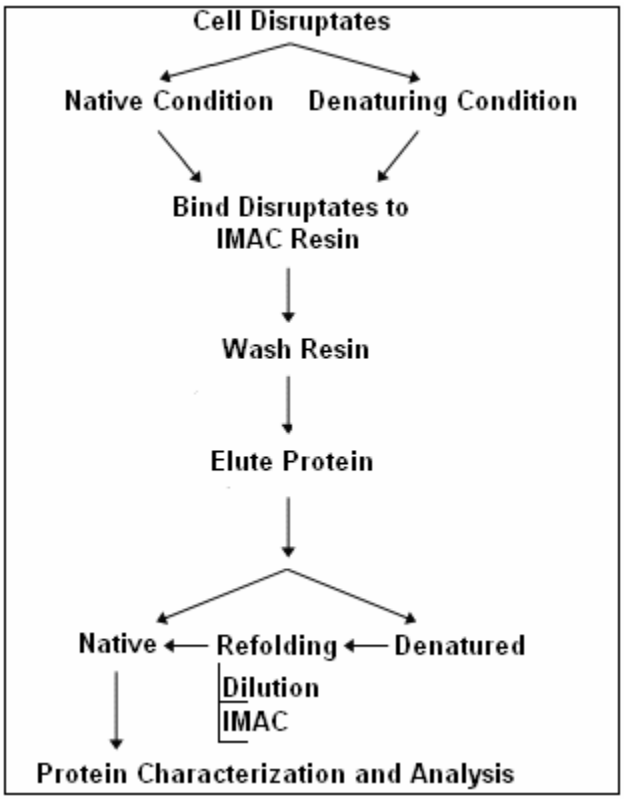


Figure 6.1 A schematic summarizing the purification and refolding procedures of 6×His-tagged recombinant gloshedobin.

Table 6.1 Summary of the relative enzymatic activity of gloshedobin obtained from various refolding processes at different protein concentrations.

Refolding process		Relative Enzymatic activity (%)*		
		100 μg/mL	500 μg/mL	2000 μg/mL
Dilution	without molecular chaperones	22.5±1.1	4.3±0.2	0.1±0.1
	with molecular chaperones	46.4±1.6	17.6±0.8	2.0±0.1
Column	without molecular chaperones	21.6±1.1	19.4±1.0	11.4±0.5
	with molecular chaperones	43.8±1.3	42.5±1.2	21.4±0.6

*Relative enzymatic activity is defined as a percentage recovery of specific enzymatic activity of refolded gloshedobin as compared to the specific enzymatic activity (98.3 U/mg) exhibited by purified native gloshedobin.

6.3.1 Purification and characterization of soluble (native) gloshedobin

Gloshedobin expressed from pET-32a(+)+TLE is highly prone to IB formation. Although a variety of efforts have been made to improve the solubility of heterologous protein expressed in *E. coli* (Yang et al., 2003a; 2003b), over-expression of several molecular chaperones to modulate *in vivo* solubility and folding of recombinant protein have become more popular during the last decade. For example, co-expression of DnaKJE chaperones proved very effective to enable marked accumulation of soluble target proteins which would otherwise have a high propensity to accumulate into IBs (Nishihara et al., 1998; De Marco et al., 2007).

As shown in Figure 6.2A, the aggregation of gloshedobin is almost completely prevented in the presence of DnaKJE and the target protein is produced mainly in the soluble form. The expression level of soluble gloshedobin from pET-32a(+)+TLE is estimated to be around 8.5%. With two-step chromatographic separation (IMAC followed by SEC (size exclusion chromatography)), the purity of gloshedobin reaches > 95% (Figure 6.2B, lanes 3 and 4).

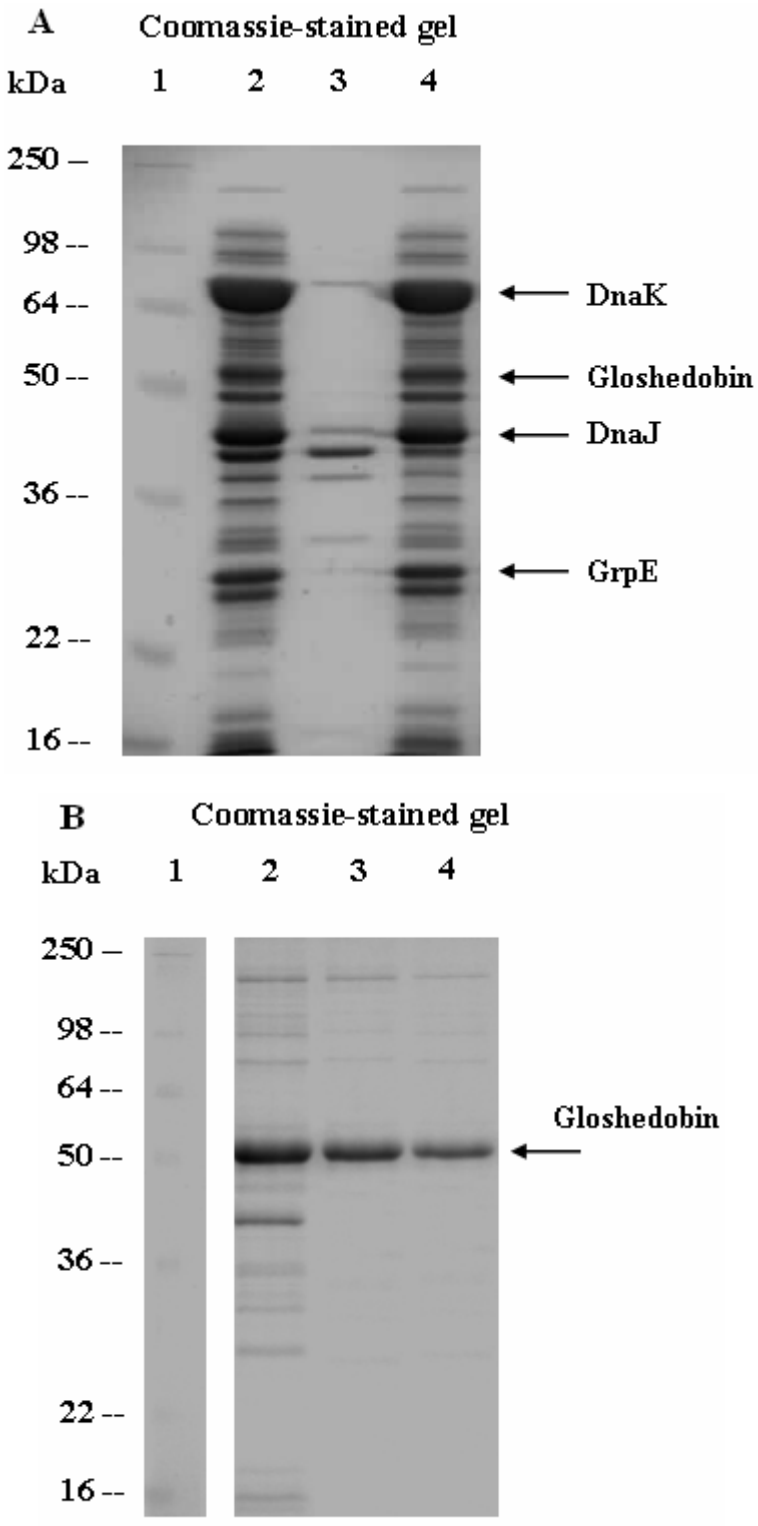


Figure 6.2 The effect of DnaKJE chaperone system co-expression on disaggregation of gloshedobin. (A) Cells harboring pET-32a(+)+TLE (expressing gloshedobin) and pKJE7 (expressing DnaKJE). Lane 1 is the molecular weight marker. Lanes 2-4 represent the whole cell extracts after high-pressure cell disruption, the insoluble and soluble fractions in the cell extracts respectively. (B) Purification of soluble gloshedobin (from cells harboring pET-32a(+)+TLE and pKJE7) under native

condition. Lane 1 is the molecular weight marker. Lane 2 is gloshedobin purified through IMAC. Lanes 3-4 are gloshedobin collected after gel filtration.

The biological activity of purified gloshedobin was determined by assessing its ability to cleave Aα chain of bovine fibrinogen (Shimokawa and Takahashi, 1995). The reduced bovine fibrinogen was separated into three main chains (Aα, Bβ and γ) with several minor polypeptide chains (Figure 6.3, lane 2). When purified gloshedobin (Figure 6.2B, lanes 3-4) was mixed with fibrinogen at a ratio of 1:3 (w/w), the Aα chain was completely degraded after 12 h incubation (Figure 6.3, lane 3). TLEs are also characterized by their strong amidolytic activity towards chromogenic substrates, such as N-(*p*-Tosyl)-Gly-Pro-Arg *p*-nitroanilide. A high enzymatic activity of 98.3 U/mg was obtained for the purified gloshedobin. This specific activity wais used as a reference to assess the efficiency of gloshedobin refolding.

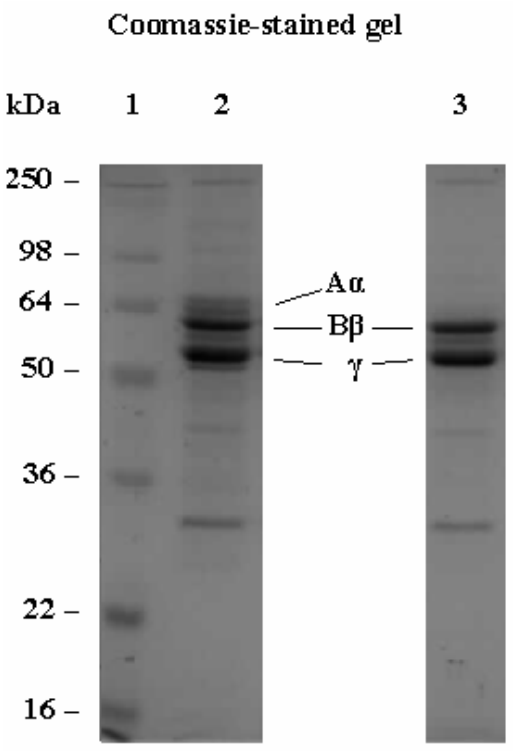


Figure 6.3 SDS-PAGE analysis of bovine fibrinogen degradation by purified gloshedobin. Lane 1 is the molecular weight marker. Lane 2 is bovine fibrinogen as a control. Lane 3 is bovine fibrinogen incubated for 12 h with 0.1 mg/mL purified gloshedobin (Figure 6.2B, lanes 3-4).

6.3.2 Purification of gloshedobin from IBs under denaturing condition

As described in Chapter 5, co-expression of ClpB with gloshedobin proved highly effective in significantly reducing its truncation product, rendering the expression of gloshedobin mostly in intact IB form (Figure 6.4A) without compromising the expression level (c.a. 11%). The extraction and solubilization of the intact gloshedobin from IBs was conducted as described in the Experimental section. Following one-step IMAC purification with the use of Ni^{2+}-IDA, the purity of denatured-solubilized gloshedobin reached approximately 99% (Figure 6.4B). The urea-denatured gloshedobin in the eluate was desalted and further incubated with 50 mM DTT to fully reduce all the disulfide bonds. However, it was reported that the presence of residual DTT hinders the correct refolding of protein, making it necessary to remove DTT prior to protein refolding (Lanckriet and Middelberg, 2003).

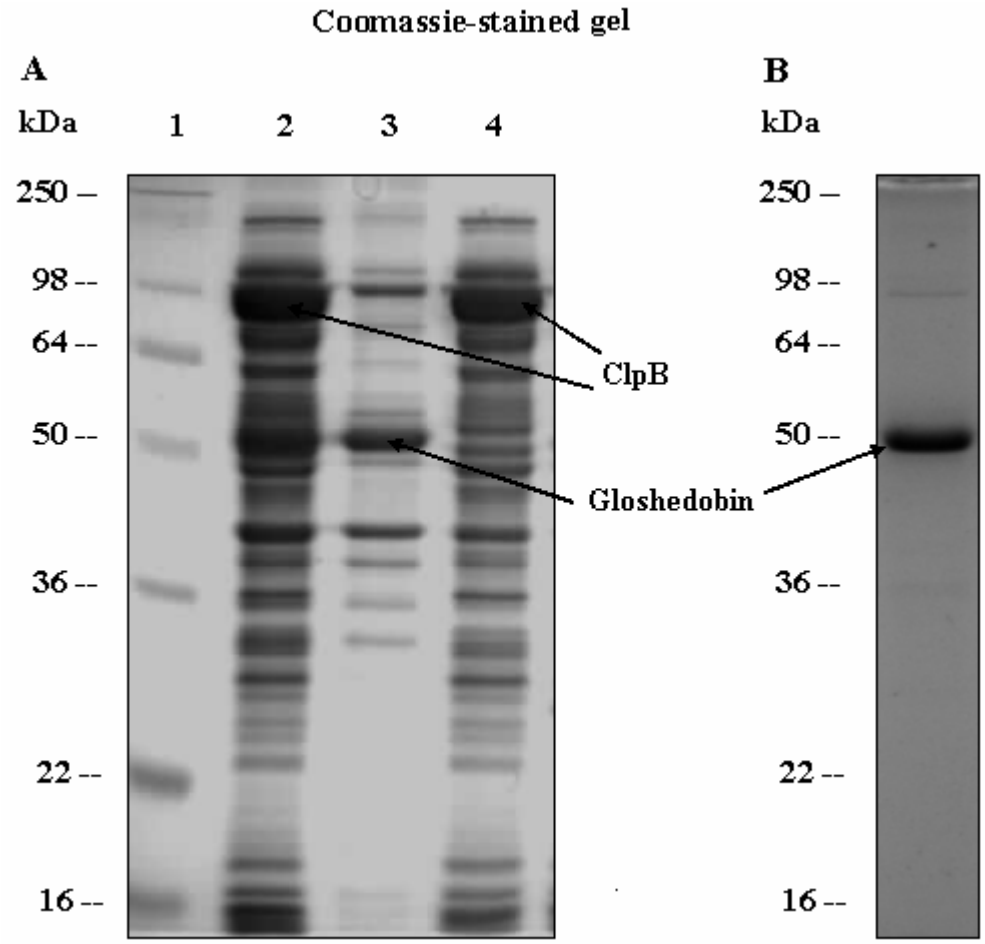


Figure 6.4 (A) SDS-PAGE analysis of gloshedobin and ClpB expressed in *E. coli* BL21(DE3). The lane description is the same as in Figure 6.2A. (B) Gloshedobin after IMAC purification under denaturing condition.

To investigate the adverse effect of DTT on the disulfide-containing TLE, we first incubated commercially available ancrod (a kind of TLE) in the standard refolding buffer (50 mM Tris, 50 mM KCl, 20 mM $MgCl_2$, pH 8) at a concentration of 10 U/mL with various concentrations of DTT. As shown in Figure 6.5A, the native ancrod loses more than 30% of its enzymatic activity in the presence of 0.5 mM DTT, indicating that the activity of this cysteine-rich protein is impeded by the presence of DTT.

