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THE EFFECT OF REFOLDING CONDITIONS ON THE PROTEIN SOLUBILITY RECOVERED FROM INCLUSION BODIES

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ABSTRACT

Recombinant proteins are expressed as inclusion bodies in bacterial enriched native-like secondary structure and thus give a great potential in biotechnological utilities. However, 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, enhanced green fluorescent protein-inclusion body 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.

KEYWORDS: *Inclusion body; protein refolding; soluble aggregate; conformation; gel electrophoresis*

1.0 INTRODUCTION

Inclusion bodies (IBs) are the misfolded insoluble proteins that fail to reach their native conformation during the overexpression in bacterial cells. They are widely exploited in the tissue engineering and biocatalysts industries because of their high protein purity and simple recovery process from cell debris (García-Fruitós, 2010). However, IBs are not efficient as soluble protein in bioreaction due to their unfolded structure and low solubility (García-Fruitós et al., 2012). Therefore, many researchers have reported the recovery methods including isolation, purification, solubilization, and refolding process to recover functional soluble protein from IBs. IBs recovery method requires the insoluble protein to be disaggregated and then refolded back to their native conformation. The conventional method of IBs solubilization uses strong denaturants such as urea and guanidine hydrochloride causes low refolding yield because the native-like secondary protein

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structures are completely disrupted during solubilization and the desired protein is aggregated during refolding. As the IBs were found to have native-like protein molecules, mild solubilization process was used for the IBs recovery. The use of high hydrostatics pressure and freeze thaw method as alternative to strong denaturants for IBs solubilization has successfully refolded the solubilized IBs to native and functional protein, and a higher refolding yield was obtained (John, Carpenter & Randolph, 1999; Lemke et al., 2015; Qi, Sun & Xiong, 2015). Other mild solubilization processes using alkaline pH, organic solvent based buffers, and detergent have been reviewed for high-throughput recovery of bioactive proteins from IBs (Singh, Upadhyay, Upadhyay, Singh & Panda, 2015).

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, Tan & Tey, 2011; Martínez-Alonso, González-Montalbán, García-Fruitós & Villaverde, 2008; Melnik, Povanitsyna & Melnik, 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 & 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. 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. 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, Li, Newton & Zhang, 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, Babaeipour, Mofid, Divsalar & Faraji, 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, Tan, Ling, Tan & Tey, 2009). Besides the fluorescence intensity data for protein conformation analysis as performed by Vera, González-Montalbán, Garcia-Fruitós, Arís & Villaverde (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). We aimed to provide researchers the better understanding of soluble aggregates formation under varying sample concentration and purity, hence the recovery of enhanced green fluorescent protein-inclusion bodies (EGFP-IBs) from *Escherichia coli* (*E. coli*) cells was conducted under different solubilization and refolding conditions. By comparing the protein bands locations in the native PAGE, the conformation of folded EGFP using different types of solubilized EGFP-IBs was identified. The refolding yield and refolded protein conformation were discussed based on the protein purity and concentration of different solubilized EGFP-IBs. The present study demonstrates the importance of protein conformation in protein refolding technology for nanobiotechnology and molecular biology applications.

2.0 MATERIALS AND METHODS

2.1 Microorganism and culture condition

E. coli BL21(DE3) carrying the pRSETEGFP plasmid encoding the EGFP gene was cultivated in Luria-Bertani broth (10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of sodium chloride at pH 7.0) containing 100 µg/mL of ampicillin at 37°C and agitated at 150 rpm. EGFP expression was induced with isopropyl β-D-1-thiogalactopyranoside at a final concentration of 1 mM when the optical density ($OD_{@600nm}$) of the culture was 0.8 to 1.0. After 16 h of protein induction, the culture was harvested by centrifugation at 4°C and 8,000 g for 10 min (Malavasi et al., 2014).

