INTEGRATED RECOVERY PROCESS FOR RECOMBINANT PROTEIN OVER-EXPRESSED AS INCLUSION BODIES IN BACTERIAL CELLS USING PREPARATIVE ELECTROPHORESIS

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ABSTRACT

INTEGRATED RECOVERY PROCESS FOR RECOMBINANT PROTEIN OVER-EXPRESSED AS INCLUSION BODIES IN BACTERIAL CELLS USING PREPARATIVE ELECTROPHORESIS

(Keywords: Inclusion bodies, fermentation, solubilization, soluble aggregate, refolding, gel electrophoresis)

Recombinant proteins can be produced in bacteria, yeast, insect cells, mammalian cells, and cell-free system. Recombinant proteins are expressed as inclusion bodies (IBs) in bacterial enriched native-like secondary structure and thus give a great potential in biotechnological utilities. IBs are produced in *Escherichia coli* cells and solubilization process is required to recover desired protein in bioactive form. In this study, the effects of solubilization methods on the recovery of soluble enhanced green fluorescent protein (EGFP) from IBs by using urea, alkyl alcohol and freeze thaw method were investigated. The present study indicates urea concentration, incubation temperature, type of alcohol and its concentration, freezing duration and freeze thaw cycles influenced the yield and purity of solubilized EGFP. Conventional method using 8 M of urea with incubation temperature of 60°C achieved the highest yield (61%) and purity (10%). Mild IBs solubilization with 6 M of n-butanol and 2 M of urea has solubilized IBs with a yield of 45% and purity of 22%. By freezing and thawing the IBs suspension in 2 M of urea, the yield (66%) and purity (9%) of solubilized EGFP were comparable to that of 8 M of urea in buffer. Hence, mild solubilization using the alkyl alcohol or freeze thaw method is applicable for IBs solubilization.

Previous studies reported the quality and nativity of refolded soluble protein from inclusion body is questionable because the refolded protein with wrong conformation will assemble to form soluble aggregates. Many studies involving proteins from inclusion bodies only assessed the protein quality based on the protein solubility and functionality, but not the protein conformation that reflects the protein aggregation tendency. In this study, EGFP-IBs was used as the model protein to investigate the soluble aggregates formation under different solubilization and refolding conditions. The present study used a gel-based imaging method to analyze the refolded soluble protein based on fluorescence intensity, charge, shape, and size of the protein. For the solubilized inclusion bodies refolding under high protein concentration and low protein conditions, aggregation can be visualized with polyacrylamide gels. Gel images showed the refolded soluble protein changed in conformation and increased in size when the solubilized inclusion bodies underwent various refolding periods. Meanwhile, the refolded soluble protein under the refolding condition of low protein concentration and high protein purity has a correct protein conformation and achieved the highest refolding yield. Studying the effects of refolding conditions using different types of solubilized inclusion bodies may provide researchers with possible

approaches to avoid soluble aggregates formation in the pharmaceutical and nanobiotechnology applications. By using PNU-PAGE for clarifying and purifying the solubilized EGFP prior to refolding process, the method has successfully recovered 2.4 µg of folded soluble EGFP with 12.4% of refolding yield and 52.2% of purity after one day of refolding incubation period.

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LIST OF ABBREVIATIONS

E. coli	*Escherichia coli*
EGFP	enhanced green fluorescent protein
EGFP-IBs	enhanced green fluorescent protein- inclusion bodies
IBs	inclusion bodies
PNU-PAGE	preparative native urea-polyacrylamide gel electrophoresis
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
UGGE	urea gradient gel electrophoresis

CHAPTER 1

INTRODUCTION

1.1 Background of study, motivation and problem statement

Escherichia coli (*E. coli*) cells have been widely used for the production of recombinant proteins because it is the most well-known species of bacteria that can be rapid grown in inexpensive and simple media. High level expression of recombinant proteins in bacterial cells may obtain large quantities of the desired protein. However, it often results in aggregation of the expressed protein molecules into inclusion bodies (IBs) (Singh et al., 2015). Previous study reported that the over-expression of recombinant protein in *E. coli* cells often leads to 70% of target protein folded into aggregates called inclusion bodies (IBs) (Yang et al., 2011). The expressed protein in IBs can be easily separated from cell debris by centrifugation after cell disruption. Hence, the IBs exhibits high protein purity that ease the recovery process. However, it cannot be directly used for bioreaction due to their misfolded structure and low solubility.

Various approaches have been reported to recover IBs as biologically active protein via four major steps: isolation of IBs from the bacterial cells, denaturation of IBs, refolding of denatured proteins and purification of refolded proteins. The denaturation process that involves the destruction of both protein secondary and tertiary structures. The conventional strategy to solubilize IBs used high concentration of denaturant (urea or guanidine HCl) and refolded into an active conformation by removing the denaturant. Using strong denaturants result in complete disruption of protein structure and lead to protein aggregation during refolding (Dill and Shortle, 1991; Panda, 2003). Previous literatures have documented that proteins in IBs have native recombinant proteins (Peternel and Komel, 2011) and can be used as biocatalysts and diagnostic tools in various bioprocess (García-Fruitós, 2010). In order to recover the native protein in IBs that is embedded in an aggregate, researches have used a mild solubilization process to facilitate the IBs solubilization (Singh et al., 2015). Mild solubilization retains the existing native-like secondary structure of protein, reduces protein aggregation during refolding and enhances the recovery of bioactive proteins from IBs (Khan et al., 1998; Singh and Panda, 2005; Singh et al., 2012).

Despite several protocols available for IBs solubilization, the performance is influenced by many factors such as the type and concentration of denaturant (Yang et al., 2011), solubilizing temperature (Day et al., 2002) and buffer pH (Berkelman et al., 2004). By using organic solvents such as alcohols, increase in chain length of alkyl alcohol exhibits variation in solvent hydrophobicity that promotes IBs solubilization (Kumari and Jaagnnadham, 2011). Singe et al. (2012) also reported that different amount of solubilized IBs was obtained when concentration of n-propanol based buffer was increased. Besides, freeze thaw process is affected by

freeze thaw cycle, sample volume, freezing temperature and incubation duration. The freeze thaw process in fast freezing rate and slow thawing rate caused protein damage (Cao et al., 2003) and the best freezing temperature of -20°C for IBs was reported by Qi et al. (2015). The influence of various factors resulting different yield and purity of the solubilized protein.

Many researchers reported that the quality and nativity of protein cannot only be determined by their solubility and functionality, but the conformation in which reflects the protein aggregation tendency is also crucial (Chew et al., 2011; Martínez-Alonso et al., 2008; Melnik et al., 2009). The recovery of soluble protein from IBs may not always lead to its native conformation. Colon and Kelly (1992) reported that the partially unfolded transthyretin induced conformational changes and triggered the aggregation into amyloid fibrils during protein refolding. Due to the environmental stresses, exposure to air-liquid or liquid-solid interfaces, and induction by other particles, the refolded soluble proteins from solubilized IBs can misfold and aggregate into amyloid-like particles (den Engelsman et al., 2011; Fink, 1998). The changes are destructive and the assembly of amyloid can lead to diseases such as Alzheimer's, Parkinson's, and diabetes (Type II) that are characterized by specific protein aggregates (Rambaran and Serpell, 2008). Aggregation in protein based pharmaceuticals such as human growth hormone can affects the amount the efficacy of the delivered drug and patients may experience undesirable immunologic responses (Rosenberg, 2006). Besides, a highly complex configuration of soluble aggregates can precipitate out from the biosensor and biodiagnostics kits and affects their application that emphasizes the protein conformation and active site (Jain, 2005).

Many refolded soluble proteins can achieve high yield and purity after the four IBs recovery steps and the quality of these proteins were analyzed by different analysis tools as summarized in Table 1.1. By using different analysis principles, various protein information including the protein size and morphology, its primary and secondary structures, and the protein activity and functional group were obtained. However, it was found that the correct protein conformation has actually been studied much less. For example, Lemke et al. (2015) confirmed the native conformation of refolded soluble protein by determining both the level of solubilization and the enzymatic activity of protein. However, the protein conformation in which reflects the protein aggregation tendency has not been studied. The refolded soluble protein does not necessarily imply that it is in the native conformation.

Table 1.1. List of analysis methods of functional soluble proteins from IBs.

Analysis method	**Analysis principle**	**Analysis result**	**References**
Chromatography (size exclusion chromatography and high performance liquid chromatography)	Separation of protein components based on molecular sieving and relative affinity	Hydrodynamic size and molecular composition	Cabanne et al., 2005; Fraga et al., 2010; Malavasi et al., 2011; Stepanenko et al., 2012
Spectroscopy (circular dichroism, infrared, fluorescence and ultraviolet-visible)	Measurement of light absorption according to the wavelength of protein molecule	Secondary structure or intensity of protein	Cabanne et al., 2005; Fraga et al., 2010; Qi et al., 2015; Shi, Zhang et al., 2014; Stepanenko et al., 2012
Microscopy (scanning electron microscopy, transmission electron microscopy, confocal microscopy and fluorescence microscopy)	Visualization of protein morphology by scanning the protein particles	Size and morphology of protein particles	Gu et al., 2002; Malavasi et al., 2011; Raghunathan et al., 2014
Western blotting and enzyme-linked immunosorbent assay	Detection of targeted protein based on the bioreaction with antibodies	Protein activity	Dehaghani et al., 2010; Fraga et al., 2010; Gu et al., 2002; Shi et al., 2014
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)	Gel separation based on the protein molecular weight under an electric field	Molecular weight of protein in primary structure	Cabanne et al., 2005; Dehaghani et al., 2010; Gu et al., 2002; Malavasi et al., 2014; Raghunathan et al., 2014; Shi et al., 2014; Qi et al., 2015
Fourier transform infrared spectroscopy	Detection of functional group in protein molecule	Functional group and component bonding in protein molecule	Malavasi et al., 2014

Refolded soluble protein from IBs with correct protein conformation is important. In this study, a gel-based imaging method was used to analyze the refolded soluble enhanced green fluorescent protein (EGFP) (Chew et al., 2009a). EGFP is used in this study as a model protein owing to its visible and stable green fluorescence (Chalfie et al., 1994). Fluorescence intensity data for protein conformation analysis as performed by Vera et al., (2006), native polyacylamide gel electrophoresis (PAGE) allowed the charge, shape, and size of EGFP to contribute to its mobility in the gel for aggregation analysis (Chew et al., 2011). Protein contaminants and concentration affect aggregation of protein (Wang & Roberts, 2010). This study aims to provide researchers the better understanding of soluble aggregates formation under varying sample concentration and purity using the gel-based imaging method, hence the IBs recovery process was conducted under different solubilization and refolding conditions.

For the recovering of functional EGFP in IBs, various approaches have been reported such as expanded bed anion exchange chromatography (Cabanne et al., 2005), chaperone-assisted metal affinity chromatography (Dong et al., 2009), and size exclusion chromatography (Lim et al., 2014). However, in all of these studies, the EGFP-IBs must be isolated and denatured in separate steps prior to the refolding and purification steps (Seras-Franzoso et al., 2015; Yang et al., 2011). Moreover, the use of gel electrophoresis technique for purifying enzyme from Dictyostelium discoideum IBs has been reported before (Ubeidat and Rutherford, 2003) in which the isolation, denaturation and refolding processes were done separately for the complete recovery process.

Using several unit operations for desired protein recovery from IBs has resulted high capital and labor costs, long processing time and high losses of product. If a convenient and efficient way of recovering desired protein molecules from IBs can be developed, it will reduce the need of extensive chromatographic purification steps. In the present research, preparative native urea-polyacrylamide gel electrophoresis (PNU-PAGE) has combined isolation and purification steps into a single unit operation for recovering EGFP-IBs from denatured cell suspension. Polyacrylamide gel has a small pore size is well suited for isolation of cells debris and separation of most protein. Chew et al. (2009b) have reported soluble protein purification from intact cells using preparative polyacrylamide gel electrophoresis. This gel column has successfully combine cells clarification, proteins concentration and purification steps into a single step. In the present study, the external electric field drift out denatured EGFP from cell suspension into porous polyacrylamide gel. By rapid electrophoresis in the porous gel, the denatured EGFP is then purified by the gel. An integrated IBs recovery system using electrophoresis concept would substantially shorten the overall processing time. This integrated system was investigated and analyzed for the aim to obtain the biologically active protein in higher yield and purity.

1.2 Research objective

1. To investigate the effects of conventional and mild solubilization methods and its process condition on the recovery of denatured EGFP from IBs.
2. To investigate the effects of solubilization and refolding conditions on the soluble aggregates formation using gel-imaging method.
3. To develop an electrophoresis-based bacterial IBs protein recovery method.

1.3 Research scope

Recombinant EGFP was overexpressed in *E. coli* strain BL21(DE3). The fermentation medium was harvested the cell pellet was lysed by freeze and thaw method to remove soluble proteins. The effects of solubilization methods on the recovery of denatured EGFP were then investigated by using urea, alkyl alcohol and freeze thaw method under varying process condition: urea concentration (0 to 8 M), incubation temperature (50 to 80°C), alkyl alcohols (methanol, ethanol, ethylene glycol, n-propanol, glycerol, and n-butanol), n-butanol concentration (0 to 6 M), freezing incubation period (0 to 4 days), freeze thaw cycle (0 to 4 cycles).

The investigation about the soluble aggregates formation during protein refolding was conducted under three different types of solubilized EGFP-IBs: solubized inclusion bodies with cell debris, solubized inclusion bodies with detergent washing, and purified solubilized inclusion bodies using PNU-PAGE. For the third condition, an electrophoresis-based IBs protein recovery method was developed to combine isolation and purification steps into a single unit operation for recovering EGFP-IBs from denatured cell suspension.

Protein analyses are including SDS-PAGE to measure denatured EGFP amount, native PAGE to determine functional EGFP amount and Bradford assay to measure total protein amount. Lastly, yield and purity of solubilized EGFP and refolded EGFP were further calculated from the collected data.

CHAPTER 2

LITERATURE REVIEW

2.1 Inclusion bodies

IBs are dense electron-refractile particles of aggregated protein that failed to reach their native conformation during the targeted gene overexpression in bacterial cell. They normally found in the cytoplasmic and periplasmic spaces of *E.coli* (Mar Carrio et al, 2000; Singh et al., 2005). The formation of IBs is anticipated when the protein consists of highly hydrophobic and disulfide bonds. This is because the reducing environment of bacterial cytosol inhibits the disulfide bonds when the proteins pass through from endoplasmic reticulum to Golgi apparatus (Kopito, 2000; Singh et al., 2005). The diameter of these pseudo spherical bacterial IBs varies from 0.5 to 1.3 μm and the density is about 1.3 mg/ml which is much higher than many cellular component (Margreiter et al., 2008). The nativity of IBs are stable when underwent ultrasonication high pressure and harsh cell disruption (García-Fruitós et al., 2012). Furthermore, the low solubility of IBs to others protein has provided a high purity properties of IBs as target protein (Kopito, 2000). Therefore, IBs are easily to be separated with other soluble protein by using high speed centrifugation after cell disruption. After IBs have been proved its active structure with 90% purity of desired protein (García-Fruitós et al., 2010), IBs are no more treated as waste product and discharged after the fermentation but widely used in industry. In biotechnology industry, IBs are solubilized or denatured to isolate from cell debris, and followed by the folding process to return their functional structure after separated from cell debris (Singh et al., 2005). Thus, a major bioprocess engineering challenge has been focused to efficiently convert the IBs into soluble and correct folded product.