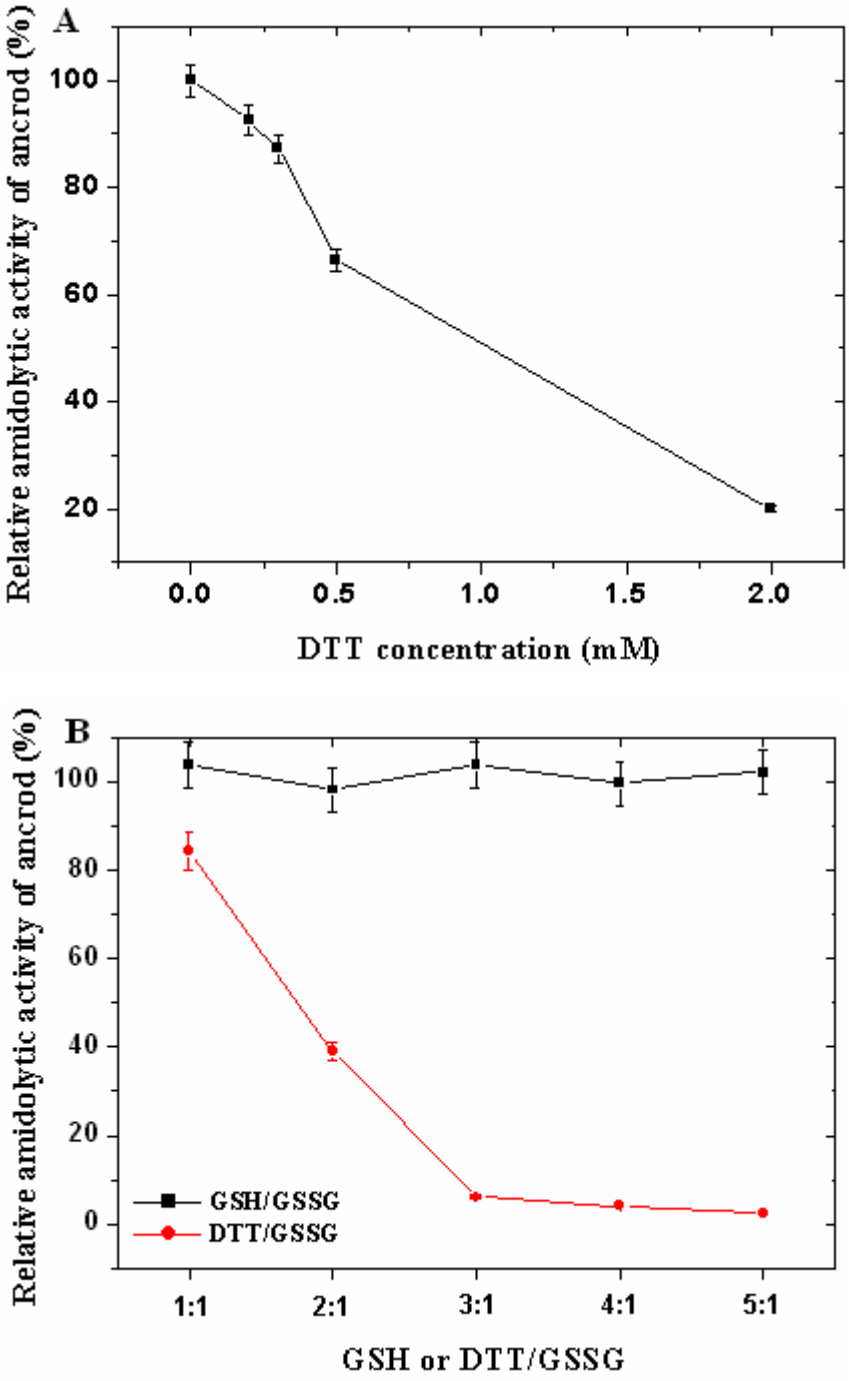


Figure 6.5 (A) Relative amidolytic activity of ancrod (a kind of TLE) in the standard refolding buffer with various concentrations of DTT. (B) The effect of GSH/GSSG and DTT/GSSG ratios on the relative amidolytic activity of ancrod. The total concentration of the thiol reagents was kept at 6 mM.

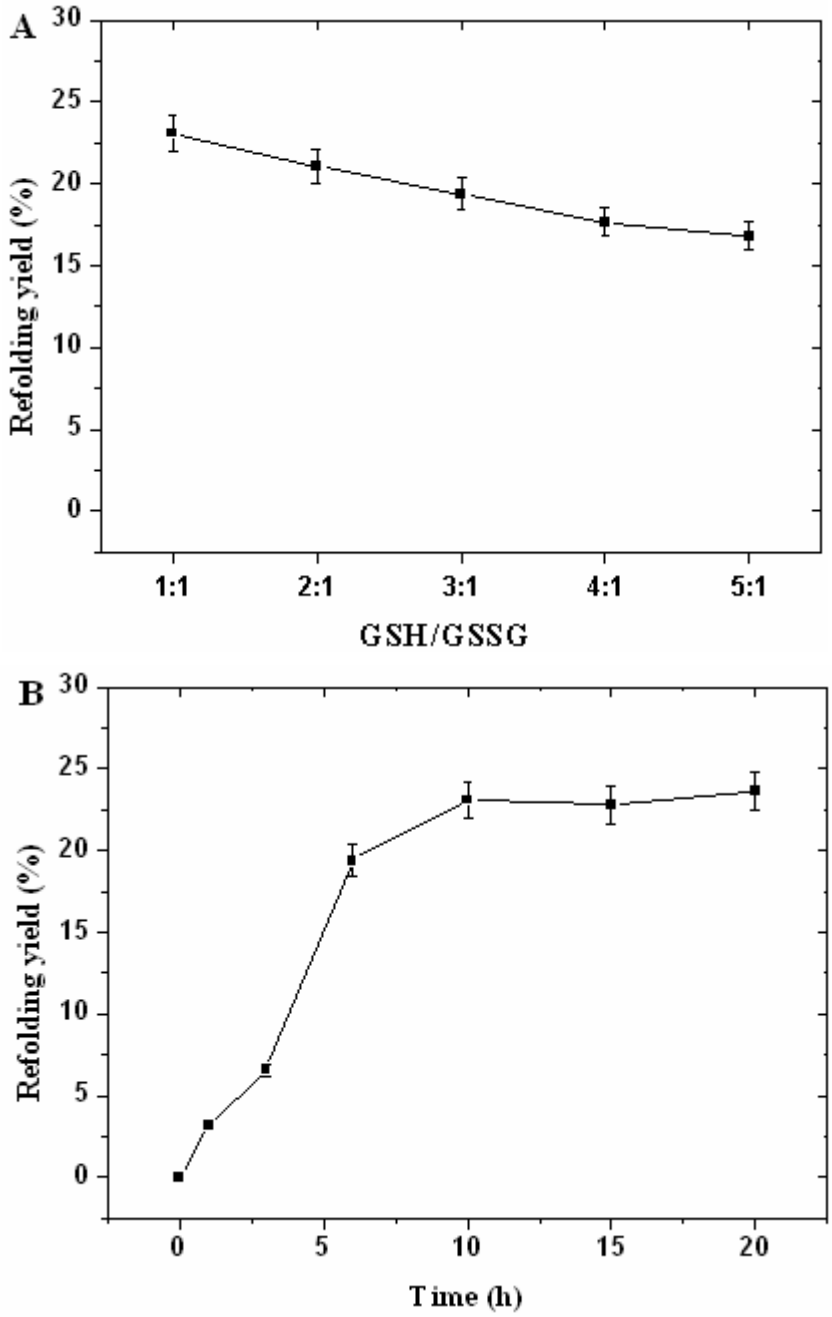
For the cysteine-rich proteins, the correct pairing of disulfide bonds is essential (Vallejo and Rinas, 2004a). The inclusion of a redox group (mixture of reduced and

oxidized thiol reagents), such as GSH and GSSG, in the refolding buffer facilitates this required oxido-shuffling process and thus increases the refolding efficiency (Fischer and Sumner, 1993; Vallejo and Rinas, 2004a). The effect of the redox group on the enzymatic activity of native ancrod was also determined. The total concentration of the thiol reagents was kept at 6 mM and the ratio of GSH or DTT to GSSG was varied. Native ancrod loses about 60% of its enzymatic activity in the presence of 6 mM GSH. With the introduction of oxidizing agent (GSSG) at the ratios studied, the enzymatic activity of ancrod is constant at around 100% (Figure 6.5B), showing that the presence of GSSG can efficiently balance the reducing effect of GSH. The enzymatic activity of native ancrod is significantly decreased by the replacement of GSH with DTT which is a stronger reducing agent (Figure 6.5B), indicating that the presence of GSSG at the tested condition was insufficient to efficiently neutralize the reducing effect of DTT.

When the purified soluble gloshedobin was mixed with DTT, it was also found that more than 30% of its enzymatic activity was lost even in the presence of 0.5 mM DTT, necessitating the removal of DTT prior to protein refolding. However, it should be noted that this DTT-deficient condition may trigger a spontaneous partial re-oxidation of protein before the initiation of protein refolding (Lanckriet and Middelberg, 2003; Leong and Middelberg, 2006).

6.3.3 Dilution refolding of gloshedobin

The dilution refolding of denatured-reduced gloshedobin was first conducted in order to provide a basis to assess the refolding efficiency of the target protein in various refolding strategies tested in the present study. The refolding yield was monitored by measuring the enzymatic activity of gloshedobin over time following dilution. It was first found that in the absence of a redox group, the refolding yield of gloshedobin at 100 μg/mL was only around 3%. Various ratios of GSH to GSSG ranging from 1:1 to 5:1 with a total thiol concentration of 6 mM were thus investigated. A GSH to GSSG ratio of 1:1 was found to be most efficient in facilitating the oxido-shuffling of the gloshedobin molecules during refolding (Figure 6.6A). With the increase in concentration of reducing agent, the refolding yield was gradually decreased, indicating the inhibition of correct disulfide bond formation by the over-reducing environment (Konishi et al., 1982). Under the optimized redox environment, the denatured gloshedobin recovered approximately 23% of its enzymatic activity after 10 h incubation (Figure 6.6A and 6.6B). There was no additional enzymatic activity recovery when refolding reaction proceeded for longer period of time. An increase in total concentration of thiol reagents (9 mM GSH and 9 mM GSSG) showed no improvement in the final refolding yield (around 22% when dilution refolding was performed at a protein concentration of 100 μg/mL), confirming a previous report that the ratio of reduced to oxidized thiol reagents affects the redox potential more significantly than the total concentration of thiol present in the refolding buffer (Qoronfleh et al., 2007).


A
Refolding yield (%)
0
5
10
15
20
25
30
1:1
2:1
3:1
4:1
5:1
GSH/GSSG
B
Refolding yield (%)
0
5
10
15
20
25
30
0
5
10
15
20
Time (h)

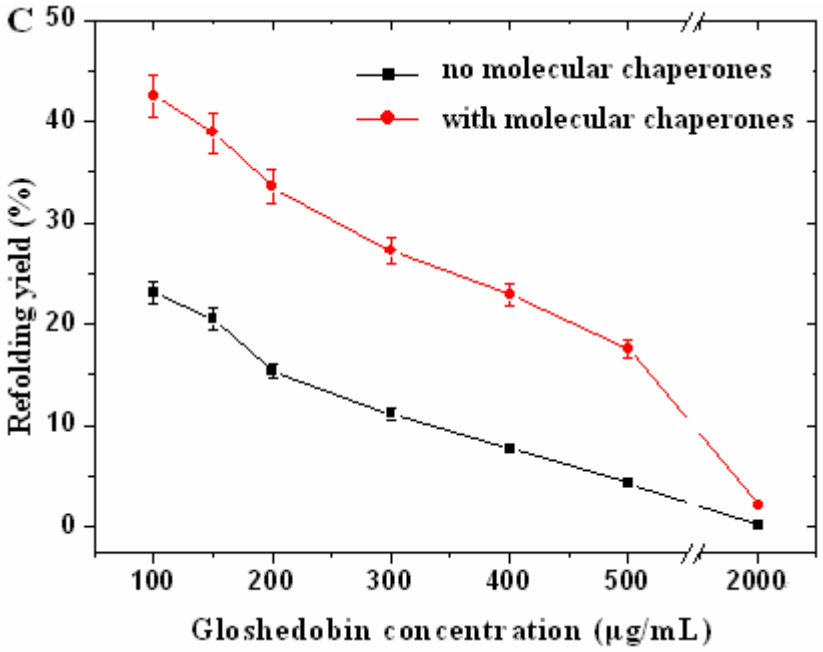


Figure 6.6 (A) The effect of different ratios of GSH to GSSG, added in the dilution refolding buffer, on the refolding yield of denatured gloshedobin at a concentration of 100 μg/mL. The refolding yields were quantified by measuring the specific amidolytic activity. The total concentration of the thiol reagents was kept at 6 mM. (B) The dilution refolding yield of gloshedobin (100 μg/mL) in the presence of GSH to GSSG ratio of 1:1 as a function of time. (C) The refolding yield of gloshedobin with or without ClpB/DnaKJE bichaperone system as a function of protein concentrations.

During the dilution refolding, the denatured protein is injected into a larger volume of refolding buffer under vigorous mixing in order to effect rapid dispersion of the denaturant molecules (Li et al., 2004). The unfolded protein molecules will thus collapse to more compact intermediate states and finally develop to correctly folded, misfolded or aggregated species. The aggregation of the protein intermediates during refolding will greatly decrease the final refolding yield. This becomes more significant at high protein concentrations where the higher-order aggregation reactions dominate over first-order refolding reactions (Kiefhaber et al., 1991). The protein concentration has to be carefully controlled at a relatively low level (usually 5-100 μg/mL) to minimize aggregation (Hevehan and Clark, 1997). To understand the effect

of protein concentration at the time of refolding reaction, dilution refolding of gloshedobin was conducted at various protein concentrations between 100 and 2000 μg/mL. The recovery of enzymatic activity showed clear dependence on the protein concentration, decreasing from 22.5% to 0.1% as the initial protein concentration increases (Figure 6.6C, Table 6.1).

Molecular chaperones have been widely studied to facilitate protein folding both *in vivo* and *in vitro*. ClpB/DnaKJE bichaperone system was found to be efficient in the disaggregation and renaturation of protein aggregates into active forms (Mogk et al., 1999; Zolkiewski, 1999; Ziętkiewicz et al., 2004; 2006). The mechanism of this system possibly relies on the extraction of individual polypeptide from the protein aggregate surface by translocation through the ClpB pore, thus initiating the unfolding of aggregated proteins. The extracted proteins are then captured and refolded by DnaKJE (Weibezahn et al., 2004b; Shorter and Lindquist, 2005; Haslberger et al., 2007). As described in Chapter 5, a refolding cocktail comprising unpurified ClpB and DnaKJE proved to be effective in rendering high efficiency refolding of heat-denatured MDH by mimicking a *de novo* protein folding scheme. As proposed in Chapter 3, the obviation of the need of purification of key molecular chaperones prior to refolding step would facilitate a large scale implementation of the developed refolding method. Based on this concept, the dilution refolding of denatured gloshedobin in the presence of ClpB/DnaKJE was investigated in this chapter. The refolding was initiated by rapid dilution of various concentrations of gloshedobin into

the optimal refolding buffer supplemented with 5 μM ClpB, 0.2 mg/mL DnaKJE and 5 mM ATP with ATP regeneration system. The reaction was allowed to proceed for 10 h. As shown in Figure 6.6C, the final refolding yield is increased to 46.4% when dilution is performed at a protein concentration of 100 μg/mL, giving a 2-fold increment compared with the condition lacking molecular chaperones. This clearly demonstrates that the presence of ClpB/DnaKJE bichaperone system is conducive to suppressing the protein aggregation and thus increases the protein refolding efficiency. As the protein concentration increases, the recovered enzymatic activity decreases gradually. When the dilution refolding is conducted at 400 μg/mL, the final refolding yield decreases to around 23%, which is however still equivalent to the efficiency of dilution refolding at 100 μg/mL without the assistance of chaperones. The benefit of using molecular chaperones in dilution refolding is clear as refolding reaction can occur at 4 times higher protein concentration without compromising the recovery of enzymatic activity.

Despite the improvement in the refolding yield, the increased complexity of refolding buffer composition and the subsequent need for further purification steps to remove added molecular chaperones may not fully justify the marginal increase in operational protein concentration at the time of refolding. It is expected that selective adsorption of the target protein with affinity tag onto the column may limit the interactions between refolding intermediates, reduce aggregation and improve refolding efficiency at high protein concentration. In addition, the specific binding between target protein

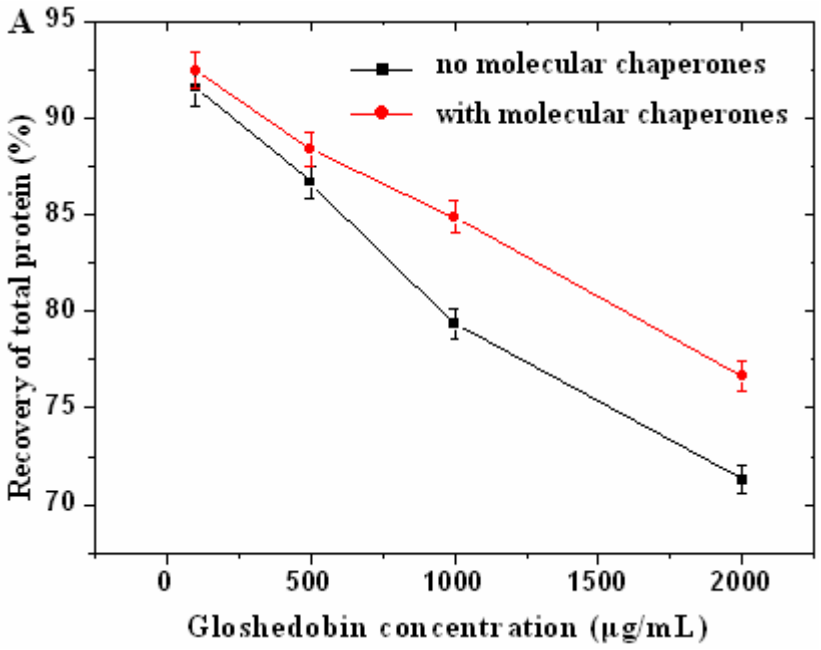
and the solid support also mitigates the demand of purification steps to remove other components present together with the target protein in the refolding buffer. It is therefore envisaged that a coupling of the refolding cocktail approach with column refolding strategy may overcome the inefficiencies encountered in the chaperone-assisted dilution refolding scheme.

