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Optimization of Human Interferon α2b Soluble Protein Overproduction and Primary Recovery of Its Inclusion Bodies

RATIH ASMANA NINGRUM, DEBBIE SOFIE RETNONINGRUM, YEYET CAHYATI, AND HENI RACHMAWATI*

School of Pharmacy, Institut Teknologi Bandung, Jalan Ganesha 10, Bandung 40132, Indonesia

The hIFN2b open reading frame has been constructed and overexpressed in *Escherichia coli* BL21(DE3). The yields of protein purified using nickel column from inclusion bodies (IB) and total soluble proteins were 3.46 mg and 2.57 mg in 1 L culture, respectively. This research was aimed to obtain optimal condition for high level overproduction of soluble hIFNα2b as well as primary recovery of hIFN2b from IB. We used two different conditions for obtaining soluble protein, i.e. induction temperatures and inducer concentrations, and three different conditions for inclusion bodies, i.e. centrifugation speeds, washing and solubilizing buffers. Induction using 0.5 mM of isopropyl thiogalactopyranoside at 25 °C yielded 8.9 mg hIFN2b in 1 L culture. The best recovery of IB was achieved when 10 000 g was applied for centrifugation, 1% Triton X-100 in 50 mM Tris Cl pH 8.0 as washing buffer, and 8M guanidine HCl in 50 mM Tris Cl pH 8.0 containing 800 mM 2-mercaptoethanol as solubilizing buffer were used. At this optimal condition the yield of hIFN2b from IB was 28.85 mg in 1 L culture. The total recovery of hIFNα2b at optimal condition was 50% from IB and 14% from soluble protein. hIFN2b from IB was refolded by 9 d dialysis in refolding buffer (0.2 mM EDTA, 0.25 mM ditiothreitol, 50 mM Tris and 0.4 M urea pH 8.0).

Key words: human interferon α2b, overproduction, soluble protein, inclusion bodies, protein refolding

Kerangka baca terbuka hIFN2b telah dikonstruksi dan diekspresikan dalam *Escherichia coli* BL21(DE3) pada penelitian sebelumnya. Jumlah protein yang dimurnikan menggunakan kolom nikel dari badan inklusi (BI) dan protein terlarut total ialah masing-masing 3.46 dan 2.57 mg dalam 1 L biakan. Penelitian ini ditujukan untuk memperoleh kondisi optimal pada overproduksi hIFNα2b terlarut dan perolehan hIFNα2b dari BI. Dua kondisi yang berbeda digunakan untuk mendapatkan protein terlarut, yaitu suhu dan konsentrasi badan penginduksi, serta tiga kondisi yang berbeda untuk BI, yaitu kecepatan sentrifugasi, dapar pencuci, dan pensolubilisasi. Penggunaan suhu 25 °C dan isopropil tiogalaktopiranosida 0.5 mM sebagai penginduksi telah menghasilkan hIFN2b sebanyak 8.9 mg dalam 1 L kultur. Perolehan terbaik dari badan inklusi diperoleh dengan menggunakan kecepatan sentrifugasi 10 000 g, dapar pencuci Triton X-100 1% dalam Tris Cl 50 mM pH 8.0, dan dapar pensolubilisasi guanidine HCl 8M yang mengandung 2-merkaptoetanol 800 mM dan Tris Cl 50 mM pH 8.0. Dengan menggunakan kondisi optimal dihasilkan hIFN2b dari BI sebanyak 28.8 mg dalam 1 L biakan. Renaturasi hIFN2b dari BI dilakukan dalam dapar perenaturasi (EDTA 0.2 mM, ditiotreitol 0.25 mM, Tris 50 mM, dan urea 0.4M pH 8.0) selama 9 hari.