2.2 Cell disruption

Cell pellet was resuspended and washed with 10% (w/v) of washing buffer (0.5 M of Tris-HCl, pH 6.8). After centrifugation at 4°C and 10,000 g for 10 min, cell pellet was collected and disrupted using freeze thaw method (Johnson & Hecht, 1994) to remove the soluble EGFP. Cell pellet was frozen at −20°C for 24 h and thawed by resuspending with 10% (w/v) of washing buffer. The supernatant containing soluble EGFP was discarded after centrifugation at the same conditions. The freezing and thawing processes were repeated for another cycle.

2.3 Solubilisation of EGFP-IBs by three different methods

The cell pellet consisted of EGFP-IBs was solubilized and folded using three different methods as described in Figure 1. 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.

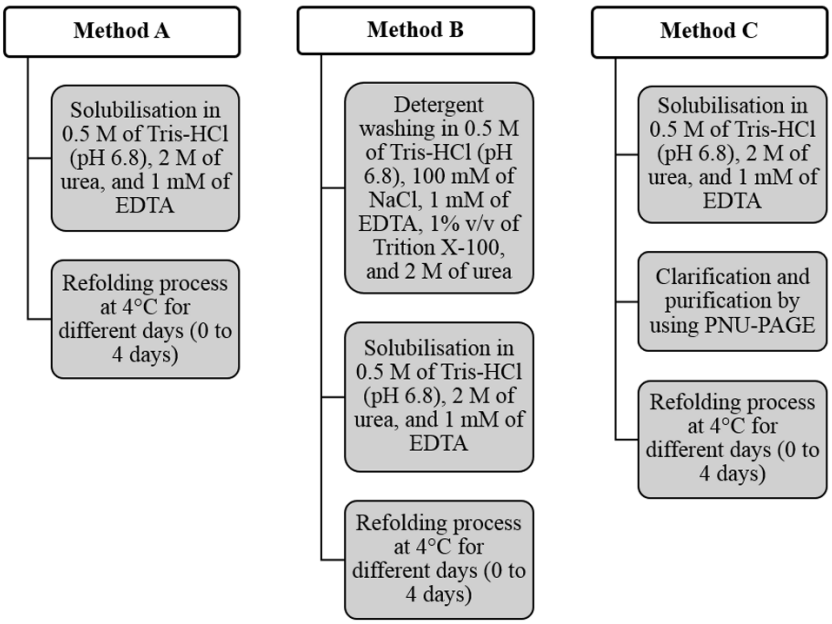

Figure 1. The comparative scheme for refolding process of three different solubilized IBs.

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.

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, Tan, Ling and Tey (2009) 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.

2.4 Native PAGE

The amount of functional EGFP was determined by gel-based imaging method using native PAGE (Chew et al., 2009). 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 image of EGFP bands on the gel was captured using FluoroChem SP imaging system (Alpha Innotec) at 3 s of exposure time. Image analysis was performed using AlphaEase FC software. The amount of functional EGFP was determined based on the fluorescent intensity of the EGFP band on the gel and a standard equation developed using pure EGFP.

2.5 SDS-PAGE

By using gel-based imaging method under SDS-PAGE (Chew et al., 2009; Laemmli, 1970), the total amount of EGFP in this experiment was determined. Polyacrylamide gel (10 × 10 × 0.2 cm) with a 4% (w/v) of stacking gel and a 15% (w/v) of resolving gel were casted by using an OmniPage minivertical system (Cleaver Scientific Ltd). The refolded protein suspension were electrophoresed under a constant current of 30 mA for 90 min. The polyacrylamide gel was then 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] overnight and destained with a destaining solution (40% [v/v] of methanol and 10% [v/v] of acetic acid). The destaining solution was exchanged for every 2 h for 5 times. The clear protein band on the gel was captured using FluoroChem SP imaging system (Alpha Innotec) at 40 ms of exposure time. Image analysis was performed using AlphaEase FC software. The total amount of EGFP was determined based on the intensity of the EGFP band on the gel which is located at 35 kDa and a standard equation developed by using pure EGFP.

2.6 Bradford assay

The total protein amount was determined by Bradford assay (Bradford, 1976) with bovine serum albumin as the protein standard. The refolded protein suspension (20 µL) were mixed with Bradford reagent (200 µL) in a 96-well transparent microplate and analyzed using a microplate reader (Infinite 200 PRO, Tecan) at 595 nm.