6.3.4 Column refolding of gloshedobin

Column refolding was performed by adsorbing denatured and reduced gloshedobin onto an IMAC column at a different protein mass per unit volume of adsorbent (100 to 2000 μg/mL). The optimized refolding buffer which gave the highest yield in dilution refolding (without molecular chaperones) was used for the column refolding study. It should be noted first that the reduction of nickel metal ions was not observed despite the presence of a redox group comprising 3 mM GSH and 3 mM GSSG. This is consistent with the information from the manufacturer (GE Healthcare) that Ni Sepharose Fast Flow is compatible with up to 10 mM GSH (Hutchinson and Chase, 2006b). In addition, no protein was detected by UV in the flow-through during column loading, in line with the binding capacity (~ 40 mg/mL of His-tagged proteins) of Ni^{2+}-charged IDA resin as reported by the manufacturer. The bound gloshedobin was renatured by a linear decreasing urea gradient (8.0-0.4 M). The final urea concentration was fixed on par with that from dilution refolding. For the chaperone-assisted column refolding, refolding cocktail containing ClpB/DnaKJE bichaperone system was circulated continuously through the column in a recycling

mode for different time periods immediately after the urea gradient. The elution was conducted with a linear 0-500 mM imidazole gradient and the fractions containing proteins were pooled prior to concentration and enzymatic activity analysis.

Total protein recovery decreased from 91.6% to 73.1% (Figure 6.7A) with the increase in protein loading from 100 to 2000 µg/mL. This is probably due to the retained aggregated proteins as higher protein loading affects the spatial isolation of protein molecules on the matrix and consequently promotes aggregation of incompletely refolded proteins (Langenhof et al., 2005). For the chaperone-assisted IMAC refolding, slight increase in the total protein recovery (92.5 and 76.7% for protein loading of 100 and 2000 µg/mL respectively (Figure 6.7A)) was observed, suggesting that the ClpB/DnaKJE chaperone system possibly acts on the aggregated species and partially re-solubilizes them.



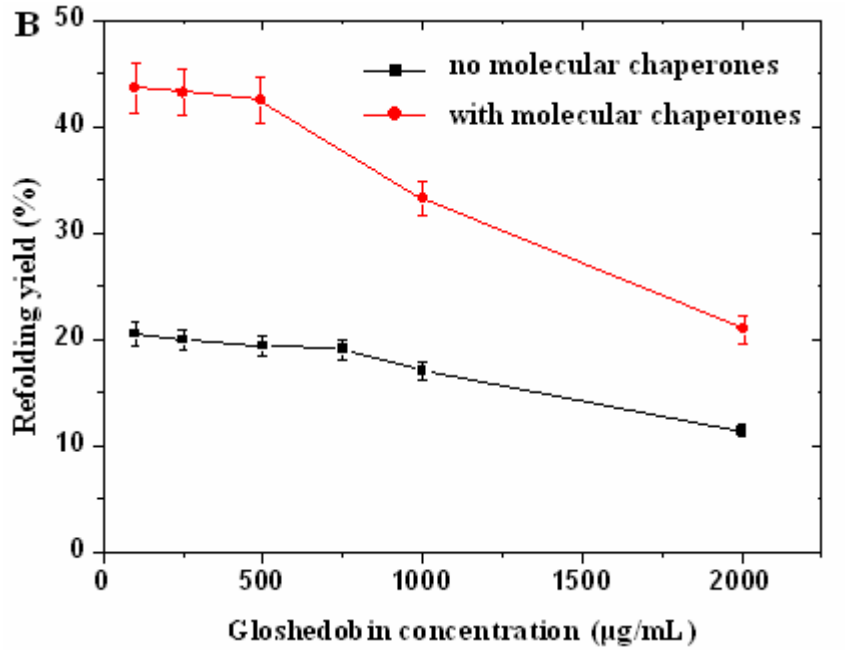


Figure 6.7 (A) Comparison of total protein recovery achieved using column refolding with or without molecular chaperones. Gloshedobin concentration is stated as the total amount of denatured and reduced gloshedobin loaded on the column per mL of adsorbent. (B) Comparison of refolding yield achieved using column refolding with or without molecular chaperones.

One of the greatest advantages of column refolding is that the refolding could be efficiently conducted at higher protein concentrations (Geng and Chang, 1992; Berdichevsky et al., 1999; Yin et al., 2003; Kweon et al., 2004). As shown in Table 6.1, similar refolding efficiency was achieved with a bichaperone-assisted column refolding process (21.4% at 2000 µg/mL) despite the 20-fold increase in protein concentration as compared to the conventional dilution refolding scheme (22.5% at 100 µg/mL). However, the total amount of buffer used for column refolding was only about 10-fold higher than in dilution refolding (Tables 6.2 and 6.3), giving an advantage of lower buffer requirement when processing an equivalent quantity of protein using column refolding. Even in the absence of molecular chaperones, the column refolding yield of 11.4% achieved at a protein concentration of 2000 µg/mL

(Figure 6.7B) was incomparably higher than that from dilution refolding (0.1%, Figure 6.6C), highlighting the superiority of column refolding approach. However, the decrease in enzymatic activity recovery along with the increased protein loading (Figure 6.7B) reconfirms that column refolding efficiency is also protein concentration dependent since the matrix-adsorbed protein molecules are more likely to be in closer proximity to one another at a higher protein loading condition. This renders the extent of aggregation reactions becoming more dominant with the increase in the loaded proteins despite the immobilization strategy, resulting in the decreased refolding efficiency. It is interesting to note that the decrease in the observed refolding yield is not significant up to average protein loading of 750 μg of denatured protein per unit volume of the resin (Figure 6.7B). This is probably because the upper part of the packed resin becomes saturated by protein molecules at a concentration of 750 μg/mL and it takes time for the protein to diffuse to the lower unoccupied region of the resin. This renders the effective concentration (i.e. the actual mass of protein molecules per unit volume of the resin) higher than the calculated one based on the averaged amount of the loaded protein for the entire resin volume, thus affecting refolding efficiencies thereafter. An alternative two-step strategy comprising 1) pre-incubation of protein molecules with the resin slurry in a batch mode; 2) subsequent column packing with the protein-loaded resin, is expected to maximize the efficacy of matrix-assisted spatial segregation of protein molecules at the time of refolding, thereby further enhancing column refolding efficiency.

Table 6.2 Summary of buffer usages in dilution refolding.

Dilution refolding	Sample preparation before dilution			Refolding buffer (mL)	Refolding buffer with molecular chaperones (mL)	Final protein concentration (mg/mL)	Total buffer used (mL)
	40 mg/mL protein sample (mL)	Desalting buffer (mL)	Protein concentration (mg/mL)				
Without molecular chaperones	0.05	0.95	2	19	N.A.	0.1	20
	0.075	0.925	3			0.15	
	0.1	0.9	4			0.2	
	0.15	0.85	6			0.3	
	0.2	0.8	8			0.4	
	0.25	0.75	10			0.5	
	1	0	40			2	
With molecular chaperones	0.05	0.95	2	N.A.	19	0.1	
	0.075	0.925	3			0.15	
	0.1	0.9	4			0.2	
	0.15	0.85	6			0.3	
	0.2	0.8	8			0.4	
	0.25	0.75	10			0.5	
	1	0	40			2	

Table 6.3 Summary of buffer usages in column refolding. Final protein concentration (mg/mL) is defined as the amount of protein (mg) per unit volume of resin used where the volume of resin used was 2.3 mL.

Column refolding	Sample preparation before loading			Denaturing buffer for equilibration (mL)	Denaturing buffer for refolding buffer gradient (mL)	Refolding buffer for refolding buffer gradient (mL)	Refolding buffer with molecular chaperones (mL)	Refolding buffer for wash (mL)	Elution buffer (mL)	Final protein concentration (mg/mL)	Total buffer used (mL)
	40 mg/mL protein sample (mL)	Desalting buffer (mL)	Protein concentration (mg/mL)								
Without molecular chaperones	0.006	0.994	0.24	20	60	60	N.A.	30	30	0.1	201
	0.015	0.985	0.575							0.25	
	0.029	0.971	1.15							0.5	
	0.043	0.957	1.725							0.75	
	0.058	0.943	2.3							1	
	0.115	0.885	4.6							2	
With molecular chaperones Method 1	0.006	0.994	0.23				20			0.1	221
	0.015	0.985	0.575							0.25	
	0.029	0.971	1.15							0.5	
	0.058	0.943	2.3							1	
	0.115	0.885	4.6							2	
With molecular chaperones Method 2	0.029	0.971	1.15			N.A.	60 (for refolding buffer gradient) + 20 (for recycling flow)			0.5	221

In order to investigate whether the presence of added molecular chaperones exhibit any synergistic effect when coupled with the column refolding strategy, the column refolding experiment was repeated with the supply of refolding cocktail containing DnaKJE/ClpB bichaperone and ATP regeneration systems. It is clear from Figure 6.7B that the presence of molecular chaperones is conducive to the target protein refolding. At a protein concentration of 2000 μg/mL, the final refolding yield, following 2 h recirculation of refolding cocktail at the end of urea gradient, reached 21.4% (1.9-fold increment compared to the case lacking molecular chaperones). Furthermore, this yield was comparable to that from the traditional dilution refolding (without molecular chaperones) conducted at 100 μg/mL of protein concentration (i.e. the same enzymatic activity was recovered by chaperone-assisted column refolding at a 20 times higher protein concentration).

Chaperone-assisted column refolding of which the outcome is presented in Figure 6.7 has two drivers to modulate refolding reaction: decrease of a chaotrope (i.e. urea) followed by chaperoning effect of the added bichaperone system during the recycling stage (Method 1). A noticeable feature in Method 1 is that the two drivers for refolding reaction were applied in a sequential manner. An alternative chaperone-assisted column refolding strategy harnessing a direct linear gradient from the denaturing washing buffer to refolding cocktail (Method 2) was investigated in order to see the effect of simultaneous application of two refolding drivers to the target protein refolding. From Method 2, the refolding yield of 35% was achieved

immediately after the gradient period (Figure 6.8), and a shorter refolding cocktail circulation time of 1 h (as compared to 2 h for Method 1) was sufficient to achieve the optimum refolding yield (Figure 6.8). Although the final refolding efficiency from either method remains unchanged, this indicates possibly that the refolding of protein through synchronized synergistic effect of two refolding drivers (i.e. chaotrope removal and chaperoning effect) is conducive to reducing process time required to reach maximum refolding yield. Another reason for the shorter recirculation time in Method 2 might be the use of more refolding cocktail from the earlier stage of refolding process. In Method 1, 20 mL of refolding cocktail was used in the recycling mode after urea removal while in Method 2, 80 mL of refolding cocktail was used (60 mL for the refolding buffer gradient and 20 mL for the flow in recirculation mode) (Table 6.3).

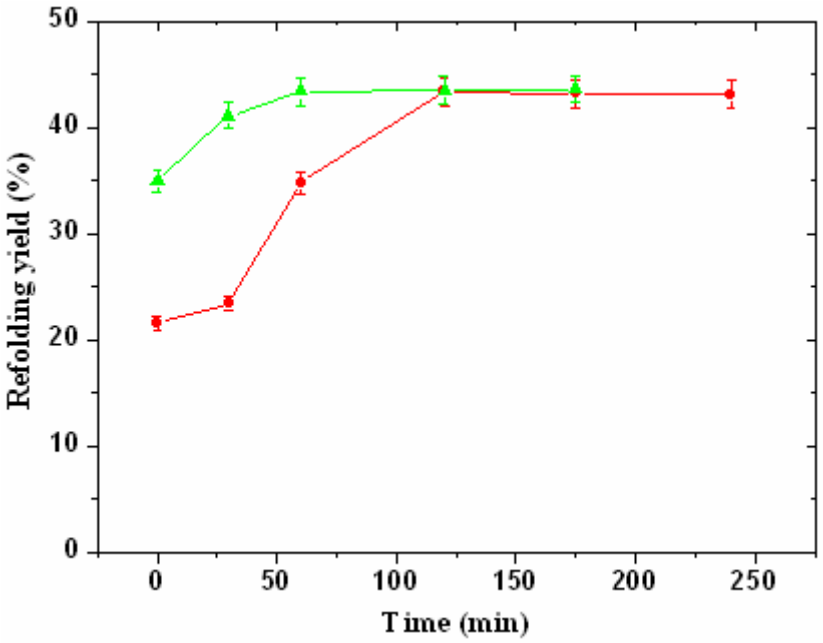


Figure 6.8 Refolding yield of gloshedobin as a function of time (after recycling flow started) allowed for the contact of the protein (500 μg/mL adsorbent) with

ClpB/DnaKJE bichaperone system. ●, recycling refolding cocktail containing molecular chaperones after the gradual removal of urea; ▲, column refolding conducted directly with a linear gradient from the denaturing washing buffer to the refolding cocktail containing molecular chaperones, followed by the recycling of refolding cocktail.

From Figure 6.7B, the refolding yield of 42.5% was obtained from chaperone-assisted column refolding conducted at a protein concentration of 500 μg/mL following Method 1, suggesting that the eluted soluble protein in the recovered fractions not only contains the correctly folded species, but also various misfolded or partially folded species lacking enzymatic activity. It was reported that for fusion proteins, solubility does not always correlate with proper folding of the passenger protein (Louis et al., 1991; Saavedra-Alanis et al., 1994; Lorenzo et al., 1997; Sachdev and Chirgwin, 1998; Nominé et al., 2001a; 2001b). The collected fractions from the chaperone-assisted column refolding were thus analyzed by RP-HPLC to analyze the profile of existing protein conformers (i.e. native, misfolded intermediate and denatured proteins) during the refolding process. For ease of tracking the individual peaks representing different refolding intermediates, the refolding experiment conducted according to Method 1 (in Figure 6.8) was chosen for the profile analysis since it showed a relatively larger time-dependent change in refolding efficiency. Figure 6.9 shows the time course of gloshedobin refolding process presented in Figure 6.8 (Method 1) immediately before the start of refolding cocktail recirculation. The HPLC chromatograms for native and denatured-reduced gloshedobin are provided for comparison. For a sample taken at 0 min, the RP-HPLC chromatogram shows a small peak at a retention time (RT) of 23 min representing the native protein and an

additional broad peak (RT = 32 min) presumably corresponding to the refolding intermediates containing misfolded or partially folded proteins (Nominé et al., 2001a; Schauer et al., 2003). At this instance, the refolding yield reached about 21.6% (Figure 6.8), indicating that only a small fraction of the denatured protein molecules recovered enzymatic activity following the completion of urea gradient while a majority still remained in the status of refolding intermediates.

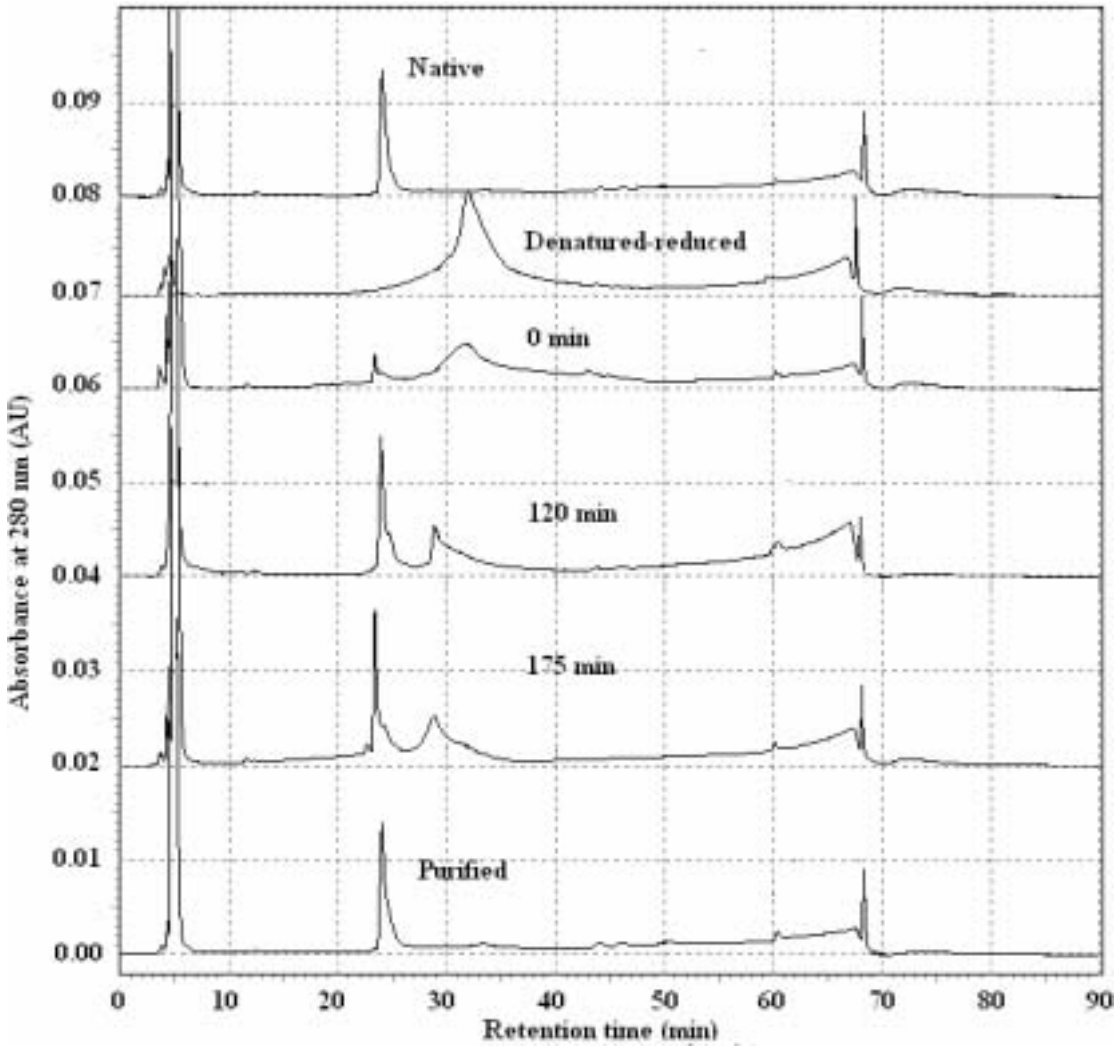


Figure 6.9 RP-HPLC chromatogram of the various components in gloshedobin protein mixture during the refolding process (where refolding cocktail containing molecular chaperones was applied after the removal of urea, Figure 6.8) from the denatured-reduced state.