2.2 Formation of inclusion bodies

Formation of IBs can be categorized under physical induced and chemical induced. For physically induced, IBs are formed by result from unbalanced equilibrium between aggregated and soluble protein of *E. coli* (Villaverde et al., 2012). Aggregation is described as specific intermolecular interaction among single type of protein molecule. Intermolecular interaction will lead to accumulation of partially folded or misfolded expressed protein. The major condition that lead to the interaction is due to non-covalent hydrophobic bond or ionic interaction between the molecules. For chemically induced IBs are formed due to the result of disulphide bond formation and non-disulphide crosslink. Many of the chemical reaction can directly crosslink the protein and thus change the hydrophobicity of the protein. Example for disulphide bond formation when free cys residues in protein can be oxidized resulting disulphide linkage such as bFGF. Table 2.1 below showing the mechanism for IBs formation.

Table 2.1: Mechanism for IBs formation.

Mechanism of formation	Type
Physical induced aggregation	Folding and unfolding intermediates
	Nucleation and growth of protein aggregates (Krishnamurthy and Manning, 2002)
	Reversibility and specificity of physical aggregation (Fink, 1998)
	Thermodynamics of protein aggregation
Chemical induced aggregation	Disulfide bond formation
	Non-disulfide crosslinking pathways

For folding and unfolding intermediate protein, hydrophobicity of the protein play the crucial role. The concept of aggregation of protein begin with the presence of patches of hydrophobic groups that act as initiator. It does determined the correct folding of polypeptide chain into functional protein. Folding and unfolding intermediate protein is not stable and poorly populated and it composed of patches of contiguous hydrophobics group that create the aggregation. In contrast for completely folded or unfolded protein do not aggregated easily. This is due to hydrophobic side chains are out of contact with water and it is scattered from each other. For the second mechanism which is nucleation and growth of protein. There are two proposed models describing the formation of inclusion bodies as a consequence of the self-assembly of non-native monomers into growing polymers of higher sizes. IBs can start from a single or limited number of nucleation sites by accumulation of misfolded intermediates. These nucleation aggregates are thermodynamically stable. On the other hands, IBs as aggregate of aggregates in which small size aggregates tend to associate themselves to give rise to one or more bigger aggregates.

Reversibility of protein aggregation depends on stage of aggregation. There are two stages of aggregation. Initial stage aggregation involves formation of soluble aggregates which may be reversible. However, it is irreversibled in the second stage of aggregates formation. Physical aggregation is a result of strong and non-specific protein-protein interactions (Durbin and Feher, 1996). For example BSA aggregates easily because of the formation of incorrect intermolecular salt bridges (Giancola et al., 1997). Yet the highlighted issue here is that aggregation may occur by specific interaction of certain conformations of protein intermediates rather than by nonspecific interactions. This statement is supported from the evidence of the fibril growth of a sequence of *E. coli*. The protein was specific, as each peptide could be nucleated by fibrils of the same peptide but not by fibrils of closely related sequences (Jarrett and Lansbury, 1993).

In thermodynamic mechanism, free energy change associated with the protein aggregation process. When there is protein aggregation, it will led to increase in overall free energy of the system. This is due to the loss of certain number of

monomer conformational and translational state. Protein with low native energies tend to have a higher energetic barrier for aggregation (Istrail et al., 1999). This condition will not help the aggregation process to take place. In chemical induced aggregation, disulfide bond is form when free cys residue in protein undergone oxidation. This process will next initiate thion disulfide exchanges which will result in protein aggregation such as bFGF and β-galactosidase. Nevertheless, without free cys disulfide-bonded protein can still undergone aggregation through disulfide exchange β-elimination. This case is valid for lyophilized insulin during storage, usually disulfide formation will cause of protein precipitation in solid state, but not always in liquid state (Costantino et al., 1995). Covalent dimers may form in the protein from non-disulfide crosslinking bond. Insulin has been proven to form transamidated dimers.

2.3 Inclusion bodies recovery

The recovery process of IBs from recombinant cell can be categorized into four parts which are isolation, solubilization, renaturation and purification (Jackson et al., 2006; Yamaguchi et al., 2012). In isolation, the dense particles IBs are normally separated from the cell debris by using low-speed centrifugation after cell lysis. The IBs are then solubilized using high concentration (6 to 8 M) of chaotropic reagents such as guanidine hydrochloride or urea (Rashid et al., 2005), and detergent such as sodium dodecyl sulphate (Suresh Chandra et al., 1985). The function of these denaturants is to damage the 3-D structure of the protein. During solubilization, reducing agents like β-mercaptoethanol (ME) or dithiothreitol (DTT) are added to prevent the formation of non-native intra- or inter-disulfide bond in high concentrated protein solution.

After solubilization of protein with the contaminant such as cell debris and other unwanted protein, the target proteins are refolded to return their native soluble structure. For efficient refolding process, the concentration of the denaturants should be reduced to a level where intra-molecular non-covalent interactions, such as hydrogen bonding, hydrophobic interactions, and salt bridges, are recovered (Yamaguchi et al., 2014). Besides, the refolding process should be performed under an appropriate oxidizing environment to form the correct disulfide bonds and low concentration of the denatured protein in the refolding buffer should be maintained to avoid intermolecular aggregation (Yamaguchi et al., 2012). The rate of aggregation is second order reaction which is relatively faster than first than order refolding rate (Jackson et al., 2006; Yamaguchi et al., 2012). Hence, the recovered native protein from IBs remains low. Furthermore, the removal of cell debris prior to refolding process is required because the hydrophobic bonds in the cell debris may attract the protease during protein refolding to degrade the targeted protein (Lilie et al., 1998).

There are three crucial considerations in the protein refolding which including the denaturant removing methods, the refolding physical conditions and the additive in the refolding buffer. The denaturants can be removed by using simple dilution,

dialysis, or solid phase methods (Yamaguchi et al., 2012). During simple dilution and dialysis, the concentration of denatured protein and denaturants will be gradually decreased respectively to prevent the intermolecular interaction and the IBs aggregation. Besides size exclusion chromatography (Saremirad et al., 2014) and microfluidic clips (Yamaguchi et al., 2014) which applying solid phase method to reduce the concentration of denaturants are widely used for the protein refolding. By varying and controlling the pore size of matrix in column and the flow rate of refolding buffer into the column, this method is able to refold protein in higher successive percentage comparing to dilution and dialysis method.

Pressure and temperature may affect the refolding process. At low temperature, the entropy contribution of hydrophobic interactions in protein to the Gibbs free energy is decreased and hydrophobic interaction is dissociated (Reddy et al., 2012; Malavasi et al., 2014). Therefore low temperature is able to suppress protein aggregation and refold the targeted protein correctly. Besides, high pressure compresses the volume of protein and leads to the suppression of hydrophobic interaction (Malavasi et al., 2014). By optimizing the temperature and pressure for the refolding process, almost 100% of refolding yield is achieved even at high protein concentration (Malavasi et al., 2014).

Beside the consideration of denaturant removing methods and the refolding physical conditions, suitable additives which act as stabilizer, enhancer, or inhibitors can enhance the protein refolding efficiency. Glycerol, ammonium sulfate, and sugars are the example of stabilizer which reduce protein surface exposing to the solvent through unfavorable interactions between protein surfaces and additives (Yamaguchi et al., 2012). The additives that act as enhancer such as protein disulfide isomerase and peptidyl prolyl cis–trans isomerase can catalyze disulfide bond formation by reacting with the thiol group of reduced target proteins. Besides, low concentration of chaotropic reagents can act as inhibitor to disrupt both intra- and inter-molecular interactions of proteins. However, these interactions are able to be recovered by gradually decreasing the concentration of chaotropic reagent and weakening the interaction of chaotropes with the hydrophobic surface of proteins. (Chen et al., 2009). The examples of inhibitors are urea, L-arginine, and detergent (Suresh Chandra et al., 1985). Recovery of native protein from IBs requires several processes, hence combination unit operation are necessary.

2.4 Mechanism of solubilization

Functionality of protein is usually existing when the protein in tertiary structure because at this state there are a few of bonds hold the protein structure to maintain its 3D shape. The stabilised tertiary structure have hydrogen bond in which this bond exist between atoms of two peptide bonds. It's also exists between atoms of a peptide bond and an amino acid side chain. Besides that, there also have hydrogen bond between two amino acids side chains. In IBs refolding process to recover the functional protein, the understanding of solubilisation mechanism is important.

Basically, solubilisation is the process to break down the aggregated interactions of IBs in the protein structure. During protein solubilisation, the internal bonding of protein which is hydrogen bonds will be broken make the tertiary structure of protein unfolded. This nature occur due to the conformation of protein structure has been changed in presence of organic solvent mainly alcohol. When put protein in polar solvent, the hydrophilic (non-polar) amino acid side chains that found on the surface of protein will be twisted to the interior of protein. At the same time the inner hydrophobic (polar) amino acid side chains will be exposed to the hydrophobic of solvent (Pace et al., 2004). This changes tend to break the hydrogen bonds between proteins amino acids and lead to unfold the unfolded protein.

2.5 Solubilization Methods

There are several IBs solubilization methods were reported. IBs have native like structure, solubilisation using mild solubilising denaturants or conditions will preserve the native-like secondary structure of the protein. Table 2.1 illustrates different solubilisation methods that has been summarized by Singh et al. (2015).

Table 2.1: Different solubilisation methods.

Methods/ Solubilizing agents	Example	Advantages	Reference
Denaturating solubilisation agents	▪ 8 M Urea ▪ 6 M Guanidine Hydrochloride	▪ Reduced incorrect disulfide bonds ▪ Resulting complete disruption of protein structure	Singh et al., (2012) Upadhyay et al., (2014)
Non-denaturing solubilisation agents	▪ Sarcosyl ▪ 5% Dimethyl sulfoxide ▪ 5% n-propanol	▪ Protect native-like secondary structure by stabilise α-helical structure of protein ▪ Modulate the protein structure in which it destabilise the tertiary structure of protein ▪ Improved recovery of proteins from IBs	(Kumari and Jagannadham , 2011)
Mild solubilisation agents	▪ High pH of buffer (>12) ▪ Organic solvents (6 M n-propanol, 6 M β-mercaptoethanol) ▪ High pressure (2–4 kbar)	▪ Improved refolding yield by retaining native-like secondary structure ▪ Organic solvents will preserve native-like secondary structure ▪ Disrupts the intermolecular interaction and disaggregates IBs	Singh et al., (2012) Chura-Chambi et al., (2013)

2.6 Gel electrophoresis

Gel electrophoresis is widely used molecular separation nowadays. Gel electrophoresis separates proteins based on their shapes, molecular weights, and isoelectric point (pI) under electrical field (Dunn, 1986). Gel electrophoresis can be categorized into one dimensional and two dimensional. One dimensional gel electrophoresis is separating protein and nucleic acid based on shapes, molecular weights, and pI in same direction while two dimensional gel electrophoresis is separating protein by their pI and then by mass in two different direction (Gallagher et al., 1997). Existing gel electrophoresis for protein recovery including urea gradient gel electrophoresis (UGGE) (Albright and Slatko, 2001) and pulsed field gel electrophoresis (Birren and Lai, 1993). To recover the protein from IBs, UGGE is widely used because this is the relatively simple, rapid and highly sensitive tool to study the properties of protein folding. The proteins will collide with the chemical inducer in polyacrylamide matrix and undergo purification and refolding process at the same time. Nowadays the UGGE using vertical slab gels is used and the protein feedstock volume is limited.

2.7 Preparative PAGE

The superior resolving power of electrophoresis on polyacrylamide gel is no longer limited to the application of analytical scale protein analysis. This technique has stimulated interest in the use for preparative purposes (Eby, 1991). In order to increase the volume of loaded protein, cylindrical gel column was designed for preparative electrophoresis (Chew et al., 2009b). For preparative PAGE, factors that affect protein separation, a method to visualize the location of protein and elution of the separated proteins from a gel column are important procedures in designing an electrophoretic device. The ultimate aim in the development of a protein recovery process is to recover the desired protein in high purity and yield.

CHAPTER 3

MATERIALS AND METHODS

3.1 Preparation of inclusion bodies

E. coli strain BL21(DE3) carrying the plasmid pRSETEGFP was used for all experiments (Chew et al., 2012). Method of recombinant protein overexpression in the cells as described by Malavasi et al. (2014) was slightly modified and used in the present study. Briefly, the cells expressing EGFP gene was grown in Luria-Bertani medium (10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of sodium chloride, 100 µg/mL of ampicillin at pH 7.0) with a liquid-to-flask volume ratio of 0.2 and inoculated with 5% (v/v) of inocula. The flask culture was carried out at a shaking frequency of 150 rpm and temperature of 30°C. The expression of EGFP was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside at the exponential phase (0.8 to 1.0 of optical density at 600 nm). After 16 h of protein induction at 37°C, bacteria were collected by centrifugation at 2,500 g and 4°C for 10 min. The pellet was washed with buffer solution [50 mM of Tris hydrochloride (pH 8.0), 50 mM of NaCl] and centrifuged at the same condition. Cell pellet was then collected and lysed by freeze thaw method (Johnson and Hecht, 1994). The pellet was frozen at -20°C for 24 h and thawed by resuspending with 10% (w/v) of buffer solution. The suspension was centrifuged at 8,000 g and 4°C for 10 min and the supernatant containing soluble EGFP was discarded. The lysing process was repeated for another cycle and the resulting IBs was stored at -20°C until further process.

3.2 Effects of solubilization methods on the recovery of denatured EGFP

3.2.1 Solubilization of inclusion bodies using urea

For the conventional urea denatured method (Qi and Xiong, 2015), IBs suspension at 10% (w/v) was prepared in buffer solution [50 mM of Tris hydrochloride (pH 8.0), 50 mM of NaCl] containing different urea concentration (0, 2, 4, 6, and 8 M). All the IBs suspension was then incubated at 50°C for 4 h and centrifuged at 8,000 g and 4°C for 10 min to get clear supernatant. The amounts of solubilized EGFP and total protein in the supernatant were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford assay, respectively. In order to study the effect of incubation temperature, the experiment was repeated by incubating IBs suspension with different molar concentration of urea at temperature of 60, 70, and 80°C.