2.7 Analyses

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. Purity is defined as

the ratio of the total EGFP amount to the total protein amount in the refolded protein suspension.

3.0 RESULTS AND DISCUSSION

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 2). 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 2A) compared to the other two methods. Surprisingly, method C provided the best refolding yield among the three methods especially at day 1 (Figure 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).

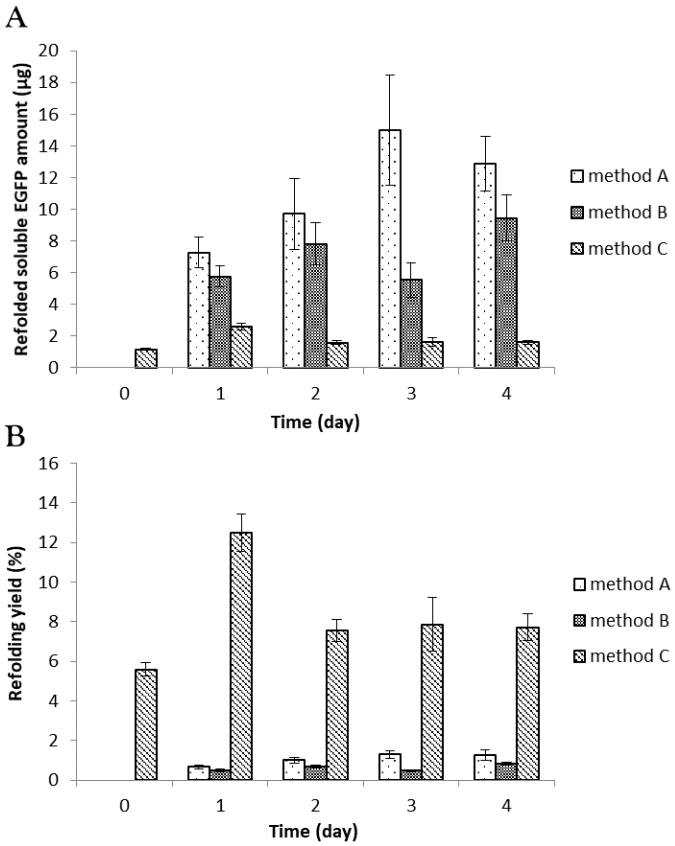

Figure 2. The recovery of refolded soluble EGFP from methods A, B, and C under various refolding incubation periods (dotted, method A; grid, method B; and downward diagonal, method C). (A) Amount of refolded soluble EGFP. (B) Refolding yield of refolded soluble EGFP. Error bars represent duplicate independent 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 2). 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 3. 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 3A) and B (Figure 3B), 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 3A). 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 shows that at day 0 of refolding period, the EGFP bands located at 35 kDa were observed for methods A and B (Figures 4A and 4B). 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 3 and 4 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 3C 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.