Following prolonged incubation with molecular chaperones for 2 h, a noticeable shift in the peak profile was observed (compare the profile at 120 min in Figure 6.8 to the profile at 0 min), indicating that interaction of the immobilized gloshedobin with the recirculated chaperones rendered more of the denatured gloshedobin to recover its native structure. The area under the peak representing the correctly refolded gloshedobin accounts for 49% of the total peak area, suggesting that 49% of the soluble gloshedobin eluted from IMAC has its native structure. Considering that the total protein recovery was around 88.7% (Figure 6.7A) for the refolding experiment conducted at 500 μg/mL using Method 1, the refolding yield (i.e. the mass ratio of final refolded to initial denatured gloshedobin) as quantified by RP-HPLC analysis is thus around 43.5%, quite close to the refolding yield (i.e. 42.5% as presented in Figure 6.8 or Table 6.1) estimated from enzymatic activity analysis.

The increase in the formation of correctly folded protein was negligible with longer contact time after 120 min, which is also consistent with the enzymatic activity assay (Figure 6.8). Despite only 2% increase in the total protein recovery for the chaperone-assisted column refolding reaction as compared with the equivalent experiment without chaperones (Figure 6.7A), the 22% increase in refolding yield (Figure 6.7B) shows that this increase mainly results from the facilitated renaturation of refolding intermediates by ClpB/DnaKJE bichaperone system present in the refolding cocktail.

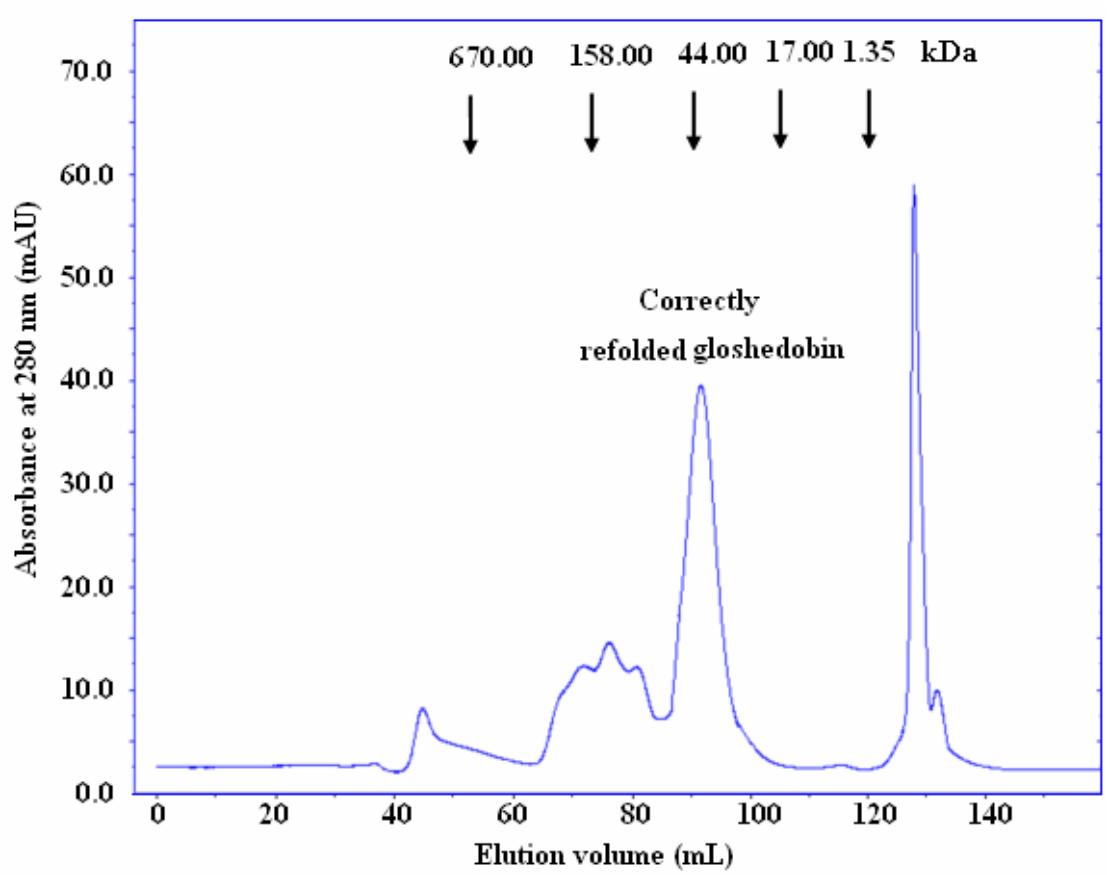


Figure 6.10 A chromatogram for gel filtration purification of refolded gloshedobin.

The refolded gloshedobin, obtained via bichaperone-assisted column refolding, was next loaded onto a gel filtration column to fractionate correctly folded protein from misfolded species and other soluble aggregates. Figure 6.10 shows that a good separation of correctly folded protein from higher molecular weight aggregates was achieved. Figure 6.9 compares RP-HPLC of refolded gloshedobin before and after gel filtration, further confirming that native protein was successfully isolated. The physical characteristics of refolded and native gloshedobin were also analyzed by CD (Figure 6.11). The secondary structure of refolded gloshedobin was determined by measuring its spectrum in the far UV region (190-250 nm). The far-UV spectrum of refolded gloshedobin was observed to overlay very closely with that of native gloshedobin, suggesting that the refolded gloshedobin has a very similar secondary

structure as the soluble native gloshedobin. The denatured gloshedobin in 8 M urea, which has an unfolded conformation, had a distinctly different CD spectrum (Figure 6.11).

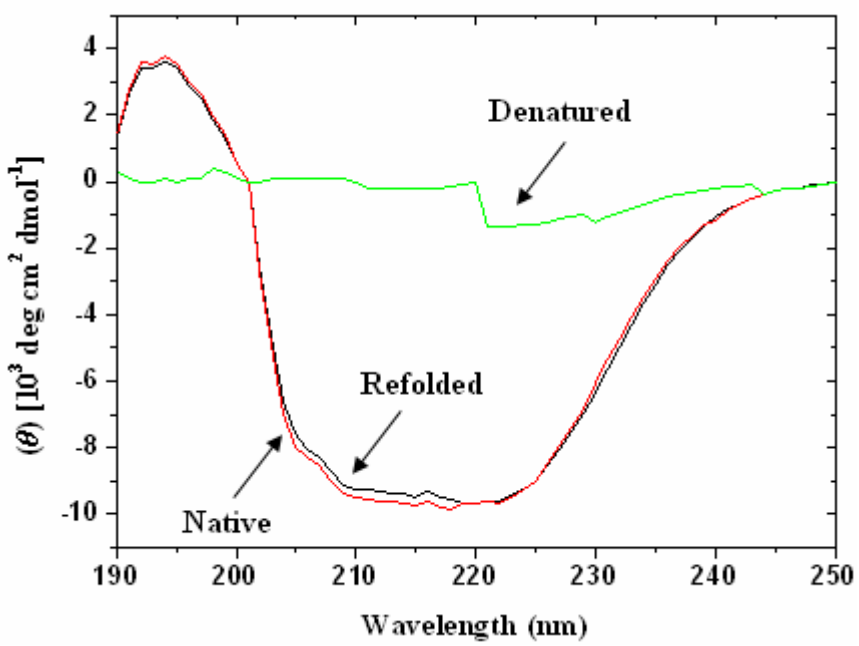


Figure 6.11 Far UV CD spectra of native, refolded and denatured gloshedobin (each analyzed at a concentration of 0.2 mg/mL).

The concurrence of both native and misfolded proteins greatly affects the biological activity of the target protein and further studies to increase the refolding efficiency should be conducted. In addition, the additional necessity to express molecular chaperones may result in the method being economically unfavorable especially for industrial-scale protein production. Therefore, it is also important to investigate the possibility of reusing the refolding cocktail for repeated column refolding processes since the reuse of the cost-prohibitive refolding buffer would significantly reduce the processing costs. Nevertheless, the advantages of molecular chaperone-assisted

column refolding with the use of refolding cocktail recirculation was clearly demonstrated to increase the enzymatic activity of refolded products at relatively high protein concentrations. The system can be potentially applied to column refolding of other chemically denatured proteins whose reactivation has been difficult to achieve through the traditional refolding strategies.

In this chapter, the new column-based refolding strategy taking advantage of ClpB/DnaKJE bichaperone system was shown to be superior to the conventional refolding methods in either batch dilution or column refolding mode especially when refolding reaction was attempted at a higher protein concentration. However, the strategy used to isolate gloshedobin IBs followed the traditionally described methods which are time-consuming and inefficient, including cell disruption by high pressure, repeated centrifugation to precipitate IBs and dissolution of IBs in high concentration of denaturant. The recovery process for gloshedobin IBs was therefore further integrated through coupling of IMAC protein purification with chemical extraction which was suggested to be an efficient way to overcome the inefficiencies associated with traditional IB recovery method. This is addressed in the following chapter (Chapter 7).

6.4 Conclusion

The refolding efficiencies of gloshedobin by dilution or column refolding strategy (with or without molecular chaperones) were studied. For column refolding without

molecular chaperones, RP-HPLC analysis shows that a large amount of protein exists in the misfolded state lacking enzymatic activity. The application of ClpB/DnaKJE bichaperone system was shown to be effective in reactivating these misfolded species to further increase the refolding yield. At a protein concentration of 2000 μg/mL, the final refolding yield, following 2 h recirculation of refolding cocktail at the end of urea gradient, reached 21.4% (1.9-fold increment compared to the case lacking molecular chaperones). The refolded protein successfully regained their native structure as ascertained by RP-HPLC and CD spectrum analysis. Furthermore, compared with traditional dilution refolding, the same enzymatic activity was recovered by the chaperone-assisted column refolding at a much higher protein concentration with lower buffer requirement. This is shown by an uncompromised refolding efficiency (c.a. 21.4%) achieved at 2000 μg/mL of initial protein concentration, which is comparable to the refolding efficiency (c.a. 22.5%) obtained at 20 times lower protein concentration (i.e. 100 μg/mL) in the conventional batch dilution refolding technique. These clearly demonstrated that the superiority of the molecular chaperone assisted column refolding developed in this study over either traditional column or dilution refolding especially when the refolding was conducted at a higher protein concentration.

Chapter 7
Polyethyleneimine-mediated chemical extraction of cytoplasmic His-tagged inclusion body proteins from *Escherichia coli*

Summary

The selectivity of polyethyleneimine (PEI) in DNA precipitation during chemical extraction was investigated. Chemical extraction was used to recover two His-tagged model proteins: gloshedobin, a thrombin-like enzyme from snake venom, and IbpA, a molecular chaperone, which were expressed mainly in the form of inclusion bodies (IBs). High DNA removal efficiency (more than 90%) was achieved at various cell densities (with OD_{600} ranging from 30 to 150) without affecting the solubility of host cell proteins. Compared to spermine-induced precipitation method reported elsewhere (Choe and Middelberg, 2001b), PEI provided a higher DNA precipitation efficiency at a significantly lower cost. Moreover, PEI obviated the use of EDTA, which has been reported to be essential for the chemical extraction methods, hence exhibiting dual roles in replacing cost-prohibitive spermine and EDTA. The residual PEI in the post-extraction mixture was efficiently counteracted by addition of Mg^{2+}, allowing the streamlined application of the extraction broth to immobilized metal affinity chromatography. Taken together, the PEI-mediated chemical extraction method provides a simpler and more economically viable processing route for the production

of recombinant proteins whose expression is hampered by IB formation.

7.1 Introduction

Traditional isolation and refolding methods for inclusion body (IB) proteins, such as cell disruption by high pressure or ultrasonic waves, differential centrifugation to remove cell debris and precipitate IBs followed by dilution or dialysis refolding, are time consuming and inefficient (Middelberg, 1995; Wong et al., 1997). To overcome the inefficiencies in traditional IB processing routes, Falconer and coworkers developed a direct chemical extraction method (Falconer Direct Extraction (FDE)) where a combination of 6 M urea and 3 mM EDTA was shown to achieve high-efficiency extraction and solubilization of cytoplasmic IB protein from *E. coli* (Falconer et al., 1997; 1998; 1999). The release of IB proteins (cytoplasmic Long-R3-IGF-I) by chemical extraction was shown to be at an equivalent level to mechanical disruption at a lab scale (Falconer et al., 1998). Moreover, the extraction efficiency was not compromised by high density cell suspension of *E. coli* (up to OD_{600} = 160) and proved highly efficient (>90%) in extracting and solubilizing His-tagged recombinant viral coat IB protein (Choe and Middelberg, 2001a). However, concomitant release of high molecular weight DNA during the extraction produced a highly viscous non-Newtonian post-extraction mixture (Choe and Middelberg, 2001b), posing a significant challenge to downstream operations (Fernández-Lahore et al., 1999).

Addition of nucleases or DNA precipitants has often been necessary, when downstream processes are complicated by the presence of host cell derived nucleic acids (DeWalt et al., 2003). Enzymatic degradation of coextracted DNA by Benzonase was employed to reduce viscosity, facilitating otherwise inefficient tangential flow filtration operation (Lee et al., 2004). However, the presence of high concentration of urea in the chemical extraction mixture prevented enzymatic approaches in FDE (Choe et al., 2006). Instead, a DNA precipitant, spermine, was used to selectively precipitate DNA during FDE (Choe and Middelberg, 2001b) where selective DNA precipitation was achieved at a ratio of 10 mg-spermine/mg-DNA (>85% DNA removal efficiency) without affecting the target protein recovery. However, due to the relatively high cost of spermine, its application at a large scale is hampered (Choe et al., 2006; Lee et al., 2006).

In order to avoid operational limitations (in terms of viscosity) and cost drawbacks, in this chapter, we focused on redesigning the FDE by replacing spermine with another polycationic agent, polyethyleneimine (PEI) which has been widely used in various bioprocesses to flocculate cellular contaminants such as nucleic acids and lipids from cell homogenates because of its ability to bind and precipitate DNA (Helander et al., 1997). The new extraction method, termed PEI-mediated chemical extraction, was investigated for two recombinant His-tagged proteins prone to IB formation: gloshedobin (Yang et al., 2002) and IbpA, a molecular chaperone (Kitagawa et al., 2002).

IbpA belongs to a small heat-shock protein (sHsp) family (Kitagawa et al., 2002) whose increased expression was shown to aid mammalian and bacterial cells to resist heat, cold, and oxidant stresses (Van den Ijssel et al., 1994; Yei et al., 1997; Sato et al., 1999). Furthermore, sHsps were demonstrated to exhibit their chaperoning activity *in vitro* by binding non-native polypeptides produced under heat-shock conditions, thereby leading to accumulation of misfolded intermediates which are protected from irreversible aggregation (Chang et al., 1996; Ehrnsperger et al., 1997; Lee et al., 1997). This reservoir effect permits the misfolded proteins to stay in a folding-competent state for an extended period of time, and allows refolding process to take place after the restoration of physiological condition in cooperation with other molecular chaperones (Ehrnsperger et al., 1997). Recently, expression of IbpA fused with a 10×His-tag was reported, but mainly in IB form (Kitagawa et al., 2002).