3.2.2 Solubilization of inclusion bodies using urea with alkyl alcohol

IBs mild solubilization using 2 M of urea with alkyl alcohol as described in Singh et al. (2012). Six different 6 M alkyl alcohols (methanol, ethanol, ethylene glycol, n-propanol, glycerol, and n-butanol) in presence of 2 M of urea, 50 mM of Tris hydrochloride (pH 8.0), and 50 mM of NaCl were employed to solubilize IBs at 10% (w/v). All the IBs suspension was then incubated at room temperature for 30 min. The IBs suspension was then separated by centrifuging at 8,000 g and 4°C for 10 min

and the supernatant was analyzed to determine the amounts of solubilized EGFP and total protein. To investigate the molar concentration of n-butanol (showed the best performance in former experiment) on the solubilization of EGFP from IBs, the experiment was repeated by solubilizing IBs pellet in buffer [50 mM of Tris hydrochloride (pH 8.0), 50 mM of NaCl, and 2 M of urea] with increasing concentration of n-butanol (0 – 6 M).

3.2.3 Solubilization of inclusion bodies using urea with freeze thaw process

Mild solubilization using 2 M of urea with freeze thaw process reported by Qi et al. (2015) was followed. To investigate the effects of freezing incubation period (0 – 4 day) and freeze thaw cycle (0 – 4 cycle) on the solubilization process, IBs suspension at 10% (w/v) was prepared in buffer solution [50 mM of Tris hydrochloride (pH 8.0), 50 mM of NaCl, and 2 M of urea] and was frozen at -20°C for different durations and cycles. The mixture was then thawed at room temperature and centrifuged at 8,000 g and 4°C for 10 min to get clear supernatant for determination of amounts of solubilized EGFP and total protein.

3.3 Effects of solubilization conditions on soluble aggregates formation during refolding process

The cell pellet consisted of EGFP-IBs was solubilized and folded using three different methods as described in Figure 3.1.

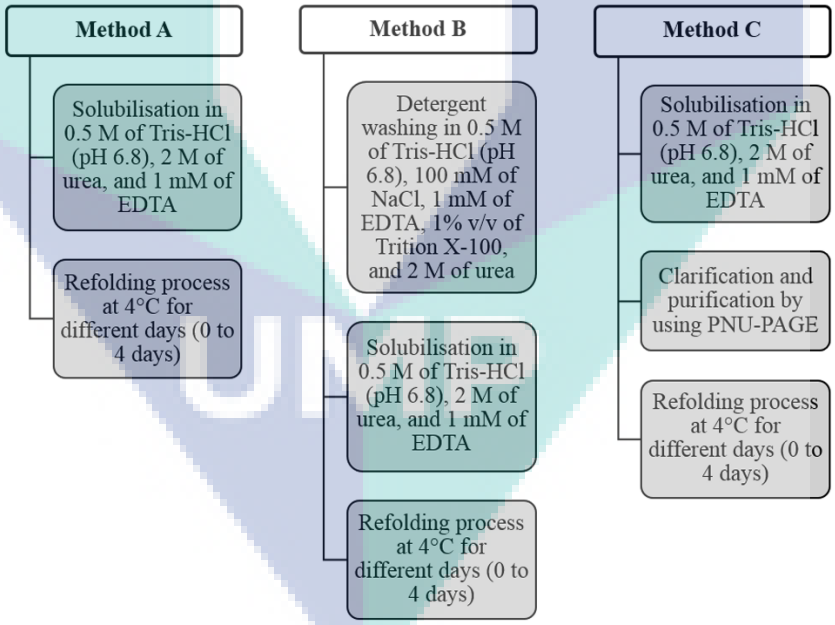


Figure 3.1. The comparative scheme for refolding process of three different solubilized IBs.

3.3.1 Method A

For method A as described by Qi et al. (2015), the cell pellet was resuspended with 15% (w/v) of solubilizing buffer (0.5 M of Tris-HCl [pH 6.8], 2 M of urea, and 1mM of EDTA) and frozen at −20°C for 24 h. The frozen cell suspensions were thawed and refolded at 4°C for various durations (0 to 4 days). After the refolding process, the cell suspensions were centrifuged at 4°C and 10,000 g for 10 min and the

supernatant was collected. The refolded protein suspension was analyzed by using SDS-PAGE and Bradford assay to determine the total EGFP amount and total protein amount, respectively. The supernatant sample was analyzed by using native PAGE to determine the amount of functional EGFP.

3.3.2 Method B

For method B, the cell pellet was washed with 15% (w/v) of detergent buffer (0.5 M of Tris-HCl [pH 6.8], 100 mM of NaCl, 1 mM of EDTA, 1% [v/v] of Trition X-100, and 2 M of urea) to remove the cell debris (Qi et al., 2015). The cell suspension was centrifuged at 4°C and 10,000 g for 10 min and the supernatant was discarded. The washing process was repeated for another three cycles until a clear supernatant was obtained and further discarded. The IBs pellet was then washed with 15% (w/v) of washing buffer to remove the detergent followed by centrifugation at the same condition. The solubilization and refolding conditions were performed as method A.

3.3.3 Method C

Mild solubilization was performed in method C as described by Qi et al. (2015) in which the cell pellets were resuspended with 15% (w/v) of solubilizing buffer and frozen at −20°C for 24 h. The frozen cell suspension was thawed at 4°C for 15 min and mixed with sample dye (5 g/L of bromophenol blue, 0.35 M of Tris-HCl, and 30% [v/v] of glycerol) in a ratio of 9:1. The mixture (100 µL) was then loaded into a preparative native urea-polyacrylamide gel electrophoresis (PNU-PAGE) column for clarification and purification processes. The preparation and operation of the PNU-PAGE was as described by Chew et al. (2009b) with modification of the stacking gel content (0.125 M of Tris-HCl [pH 6.8], 2 M of urea, 1 mM of EDTA, 4% [w/v] of acrylamide, 0.05% [w/v] of ammonium persulfate, and 0.001% [v/v] of TEMED) and the resolving gel content (0.375 M of Tris-HCl [pH 8.8], 2 M of urea, 1 mM of EDTA, 12% [w/v] of acrylamide, 0.05% [w/v] of ammonium persulfate, and 0.001% [v/v] of TEMED). Briefly, 1 cm of stacking and 2 cm of resolving gels were prepared in a glass column with 1.7 cm inner diameter. Using 0.025 M of Tris and 0.192 M of glycine of electrode buffer, the prepared cell suspension mixture was clarified and purified under a constant current of 30 mA. The eluted samples were collected using a dialysis tube for 2 h with 15-min intervals and refolded at 4°C for various days (0 to 4 days). After the refolding process, the samples were centrifuged at 4°C and 10,000 g for 10 min and the supernatant was collected. The refolded protein suspension was analyzed using SDS-PAGE and Bradford assay to determine the total EGFP amount and total protein amount, respectively. The supernatant sample was analyzed using native PAGE to determine the amount of functional EGFP.

3.4 Protein analyses

3.4.1 SDS-Polyacrylamide gel electrophoresis analysis

Measurement of denatured EGFP amount in protein samples were based on gel-based imaging using SDS-PAGE (Chew et al., 2009a; Laemmli, 1970). Protein

samples were mixed with equal volume of 2× SDS sample buffer [125 mM of Tris hydrochloride (pH 6.8), 20% (w/v) of glycerol, 4% (w/v) of SDS, 200 mM of β-mercaptoethanol, and 0.01% (w/v) of bromphenol blue] and boiled for 10 min to denature proteins. Samples were then electrophoresed on a 15% (w/v) of SDS polyacrylamide gel under a constant current of 30 mA for 90 min. After electrophoresis, the gel was stained with a staining solution [0.1% (w/v) of Coomassie Brilliant Blue R-250, 52.5% (v/v) of methanol, and 10.5% (v/v) of acetic acid] and destained with a destaining solution [40% (v/v) of methanol, and 10% (v/v) of acetic acid] until clear protein bands on the gel were obtained. The gel was captured using FluoroChem SP imaging system (Alpha Innotec) and the total amount of denatured EGFP was determined by densitometry analysis of corresponding band using AlphaEase FC software and a standard equation developed by using pure EGFP.

3.4.2 Native Polyacrylamide gel electrophoresis analysis

For the determination of functional EGFP amount based on the protein fluorescent intensity, protein samples were remained native and electrophoresed in native polyacrylamide gel as described in Chew et al. (2009a). By using an OmniPage minivertical system (Cleaver Scientific Ltd), 4 and 15% (w/v) of stacking and resolving gels, respectively, were casted with as gel size of 10 × 10 × 0.2 cm (length × width × thickness). The supernatant samples were electrophoresed under a constant current of 30 mA for 90 min. After electrophoresis, the fluorescent intensity of EGFP band on the gel was captured and measured using an imaging system. The intensity value was then compared with a standard equation developed by using pure EGFP to determine the total amount of functional EGFP.

3.4.3 Bradford assay

The amount of total protein was determined by following Bradford (1976) with bovine serum albumin as the protein standard. Protein samples (20 µL) were mixed with 200 µL of Brafdord reagent [0.05% (w/v) Coomasie Brilliant Blue G-250, 23.75% (v/v) ethanol, and 42.5% (v/v) ortho-phosphoric acid] and analyzed with a microplate reader (Infinite 200 PRO, Tecan) under an absorbance value at wavelength of 595 nm.

3.4.4 Calculation

The yield of solubilization was calculated as the total amount of EGFP in supernatant sample to the total amount EGFP in IBs suspension. The purity of solubilized EGFP was calculated as the total EGFP amount to the total protein amount in supernatant sample. Refolding yield is defined as the ratio of the functional EGFP amount in the supernatant sample to the total EGFP amount in the refolded protein suspension. The purity of refolded EGFP is defined as the ratio of the total EGFP amount to the total protein amount in the refolded protein suspension.

CHAPTER 4

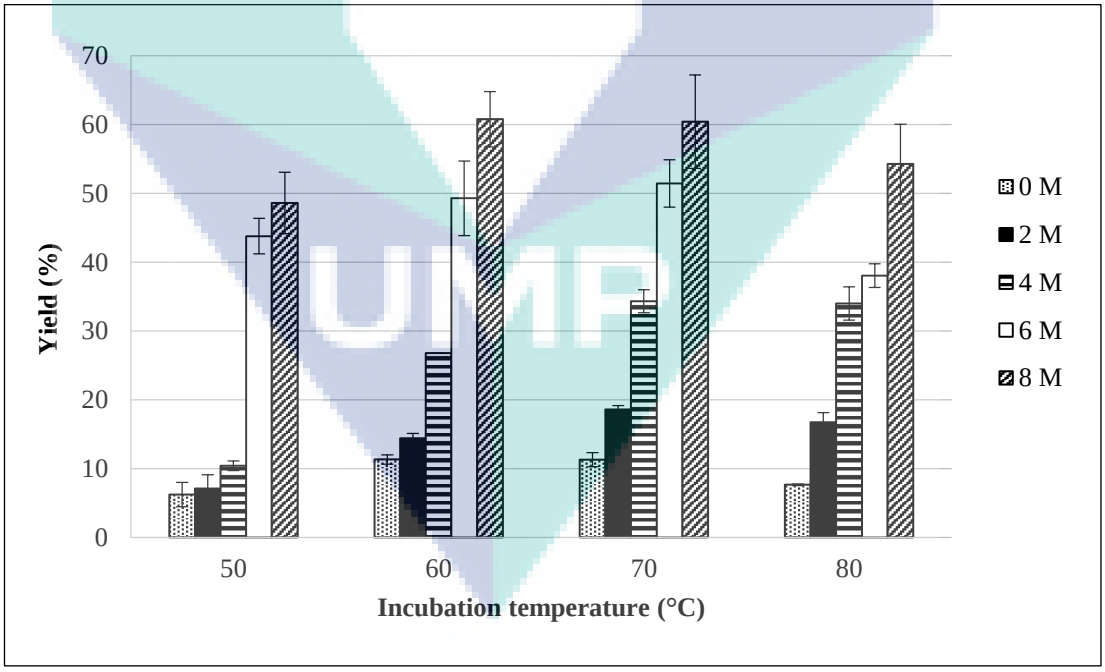
RESULTS AND DISCUSSION

4.1 Effects of solubilization methods on the recovery of denatured EGFP

4.1.1 Solubilization of inclusion bodies using urea

Recovery of functional protein from IBs requires solubilization proses. By using conventional IBs solubilization method, different concentrations of urea (0 to 8 M) and incubation temperatures (50 to 80°C) were applied to denature the EGFP IBs and the yield and purity were determined (Figure 4.1). These two factors have a marked influence on the yield and purity of solubilized EGFP. Figure 4.1a shows that 8 M of urea dissolved most of the IBs. The yield of solubilized EGFP was increased as the concentration of urea increased from 0 to 8 M for every incubation temperature. The present findings are as reported by Zou et al. (1998). The high level intermolecular β-sheet structure in IBs is formed by hydrophobic bonding. Urea as denaturant tends to interact with polar group of protein and this cause the disruption of hydrophobic interaction within the protein structure (Tanford, 1970). After the disruption, hydrogen bonding will be formed between the urea and amino side chain that turns protein structure becomes primary structure (Almarza et al., 2009). The presence of urea denatures the IBs protein by decreasing the hydrophobic effect which linearly depends on the urea concentration.

(a)



(b)

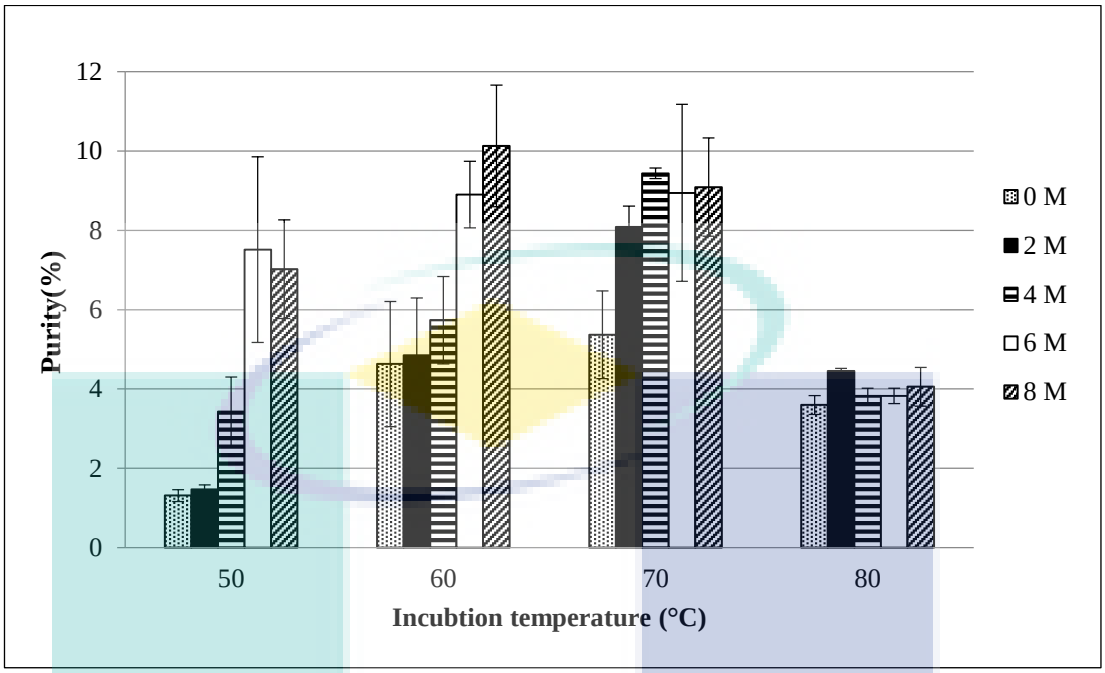


Figure 4.1. Effects of urea concentration and incubation temperature on the solubility of EGFP IBs. (a) Yield of solubilized EGFP. (b) Purity of solubilized EGFP. Error bars represent duplicate independent experiments with duplicate measurements.