Kata kunci: interferon 2b manusia, overproduksi, protein terlarut, badan inklusi, pelipatan kembali protein

Interferon (IFN) is a cytokine produced and secreted by almost all eukaryotic cells as a response to viral, bacterial, antigen, or mitogen stimuli. Based on their receptor types on the cell membrane surface, IFN is classified into type I and type II. Type I consists of IFNα, IFNβ, IFNτ, and IFNω. While type II consists of IFNγ (Wang *et al*. 2002; Gao *et al*. 2004). IFNα has wide range of biological activities ranging as antiproliferation, immunomodulation, and antivirus (Samuel 2001). Human IFN2b (hIFN2b), as a subclass of IFNα, is a glycoprotein consisting of 165 amino acids with size of 19 271 Dalton. The molecule's O-glycosylation at threonine position 106 is not important for its biological activity (Nyman *et al.* 1998). hIFN2b has two disulfide bridges formed by cysteins (between positions 1 and 98, and between 29 and 138). Previous study reported that disruption of disulfide bridges formed by cystein 1 and 98 resulted in higher antiviral activity (Neves *et al.* 2004). So far hIFNα2b is used as a therapeutic protein for hepatitis B and hepatitis C treatments, both as a single therapy or in combination with other nucleoside analogs (Jonasch and Haluska 2001). Its use to treat several types of cancer, i.e. multiple myeloma, chronic myeloid leukemia, non-Hodgkin's lymphoma, renal cell carcinoma, epidermoid cervical cancer, head and neck tumours, melanoma and medullary thyroid carcinoma, had also been reported as well (Wang *et al*. 2002).

In vitro DNA manipulation has been applied to express many eukaryotic genes in prokaryotes, such as *Escherichia coli*. However, the use of *E. coli* as an expression host often results in the formation of insoluble protein in inclusion bodies (IB). For proteins containing disulfide bridges, IB formation tends to be higher than those without disulfide bridges. In order to recover its activity several procedures including solubilization, refolding, and purification is absolutely

*Corresponding author: Phone: +62-22-2504852, Fax: +62-22-2504852 Email: h_rachmawati@fa.itb.ac.id

necessary (Rabhi-Essafi *et al.* 2007). Two factors that influence IB formation are protein properties (average charge, cysteine and proline contents, hydrophilicity, and total number of residues) and environmental or culture condition (temperature, pH, and nutrient supply) (Fischer and Sumner 1993; Rabhi-Essafi *et al.* 2007). Several publications have reported cloning and overexpression of hIFN-2b gene in *E. coli* with various yields (Neves *et al.* 2004; Srivasta *et al.* 2005; Valente *et al.* 2006). In addition, optimization of IB primary recovery of hIFN-2b expressed in *E. coli* has been reported. The recovery of IB from optimal condition ranged from 61% to 68% (Valente *et al.* 2006).

In our previous work (Retnoningrum *et al*. 2010), we assembled synthetic open reading frame (ORF) encoding for hIFN-2b using thermodynamically balanced inside out method. The ORF was cloned and overexpressed in *E. coli* BL21(DE3). The recombinant hIFN-2b was produced as a fusion protein of 37 kDa, containing thioredoxin and polihistidine tag at its N terminus. The protein was confirmed to be hIFN-2b by Nano LC MS/MS resulting 80% amino acids coverage. Therefore, this research aimed to obtain optimal condition for overproduction of hIFNα2b in soluble form and primary recovery of hIFN2b from IB. Optimal condition will be used to obtain highest expression level in the future.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Media. *E. coli* BL21(DE3) harbouring pET32b-hifnα2b recombinant plasmid from previous work was used for gene expression (Retnoningrum *et al.* 2010).