Following the assessment of PEI-mediated chemical extraction of gloshedobin and IbpA, we further demonstrated that the denatured-solubilized proteins in the extraction suspension were efficiently processed by immobilized metal affinity chromatography (IMAC) under denaturing condition, hence providing a new IB processing strategy coupling PEI-mediated chemical extraction and IMAC.

7.2 Materials and methods

7.2.1 Plasmids

pET-32a(+)+TLE, a plasmid encoding His-tagged gloshedobin-thioredoxin, was

kindly provided by Dr Qing Yang from Dalian University of Technology, Dalian, Liaoning, China. The plasmid encoding His-tagged IbpA (pET-19b+IbpA) was kindly provided by Dr Tetsuaki Tsuchido (Kansai University, Osaka, Japan).

7.2.2 Protein expression

E. coli BL21(DE3) cells were transformed with pET-32a(+)+TLE to enable the expression of His-tagged gloshedobin with thioredoxin partner. The transformed cells were grown at 37°C to OD_{600} ~ 0.8 in LB broth containing 0.1 mg/mL ampicillin. Protein expression was induced with 1 mM IPTG for 4 h during the exponential growth phase. The cell suspension, harvested at its stationary growth phase, was centrifuged at 5000*g* and 4°C for 20 min. The cell pellet was immediately subjected to chemical extraction or high pressure cell disruption.

The expression of His-tagged IbpA was achieved according to Kitagawa et al. (2002). *E. coli* BL21(DE3) cells (Novagen) transformed with pET-19b+IbpA were grown at 28°C to OD_{600} ~ 0.8 in LB medium containing 0.1 mg/mL ampicillin. Protein expression was induced with 0.3 mM IPTG for 4 h during the exponential growth phase. The cell pellets were collected and processed as described above.

7.2.3 Protein extraction by high pressure cell disruption

The cell pellets were resuspended in Tris-NaCl buffer (0.1 M Tris, 50 mM NaCl, pH 8.0) to give OD_{600} = 60. One Shot Cell Disrupter (Constant Cell Disruption System,

UK) was used to disrupt the cells at 21.0 Kpsi. Following the disruption, the cell disruptates were centrifuged at 10000*g* and 4°C for 30 min. The soluble and insoluble fractions and the whole cell disruptates were separately stored at -20°C for further analysis.

7.2.4 The effect of PEI on selective DNA precipitation

Solutions containing 0.1 M Tris and 640 mg/L Calf thymus DNA (D1501, Sigma) at various pH (7, 8, 9, 10, 11 and 12) were prepared. PEI solution (40,872-7, Aldrich), with high molecular weight of 25000 at a density of 1.03 g/mL, was next diluted in Tris solution to give a final PEI concentration of 100 mg/mL in 0.1 M Tris. 750 µL of prepared DNA solution was mixed with different amount of PEI solution according to Table 7.1 to give a final DNA concentration of 480 mg/L and the desired PEI/DNA ratio (mg/mg). The mixture was then vortexed and incubated for 0.5 h. After centrifugation at 15000*g* for 25 min, the supernatant was immediately analyzed for residual DNA concentration as described in the Analytical methods section.

Table 7.1 Experimental design for DNA precipitation by PEI.

DNA volume (µL)	PEI volume (µL)	0.1 M Tris volume (µL)	Total volume (µL)	DNA concentration (mg/L)	PEI concentration. (mg/L)	PEI/DNA ratio (mg/mg)
750	0	250	1000	480	0	0
750	0.5	249.5	1000	480	50	0.1
750	1	249	1000	480	100	0.2
750	2	248	1000	480	200	0.4
750	3	247	1000	480	300	0.6
750	4	246	1000	480	400	0.8
750	5	245	1000	480	500	1.1
750	10	240	1000	480	1000	2.1
750	20	230	1000	480	2000	4.2
750	30	220	1000	480	3000	6.3
750	40	210	1000	480	4000	8.3
750	50	200	1000	480	5000	10.4
750	100	150	1000	480	10000	20.8
750	110	140	1000	480	11000	22.7
750	120	130	1000	480	12000	25.0
750	130	120	1000	480	13000	27.0
750	140	110	1000	480	14000	29.4
750	150	100	1000	480	15000	31.3
750	200	50	1000	480	20000	41.7
750	250	0	1000	480	25000	52.6

Next, PEI-induced DNA precipitation was studied in the presence of urea in order to determine the effect of urea, if any, on the precipitation of DNA. DNA solutions were prepared as above in 0.1 M Tris solution containing 8 M urea at the same pH conditions as above. Different amounts of PEI solution were added to 750 µL of DNA solution according to Table 7.1 to give a final DNA concentration of 480 mg/L and urea concentration of 6 M. The mixture was then processed as above.

In order to mimic the actual chemical extraction process, BSA was selected as a representative protein to investigate the effect of PEI-induced DNA precipitation on protein recovery. The DNA solution was prepared in the presence of urea and BSA to

give a final concentration of 0.1 M Tris, 8 M urea, 640 mg/L DNA and 20 g/L BSA at different pH ranging from 7 to 12. 750 µL of this solution containing urea, DNA and BSA was mixed with PEI solution according to Table 7.1 to give a final urea concentration of 6 M, DNA concentration of 480 mg/L and BSA concentration of 15 g/L. After incubation and centrifugation at the same conditions as above, the DNA and BSA content in the supernatant was analyzed (see Analytical methods section).

7.2.5 Chemical extraction of IB proteins and precipitation of coextracted DNA by PEI

The cell suspension, harvested at its stationary growth phase, was centrifuged at 5000*g* and 4°C for 25 min. The collected cell pellets were washed with PBS buffer and recentrifuged as above. To investigate the effect of PEI on DNA precipitation during the chemical extraction, the washed cell pellets were resuspended in Tris-NaCl-urea buffer (0.1 M Tris, 50 mM NaCl, 8 M urea, pH 8.0) to give a suspension of OD_{600} = 80. Chemical extraction was conducted at a final cell density of OD_{600} = 60 using the following two conditions. In the first condition, 0.1 mL of solution of varying PEI concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg/mL) in Tris buffer (0.1 M Tris, pH 8.0) was added to 0.75 mL of cell suspension in order to give final PEI concentrations in the range of 0 to 10 mg/mL. In the second condition, 0.1 mL of PEI solution prepared as above and 0.1 mL of 30 mM EDTA solution were added to the 0.75 mL of cell suspension (OD_{600} ~ 80) in order to give final PEI concentrations in the range of 0 to 10 mg/mL and fixed EDTA concentration

of 3 mM. For both conditions, the total volume was maintained at 1 mL with the addition of Tris-NaCl buffer. After incubation for 6 h, the extraction mixture was centrifuged at 15000*g* for 25 min. The supernatant was analyzed by SDS-PAGE and residual DNA concentration was measured as described in the Analytical methods section. The aforementioned procedure was repeated for cell suspensions of varying cell densities in Tris-NaCl-urea buffer.

7.2.6 IMAC purification of His-tagged proteins

Following the extraction, $MgCl_2$ was added to the extraction mixture at a final concentration of 100 mM in order to counteract residual PEI, which was essential for extraction but detrimental to IMAC operation. The mixture was incubated for 0.5 h and centrifuged at 15000*g* for 0.5 h. The supernatant collected was applied at a flow rate of 1 mL/min to a 2.5 cm i.d.×15 cm column (Econo-Column Chromatography Column, 737-1517, Bio-Rad) packed with 5 mL of Ni^{2+}-charged iminodiacetic acid (IDA) resin following equilibration with a binding buffer (50 mM Tris, 150 mM NaCl, 6 M urea, 20 mM imidazole, pH 8.0). The resin was then washed with 10 column volume (CV) of washing buffer (50 mM Tris, 150 mM NaCl, 6 M urea, 40 mM imidazole, pH 8.0) to eliminate weakly or nonspecifically bound proteins. The bound proteins were eluted by gradually replacing the washing buffer with elution buffer (50 mM Tris, 150 mM NaCl, 6 M urea, 500 mM imidazole, pH 8.0) over 60 min. The protein content in the eluent was analyzed by SDS-PAGE. All the chromatographic purifications were conducted using BioLogic LP from Bio-Rad (731-8300, 731-8301).

7.2.7 Analytical methods

Protein detection and quantification: Total protein concentration determination and SDS-PAGE were conducted as described in Chapter 3.

DNA measurement: Residual DNA in the supernatant was measured using Quant-iT™ Oligreen® ssDNA Quantitation Kit (O-7582, Molecular Probes™). All samples were assayed in a final volume of 200 µL (100 µL of sample and 100 µL of the diluted Quant-iT™ Oligreen® Reagent in 96-well microplate). Samples were excited at 485 nm and fluorescence intensity was measured at 535 nm with a fluorescence microplate reader (Genios Multi-Detection Microplate Reader, Tecan).

When urea was not used, the dsDNA should be first converted to ssDNA for Oligreen assay (Choe and Middelberg, 2001b). The collected supernatants were autoclaved at 121°C for 15 min to convert dsDNA to ssDNA before dilution of the sample. Autoclaved samples and standard calf thymus DNA samples were removed from the autoclave at 95°C and were cooled rapidly on ice to minimize DNA annealing. When urea was present in the samples, the samples were directly assayed using 6 M urea treated calf thymus DNA as a standard.

Cellular components in the crude protein mixture after chemical extraction might exert an adverse effect on the DNA analytical assay, and thus relative solubility of DNA was reported in the Results and discussion instead of absolute concentration of

DNA. By taking the ratio of two DNA concentrations, relative solubility of DNA would allow the normalization of assay error (if any). For consistency, recovery of proteins was also presented in relative form.

7.3 Results and discussion

7.3.1 Expression of recombinant gloshedobin and IbpA

pET-32a(+)+TLE and pET-19b+IbpA were separately transformed into *E. coli* BL21(DE3). The expression of recombinant gloshedobin and IbpA relied on IPTG induction. As shown in Figure 7.1, both proteins were expressed mainly in the form of IBs (lane 6 in panel A or lane 6 in panel B). The expression levels estimated by densitometry are around 13% and 10% for gloshedobin and IbpA, respectively.

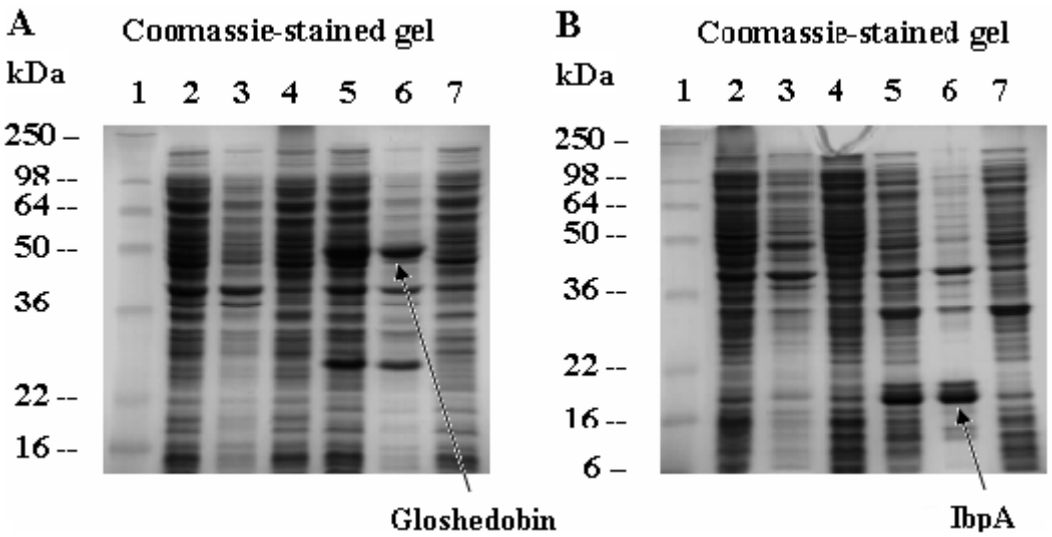


Figure 7.1 Expression profiles of recombinant gloshedobin and IbpA. (A) SDS-PAGE for the analysis of gloshedobin expressed in *E. coli* BL21(DE3) harboring pET-32a(+)+TLE. Molecular weight marker was loaded in lane 1. Lanes 2-4 are from the uninduced cells: the whole cell extracts (lane 2), the insoluble fraction (lane 3) and the soluble fraction (lane 4) in the cell extracts after high pressure cell disruption. Lanes 5-7 are from the induced cell: the whole cell extracts (lane 5) the insoluble fraction (lane 6) and the soluble fraction (lane 7) in the cell extracts. (B) SDS-PAGE

for the analysis of IbpA expressed in *E. coli* BL21(DE3) harboring pET-19b+IbpA. The lane description is the same as in (A).

Processes with a long downstream cascade generally result in a lower product recovery, while incurring more time, labor and cost due to the increased process steps and also to process incompatibility between each step (Choe et al., 2006). These inefficiencies are inherent in the conventional IB processing route which is characterized by multiple unit operations including cell disruption, the initial fractionation of IB proteins often entailing repeated washing steps, solubilization of IBs and subsequent various chromatographic procedures to purify the solubilized proteins prior to refolding. Although the development of alternative IB protein extraction methods (Falconer et al., 1997; 1998; 1999) obviated the need to separate micronized cell debris and has thus addressed some inefficiencies residing in the mechanical cell disruption based IB extraction methods, an excessive viscosity increase in the resulting extraction suspension due to the co-released non-sheared DNA necessitated further study to provide a more economically viable milieu to harness the advantages of chemical extraction route for IB processing. Thus the present study investigated the possibility of selectively precipitating DNA (thereby reducing the viscosity) by incorporating a widely known polycationic DNA precipitant PEI during the extraction of two model proteins (gloshedobin and IbpA) expressed primarily as IBs.

7.3.2 Effect of PEI on selective DNA precipitation

The effect of pH on PEI precipitation of DNA was first investigated at a fixed DNA concentration (480 mg/L). The PEI concentration at each pH was varied to give predetermined PEI to DNA ratio (mg/mg) (Table 7.1). As shown in Figure 7.2, PEI-induced DNA precipitation was more effective at lower pH conditions (pH 7-10). This is in good agreement with previous findings where the amine groups within PEI gradually lost their positive charges due to deprotonation with increasing pH and thus became less efficient in DNA precipitation (Cordes et al., 1990).

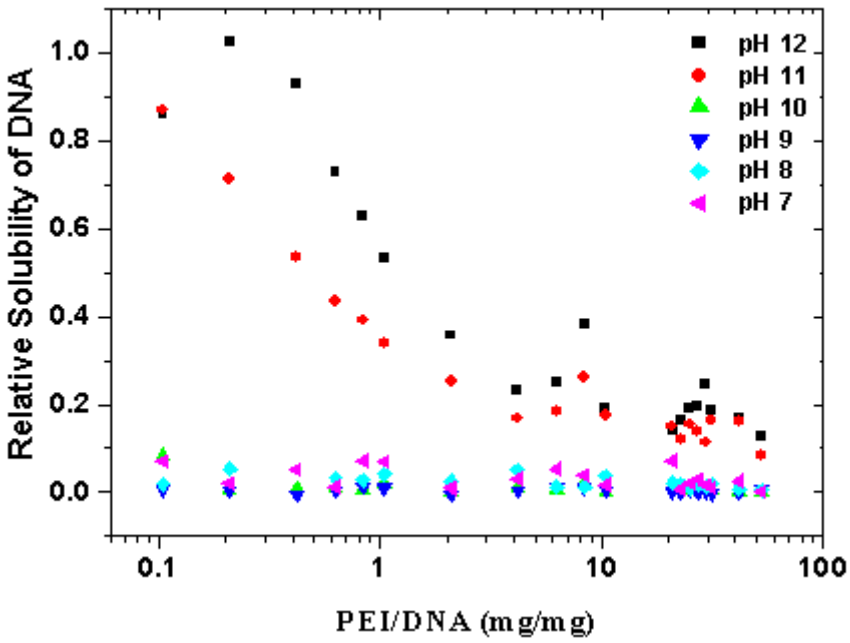


Figure 7.2 Solubility profiles of calf thymus DNA in 0.1 M Tris at various pH conditions ranging from pH 7 to 12. Initial DNA concentration was 480 mg/L.