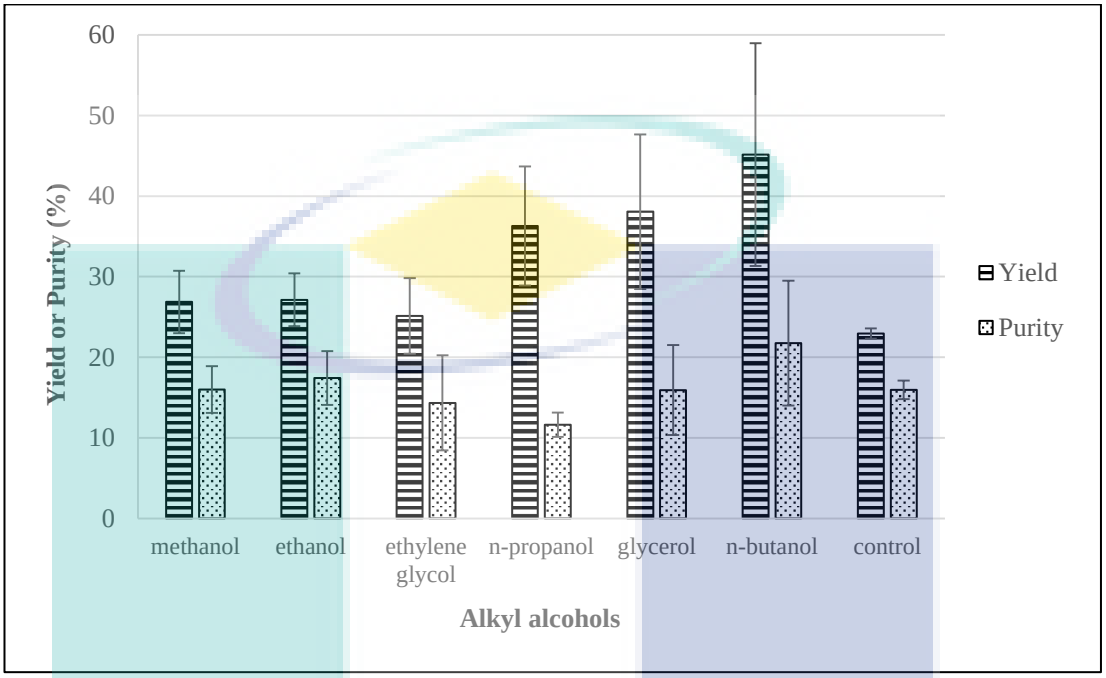
For the incubation temperature, the yield and purity of solubilized EGFP increased from 50 to 70°C (Figure 4.1). Under high incubation temperature, IBs are heated and certain amount of energy is absorbed. High incubation temperature increases the enthalpic reaction of non-polar group of protein. The absorbed energy causes the protein molecules start to vibrate rapidly and violently which results in protein structure disruption. When the temperature was increased to 80°C, there were a slight decrease in the yield obtained and the solubilized protein purities are low. This might be due to the huge amount of heat that has changed and broken the covalent bond within the protein structure and causes protein degradation. Hence, IBs solubilization with 8 M of urea concentration and incubated at 60°C were found to be the best condition achieved, wherein high values of yield (61%) and purity (10%) were achieved.

4.1.2. Solubilization of inclusion bodies using urea with alkyl alcohol

IBs proteins have been reported to have native-like secondary structure (Peternel et al., 2008; García-Fruitós et al., 2005; Peternel and Komel, 2011) and the structure can be protected by using mild solubilization. The solubilizing buffer in the mild solubilization containing 2 M of urea which serves in physical separation of the water and protein molecules by disrupting the hydrophobic interactions (Patra et al., 2000). In this study, the solubilizing effect of buffer containing 2 M of urea with different 6 M of alkyl alcohols on EGFP IBs was compared. Figure 4.2a revealed that all types of alkyl alcohols contributed to IBs solubilization with obtained yields

higher than the control sample and n-butanol was the best alkyl alcohol for solubilization of EGFP IBs with high value of yield (45%) and purity (22%).

(a)



(b)

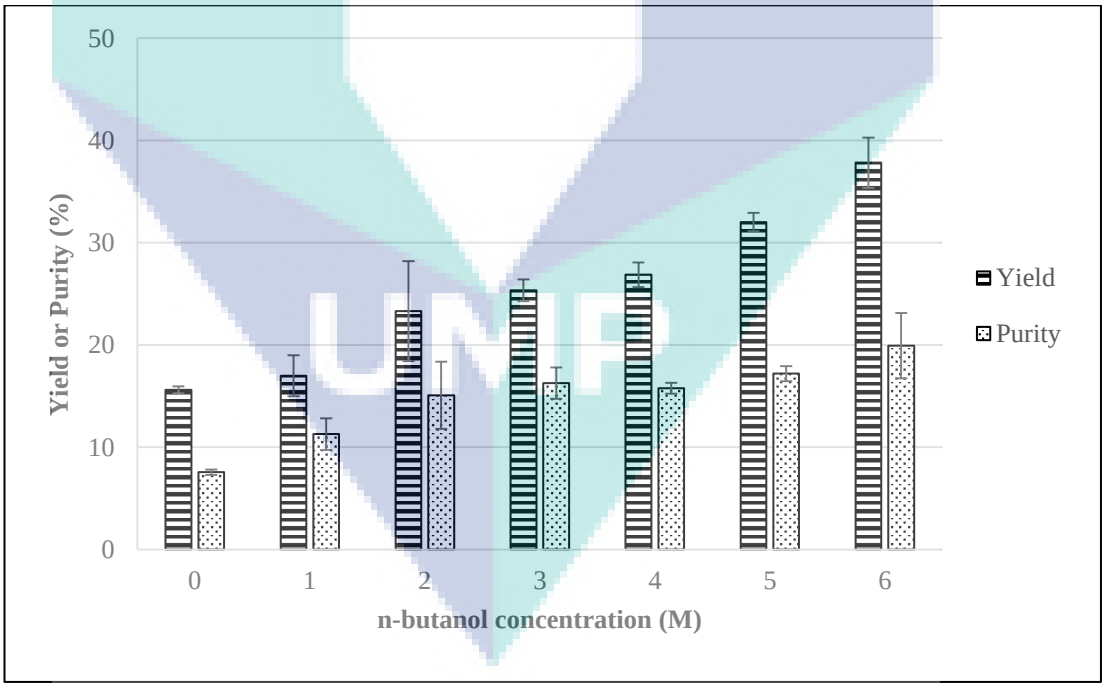


Figure 4.2. Solubilization of EGFP IBs using 2 M of urea with alkyl alcohol. (a) Effect of different alkyl alcohols and (b) Effect of concentration of n-butanol on the yield and purity of solubilized EGFP. Error bars represent duplicate independent experiments with duplicate measurements.

IBs solubilization using alkyl alcohols which have alpha helix stabilizing properties tend to unfold the proteins but remain the existing native-like secondary structure of protein (Kumari and Jagannadham, 2011). By adding IBs into Tris buffer containing alcohol, the hydrophilic (polar) amino acid side chains of protein that dislike alcohol tend to be tucked away and moving toward the protein's interior, while the inner hydrophobic (non-polar) amino acid side chains of protein become exposed to solvent (Pace et al., 2004). The exchange of protein structures tends to break the hydrogen bonds between amino acids of proteins and unfold the IBs. The hydrogen bond from the protein was then start to produce new link to alcohol molecules during the alcohol denaturation. Higher chain length of carbons increase the hydrophobicity of solvent and able to attracts more inner hydrophobic amino acid side chains of protein for denaturation. Hence, the yield of solubilized EGFP increased when a higher the carbon chain length solvent was applied.

Methanol, ethanol and ethylene glycol only slightly contribute in IBs solubilization (about 10 to 18% higher than control sample). The results are about similar as reported by Singh et al. (2012) where Tris buffer containing these alkyl alcohols failed to solubilize recombinant human growth hormone IBs. Effect of ethanol on the stability of bovine serum albumin was investigated by Yoshikawa et al. (2012) and discovered that the favourable interaction of ethanol with hydrophobic residues causes protein denaturation, but the unfavourable interaction with charged groups exposed to the solvent causes reduction of protein solubility. When protein in denatured state, the protein consists of 83% of non-polar chains are exposed to solvent (Pace et al., 2004). Alkyl alcohols with lower chain length have higher polarity value will not interact with the exposed non-polar chains of protein due to the difference in molecular polarity. Interaction among the non-polar chains of denature proteins increase the likelihood of aggregation and facilitate precipitation. Ethanol has the ability for denaturation, however it does not lead to better solubility of the denatured protein (Pace et al., 2004; Yoshikawa et al., 2012). Hence, contributions of the low chain length alcohol based buffers in solubility are lesser compared with n-butanol based buffer.

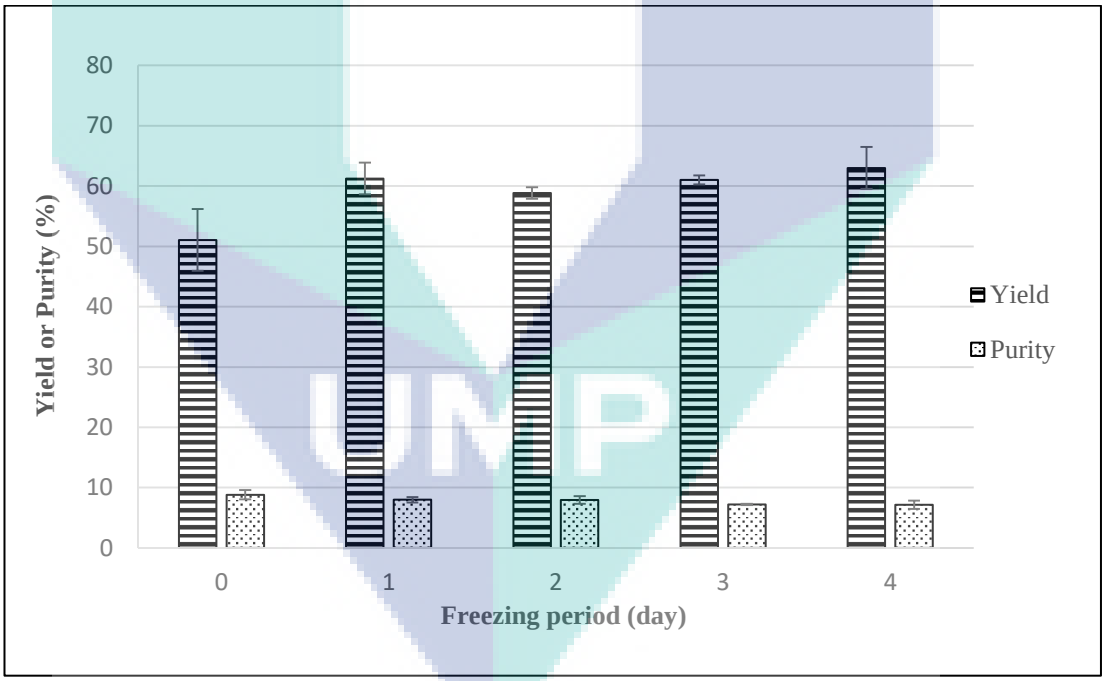
Besides the effect of increasing hydrocarbon content, effect of increasing hydroxyl group on solubilizing potential of a solvent was investigate by using ethylene glycol and glycerol which consist of two hydroxyl groups with two carbon atoms and three hydroxyl groups with three carbon atoms, respectively. The yield of solubilized EGFP of ethylene glycol was compared with ethanol and the yield of solubilized EGFP of glycerol was compared with propanol as shown in Figure 4.2a. The increments of hydroxyl content of ethylene glycol and glycerol only slightly contribute to the yields of solubilized EGFP. The addition of hydroxyl groups increased the buffer hydrophilicity, however did not much contributed to the IBs solubilization.

The effect of n-butanol concentration (0 to 6 M) on the IBs solubilization was also evaluated (Figure 4.2b). It was found that enhancement of n-butanol concentration in the mixture resulted in the improvement of IBs solubilization. Similar result was reported by Stigter and Dill (1993) in which a higher solvent concentration during denaturation enhanced the protein solubility. High solvent concentration may attracts more inner hydrophobic amino acid side chains of protein, make the hydrophilic amino acid side chains on protein surface twist to the internal protein area and lastly break the hydrogen bonds between protein amino acids.

4.1.3 Solubilization of inclusion bodies using urea with freeze thaw process

Mild solubilization using 2 M of urea with freeze thaw process was conducted by varying the freezing duration and number of freeze thaw cycle. By freezing the IBs suspension for one day and one freeze thaw cycle, higher yield of solubilized EGFP from IBs was observed (Figure 4.3). Further increase the freezing duration and number of freeze thaw cycle did not help much in solubilizing higher yield of solubilized EGFP. The yield of solubilized EGFP was comparable to that of 8 M of urea in buffer. However, addition of increasing freezing period and freeze thaw cycle had no effect on purity of solubilized EGFP.

(a)



(b)

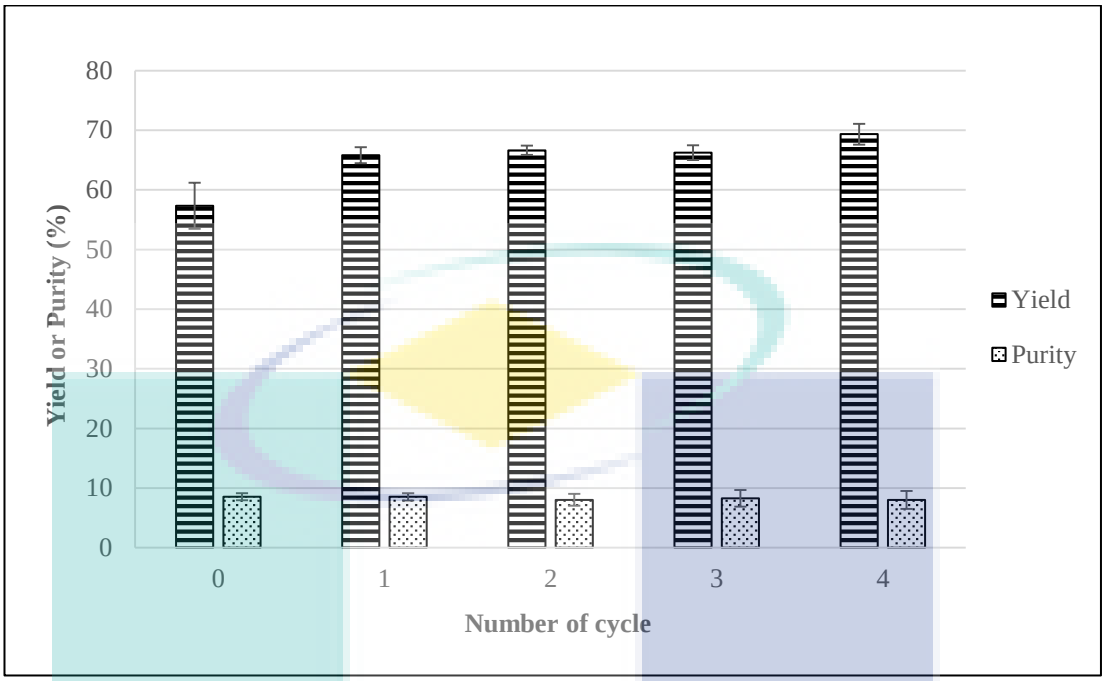


Figure 4.3. Solubilization of EGFP IBs using 2 M of urea with freeze thaw process. . (a) Effect of freezing period and (b) Effect of freeze thaw cycle on the yield and purity of solubilized EGFP. Error bars represent duplicate independent experiments with duplicate measurements.