Overproduction of hIFN-2b. *E. coli* BL21(DE3) pET32b-*ifn2b* overnight culture was added (3% v v^{-1}) to 100 mL of LB broth containing 100 µg mL^{-1} of ampicillin. The culture was incubated for about 1.5 h in a shaking incubator at 37 °C 200 rpm. Three final concentrations of IPTG (0.25, 0.5, and 1.0 mM) were each added to midlog phase of cell culture. Incubation was continued for an additional 3 h. Cell pellet was harvested by centrifugation at 5000 g for 10 min. The cells were resuspended in lysis buffer (50 mM NaCl and 1 mM EDTA), the composition that was reported by Valente *et al.* (2006). The cells were lyzed by sonication at 2.5 Hertz in the presence of 1 mM of phenylmethyl sulphonyl fluoride. To prevent temperature elevation, the cells were sequentially sonicated and cooled on ice for 10 times, each time for 30 s. Soluble recombinant protein was separated from IB by centrifugation at 10 000 g. The soluble protein was purified using nickel column according to the manufacturer's protocol (Protino, Germany). The protein isolation and overproduction steps were monitored using 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). To determine the yield, protein concentration was measured based on coomassie blue staining using Bradford method with standard concentrations of bovine serum albumin were ranging from 125 to 1000 µg mL^{-1}.

Optimization of Inclusion Bodies Primary Recovery. Optimization of inclusion bodies primary recovery referred to condition as reported by Valente *et al*. (2006) with some modifications. After sonication, IB was separated from soluble protein by centrifugation for 10 min. Five centrifugation speeds 500, 1000, 2500, 5000, and 10 000 g were tested in this experiment. The cells were washed with washing buffer (750 µL of buffer for each 0.1 g of pellet), incubated at room temperature for 5 min and centrifuged at 12 000 g for 5 min. The washing step was repeated to remove impurities. Two washing buffers were studied, i.e. buffer A (50 mM Tris Cl pH 8 containing 1% Triton X-100) and buffer B (lysis buffer containing 0.5% Triton X-100 pH 7.2). The washed pellet was resuspended in solubilizing buffer (750 µL of buffer for each 0.1 g of pellet) for 30 min at room temperature. Two solubilizing buffers were tested, guanidine HCl (GdnHCl) buffer consisted of 6 M GdnHCl in 50 mM Tris-HCl pH 8 containing either 2-mercaptoethanol (2-ME) or dithiotreitol (DTT). To observe the effect of reducing agents, various concentrations of 2-ME (100, 200, 400, 650, and 800 mM) and DTT (20, 50, 100, 200, and 300 mM) were tested. Subsequently, solubilized pellet was centrifugated at 15 000 g for 15 min and 4 °C to separate hIFNα2b and unsolubilized material. hIFNα2b from IB was characterized using SDS-PAGE and its concentration was measured.

Protein Refolding. Dialysis was perfomed to refold hIFNα2b from IB. Two steps were applied, first step to remove denaturant using denaturing removal buffer and the second step to renaturate hIFNα2b from IB using refolding buffer (Valente *et al*. 2006). To remove the denaturant, 0.6 mg of IB was dialyzed overnight in the cellulose ester membrane (Spectra/Por® CE, 1 mL volume capacity, MWCO 1000) against 250 mL denaturing removal buffer (0.2 mM EDTA in 50 mM Tris, pH8.0) at 4 °C with gentle agitation. To renaturate the hIFNα2b from IB, the hIFNα2b was dialyzed in 250 mL refolding buffer (0.4 M urea, 0.2 mM EDTA, 0.25 mM DTT in 50 mM Tris, pH 8.0) for 9 d with buffer changes every

2 d. To monitor the renaturation process, refolded hIFNα2b was analyzed using non reducing SDS-PAGE.

RESULTS

Overproduction of hIFN-2b. The expression profile of soluble hIFN2b showed that the best expression level was achieved at 25 °C with 0.5 mM IPTG induction (Fig 1). The purity of hIFN2b after purification using nickel affinity chromatography was analyzed using 15% polyacrylamide gel as shown in

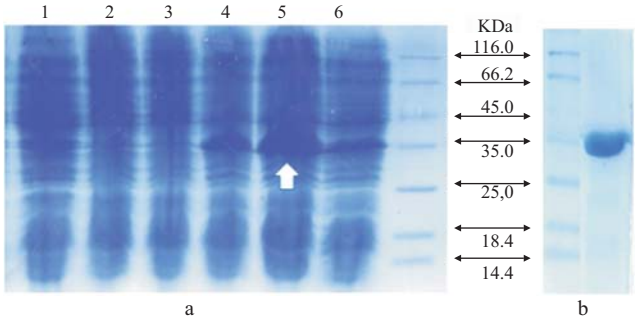

Fig 1 SDS-PAGE of human interferon 2b (hIFN2b) soluble protein. a, Lanes 1-3: total soluble protein produced at 37 °C with 0.25, 0.5, and 1.0 mM of IPTG. Lanes 4-6: total soluble protein produced at 25 °C with 0.25, 0.5, and 1.0 mM of IPTG. b, Purified hIFN2b soluble protein obtained from optimal condition of overproduction (at 25 °C with 0.5 mM IPTG).