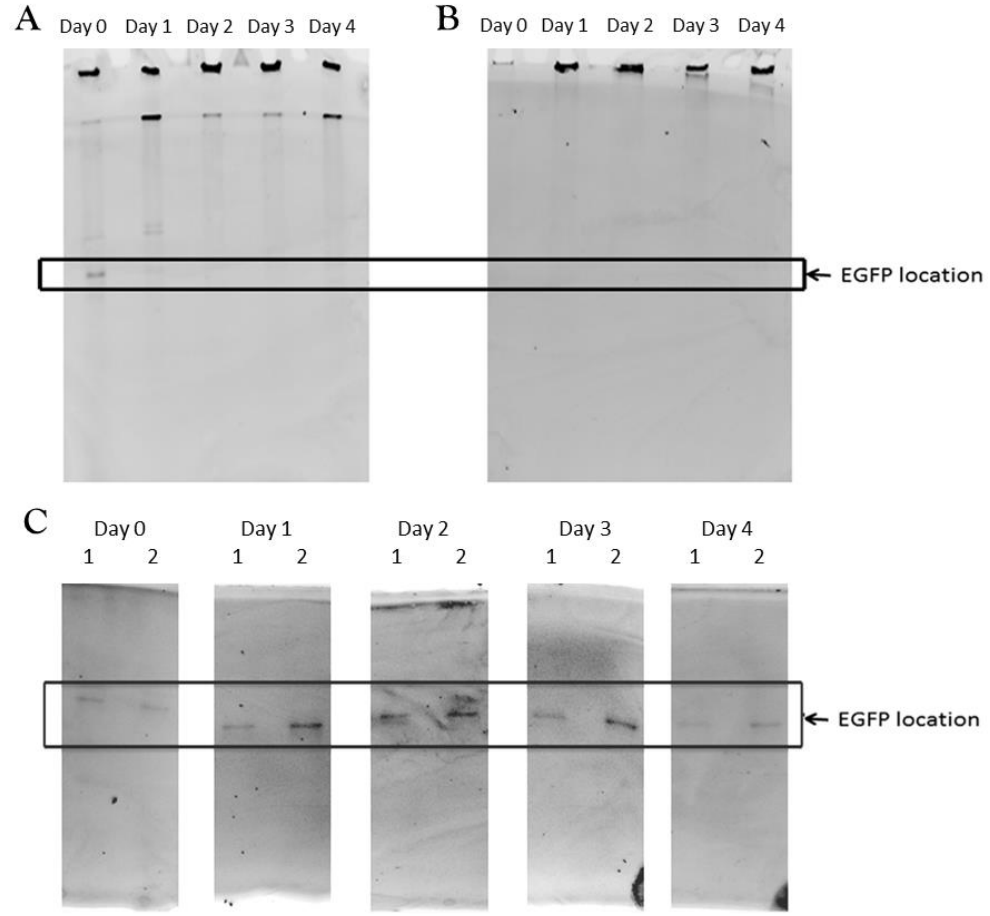

Figure 3. Native PAGE analysis of refolded soluble EGFP for methods A, B, and C under various refolding incubation periods. (A) Method A. (B) Method B. (C) Method C (lane 1: elute sample collected between 75 to 90 min and lane 2: elute sample collected between 90 to 105 min). The rectangular boxes show the location of EGFP.

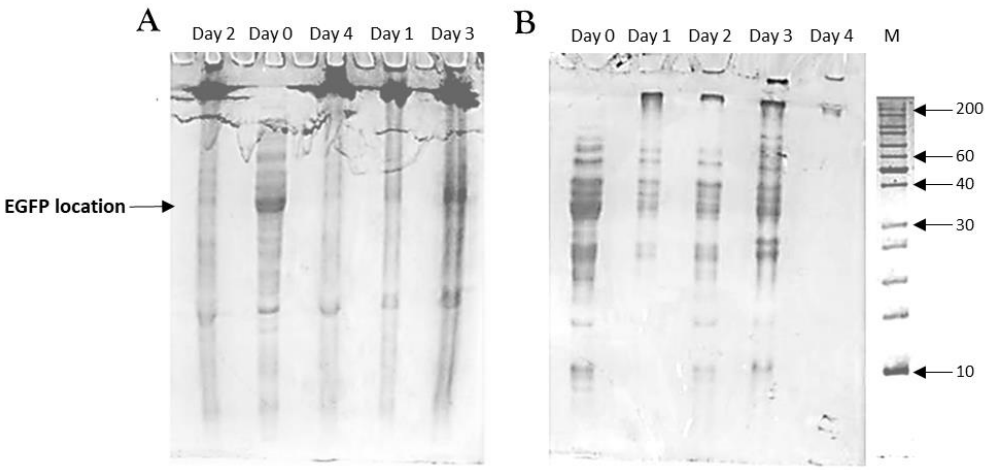

Figure 4: SDS-PAGE analysis of refolded soluble EGFP for methods A, and B under various refolding incubation periods. (A) Method A. (B) Method B. Molecular weight marker (M) with protein sizes in kilodaltons is indicated on the right.

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 3 and 4). 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 & 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) 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.

4.0 CONCLUSION

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. This process can be further optimised for better recovery and applied in other IBs recovery process.

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