Freeze thaw process affects the protein stability through physical and chemical stresses. During the freezing process, water is converted to ice crystal in which a physical stress is applied for denaturing the IBs. Formation of ice reduces the water content and increases the solutes concentration in the IBs mixture. The concentrated urea and protein mixture may affect protein structure through changing in pH and ionic strength which cause chemical degradation (Cao et al., 2003). The freeze concentration causes severe stresses to protein stability. The freezing duration determines the amount of applied stress on the frozen IBs mixture and the IBs are denatured during the thawing process.

Freeze thaw process is a gentle process for cell disruption where the cell envelope is damaged by repeating freezing and thawing processes to release the desired protein from cells (Johnson and Hecht, 1994). In this study, freezing condition results physical and chemical stresses on IBs. After thawing, IBs protein is further stressed by recrystallization. Increasing the freeze thaw cycles exert additional interfacial tension or shear on the entrapped proteins and cause protein denaturation. Repeated cycles of freezing and thawing disrupt cells were applied in this study by forming ice on the cell membrane in breaking down the cell membrane. The produced IBs might consist with undisrupted cell membrane which was further broken down in solubilization process. While slight increase in yield of solubilized EGFP, the destroyed cell membrane might increase the level of contaminants in the sample and causing the same purity reading in Figure 4.3.

4.1.4 Comparative solubilization of inclusion bodies

IBs solubilization is a part of protein production processes for recombinant proteins that are overexpressed in the bacterial host systems. Solubilization using high concentration of urea will cause a complete disruption of protein structure. The conventional urea solubilization method generates random coil structure where the hydrophobic amino acid patches are exposed and this leads aggregation of protein molecule during refolding process (Singh et al., 2015; Qi et al., 2015). Mild solubilization was reported in which the IBs are solubilized under low concentration of urea with addition physical and chemical stresses. Solubilize IBs in mild condition prevents hydrophobic interaction during the initial stage of refolding and enhances the recovery of active protein from IBs (Singh et al., 2015).

The mild solubilization methods described in this work could be applied for enhancing the recovery of important proteins expressed as IBs in *E. coli*. The interaction between alcohol and protein molecules have been widely studied because alcohol has potential to modulate the protein structure. Alcohol denatures proteins by disrupting the side chain intramolecular hydrogen bonding. Freeze thaw process affects the protein stability through external stresses. Low concentration of urea reduces hydrophobic interaction and its combination with alcohol or freeze and thaw process may uses as IBs solubilizing agent which destabilise the tertiary structure of protein as well as protect native-like secondary protein structure. These combine effects of this mild denaturant can enhance recovery of desired protein from IBs and decrease protein aggregation during refolding (Singh and Panda, 2005).

Solubilization potential of alkyl alcohol based buffers were compared with that of various urea concentration buffers. The 6 M n-butanol based solubilization buffer may solubilized EGFP from the IBs with results comparable to 6 M urea buffer (Figures 4.1 and 4.2). Besides, the result of mild solubilization using 2 M of urea with freeze thaw process for one day and one freeze thaw cycle was comparable to the traditional 8 M urea denaturation method (Figures 4.1 and 4.3). It was observed that even though the yield of recombinant protein solubilized in n-butanol based buffer was lower than that achieved with 8 M of urea buffer, the obtained purity was better when solubilized in n-butanol based buffer. Chaotropic agent such as urea is used for cell lysis by breaking hydrophobic and hydrophilic interactions of cells (Islam et al., 2017). The IBs undisrupted cell membrane may further destroyed can contribute impurities for low purity. Under the different solubilization temperature and urea concentration, the best obtained purity was around 10% in comparison that of 20% observed for 6 M n-butanol based buffer. Solubilized EGFP with lesser contaminating proteins may reduce aggregation during refolding and enhance the overall renaturation yield.

4.2 Effects of solubilization conditions on soluble aggregates formation during refolding process

Soluble EGFP and EGFP-IBs were produced during the fermentation. After two cycles of freezing and thawing process, more than 95% of soluble EGFP were removed from cells (data not shown). In this study, quantitation of refolded soluble EGFP was based on the EGFP fluorescence intensity. Based on the fluorescence intensities under native PAGE analysis, the amounts and refolding yields of the refolded soluble EGFP from three different solubilized FGFP-IBs with refolding day 0 to 4 were determined (Figure 4.4). Due to the sensitivity limitation of the native PAGE analysis, the refolded soluble EGFP fluorescence intensities for methods A and B at day 0 were not able to be determined and assumed as zero. Method A successfully recovered the highest amount of refolded soluble EGFP from day 1 to 4 (Figure 4.4A) compared to the other two methods. Surprisingly, method C provided the best refolding yield among the three methods especially at day 1 (Figure 4.2B). The amount of refolded soluble EGFP and the refolding yield varied as the refolding period was increased. For method A, the highest yield and amount of refolded soluble EGFP were obtained after three days of refolding incubation period. For method B, the best refolding incubation period was two days. The amount of refolded soluble EGFP and refolding yield remained constant up to nine days of incubation (data not shown).

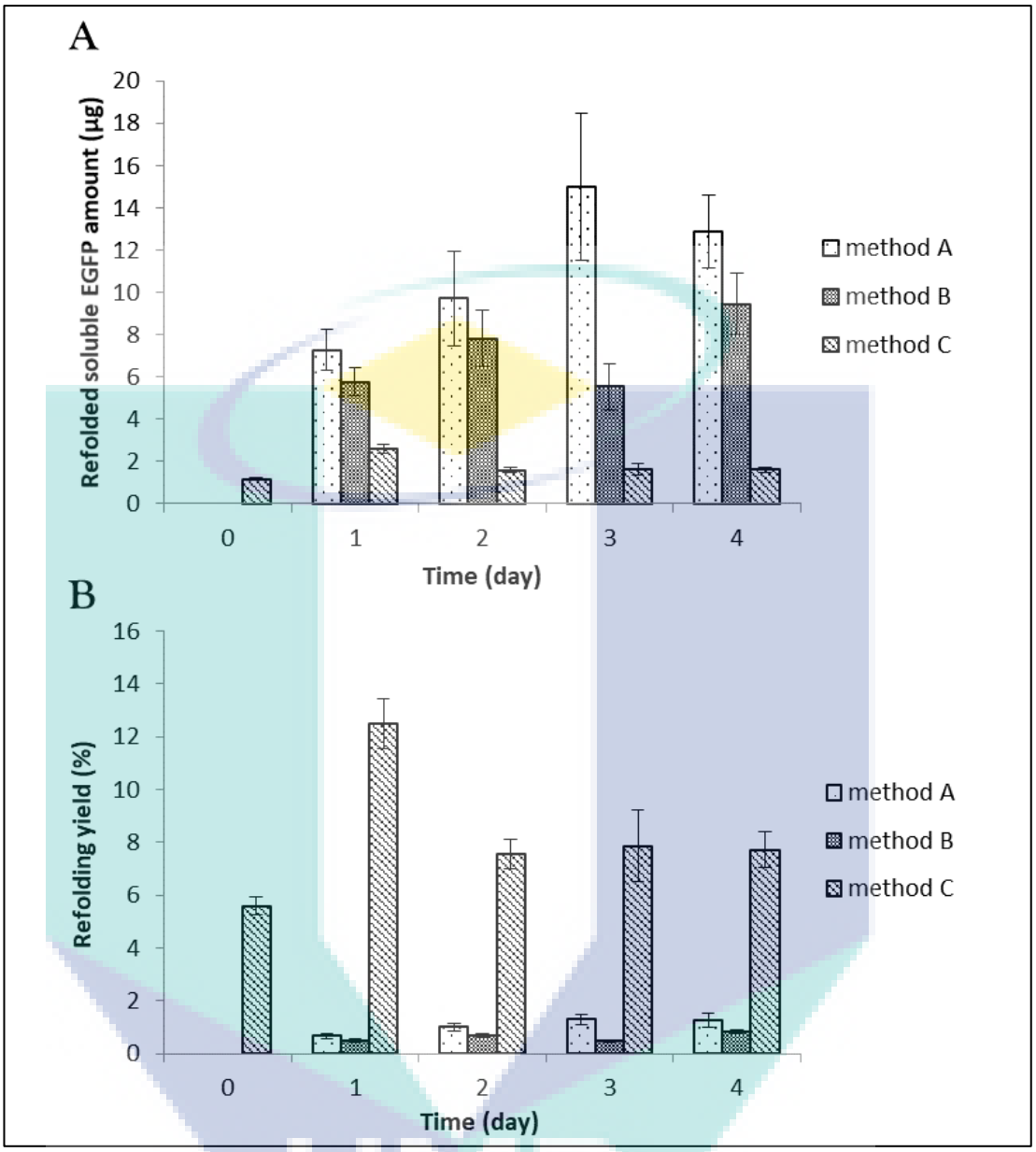


Figure 4.4. The recovery of refolded soluble EGFP from methods A, B, and C under various refolding incubation periods (dotted, method A; grid, method B; and downward diagonal, method C). (A) Amount of refolded soluble EGFP. (B) Refolding yield of refolded soluble EGFP. Error bars represent duplicate independent experiments with duplicate measurements.

After the cell disruption, a small amount of soluble EGFP remained and captured in between the cell pellet. In method A, the soluble EGFP was solubilized together with the EGFP-IBs in solubilising buffer and refolded into the functional EGFP (Figure 4.4). This may be the reason that contributed to the high amount of refolded soluble EGFP. In method B, cell pellets were washed with Triton X-100 which further removed the trapped soluble EGFP and cell membrane. Thus, pure EGFP-IBs was obtained for solubilisation and refolding processes. Under the same sample preparation concentration of 15% (w/v) with that in method A, the EGFP-IBs concentration in the suspension (method B) was high. A mild solubilisation with 2 M

of urea was applied in this study to preserve the existing native-like secondary structure during refolding (Singh, Upadhyay and Panda, 2015). However, solubilisation using 2 M of urea appeared insufficient to solubilize the high amount of EGFP-IBs in the mixture. Hence, low refolded soluble EGFP amount and refolding yield were obtained in method B.

The solubilized suspension was clarified and purified using the PNU-PAGE in method C. The insoluble components such as cell debris and insolubilized EGFP-IBs were stuck on top of the stacking gel and solubilized EGFP were purified through the resolving gel. Only a small amount of pure solubilized EGFP was eluted from the gel column. Thus, the amount of refolded soluble EGFP was the least in method C compared to the other two methods. Moreover, the amount of refolded soluble EGFP and the refolding yield decreased after day 1 of refolding incubation period. This might be due to the precipitation of refolded soluble EGFP with the negatively charged polyacrylate from polyacrylamide gel. Hilbrig and Freitag (2003) reported that the negatively charged polyacrylate could be formed and precipitated at a pH between 6.5 and 8.9. The eluted polyacrylate and EGFP after a day refolding period may be precipitated and acted as contaminants that has increased the turbidity of the collected sample and lowered the protein purity.

In native PAGE analysis, the images of native gels were captured using a gel imaging system and the gel images are shown in Figure 4.5. Using different types of solubilized EGFP-IBs with various refolding incubation periods, the refolded soluble EGFP bands appeared at different locations of the gel. For methods A (Figure 4.5A) and B (Figure 4.5B), all the EGFP fluorescent bands were located at the top of the resolving and stacking gels except day 0 sample for method A. For method A, day 0 EGFP fluorescent band after electrophoresis were located at the correct EGFP band location as that of native soluble EGFP (Figure 4.5A). The gel images with fluorescent bands located at the top of the resolving and stacking gels show that the refolded soluble EGFP formed soluble aggregate after refolding process. The large protein structure of aggregated EGFP prevented its migration into the stacking and resolving gels. These samples (refolded soluble EGFP) were further analyzed by using SDS-PAGE analysis. Figure 4.6 shows that at day 0 of refolding period, the EGFP bands located at 35 kDa were observed for methods A and B (Figures 4.6A and 4.6B). However, the protein bands from day 1 to 4 were located at the top of stacking and resolving SDS gels for both methods, similar to the native PAGE analysis. Proteins separation by SDS-PAGE is based on their molecular weight. The aggregated EGFP with its high molecular weight has prevented its migration into the stacking and resolving gels. The results in Figures 4.5 and 4.6 confirmed that the refolded soluble EGFPs for methods A and B were not only changed in protein structure but also increased in molecular weight. For the refolding process using method C, the native PAGE analysis in Figure 4.5C shows that the EGFP fluorescent bands were located at the proper EGFP location for all incubation periods. The

refolded soluble EGFP maintained its protein conformation as the native soluble EGFP.