When pH was 10 or lower, PEI-induced precipitation of DNA appeared to be highly efficient, as a fast transition from soluble to insoluble DNA was observed at a PEI/DNA ratio of approximately 0.1 mg/mg. Precipitation of DNA by PEI was almost

complete and fast, as no detectable residual DNA was found in the solution for a wide range of PEI/DNA ratios. It was reported that FDE efficiency was strongly pH-dependent and increased with pH (Falconer et al., 1997). However, a typical pH for chemical extraction has often been fixed around 9.0, since this condition enabled almost complete release of cytoplasmic proteins including IBs from *E. coli* while minimizing the danger of protein modification at alkaline condition (Falconer et al., 1997). In this context, the optimum pH range (7-10) for PEI-induced DNA precipitation would not compromise the extraction efficiency. Furthermore, compared to the previously reported spermine-induced DNA precipitation (Choe and Middelberg, 2001b) where a spermine to DNA ratio of 0.5 was required to precipitate DNA, five times less PEI was sufficient to achieve an equivalent extent of DNA precipitation in the same condition. In addition, the cost of spermine is approximately 48 times more than PEI, suggesting that PEI is a more economically viable DNA precipitant than spermine.

Besides the pH factor, the presence of chemicals commonly present in chemical extraction condition might also affect the PEI-induced DNA precipitation. EDTA and urea are the two major chemicals in FDE (Falconer et al., 1997; 1998; 1999). EDTA was reported to play an important role for the effective extraction of intracellular proteins by chelating the divalent cations that maintain the integrity of the *E. coli* outer membrane (OM) (Brown, 1963; Leive, 1965; Asbell and Eagon, 1966; Leive, 1973). It was found that the presence of EDTA (3 mM) did not affect PEI-induced

DNA precipitation (data not shown), and this is in good agreement with the previous study where spermine was used as a DNA precipitant (Choe and Middelberg, 2001b).

The use of urea in FDE was also reported to be essential for the effective permeabilization of *E. coli* inner membrane (IM) by solubilizing membrane proteins (Falconer et al., 1997; 1998; 1999). Since urea increases the dielectric constant of the solution, the reduction of DNA precipitation efficiency is expected in the presence of urea. Urea interference with DNA precipitation was confirmed by a noticeable shift in the spermine/DNA ratio required for DNA precipitation from 0.5 (in the absence of urea) to 5.0 (in the presence of urea) (Choe and Middelberg, 2001b). The interference of urea was also observed in PEI-induced DNA precipitation and the PEI/DNA ratio required for almost complete removal of DNA increased to approximately 0.5, suggesting that more than 5 times of PEI was required to achieve complete removal of DNA in the presence of urea compared with the conditions lacking urea (Figure 7.3). The pH effect on DNA precipitation was independent of the urea interference since the same pH-dependency in DNA precipitation efficiency was found: DNA precipitation was negligible at high pH conditions (>10), but almost complete DNA precipitation was achieved at PEI/DNA ratio higher than 5 at low pH conditions (<10) and the precipitation efficiency was indistinguishable from that achieved in the absence of urea (Figure 7.3). At pH 10, resolubilization of DNA occurred at a PEI/DNA ratio of around 11. A similar phenomenon was also observed in the spermine-induced DNA precipitation (Choe and Middelberg, 2001b) where the

specific spermine concentration for DNA precipitation is 5-10, while resolubilization of DNA was found at a spermine/DNA ratio of around 100. Raspaud et al. (1998) also reported that the required spermine concentration for DNA precipitation increased with the DNA concentration, while a redissolution of DNA in excess of spermine is nearly independent of DNA concentration.

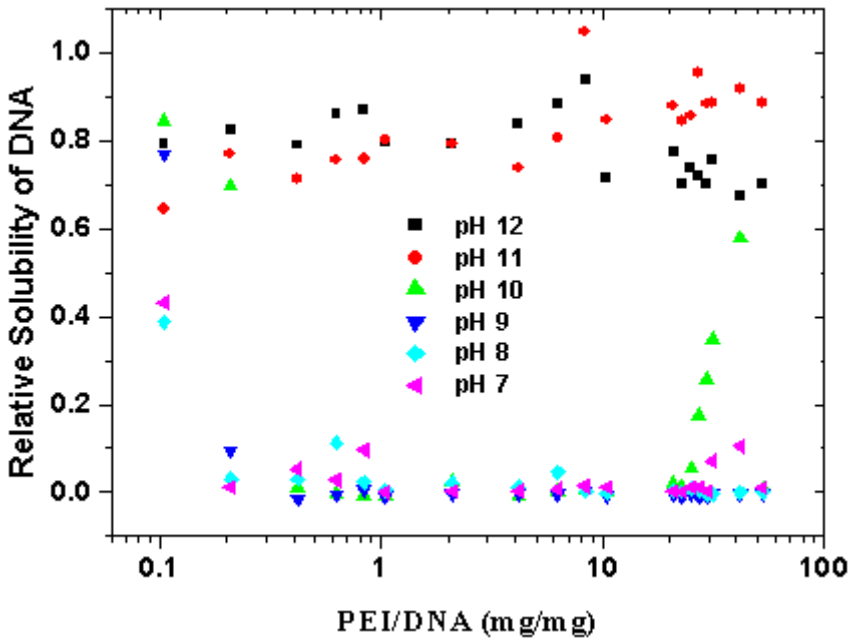


Figure 7.3 Solubility profiles of calf thymus DNA in the presence of 6 M urea at various pH conditions ranging from pH 7 to 12. Initial DNA concentration was 480 mg/L.

To mimic the actual chemical extraction environment and also to investigate the selectivity of PEI-induced DNA precipitation, BSA was chosen as a model protein and urea (commonly present in chemical extraction medium) was added separately or in combination with BSA and was incubated with PEI and DNA. As the presence of PEI would interfere with the commonly employed protein assays (Gupta et al., 2000), the recovered BSA was measured by densitometric method using GeneTools after

running SDS-PAGE. PEI was found to interact selectively with DNA, and negligible protein precipitation was found. Complete recovery of BSA was achieved for a wide range of PEI concentrations (Figure 7.4), indicating that PEI interacted selectively with DNA without affecting BSA solubility (DNA precipitation efficiencies were similar to those in Figure 7.2). This high-selectivity interaction of PEI with DNA would be promising for minimizing the loss in protein recovery during the extraction process. The pH-dependent DNA precipitation by PEI was confirmed again in the presence of BSA and urea since high-efficiency DNA precipitation occurred only in the pH range from 7 to 10 (Figure 7.5).

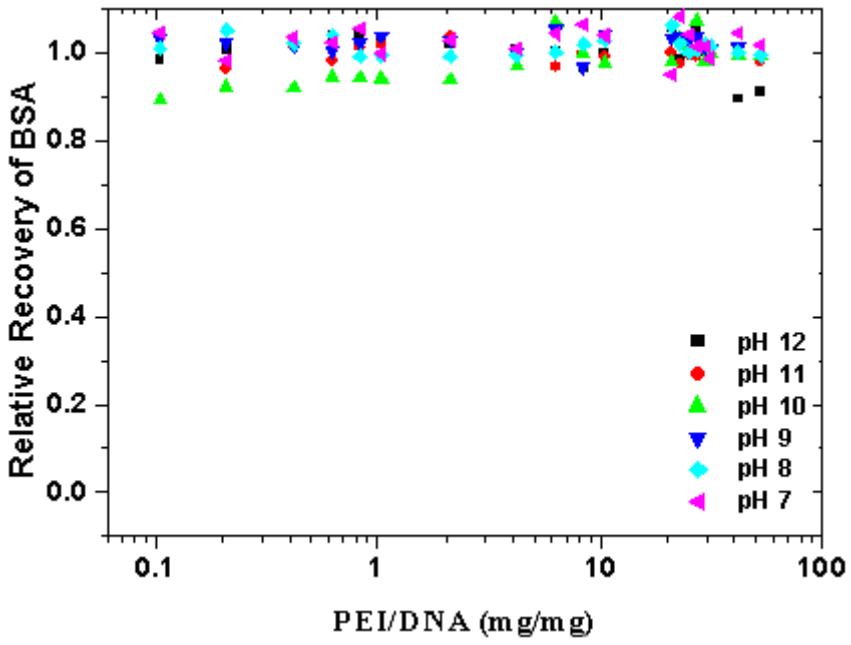


Figure 7.4 BSA recovered in the supernatant at various pH conditions. Initial DNA concentration was 480 mg/L. Initial BSA concentration was 15 g/L.

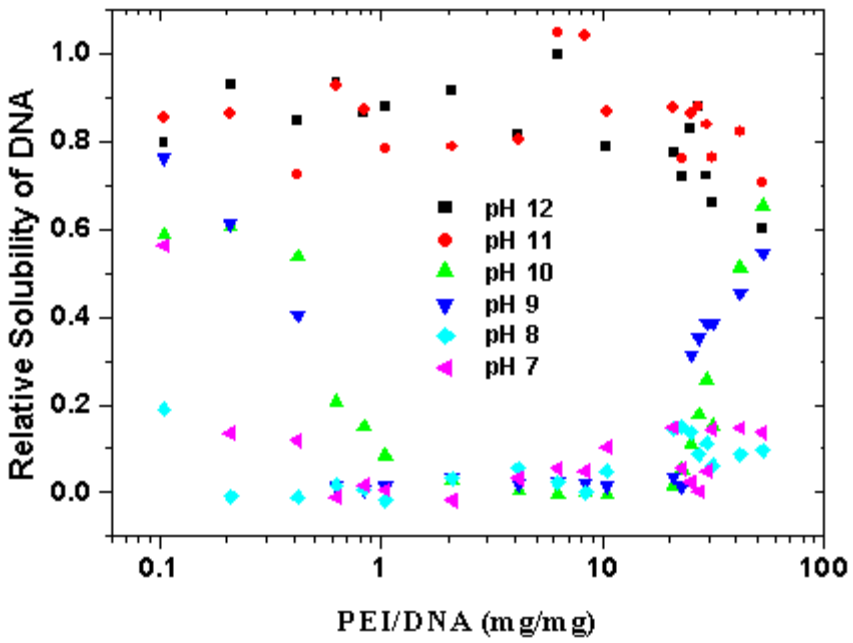


Figure 7.5 Solubility profiles of calf thymus DNA in the presence of 6 M urea and 15 g/L BSA at various pH conditions. Initial DNA concentration was 480 mg/L.

The concentrations of DNA and protein tested in the present study are those typically employed for the chemical extraction of *E. coli* cell suspension of OD_{600} = 60-80. The demonstrated high selectivity of PEI to DNA is very promising for achieving selective removal of DNA from the extraction broth without affecting the target protein recovery. The optimum pH conditions for PEI-induced DNA precipitation (i.e., pH range of 7-10) are also conducive to maximizing the pH-dependent extraction efficiency reported previously (Falconer et al., 1997; 1998; 1999; Choe and Middelberg, 2001b).

7.3.3 Extraction of gloshedobin and precipitation of coextracted DNA using PEI

Although PEI proved effective in selectively precipitating DNA without affecting the

protein solubility, its effect on the chemical extraction efficiency should be assessed in order to justify the use of PEI as a low-cost DNA precipitant during the extraction. Therefore, we introduced PEI in the extraction processes at various pH conditions ranging from 7 to 10. Traditionally used 3 mM EDTA in FDE was also included to see whether the presence of EDTA will affect the efficiency of extraction or DNA precipitation during PEI-mediated chemical extraction. As shown in Figure 7.6, the cytoplasmic proteins were almost completely released and recovered in the supernatant in a wide range of PEI concentrations and the efficiency of chemical extraction estimated by total protein release showed no significant difference for all the pH conditions tested.

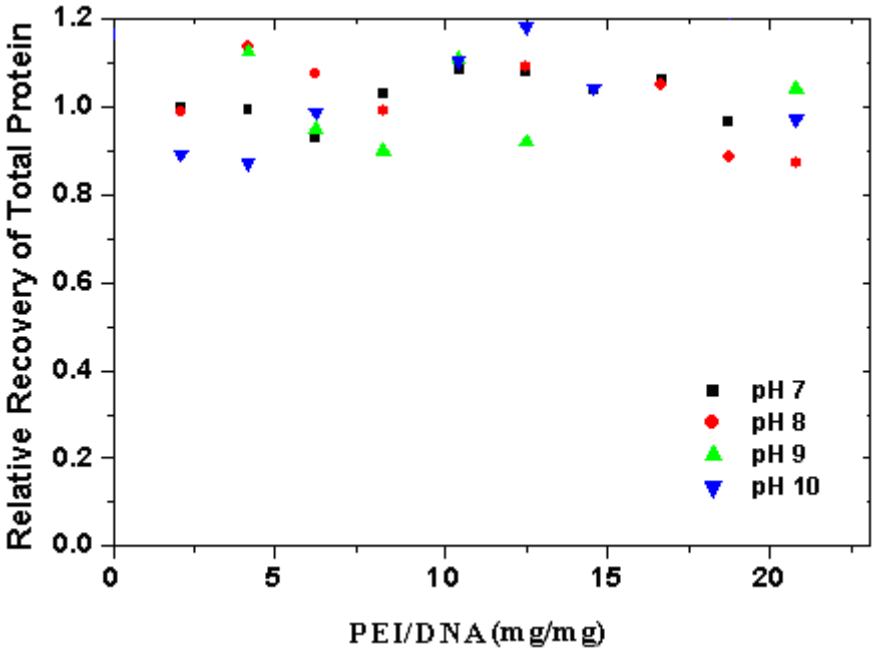


Figure 7.6 Total protein recovery following PEI-mediated chemical extraction of recombinant *E. coli* BL21(DE3) expressing gloshedobin (mostly as IBs) without the use of EDTA. Extraction was conducted at a cell suspension of OD_{600} = 60. Total protein release following the high pressure cell disruption at the same OD was set as 1.

Interestingly, compared to traditional FDE, the employment of EDTA was not necessary for the extraction of gloshedobin from the recombinant *E. coli* strain BL21(DE3) since the addition of 3 mM EDTA showed no further improvement in the extraction efficiency (Figure 7.7). It is well-known that the presence of an OM in all Gram-negative bacteria forms a permeability barrier against hydrophobic substances and macromolecules (Nikaido, 1989). For this reason, Gram-negative bacteria exhibit higher resistance to detergents and hydrophobic antibiotics than Gram-positive bacteria (Nikaido, 1989). Therefore, our experimental data implied that PEI (a good metal chelating agent) mimicked the role of well-known permeabilizer EDTA in terms of chelating divalent cations essential for the stabilization of the OM of Gram-negative bacteria including *E. coli* in addition to working as a selective DNA precipitant.

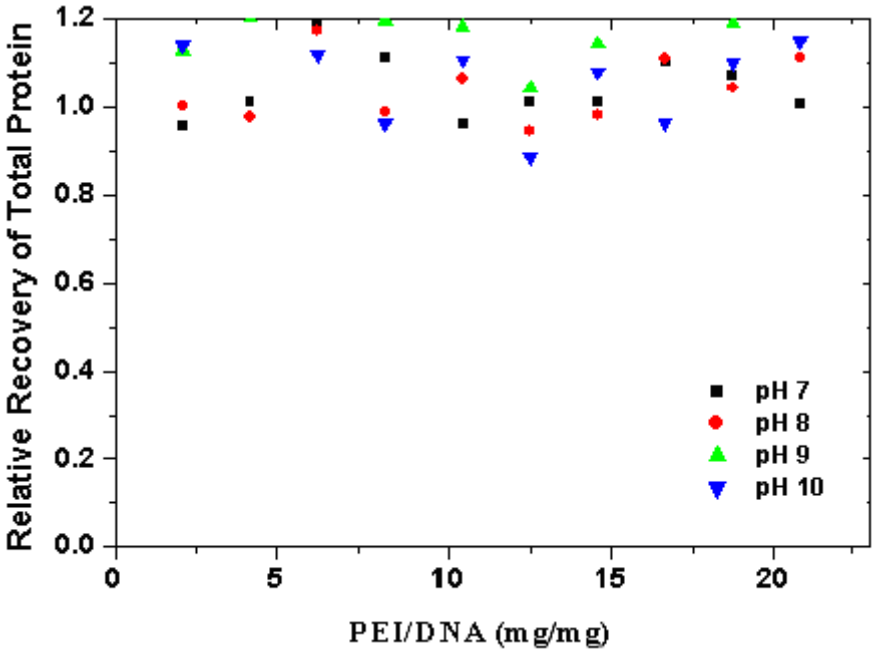


Figure 7.7 Total protein recovery following PEI-mediated chemical extraction with the use of 3 mM EDTA and 6 M urea. Extraction was conducted at a cell suspension

of OD_{600} = 60. Total protein release following the high pressure cell disruption at the same OD was set as 1.