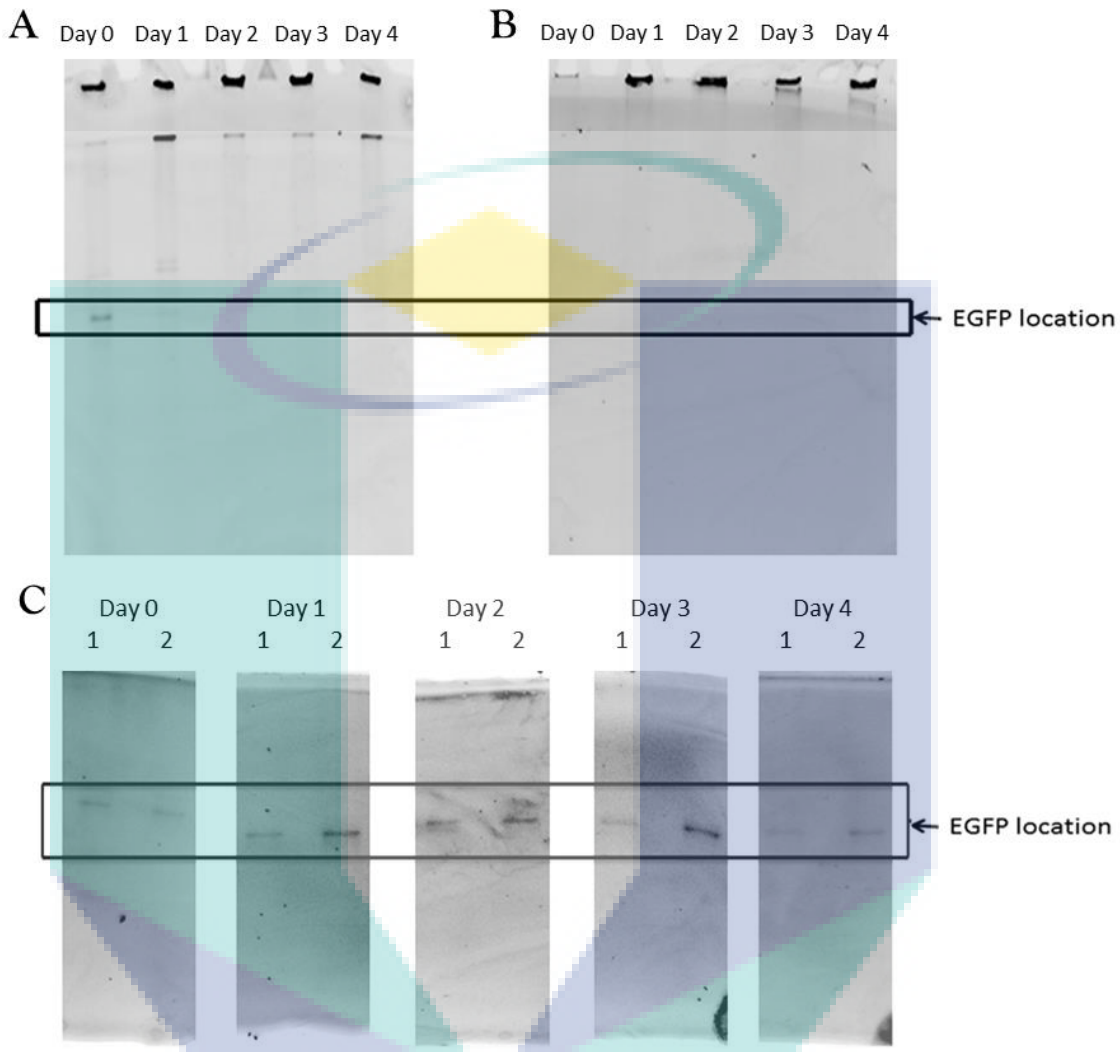


Figure 4.5. Native PAGE analysis of refolded soluble EGFP for methods A, B, and C under various refolding incubation periods. (A) Method A. (B) Method B. (C) Method C (lane 1: elute sample collected between 75 to 90 min and lane 2: elute sample collected between 90 to 105 min). The rectangular boxes show the location of EGFP.

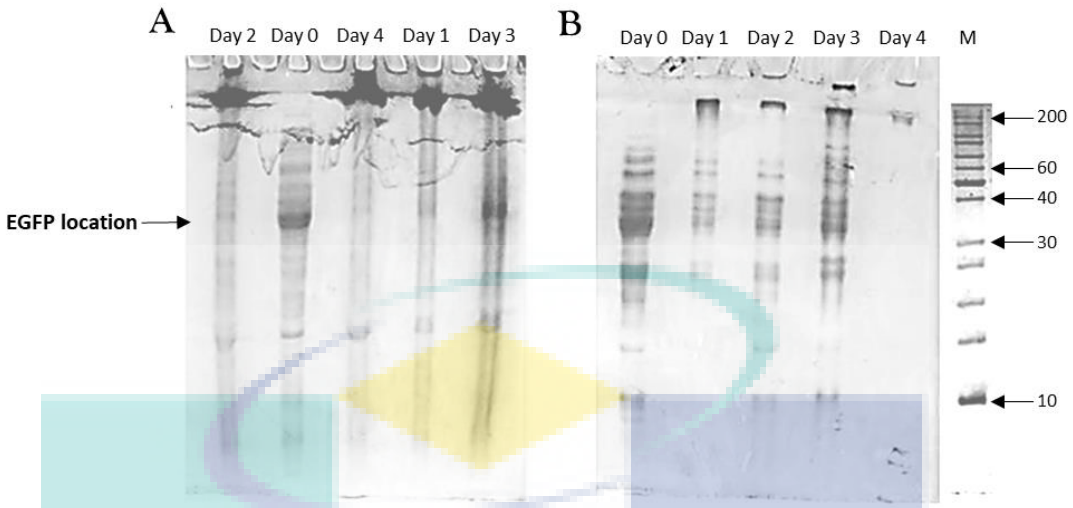


Figure 4.6: SDS-PAGE analysis of refolded soluble EGFP for methods A, and B under various refolding incubation periods. (A) Method A. (B) Method B. Molecular weight marker (M) with protein sizes in kilodaltons is indicated on the right.

IBs consist of high level intermolecular β-sheet structure which are formed by hydrophobic bonding (Carrió, González-Montalbán, Vera, Villaverde & Ventura, 2005). To return the structure of IBs back to the structure of soluble protein, this bonding has to be rearranged. Normally during the protein refolding, the noncovalent interactions of IBs such as hydrogen bonding and hydrophobic interaction are affected by chaotropic agent (Yamaguchi, Yamamoto, Mannen & Nagamune, 2012) or temperature shift (Malavasi et al., 2014). In this study, the solubilized proteins experienced a temperature shift during refolding process which was −20°C and 4°C of freeze thaw method. Under low temperature incubation, the enthalpy of the hydrogen bonding and hydrophobic interaction are reduced and IBs will unfold to their primary structure. The temperature increase during thawing provides the energy for bonding formation. In a correct refolding process, the hydrophobic surface of the protein structure will interact in an intramolecular manner to form native conformation (Fink, 1998; Yamaguchi et al., 2012). However, refolding process under methods A and B caused soluble aggregates formation. This might be due to the unsteady monomeric protein that improperly interacted with other proteins' free ends and formed misfolded proteins which were morphologically similar to amyloid fibrils (Rambaran & Serpell, 2008). These amyloid-like soluble proteins may act as the seed for further aggregation under high protein concentration condition.

The purities of the refolded protein suspension for methods A, B, and C were determined as 26.01%, 34.55%, and 52.16%, respectively. For methods A and B, the refolding process under low protein purity develop a high complex protein structure (Figures 4.5 and 4.6). Previous studies have reported that the unsteady misfolded protein may speed up the formation of mature fibrils or even dense particles such as IBs (Fink, 1998; Jain, 2005; Rambaran and Serpell, 2008). The fibrils assemble to form insoluble fibers in soluble protein. Singh and Panda (2005) reported that protein

aggregation is a higher order reaction whereas protein refolding is a first order reaction. Hence, protein aggregation is more preferable in the high protein concentration and low protein purity conditions. The solubilized IBs in method C is the best condition for protein refolding among the three methods as the refolding occurred under the condition of low protein concentration and high protein purity.

A correct protein conformation that reflects the protein quality is important in the pharmaceutical and nanobiotechnology applications. In fact, the existing methods (Table 1.1) have a high recovery of refolded protein at around 50% to 90%. Nonetheless, the performance of refolding was calculated based on the protein molecular weight and size, protein activity, and functional group in the protein molecules. The monitoring of correct conformation of refolded soluble protein was always ignored in the previous studies. In addition, the protein analyses were conducted within one day after the protein had refolded. In this study, functional soluble EGFP aggregated after day 1 of incubation. This shows protein aggregation might happen and change the protein conformation if the refolding period is extended. This unwanted aggregation process can have severe consequences in human diseases such as Alzheimer's and Parkinson's diseases, and in the manufacturing, storage and delivery of protein based pharmaceuticals. In addition, Yang, Moss and Philips (1996) reported that the amyloid-like protein was able to provide the same properties as the native protein, but not the protein conformation. The present study demonstrates that low protein concentration and high protein purity are the important conditions to refold proteins correctly into their native conformation. Among three refolding methods studied, method C successfully refolded native EGFP with correct protein conformation.

4.3 Development of PNU-PAGE for EGFP-IBs recovery

PNU-PAGE was applied for clarifying and purifying the solubilized EGFP prior to refolding process in method C. This electrophoresis-based process has successfully recovered 2.4 µg of folded soluble EGFP with 12.4% of refolding yield after one day of refolding incubation period. In purity analysis, the same total amount of protein samples for methods A, B, and C were analyed using SDS-PAGE (Figure 4.7). For the samples from methods A (lane 2) and B (lane 1), the protein samples had impurities bands as shown on the SDS gel images. The purities of the protein samples for methods A, B, and C were 26.01%, 34.55%, and 52.16%, respectively. Even though method C gave the highest purity, the refolded EGFP band after the treatment of PNU-PAGE was not located at 35 kDa. Figure 4.7 shows the EGFP band was located between 60 and 70 kDa (lane 3). It is suspected that a dimer was formed. Yang (1997) reported that dimerization of EGFP is preferable if the amount of hydrophobic bond is high. In this study, the overexpression of EGFP provided a high amount of hydrophobic bonding in the protein structure. Therefore, the solubilized EGFP has the potential to form a dimer after refolding process.

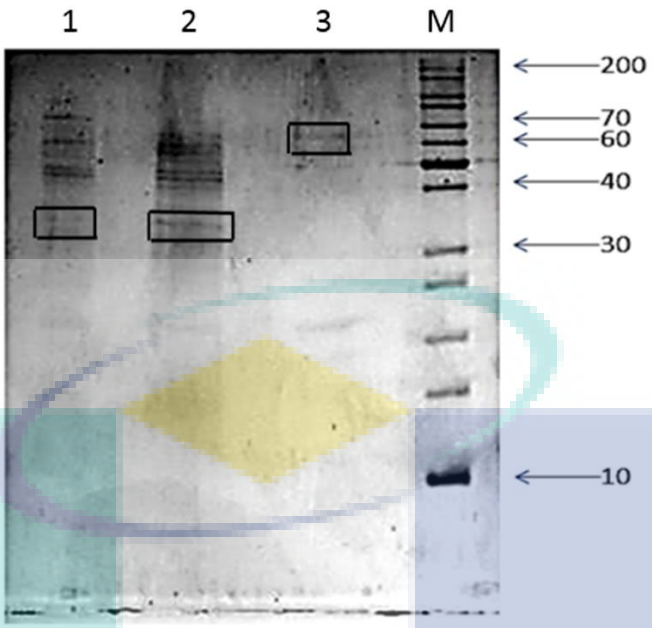


Figure 4.7: SDS-PAGE analysis of refolded EGFP for methods A, B, and C at zero day of refolding. Lane 1: method B, lane 2: method A, lane 3: elute sample collected between 75 to 90 min by using method C, lane M: molecular mass markers in kDa. Total protein amounts were adjusted to 0.005 µg. The rectangular boxes show the location of EGFP.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Conventional and mild solubilization methods were conducted by varying its process condition to investigate the effects of the solubilization methods on denaturation of EGFP from IBs. Incubation of IBs suspension at temperature of 60°C with 8 M of urea based buffer achieved a yield of 61% and purity of 10%. However, mild solubilization of IBs protein using the alkyl alcohol and freeze thaw methods generally applicable. The yield and purity of solubilized EGFP in 6 M of n-butanol and 2 M of urea based buffer were 45% and 22%, respectively. Besides, the yield (66%) and purity (9%) of mild solubilization using 2 M of urea with freeze thaw process was comparable to the conventional 8 M urea denaturation method.

Protein refolding using different types of solubilized EGFP-IBs that were varied in the sample purity and concentration affected the refolded soluble EGFP conformation and caused soluble aggregate generation. By using the native PAGE and SDS-PAGE analyses, the refolded soluble EGFPs of methods A and B were found turned to soluble aggregates. By clarifying and purifying the solubilized EGFP-IBs using PNU-PAGE (method C), the solubilized EGFP was able to refold correctly with high protein refolding yield after one day of incubation with 2.4 µg of folded soluble EGFP, 12.4% of refolding yield, and 52.2% of purity.

5.2 Recommendations

There are some recommendations for future research:

a. The solubilized EGFP using mild solubilization methods can be refolded to further confirm the refolding yield and purity of functional EGFP.
b. The mild solubilization methods can be utilized to prepare large qualities of active soluble proteins from IBs for research and industrial purpose.
c. Gel-imaging method can be used to determine the refolded protein conformation for nanobiotechnology and molecular biology applications.
d. PNU-PAGE process can be further optimised for better recovery and applied in other IBs protein recovery process

REFERENCES

Albright LM, Slatko BE. (2001). Denaturing polyacrylamide gel electrophoresis. Current Protocol Nucleic Acid Chem. 3.

Almarza J, Rincon L, Bahsas A, Brito F. (2009). Molecular mechanism for the denaturing of protein by urea. Biochemistry 48: 7608-7613.

Berkelman T, Brubacher MG, Chang H. (2004). Important factors influencing protein solubility for 2-D electrophoresis. Bio-Rad laboratories. 114:30-32.

Birren B, Lai E. (1993). Pulsed field gel electrophoresis. Academic Press, INC.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72: 248-54.

Cabanne C, Noubhani AM, Hocquellet A, Dole F, Dieryck W, Santarelli X. (2005). Purification and on-column refolding of EGFP overexpressed as inclusion bodies in Escherichia coli with expanded bed anion exchange chromatography. Journal of Chromatography B. 818: 23-27.

Cao E, Chen Y, Cui Z, Foster PR. (2003). Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. Biotechnology and Bioengineering 82:684-690.

Carrió M, González-Montalbán N, Vera A, Villaverde A, Ventura S. (2005). Amyloid-like properties of bacterial inclusion bodies. Journal of Molecular Biology 347:1025-1037.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. (1994). Green fluorescent protein as a marker for gene expression. Science. 263: 802-805.

Chen J, Liu Y, Li X, Wang Y, Ding H, Maa G, Su Z. (2009). Cooperative effects of urea and L-arginine on protein refolding. Protein Expression and Purification 66: 82–90.

Chew FN, Tan WS, Boo HC, Tey BT. (2012). Statistical optimization of green fluorescent protein production from Escherichia coli BL21 (DE3). Preparative Biochemistry & Biotechnology 42:535-550.

Chew FN, Tan WS, Ling TC, Tan CS, Tey BT. (2009a). Quantitation of green fluorescent protein using a gel-based imaging method. Analytical Biochemistry. 384: 353-355.

Chew FN, Tan WS, Ling TC, Tey BT. (2009b). Single-step purification of the recombinant green fluorescent protein from intact Escherichia coli cells using preparative PAGE. Electrophoresis 30: 3017-3023.

Chew FN, Tan WS, Tey BT. (2011). Fluorescent quantitation method for differentiating the nativity of green fluorescent protein. Journal of Bioscience and Bioengineering 111:246-248.

Chura-Chambi RM, Cordeiro Y, Malavasi NV, Lemke LS, Rodrigues D, Morganti L. (2013). An analysis of the factors that affect the dissociation of inclusion bodies and the refolding of endostatin under high pressure. Process Biochemistry 48: 250–259.

Colon W, Kelly JW. (1992). Partial denaturation of transthyretin in sufficient for amyloid fibril formation in vitro. Biochemistry 31:8654-8660.

Costantino HR, Langer R, Klibanov AM. (1995). Aggregation of a lyophilized pharmaceutical protein, recombinant human albumin: Effect of moisture and stabilization by excipients. Nature Biotechnology 13:493-496.

Day R, Bennion BJ, Ham S, Dagget V. (2002). Increasing temperature accelerates protein unfolding without changing the pathway of unfolding. Journal of Molecular Biology. 322:189-203.

Dehaghani SA, Babaeipour V, Mofid MR, Divsalar A, Faraji F. (2010). An efficient purification method for high recovery of recombinant human granulocyte colony stimulating factor from recombinant *E. coli*. International Journal of Environmental Science and Development 1:111-114.

den Engelsman J, Garidel P, Smulders R, Koll H, Smith B, Bassarab S, Seidi A, Hainzi O, Jiskoot W. (2011). Strategies for the assessment of protein aggregates in pharmaceutical biotech product development. Pharmaceutical Research 28:920-933.

Dill KA, Shortle D. (1991). Denatured states of proteins. Annual Review of Biochemistry 60:795-825.

Durbin SD, Feher G. (1996). Protein crystallization. Annual review of physical chemistry 47:171-204.

Eby MJ. (1991). Prep-phoresis: a wealth of novel possibilities. Nature Biotechnology 9: 528-530.

Fink AL. (1998). Protein aggregation: folding aggregates, inclusion bodies and amyloid. Folding and Design 3:9-23.

Fraga TR, Chura-Chambi RM, Goncales AP, Morais ZM, Vascocellos SA, Morganti L, Martins EAL. (2010). Refolding of the recombinant protein OmpA70 from *Leptospira interrogans* from inclusion bodies using high hydrostatic pressure and partial characterization of its immunological properties. Journal of Biotechnology 148:156-162.

García-Fruitós E, González-Montalbán N, Morell M, Vera A, Ferraz R, Arís A, Ventura S, Villaverde A. (2005). Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. Microbial Cell Factories 4:27.

García-Fruitós E, Vazquez E, Dıez-Gil C, Corchero JL, Seras-Franzoso J, Ratera I, Veciana J, Villaverde A. (2011). Bacterial inclusion bodies: making gold from waste. Trends in Biotechnology 30:65-70.

García-Fruitós E, Vázquez E, Díez-Gil C, Corchero JL, Seras-Franzoso J, Ratera I, Veciana J, Villaverde A. (2012). Bacterial inclusion bodies: making gold from waste. Trends in Biotechnology 30:65-70.

García-Fruitós E. (2010). Inclusion bodies: a new concept. Microbial Cell Factories 9:80.

Giancola C, De Sena C, Fessas D, Graziano G, & Barone G. (1997). DSC studies on bovine serum albumin denaturation effects of ionic strength and SDS concentration. International journal of biological macromolecules 20:193-204.

Gu Z, Weidenhaupt M, Ivanova N, Pavlov M, Xu B, Su ZG, Janson JC. (2002). Chromatographic methods for the isolation of, and refolding of proteins from, *Escherichia coli* inclusion bodies. Protein Expression and Purification 25:174-179.

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

Islam MS, Aryasomayajula A, Selvaganapathy PR. (2017). A review on macroscale and microscale cell lysis methods. Micromachines 8:83.

Istrail S, Schwartz R, King J. (1999). Lattice simulations of aggregation funnels for protein folding. Journal of Computational Biology 6:143-162.

Jackson SE, Craggs TD, Huang JR. (2006). Understanding the folding of GFP using biophysical techniques. Expert Rev. Proteomics 3:545-559.

Jain KK. (2005). Nanotechnology in clinical laboratory diagnostics. Clinica Chimica Acta 358:37-54.

Jarrett JT, Lansbury Jr PT (1992). Amyloid fibril formation requires a chemically discriminating nucleation event: studies of an amyloidogenic sequence from the bacterial protein OsmB. Biochemistry 31:12345-12352.

John RJS, Carpenter JF, Randolph TW. (1999). High pressure fosters protein refolding from aggregates at high concentrations. Proceedings of the National Academy of Sciences of the United States of America 96: 13029-13033.

Johnson BH, Hecht MH. (1994). Recombinant proteins can be isolated from *E. coli* cells by repeated cycles of freezing and thawing. Nature Biotechnology 12:1357-1360.

Khan RH, Rao KB, Eshwari AN, Totey SM, Panda AK. (1998). Solubilization of recombinant ovine growth hormone with retention of native-like secondary structure and its refolding from the inclusion bodies of *Escherichia coli*. Biotechnology Progress 14:722-728.

Kopito RR. (2000). Aggresomes, inclusion bodies and protein aggregation. Cell Biology 10:524-530.

Krishnamurthy R, Manning MC. (2002). The stability factor: importance in formulation development. Current pharmaceutical biotechnology 3:361-371.

Kumari NKP, Jagannadham MV. (2011). Organic solvent induced refolding of acid denatured heynein: Evidence of domains in the molecular structure of the protein and their sequential unfolding. Journal of Protein and Proteomics 2:11-21.

Laemmli UK. (1970). Cleavege of structural protein during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lemke LS, Chura-Chambi RM, Rodrigues D, Cussiol JRR, Malavasi NV, Alegria TGP, Netto LES, Morganti L. (2015). Investigation of solubilization protocols in the refolding of the thioredoxin TsnC from *Xylella fastidiosa* by high hydrostatic pressure approach. Protein Expression and Purification 106: 72-77.

Lilie H, Schwarz E, Rudolph R. (1998) Advances in refolding of proteins produced in *E. coli*. Current Opinion Biotechnol 9:497-501

Lim S., Chundawat SPS, Fox BG. (2014). Expression, purification and characterization of a functional carbohydrate-binding module from Streptomyces sp. SirexAA-E. Protein Expression and Purification. 98: 1-9.

Malavasi NV, Cordeiro Y, Rodrigues D, Chura CRM. (2014). The effect of temperature on protein refolding at high pressure: Enhance green fluorescent as a model. Process Biochemistry 49:54-60.

Malavasi NV, Foguel D, Bonafe CFS, Braga CA, Chura-Chambi RM, Vieira JM, Morganti L. (2011). Protein refolding at high pressure: optimization using eGFP as a model. Process Biochemistry 46:512-518.

Mar Carrio M, Cubarsib R, Villaverdea A. (2000). Fine architecture of bacterial inclusion bodies. Federation of European Biochemical Societies 471:7-11.

Margreiter G, Gross-Selbeck S, Obinger C, Bayer K. (2008). Fast Quantification of recombinant protein inclusion bodies within intact cells by FT-IR spectroscopy. Biotechnology Program 23:762-766.

Marston FAO, Hartley DL. (1990). Solubilization of protein aggregates. Methods Enzymology 182:264-276.

Martínez-Alonso M, González-Montalbán N, García-Fruitós E, Villaverde A. (2008). The functional quality of soluble recombinant polypeptides produced in *Escherichia coli* is defined by a wide conformational spectrum. Applied and Environmental Microbiology 74:7431-7433.

Melnik BS, Povanitsyna TV, Melnik TN. (2009). Can the fluorescence of green fluorescnt protein chromophore be related directly to the nativity of protein structure? Biochemical and Biophysical Research Communications 390:1167-1170.

Pace C, Treviño S, Prabhakaran E, Scholtz J. (2004). Protein structure, stability and solubility in water and other solvents. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 359:1225-1235.

Panda AK. Bioprocessing of therapeutic proteins from the inclusion bodies of *Escherichia coli*. (2003). Advances in Biochemical Engineering /Biotechnology 85:43-93.

Patra A, Mukhopadhyay R, Mukhija R, Krishnan A, Garg L, Panda A. (2000). Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coil*. Protein Expression and Purification 18:182-192.

Peternel Š, Jevševar S, Bele M, Gaberc-Porekar V, Menart V. (2008). New properties of inclusion bodies with implications for biotechnology. Biotechnology and Applied Biochemistry 49:239-246.

Peternel S, Komel R. (2011). Active protein aggregates produced in *Escherichia coli*. International Journal of Molecular Sciences 12:8275-8287.

Qi X, Sun Y, Xiong S. (2015). A single freeze-thawing cycle for highly efficient solubilization of inclusion body proteins and its refolding into bioactive form. Microbial Cell Factories 14:24-36.

Raghunathan G, Munussami G, Moon HJ, Paik HJ, An SS, Kim YS, Kang SB, Lee SG. (2014). A variant green fluorescent protein exclusively deposited to active intracellular inclusion bodies. Microbial Cell Factories 13:68-79.

Rambaran RN, Serpell LC. (2008). Amyloid fibrils: abnormal protein assembly. Prion 2:112-117.

Rashid F, Sharma S, Bano B (2005). Comparison of guanidine hydrochloride (GdnHCl) and urea denaturation on inactivation and unfolding of human placental. The Protein Journal 24:283-292.

Reddy G, Liu Z, Thirumalai D (2012). Denaturant-dependent folding of GFP. PNAS 109:17832-17838.

Rosenberg AS. (2006). Effects of protein aggregates: An immunologic perspective. Journal of the American Association of Pharmaceutical Scientists 8: E501-E507.

Saremirad P, Wood JA, Zhang Y, Ray AK. (2014). Oxidative protein refolding on size exclusion chromatography at high loading concentrations: Fundamental studies and mathematical modeling. Journal of Chromatography A 1370:147-155.

Seras-Franzoso J, Peternel S, Cano-Garrido O, Villaverde A, Garcia-Fruitos E. (2015). Bacterial inclusion body purification. Methods in Molecular Biology. 1258: 293-305.

Shi TL, Zhang LC, Li ZY, Newton IP, Zhang QB. (2014). Expression, purification and renaturation of truncated human integrin β1 from inclusion bodies of *Escherichia coli*. Protein Expression and Purification 107:13-19.

Singh A, Upadhyay V, Panda AK. (2015). Solubilization and refolding of inclusion body proteins. Methods in Molecular Biology. 1258: 283-291.

Singh A, Upadhyay V, Upadhyay A, Singh S, Panda A. (2015). Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. Microbial Cell Factories 14:41.

Singh S., Sharma A., Upadhyay A., Singh A., Garg L, Panda, A. (2012). Solubilization of inclusion body proteins using n-propanol and its refolding into bioactive form. Protein Expression and Purification 81:75-82.

Singh SM, Panda AM. (2005). Solubilization and refolding of bacterial inclusion body proteins. Journal of Bioscience and Bioengineering 99:303-310.

Stepanenko OV, Stepanenko OV, Kuznetsova IM, Shcherbakova DM, Verkhusha VV, Turoverov KK. (2012). Distinct effects of guanidine thiocyante on the structure of superfolder GFP. PLOS ONE 7:e48809.

Stigter D, Dill KA. (1993). Theory for protein solubilities. Fluid Phase Equilibria 1993; 82:237-249.

Suresh Chandra BR, Prakash V, Narasinga Rao MS. (1985). Association-dissociation of glycinin in urea, guanidine hydrochloride and sodium dodecyl sulphate solutions. Journal Bioscience 9:177-184.

Tanford C. (1970). Protein denaturation. Advance Protein Chemicals 23: 121-282.

Ubeidat M., Rutherford CL. (2003). Purification and renaturation of Dictyostelium recombinant alkaline phosphatase by continuous elution electrophoresis. Protein Expression and Purification. 27: 375-383.

Upadhyay AK, Singh A, Mukherjee K J, Panda AK. (2014). Refolding and purification of recombinant L-asparaginase from inclusion bodies of *E. coli* into active tetrameric protein. Frontiers in Microbiology 5:486.

Vera A, González-Montalbán N, Garcia-Fruitós E, Arís A, Villaverde A. (2006). Low growth temperatures improve the conformational quality of aggregation prone recombinant proteins in both soluble and insoluble *E. coli* cell fractions. Microbial Cell Factories 5:P7.

Villaverde A, García-Fruitós ., Rinas U, Seras-Franzoso J, Kosoy A, Corchero JL, Vazquez E. (2012). Packaging protein drugs as bacterial inclusion bodies for therapeutic applications. Microbial cell factories 11:76.

Wang W, Roberts CJ. (2010). Aggregation of therapeutic proteins. New York: John Wiley and Sons, Inc.

Yamaguchi H, Miyazaki M. (2014). Refolding techniques for recovering biologically active recombinant proteins from inclusion bodies. Biomolecules 4:235-251.

Yamaguchi S, Yamamoto E, Mannen T, Nagamune T. (2012). Protein refolding using chemical refolding additives. Biotechnology Journal 8:17-31.

Yang F, Moss LG, Philips JGN. (1996). The molecular structure of green fluorescent protein. Nature Biotechnology 14:1246-11251.

Yang F. (1997). The molecular structure of green fluorescent protein. PhD Thesis, Rice University, Texas, US.

Yang Z, Zhang L, Zhang Y, Zhang T, Feng Y, Lu X, Lan W, Wang J, Wu H, Cao C, Wang X. (2011). Highly efficient production of soluble proteins from insoluble inclusion bodies by a two-step-denaturing and refolding method. PLOS One 6:1-8.

Yoshikawa H, Hirano A, Arakawa T, Shiraki K. (2012). Effects of alcohol on the solubility and structure of native and disulfide-modified bovine serum albumin. International Journal of Biological Macromolecules 50:1286-1291.

Zou Q, Habermann-Rottinghaus S, Murphy K. (1998). Urea effects on protein stability: Hydrogen bonding and the hydrophobic effect. Proteins: Structure, Function, and Bioinformatics 31:107-115.

REFERENCES

Albright LM, Slatko BE. (2001). Denaturing polyacrylamide gel electrophoresis. Current Protocol Nucleic Acid Chem. 3.

Almarza J, Rincon L, Bahsas A, Brito F. (2009). Molecular mechanism for the denaturing of protein by urea. Biochemistry 48: 7608-7613.

Berkelman T, Brubacher MG, Chang H. (2004). Important factors influencing protein solubility for 2-D electrophoresis. Bio-Rad laboratories. 114:30-32.

Birren B, Lai E. (1993). Pulsed field gel electrophoresis. Academic Press, INC.

Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 72: 248-54.

Cabanne C, Noubhani AM, Hocquellet A, Dole F, Dieryck W, Santarelli X. (2005). Purification and on-column refolding of EGFP overexpressed as inclusion bodies in Escherichia coli with expanded bed anion exchange chromatography. Journal of Chromatography B. 818: 23-27.

Cao E, Chen Y, Cui Z, Foster PR. (2003). Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. Biotechnology and Bioengineering 82:684-690.

Carrió M, González-Montalbán N, Vera A, Villaverde A, Ventura S. (2005). Amyloid-like properties of bacterial inclusion bodies. Journal of Molecular Biology 347:1025-1037.

Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. (1994). Green fluorescent protein as a marker for gene expression. Science. 263: 802-805.