The chelation of metal ions by PEI at alkaline pH is due to the interaction of lone-pair electrons of nitrogen atoms on PEI with the electron-deficient, positively charged metal ions (Geckeler and Volchek, 1996; Molinari et al., 2004). At low solution pH, nitrogen atoms on PEI are protonated (positively charged), and hence no lone-pair electrons are available to interact with the metal ions. As the solution pH increases, the extent of protonation for the nitrogen atoms on PEI decreases, rendering more lone-pair electrons available. Although PEI (MW 6×10^5-1×10^6) has a net positive charge up to pH 11 (Erim et al., 1995), the presence of lone-pair electrons on the nitrogen enables PEI to interact with the transition metal ions. In contrast, EDTA has two nitrogen atoms and the four carboxylic acid groups and the degree of their protonation is inversely proportional to pH value. Hence, EDTA is positively charged at low pH, whereas at high pH it is negatively charged due to the presence of deprotonated carboxylate group (COO-). At alkaline pH, EDTA chelates metal ions through both the nitrogen lone pair and carboxylate group. The presence of a carboxylate group in EDTA but not in PEI probably allows alkali and alkaline earth metals to bind to EDTA but not to PEI at alkaline pH.

The OM binds various metal ions such as alkali metal (Na, K), alkaline earth metal (Mg, Ca), and transition metal (Hoyle and Beveridge, 1983). It is known that chemical extraction requires EDTA to disintegrate the OM by chelating the metal ions

(Brown, 1963; Leive, 1965; Asbell and Eagon, 1966; Leive, 1973). In the present study, the chemical extraction proceeded successfully despite the replacement of EDTA with PEI, indicating that efficient chelation of transition metal ions present in the OM is sufficient for OM disintegration. However, it was reported that the efficiencies of FDE methods were strongly pH-dependent and increased with pH (Falconer et al., 1997) due to the proportionally increasing chelating efficacy of EDTA. On the contrary, PEI-mediated extraction efficiency was not pH-dependent for the pH range tested (from 7 to 10), exhibiting a highly efficient protein release regardless of the extraction pH (Figures 7.6 and 7.7). The use of PEI during the extraction also proved very effective in extracting IB protein (i.e., gloshedobin). As shown in Figure 7.8, the efficiencies of target protein release in PEI-mediated chemical extraction (without EDTA) at various pH conditions were equivalent to the mechanical cell disruption efficiency.

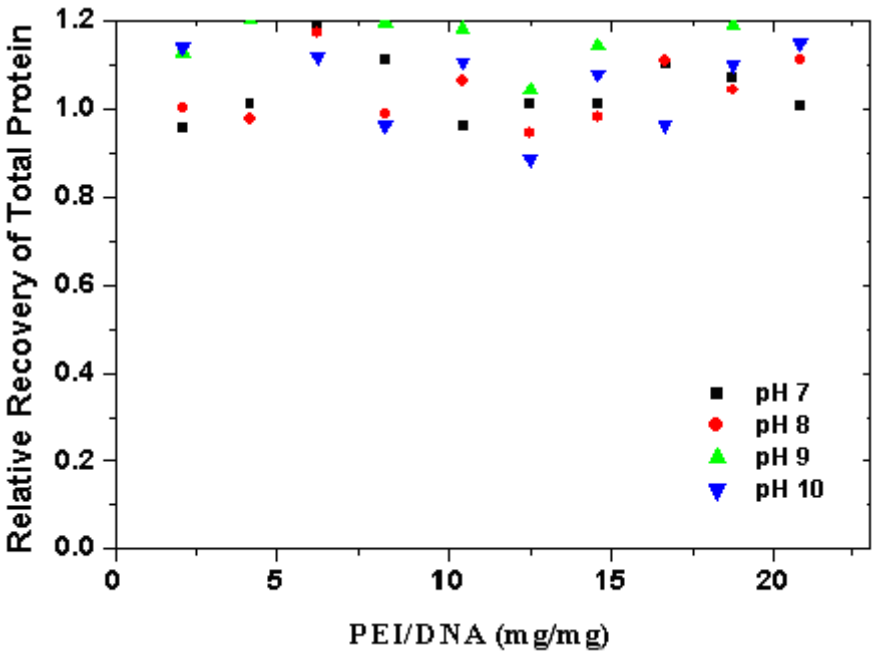


Figure 7.8 Recovery of gloshedobin after direct chemical extraction of recombinant *E. coli* BL21(DE3) expressing gloshedobin (mostly as IBs). Extraction was conducted at a cell suspension equivalent to OD_{600} = 60. The concentration of gloshedobin was estimated from the corresponding bands from SDS-PAGE gels by densitometric analysis. The release of gloshedobin following the high pressure cell disruption at the same OD was set as 1.

This broad optimum pH range would be an additional advantage of PEI-mediated extraction, in addition to replacing EDTA and cost-prohibitive spermine. Moreover, besides the chelating effect, PEI was reported to render Gram-negative bacteria permeable to detergents and to hydrophobic antibiotics at acidic and neutral pH conditions (Helander et al., 1997; 1998). The potential role of PEI for this enhanced permeability of PEI-treated Gram-negative bacteria was suggested to arise from its binding to anionic lipopolysaccharide (LPS) on the OM surface, thereby causing impairment in the protective function of the OM (Helander et al., 1998). Various divalent cations and LPS are abundant in the OM and known to be essential for OM integrity. It was therefore postulated that the putative function of PEI in the PEI-mediated extraction could be to weaken the OM, the first permeability barrier in *E. coli*, by scavenging divalent cations (via chelation) and/or extracting LPS (via electrostatic or affinity binding). This proposed individual or synergistic action of PEI could lead to the disruption of the OM and thus make the chemical extraction work in a wider pH range compared to the traditional FDE.

From Figure 7.9, it is clear that PEI-mediated precipitation of coextracted DNA was efficient in the pH range of 7-9 in the chemical extraction condition using PEI without

the use of EDTA. Compared with Choe and Middelberg's work (2001b) in which a spermine/DNA ratio of 10 mg/mg was needed to precipitate 85% of DNA, only 7.5 mg/mg of PEI/DNA was required to achieve more than 90% precipitation of DNA from the extraction mixture. It is noted that the pH 10 condition, which enabled almost complete precipitation of calf thymus DNA while achieving high-efficiency protein extraction, gave rise to significantly compromised DNA precipitation, probably due to the decreased PEI-DNA electrostatic interaction via increased screening effect as expected from a highly complex solution such as cell extracts. Even for pH 7-9 conditions, the complete precipitation of DNA was not realized since approximately 10% DNA was still soluble, indicating that other cellular components might exert an adverse effect on PEI-mediated DNA precipitation. Alternatively, as reported by Choe and Middelberg (2001b), this might be caused by a certain fraction of short DNA fragments in the extraction medium that were difficult to condense using PEI. Widom and Baldwin (1980) also found that the condensation of shorter DNA fragments by hexamine cobalt (III) required longer incubation time. In addition, Hoopes and McClure (1981) reported a threshold of DNA fragment length (~200 bp) for effective precipitation and found that this effect was important primarily under moderate salt conditions. It is thus likely that the reduced efficiency of DNA precipitation is mainly due to the population of short DNA fragments (in the chemical extraction mixture) that are not present in calf thymus DNA solution.

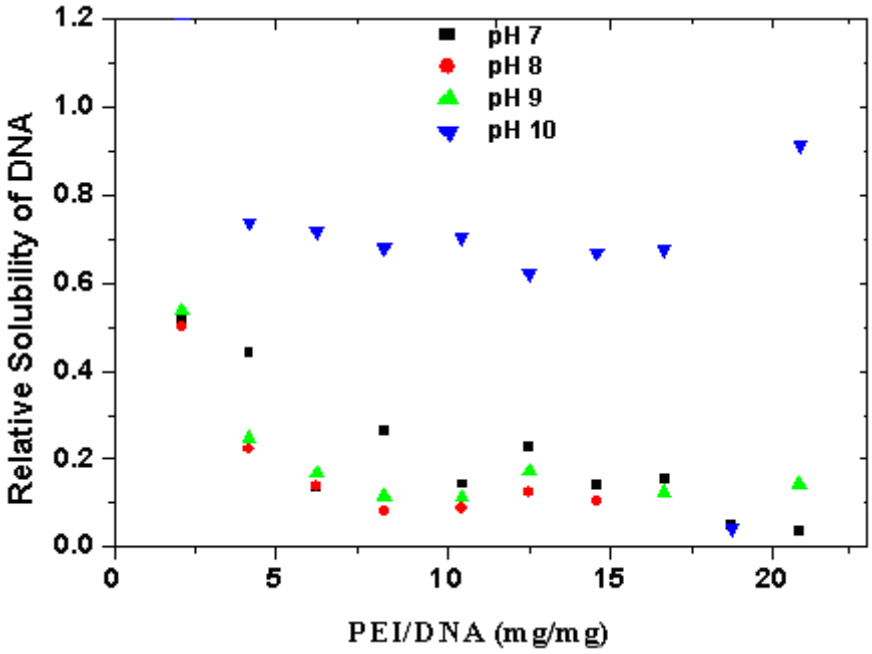


Figure 7.9 Solubility profiles of DNA following PEI-mediated chemical extraction of recombinant *E. coli* BL21(DE3) expressing gloshedobin (mostly as IBs). Extraction was conducted at a cell suspension of OD_{600} = 60. The concentration of DNA from the extraction condition lacking PEI was set as 1.

7.3.4 PEI-mediated chemical extraction and selective precipitation of DNA at high cell densities

Mechanical disruptions at various cell densities were conducted as control experiments to demonstrate the efficiency of chemical extraction. As shown in Figure 7.10, the efficiency of PEI-mediated chemical extraction was comparable to that of mechanical disruption even at OD_{600} = 150. At each OD, more than 90% of DNA was removed when PEI was applied at a final concentration of 10 mg/mL. The selected PEI concentration was equivalent to approximately 20 mg/mg of PEI/DNA at OD_{600} = 60 (Figure 7.9) and about 2.7 times higher than the optimal ratio determined at OD_{600} = 60 (7.5 mg/mg). The excess use of PEI was to account for the proportionally increasing DNA concentration at high cell densities (note that DNA release at OD_{600} =

150 is 2.5 times that at OD_{600} = 60). The data summarized in Figures 7.7-7.10 demonstrated that PEI is highly efficient for selective precipitation of DNA from the chemical extraction mixture while exhibiting its function in a wide range of cell densities (OD_{600} = 30-150) as an effective extraction chemical capable of replacing EDTA.

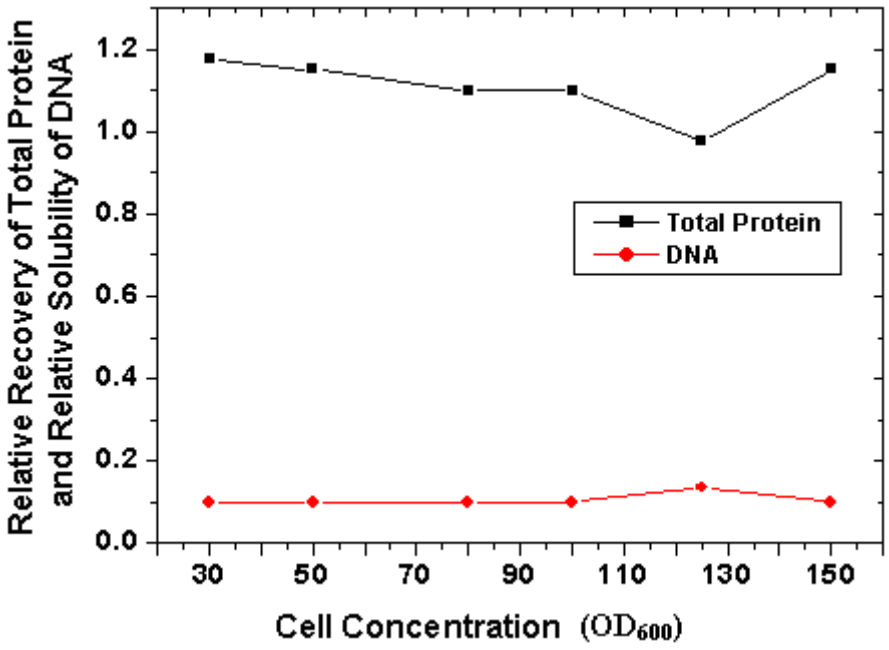


Figure 7.10 Recovery of total protein and solubility profile of DNA following PEI-mediated chemical extraction of recombinant *E. coli* BL21(DE3) expressing gloshedobin at various cell densities. The PEI concentration at each OD was 10 mg/mL.

7.3.5 Chemical extraction of IbpA and precipitation of coextracted DNA by PEI

We further tested the efficacies of PEI-mediated chemical extraction and DNA precipitation of another recombinant protein IbpA which was also expressed mainly as IBs (Figure 7.1B) to verify that the PEI effect on the chemical extraction and

selective DNA precipitation is not protein-specific. For the case of IbpA, the extraction was conducted for a cell suspension of OD_{600} = 80. Despite the change of model protein, the efficiency of total or target protein extraction and the efficacy of selective DNA precipitation remained almost the same as those observed for gloshedobin extraction (data not shown).

7.3.6 IMAC purification of His-tagged gloshedobin and IbpA

As our target proteins (both gloshedobin and IbpA) were expressed as fusion proteins with a 6×His-tag at their N-terminal, i.e., thioredoxin-6×His-tag-gloshedobin and 10×His-tag-IbpA, IMAC provides a convenient post extraction purification and potential refolding step. The supernatant after chemical extraction was loaded onto a Ni^{2+}-charged IMAC column. The trace amount of PEI left in the supernatant, after screening PEI-metal ion interaction by Mg^{2+} addition (Juang and Chiou, 2000), had no significant effect on the Ni^{2+}-charged resin or the binding of target protein to the resin. This simple strategy of Mg^{2+} addition was effective to counteract PEI, which is essential at the time of extraction but detrimental to the subsequent IMAC process, thereby facilitating direct coupling of PEI-mediated extraction with IMAC purification. Following the adsorption of target protein and extensive washing, the bound protein was eluted using a linear gradient spanning from washing buffer to elution buffer containing 0.5 M imidazole. The fractions containing the His-tagged gloshedobin or His-tagged IbpA were analyzed by SDS-PAGE, followed by Coomassie blue-staining (Figure 7.11). The purity of IbpA following IMAC

purification was over 95% as determined by densitometric quantification. The purified gloshedobin, however, was significantly contaminated by an expression product (bands with molecular weight of 27.5 kDa in Figure 7.11A) and the purity of full-length gloshedobin was approximately 60%. Comparing the insoluble fraction from uninduced cells (Figure 7.1A, lane 3) to the insoluble fraction from induced cells (Figure 7.1A, lane 6), we observe that the lower band for gloshedobin only appears in the insoluble fraction from induced cells. Moreover, this lower band contains the 6×His-tag as it could be purified by IMAC (Figure 11A). These show that the lower band is a truncated product, which might be the thioredoxin-6×His-tag containing N-terminal fraction of intact gloshedobin, and therefore it could be co-purified by IMAC together with the full-length target protein.

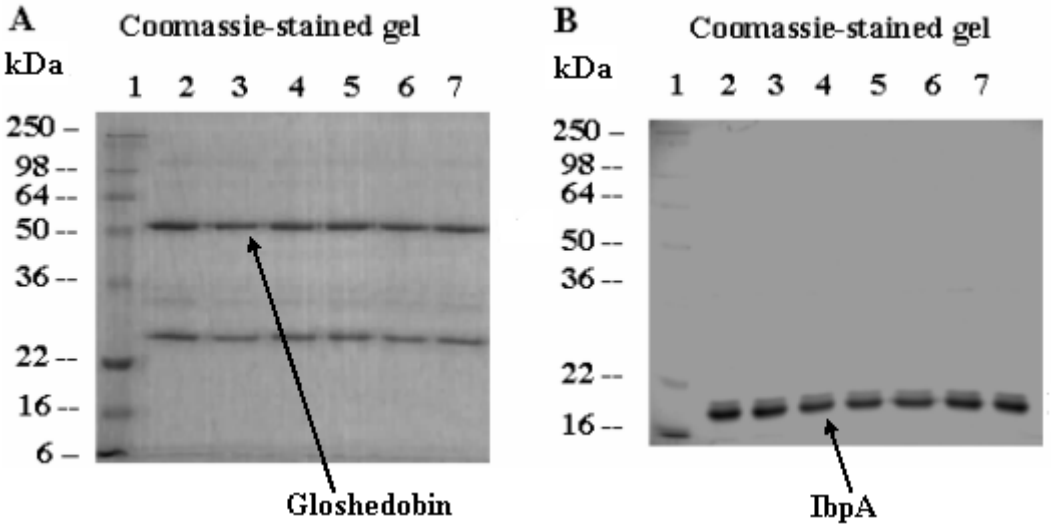


Figure 7.11 (A) Purified gloshedobin (A) and IbpA (B) by IMAC following their extraction from the expression hosts using PEI-mediated extraction method. The bound proteins were eluted by a liner gradient of imidazole (0-0.5 M). Fractions containing proteins (gloshedobin or IbpA) were collected and analyzed with SDS-PAGE. (Lane 1, molecular weight marker; Lanes 2-7, selected elution fractions collected during IMAC purification).

In this chapter, we demonstrated that direct single-step PEI-mediated extraction was more efficient for the extraction of cytoplasmic IB proteins compared to mechanical disruption or other chemical extraction methods reported previously. High molecular weight DNA was efficiently precipitated by simple addition of PEI without affecting the target protein recovery. In addition, PEI obviated the need of using EDTA or cost-prohibitive spermine at the time of extraction, and hence PEI proved to serve at least a dual purpose in the new chemical extraction protocol presented. This facilitates direct coupling of chemical extraction step with IMAC or other subsequent processing step, simplifying protein purification process.

7.4 Conclusion

The present study demonstrated that PEI is a very efficient agent to precipitate DNA from the extraction broth without affecting the solubility of target protein. Compared to spermine-induced precipitation of DNA reported elsewhere (Choe et al., 2001b), PEI-induced DNA precipitation not only showed higher efficiency, but also involved lower cost. The traditionally used EDTA was not necessary for the PEI-mediated chemical extraction and the use of only urea and PEI in Tris buffer was sufficient to obtain almost complete recovery of IBs from the cytoplasm of *E. coli*. This selective removal of DNA by PEI is fully compatible with the extraction method, and is simple to implement. The PEI-mediated chemical extraction therefore allows direct coupling of chemical extraction at a high cell density with IMAC and thus greatly simplifies the IB purification process in an economically viable way.

Chapter 8
Conclusions and future work

Summary

Throughout the research work presented in Chapters 3-7, gloshedobin, a kind of thrombin-like enzyme (TLE), expressed mainly as inclusion bodies (IBs) in *E. coli*, was successfully produced as intact form with significant amount of recovered activity. The contamination of truncated expression products associated with the full-length gloshedobin expression was eliminated by co-expression of a molecular chaperone, ClpB. A folding-like-refolding strategy harnessing unpurified ClpB and DnaK/DnaJ/GrpE (DnaKJE) bichaperone system was developed using a model protein (heat-denatured malate dehydrogenase (MDH)) and successfully applied to enhance the column-based (with the use of immobilized metal affinity chromatography (IMAC)) refolding of full-length gloshedobin from cell disruptates. The new refolding method showed significant improvement on the protein refolding compared with the traditional protein refolding strategies in either dilution or on-column mode. Further process intensification for recovery of gloshedobin IBs was achieved by incorporation of PEI-mediated chemical extraction with IMAC protein purification to overcome the inefficiencies associated with the traditionally used IB recovery strategy, such as mechanical cell disruption. This novel process intensification method, together with the approach to reduce the truncated expression products and the folding-like-refolding strategy are thus expected to lead to a more

efficient and economically viable processing route for the large-scale production of refolding-recalcitrant IB proteins.

To maximize the benefits of the novel process developed in this work, some points requiring further investigation are addressed. In this study, although the unpurified ClpB/DnaKJE bichaperone system was demonstrated to efficiently refold heat-denatured protein (MDH), the application of this system is still constricted considering the highly complex refolding cocktail used in the refolding process. To overcome this problem, addition of affinity tags (e.g. 6×His-tag) to each molecular chaperone may facilitate the subsequent purification of target proteins after the refolding reaction. An alternative refolding strategy harnessing chaperone-tagged beads can also be investigated in order to facilitate the downstream protein processing with ease separation of target proteins from molecular chaperones. In addition, the mechanism of ClpB in the reduction of truncated expression need to be further investigated. Strategies relying on the mutation of different domains on ClpB may be conducted to understand its detailed function. Finally, the folding-like-refolding strategy developed in this study can be further extended to other refolding techniques, such as expanded bed adsorption (EBA), high gradient magnetic separation (HGMS) or high-pressure fostered protein refolding. To prove the general applicability of the demonstrated strategy to IB processing, more IB proteins with different characteristics should be tested.

8.1 Main conclusions

In general, *Escherichia coli* is one of the most extensively used prokaryotic organisms for genetic manipulations and industrial production of proteins (Swartz, 2001). However, many recombinant proteins exist in insoluble protein aggregates called inclusion bodies (IBs) when overexpressed in *E. coli*. Traditional isolation and refolding methods for IBs, such as cell disruption by high pressure or ultrasonic waves and differential centrifugation to remove cell debris and precipitate IBs followed by dilution or dialysis refolding, are time-consuming and inefficient (Middelberg, 1995; Falconer et al., 1997; Wong et al., 1997). Moreover, the large amount of truncated expression products significantly complicated the following purification and refolding of full-length target protein, gloshedobin (a thrombin-like enzyme (TLE) recently isolated from snake venom) (Yang et al., 2002).

This research aims to design an efficient protein recovery scheme so as to produce intact gloshedobin with biological activity. The selection of strategies and unit operations for this purpose were done following a review of recent advances in downstream protein purification and refolding detailed in Chapter 2. The molecular chaperones mediated protein refolding (Weibezahn et al., 2004b), column-based protein purification/refolding (Ueda et al., 2003), and the direct chemical extraction (Falconer et al., 1997; 1998; Choe and Middelberg, 2001a; 2001b) were selected as promising processing steps for simplified and more efficient IB recovery strategy. Multitasking of a single unit operation, some industrial heuristics and feasibility of

large-scale implementation were emphasized during the study.

In Chapter 3, a novel folding-like-refolding strategy harnessing a bichaperone-based refolding cocktail comprising unpurified *E. coli* heat-shock proteins ClpB and DnaK/DnaJ/GrpE (DnaKJE), was first developed to mimic the exquisite folding mechanism afforded by protein quality control network reported elsewhere (Schlieker et al., 2002; Baneyx and Mujacic, 2004). The concept of using unpurified proteins obviated the demand of main cost-inhibitive steps to express and purify each of these molecular chaperones. A plasmid encoding ClpB with a 6×His-tag at its C-terminus (His-ClpB) was first constructed to facilitate its purification through IMAC. A different plasmid capable of expressing the DnaKJE was used to obtain a cell extract containing unpurified DnaKJE. The effect of purified His-ClpB and unpurified DnaKJE on the refolding of a model protein (heat-denatured malate dehydrogenase (MDH)) was investigated, and proved to be highly efficient. Furthermore, the use of both unpurified His-ClpB and DnaKJE available in the cell extract enabled highly successful refolding of the heat-denatured MDH with efficacy comparable to the case where the purified His-ClpB was used, thus providing a practical and economically viable way of implementing a large-scale folding-like-refolding strategy.

In Chapter 4, we further found that the use of polyethylene glycol (PEG) as a refolding additive to the refolding cocktail comprising ClpB/DnaKJE bichaperone system significantly enhanced the chaperone-mediated refolding of heat-denatured

MDH. The binding of PEG onto the refolding intermediates initiated by chaperoning activity of ClpB/DnaKJE might inhibit the reaggregation of these intermediates. The critical factor to affect the refolding yield is the time point of introducing PEG and the refolding efficiency reached approximately 90% only when PEG was added at the beginning of refolding reaction. The synergistic coordination of an inexpensive refolding additive PEG with the ClpB/DnaKJE bichaperone system may provide an economical route to further enhance the efficacy of ClpB/DnaKJE refolding cocktail approach, facilitating its implementation in large-scale refolding processes.

Prior to confirming the applicability of the proposed folding-like-refolding strategy to the refolding of gloshedobin IBs, in Chapter 5, it was observed that the expression of gloshedobin was strongly dependent on the expression host. The truncated expression was reduced by 25% when the protein was expressed in *E. coli* BL21(DE3)pLysS instead of BL21(DE3). It was also demonstrated that co-expression of ClpB in BL21(DE3) enabled the expression of gloshedobin mostly in intact form without compromising expression level, while almost completely eliminating its truncation products. Following extraction and solubilization of the IBs from the cell disruptates, one-step IMAC purification produced highly purified (>99%) denatured-solubilized full-length gloshedobin ready to enter the subsequent refolding process. However, the traditional dilution or column refolding strategy, based on gradual denaturant removal, was found to be inefficient for the recovery of protein activity.

In Chapter 6, a novel column-based (IMAC) refolding strategy employing unpurified ClpB/DnaKJE bichaperone system as developed in Chapter 3 was demonstrated to be superior to the conventional refolding methods in either batch dilution or column refolding mode. The application of the bichaperone system was shown to be effective in reactivating these misfolded species to further increase the refolding yield and the refolded protein successfully regained their native structure as ascertained by RP-HPLC and CD spectrum analysis. Furthermore, compared with traditional dilution refolding, the same enzymatic activity was recovered by the chaperone-assisted column refolding at a much higher protein concentration with lower buffer requirement. However, the strategy used in this chapter to isolate gloshedobin IBs followed the traditionally utilized methods which are time-consuming and inefficient, including cell disruption by high pressure, repeated centrifugation to precipitate IBs and dissolution of IBs in high concentration of denaturant.

With the limitations realized, the recovery process for gloshedobin IBs was therefore further integrated through coupling of IMAC protein purification with chemical extraction (Falconer et al., 1997; 1998; 1999; Choe and Middelberg, 2001a) which was suggested to be an efficient way to overcome the inefficiencies associated with traditional IB recovery method. In Chapter 7, a more efficient PEI-mediated chemical extraction was developed. The selectivity of PEI in DNA precipitation during chemical extraction was investigated in order to reduce the high viscosity of the cell extracts resulted from the presence of high molecular weight DNA. High DNA

removal efficiency (more than 90%) was achieved at various cell densities (with OD_{600} up to 150) without affecting the solubility of host cell proteins. Compared to spermine-induced precipitation method reported elsewhere (Choe and Middelberg, 2001b), PEI provided a higher DNA precipitation efficiency at a significantly lower cost. Moreover, PEI obviated the use of EDTA, which has been reported to be essential for the chemical extraction methods, hence exhibiting dual roles in replacing cost-prohibitive spermine and EDTA. The residual PEI in the post-extraction mixture was efficiently counteracted by addition of Mg^{2+}, allowing the streamlined application of the extraction broth to IMAC protein purification.

Overall the research presented in this thesis established new concepts for IB processing. A novel folding-like-refolding strategy harnessing unpurified ClpB/DnaKJE molecular bichaperone system provided a straightforward way to apply this system in more operations, especially for large-scale protein production. Through the initial reduction of truncated expression products by co-expression of ClpB, the quality of protein expression can be significantly improved, which may greatly facilitate the following protein purification and refolding. The subsequent bichaperone-mediated column refolding strategy provided an effective tool for refolding-recalcitrant proteins whose expression is otherwise difficult to achieve. Finally the PEI-mediated chemical extraction coupled with IMAC protein purification provided a more economically viable way for production of recombinant proteins whose expression is hampered by IB formation. This offers the potential for further

process intensification. Therefore, a totally new IB processing scheme which may enable the more efficient recovery of IB proteins especially for those contaminated with truncated expression products was proposed as shown in Figure 8.1.

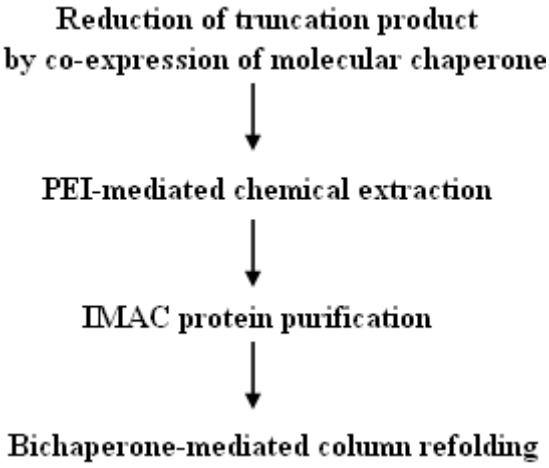


Figure 8.1 A more efficient and simplified IB scheme as proposed in this study.

8.2 Suggestions for future work

In this study, we demonstrated the refolding of heat-denatured protein (MDH) by unpurified ClpB/DnaKJE bichaperone system. However, application of this system in this way is still constricted considering the highly complex refolding cocktail used in the refolding process. Additional purification steps must be incorporated in order to recover the refolded target protein. To overcome this problem, we can either add fusion tags (e.g. 6×His-tag) to the target protein or to the molecular chaperones (e.g. His-DnaK, His-DnaJ and His-GrpE) to facilitate the IMAC purification of target protein after complete or partial refolding. Chromatographic separation using size exclusion chromatography (SEC) can be used subsequently to fractionate the correctly

folded target protein from other refolding intermediates. Those molecular chaperones captured by the metal affinity resin may be eluted and the potential of recycling the recovered molecular chaperones for the next batch refolding process need to be further investigated.

An alternative refolding strategy harnessing chaperone-tagged beads can also be explored to facilitate downstream native protein isolation. In this refolding scheme, stoichiometric amounts of different kinds of His-tagged molecular chaperones are first immobilized on the metal chelating beads (or magnetic beads functionalized to capture His-tagged chaperones) and the refolding process is then initiated by introducing the target protein into the bead suspension. The purification of refolded target protein in the bulk solution is achieved by a low-speed centrifugation or filtration followed by SEC fractionation to separate the chaperone-tagged beads from the soluble, refolded target protein. The separated chaperone-tagged beads are ready to be re-used for the next batch of refolding reaction.

The additional benefit through harnessing His-tagged chaperones is that by using the purified His-DnaK, His-DnaJ and His-GrpE described as above, the investigation on the effects of each individual molecular chaperone on the protein refolding can be achieved. The yield of heat-denatured MDH refolding was demonstrated to be dependent on the combined concentration of DnaKJE system in Chapter 3. Since DnaK, DnaJ and GrpE were co-translated from a single plasmid (pKJE7), the ratio of

these three chaperones kept unchanged and the individual contribution of each chaperone for the refolding of target protein could not be quantitatively assessed in the present study.

Moreover, the inhibition mechanism through co-expression of ClpB on the formation of truncated expression products in Chapter 5 needs to be further studied. Some plausible scenarios which may be conducive to understand the roles of ClpB include: i) Co-expression ClpB with other kind of proteins whose expression was also impeded by the formation of truncation products. To achieve this, the gene encoding ClpB can be cloned and inserted to the plasmid expressing the target protein to realize the co-expression. An alternative way is to construct a different plasmid encoding ClpB but with other kind of compatible promoters. Both plasmids encoding ClpB and target protein can then be easily co-transformed to *E. coli* cells to achieve the co-expression, ii) Mutations on different domains of ClpB can be performed and co-expression with target protein is then conducted to investigate the effect of different domains on the reduction of truncation products, and iii) The protein sequence of the truncation products can be compared with the full-length protein to identify the proteases which cause the truncation. Then *in vitro* experiments may be conducted to see the interplay between ClpB and those specific proteases.

Finally, the developed folding-like-refolding strategy may be further coupled with other refolding techniques, such as expanded bed adsorption (EBA), high gradient

magnetic separation (HGMS) or high-pressure fostered protein refolding. High-pressure protein refolding was demonstrated to be efficient in protein refolding. IB proteins dissociate under high pressure to unfolded form and with the decrease of pressure, the proteins gradually regain their native state. Considering the DnaKJE system in the protein refolding step assists the proper folding of unfolded polypeptide, the incorporation of DnaKJE system in high-pressure protein refolding may further improve the refolding efficiency.

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Appendix I
List of publications

Journal publication:

- Nian R, Tan L, and Choe WS. Polyethylene glycol (PEG) facilitates ClpB/DnaKJE-mediated disaggregation of MDH. Biotechnol. Prog. (In press)

- Nian R, Tan L, Yoo Ik-Keun and Choe WS. Molecular chaperones enhanced IMAC refolding of gloshedobin, a thrombin-like enzyme from snake venom. J. Chromatogr. A. 2008, **1214**, 47-58.

- Nian R, Tan L, and Choe WS. Folding-like-refolding of heat-denatured MDH using unpurified ClpB and DnaKJE. Biochem. Eng. J. 2008, **40**, 35-43.

- Nian R, Tan L, and Choe WS. Effective reduction of truncated expression of gloshedobin in *Escherichia coli* using molecular chaperone ClpB. Chem. Eng. Sci. 2008, **63**, 2875-2880.

- Nian R, Tan L, and Choe WS. Polyethyleneimine-mediated chemical extraction of cytoplasmic His-tagged inclusion body proteins from *E. coli*. Biotechnol. Prog. 2008, **24**, 417-425.

- Choe WS, Nian R, and Lai WB. Recent advances in biomolecular process intensification. Chem. Eng. Sci. 2006, **61**, 886-906.