Chen J, Liu Y, Li X, Wang Y, Ding H, Maa G, Su Z. (2009). Cooperative effects of urea and L-arginine on protein refolding. Protein Expression and Purification 66: 82–90.

Chew FN, Tan WS, Boo HC, Tey BT. (2012). Statistical optimization of green fluorescent protein production from Escherichia coli BL21 (DE3). Preparative Biochemistry & Biotechnology 42:535-550.

Chew FN, Tan WS, Ling TC, Tan CS, Tey BT. (2009a). Quantitation of green fluorescent protein using a gel-based imaging method. Analytical Biochemistry. 384: 353-355.

Chew FN, Tan WS, Ling TC, Tey BT. (2009b). Single-step purification of the recombinant green fluorescent protein from intact Escherichia coli cells using preparative PAGE. Electrophoresis 30: 3017-3023.

Chew FN, Tan WS, Tey BT. (2011). Fluorescent quantitation method for differentiating the nativity of green fluorescent protein. Journal of Bioscience and Bioengineering 111:246-248.

Chura-Chambi RM, Cordeiro Y, Malavasi NV, Lemke LS, Rodrigues D, Morganti L. (2013). An analysis of the factors that affect the dissociation of inclusion bodies and the refolding of endostatin under high pressure. Process Biochemistry 48: 250–259.

Colon W, Kelly JW. (1992). Partial denaturation of transthyretin in sufficient for amyloid fibril formation in vitro. Biochemistry 31:8654-8660.

Costantino HR, Langer R, Klibanov AM. (1995). Aggregation of a lyophilized pharmaceutical protein, recombinant human albumin: Effect of moisture and stabilization by excipients. Nature Biotechnology 13:493-496.

Day R, Bennion BJ, Ham S, Dagget V. (2002). Increasing temperature accelerates protein unfolding without changing the pathway of unfolding. Journal of Molecular Biology. 322:189-203.

Dehaghani SA, Babaeipour V, Mofid MR, Divsalar A, Faraji F. (2010). An efficient purification method for high recovery of recombinant human granulocyte colony stimulating factor from recombinant *E. coli*. International Journal of Environmental Science and Development 1:111-114.

den Engelsman J, Garidel P, Smulders R, Koll H, Smith B, Bassarab S, Seidi A, Hainzi O, Jiskoot W. (2011). Strategies for the assessment of protein aggregates in pharmaceutical biotech product development. Pharmaceutical Research 28:920-933.

Dill KA, Shortle D. (1991). Denatured states of proteins. Annual Review of Biochemistry 60:795-825.

Durbin SD, Feher G. (1996). Protein crystallization. Annual review of physical chemistry 47:171-204.

Eby MJ. (1991). Prep-phoresis: a wealth of novel possibilities. Nature Biotechnology 9: 528-530.

Fink AL. (1998). Protein aggregation: folding aggregates, inclusion bodies and amyloid. Folding and Design 3:9-23.

Fraga TR, Chura-Chambi RM, Goncales AP, Morais ZM, Vascocellos SA, Morganti L, Martins EAL. (2010). Refolding of the recombinant protein OmpA70 from *Leptospira interrogans* from inclusion bodies using high hydrostatic pressure and partial characterization of its immunological properties. Journal of Biotechnology 148:156-162.

García-Fruitós E, González-Montalbán N, Morell M, Vera A, Ferraz R, Arís A, Ventura S, Villaverde A. (2005). Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. Microbial Cell Factories 4:27.

García-Fruitós E, Vazquez E, Díez-Gil C, Corchero JL, Seras-Franzoso J, Ratera I, Veciana J, Villaverde A. (2011). Bacterial inclusion bodies: making gold from waste. Trends in Biotechnology 30:65-70.

García-Fruitós E, Vázquez E, Díez-Gil C, Corchero JL, Seras-Franzoso J, Ratera I, Veciana J, Villaverde A. (2012). Bacterial inclusion bodies: making gold from waste. Trends in Biotechnology 30:65-70.

García-Fruitós E. (2010). Inclusion bodies: a new concept. Microbial Cell Factories 9:80.

Giancola C, De Sena C, Fessas D, Graziano G, & Barone G. (1997). DSC studies on bovine serum albumin denaturation effects of ionic strength and SDS concentration. International journal of biological macromolecules 20:193-204.

Gu Z, Weidenhaupt M, Ivanova N, Pavlov M, Xu B, Su ZG, Janson JC. (2002). Chromatographic methods for the isolation of, and refolding of proteins from, *Escherichia coli* inclusion bodies. Protein Expression and Purification 25:174-179.

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

Islam MS, Aryasomayajula A, Selvaganapathy PR. (2017). A review on macroscale and microscale cell lysis methods. Micromachines 8:83.

Istrail S, Schwartz R, King J. (1999). Lattice simulations of aggregation funnels for protein folding. Journal of Computational Biology 6:143-162.

Jackson SE, Craggs TD, Huang JR. (2006). Understanding the folding of GFP using biophysical techniques. Expert Rev. Proteomics 3:545-559.

Jain KK. (2005). Nanotechnology in clinical laboratory diagnostics. Clinica Chimica Acta 358:37-54.

Jarrett JT, Lansbury Jr PT (1992). Amyloid fibril formation requires a chemically discriminating nucleation event: studies of an amyloidogenic sequence from the bacterial protein OsmB. Biochemistry 31:12345-12352.

John RJS, Carpenter JF, Randolph TW. (1999). High pressure fosters protein refolding from aggregates at high concentrations. Proceedings of the National Academy of Sciences of the United States of America 96: 13029-13033.

Johnson BH, Hecht MH. (1994). Recombinant proteins can be isolated from *E. coli* cells by repeated cycles of freezing and thawing. Nature Biotechnology 12:1357-1360.

Khan RH, Rao KB, Eshwari AN, Totey SM, Panda AK. (1998). Solubilization of recombinant ovine growth hormone with retention of native-like secondary structure and its refolding from the inclusion bodies of *Escherichia coli*. Biotechnology Progress 14:722-728.

Kopito RR. (2000). Aggresomes, inclusion bodies and protein aggregation. Cell Biology 10:524-530.

Krishnamurthy R, Manning MC. (2002). The stability factor: importance in formulation development. Current pharmaceutical biotechnology 3:361-371.

Kumari NKP, Jagannadham MV. (2011). Organic solvent induced refolding of acid denatured heynein: Evidence of domains in the molecular structure of the protein and their sequential unfolding. Journal of Protein and Proteomics 2:11-21.

Laemmli UK. (1970). Cleavege of structural protein during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lemke LS, Chura-Chambi RM, Rodrigues D, Cussiol JRR, Malavasi NV, Alegria TGP, Netto LES, Morganti L. (2015). Investigation of solubilization protocols in the refolding of the thioredoxin TsnC from *Xylella fastidiosa* by high hydrostatic pressure approach. Protein Expression and Purification 106: 72-77.

Lilie H, Schwarz E, Rudolph R. (1998) Advances in refolding of proteins produced in *E. coli*. Current Opinion Biotechnol 9:497-501

Lim S., Chundawat SPS, Fox BG. (2014). Expression, purification and characterization of a functional carbohydrate-binding module from Streptomyces sp. SirexAA-E. Protein Expression and Purification. 98: 1-9.

Malavasi NV, Cordeiro Y, Rodrigues D, Chura CRM. (2014). The effect of temperature on protein refolding at high pressure: Enhance green fluorescent as a model. Process Biochemistry 49:54-60.

Malavasi NV, Foguel D, Bonafe CFS, Braga CA, Chura-Chambi RM, Vieira JM, Morganti L. (2011). Protein refolding at high pressure: optimization using eGFP as a model. Process Biochemistry 46:512-518.

Mar Carrio M, Cubarsib R, Villaverdea A. (2000). Fine architecture of bacterial inclusion bodies. Federation of European Biochemical Societies 471:7-11.

Margreiter G, Gross-Selbeck S, Obinger C, Bayer K. (2008). Fast Quantification of recombinant protein inclusion bodies within intact cells by FT-IR spectroscopy. Biotechnology Program 23:762-766.

Marston FAO, Hartley DL. (1990). Solubilization of protein aggregates. Methods Enzymology 182:264-276.

Martínez-Alonso M, González-Montalbán N, García-Fruitós E, Villaverde A. (2008). The functional quality of soluble recombinant polypeptides produced in *Escherichia coli* is defined by a wide conformational spectrum. Applied and Environmental Microbiology 74:7431-7433.

Melnik BS, Povanitsyna TV, Melnik TN. (2009). Can the fluorescence of green fluorescnt protein chromophore be related directly to the nativity of protein structure? Biochemical and Biophysical Research Communications 390:1167-1170.

Pace C, Treviño S, Prabhakaran E, Scholtz J. (2004). Protein structure, stability and solubility in water and other solvents. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences 359:1225-1235.

Panda AK. Bioprocessing of therapeutic proteins from the inclusion bodies of *Escherichia coli*. (2003). Advances in Biochemical Engineering /Biotechnology 85:43-93.

Patra A, Mukhopadhyay R, Mukhija R, Krishnan A, Garg L, Panda A. (2000). Optimization of inclusion body solubilization and renaturation of recombinant human growth hormone from *Escherichia coil.* Protein Expression and Purification 18:182-192.

Peternel Š, Jevševar S, Bele M, Gaberc-Porekar V, Menart V. (2008). New properties of inclusion bodies with implications for biotechnology. Biotechnology and Applied Biochemistry 49:239-246.

Peternel S, Komel R. (2011). Active protein aggregates produced in *Escherichia coli*. International Journal of Molecular Sciences 12:8275-8287.

Qi X, Sun Y, Xiong S. (2015). A single freeze-thawing cycle for highly efficient solubilization of inclusion body proteins and its refolding into bioactive form. Microbial Cell Factories 14:24-36.

Raghunathan G, Munussami G, Moon HJ, Paik HJ, An SS, Kim YS, Kang SB, Lee SG. (2014). A variant green fluorescent protein exclusively deposited to active intracellular inclusion bodies. Microbial Cell Factories 13:68-79.

Rambaran RN, Serpell LC. (2008). Amyloid fibrils: abnormal protein assembly. Prion 2:112-117.

Rashid F, Sharma S, Bano B (2005). Comparison of guanidine hydrochloride (GdnHCl) and urea denaturation on inactivation and unfolding of human placental. The Protein Journal 24:283-292.

Reddy G, Liu Z, Thirumalai D (2012). Denaturant-dependent folding of GFP. PNAS 109:17832-17838.

Rosenberg AS. (2006). Effects of protein aggregates: An immunologic perspective. Journal of the American Association of Pharmaceutical Scientists 8: E501-E507.

Saremirad P, Wood JA, Zhang Y, Ray AK. (2014). Oxidative protein refolding on size exclusion chromatography at high loading concentrations: Fundamental studies and mathematical modeling. Journal of Chromatography A 1370:147-155.

Seras-Franzoso J, Peternel S, Cano-Garrido O, Villaverde A, Garcia-Fruitos E. (2015). Bacterial inclusion body purification. Methods in Molecular Biology. 1258: 293-305.

Shi TL, Zhang LC, Li ZY, Newton IP, Zhang QB. (2014). Expression, purification and renaturation of truncated human integrin β1 from inclusion bodies of *Escherichia coli*. Protein Expression and Purification 107:13-19.

Singh A, Upadhyay V, Panda AK. (2015). Solubilization and refolding of inclusion body proteins. Methods in Molecular Biology. 1258: 283-291.

Singh A, Upadhyay V, Upadhyay A, Singh S, Panda A. (2015). Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. Microbial Cell Factories 14:41.

Singh S., Sharma A., Upadhyay A., Singh A., Garg L, Panda, A. (2012). Solubilization of inclusion body proteins using n-propanol and its refolding into bioactive form. Protein Expression and Purification 81:75-82.

Singh SM, Panda AM. (2005). Solubilization and refolding of bacterial inclusion body proteins. Journal of Bioscience and Bioengineering 99:303-310.

Stepanenko OV, Stepanenko OV, Kuznetsova IM, Shcherbakova DM, Verkhusha VV, Turoverov KK. (2012). Distinct effects of guanidine thiocyante on the structure of superfolder GFP. PLOS ONE 7:e48809.

Stigter D, Dill KA. (1993). Theory for protein solubilities. Fluid Phase Equilibria 1993; 82:237-249.

Suresh Chandra BR, Prakash V, Narasinga Rao MS. (1985). Association-dissociation of glycinin in urea, guanidine hydrochloride and sodium dodecyl sulphate solutions. Journal Bioscience 9:177-184.

Tanford C. (1970). Protein denaturation. Advance Protein Chemicals 23: 121-282.

Ubeidat M., Rutherford CL. (2003). Purification and renaturation of Dictyostelium recombinant alkaline phosphatase by continuous elution electrophoresis. Protein Expression and Purification. 27: 375-383.

Upadhyay AK, Singh A, Mukherjee K J, Panda AK. (2014). Refolding and purification of recombinant L-asparaginase from inclusion bodies of *E. coli* into active tetrameric protein. Frontiers in Microbiology 5:486.

Vera A, González-Montalbán N, Garcia-Fruitós E, Arís A, Villaverde A. (2006). Low growth temperatures improve the conformational quality of aggregation prone recombinant proteins in both soluble and insoluble *E. coli* cell fractions. Microbial Cell Factories 5:P7.

Villaverde A, García-Fruitós ., Rinas U, Seras-Franzoso J, Kosoy A, Corchero JL, Vazquez E. (2012). Packaging protein drugs as bacterial inclusion bodies for therapeutic applications. Microbial cell factories 11:76.

Wang W, Roberts CJ. (2010). Aggregation of therapeutic proteins. New York: John Wiley and Sons, Inc.

Yamaguchi H, Miyazaki M. (2014). Refolding techniques for recovering biologically active recombinant proteins from inclusion bodies. Biomolecules 4:235-251.

Yamaguchi S, Yamamoto E, Mannen T, Nagamune T. (2012). Protein refolding using chemical refolding additives. Biotechnology Journal 8:17-31.

Yang F, Moss LG, Philips JGN. (1996). The molecular structure of green fluorescent protein. Nature Biotechnology 14:1246-11251.

Yang F. (1997). The molecular structure of green fluorescent protein. PhD Thesis, Rice University, Texas, US.

Yang Z, Zhang L, Zhang Y, Zhang T, Feng Y, Lu X, Lan W, Wang J, Wu H, Cao C, Wang X. (2011). Highly efficient production of soluble proteins from insoluble inclusion bodies by a two-step-denaturing and refolding method. PLOS One 6:1-8.

Yoshikawa H, Hirano A, Arakawa T, Shiraki K. (2012). Effects of alcohol on the solubility and structure of native and disulfide-modified bovine serum albumin. International Journal of Biological Macromolecules 50:1286-1291.

Zou Q, Habermann-Rottinghaus S, Murphy K. (1998). Urea effects on protein stability: Hydrogen bonding and the hydrophobic effect. Proteins: Structure, Function, and Bioinformatics 31:107-115.