Fig 1b. Under optimal overproduction condition, the yield of purified hIFN2b was 8.9 mg protein in 1 L culture. The total recovery of purified hIFN2b was 14% from total soluble protein.

Optimization of IB Primary Recovery. The solubilization step showed minor impurities in all centrifugation speeds used (Fig 2a). The highest amount of hIFNα2b from IB was achieved at 10 000 g (Fig 2b). We studied two washing buffers to compare its ability to remove impurities. The impurity profiles of the two buffers looked the same (Fig 3a) but buffer A showed higher IB recovery than buffer B (Fig 3b).

We used GdnHCl buffer containing various concentration of 2-ME or DTT in solubilization step. By this mean the impurity profiles did not differ (Fig 4a and b). However, higher IB recovery was achieved when highest concentration of 2-ME was used (Fig 4d). Under this optimal condition the hIFNα2b from IB that can be recovered was 28.85 mg L^{-1} (50%), hence made up 64% total yield of hIFNα2b protein (14% from soluble protein and 50% from IB).

Non-reducing SDS-PAGE was used to check the renaturation result. This method can distinguish protein electrophoretic mobility based on its disulfide bond numbers. One upper band (Fig 5 lane 2) had the same size with solubilized IB and reduced soluble

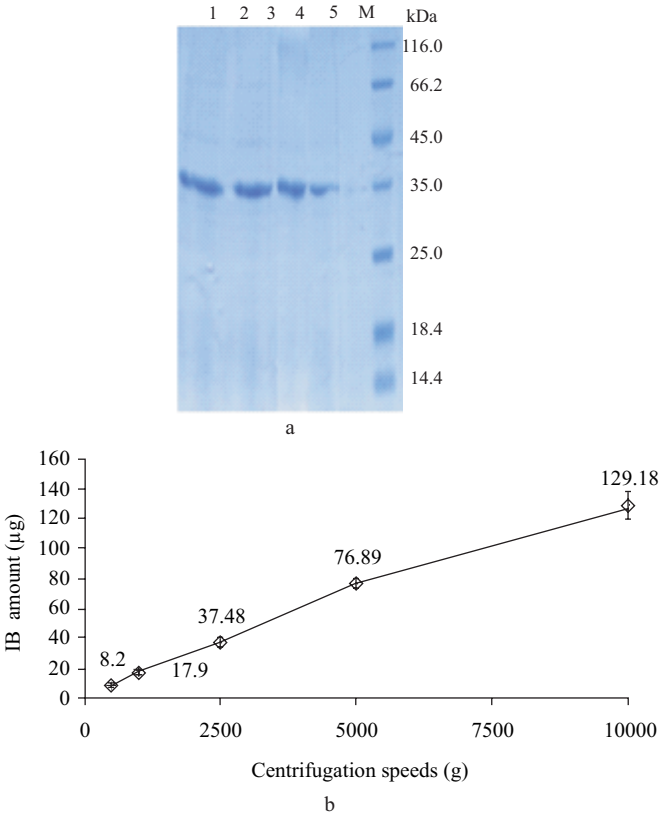

Fig 2 The effect of centrifugation speeds on human Interferon 2b (hIFN2b) inclusion bodies (IB) yield. a, Lanes 1-5: IB sedimented at 10 000; 5000; 2500; 1000; and 500 g. Pellet weight was 0.1 g and loaded volume was 5 µL. b, Amount of IB with different centrifugation speed.

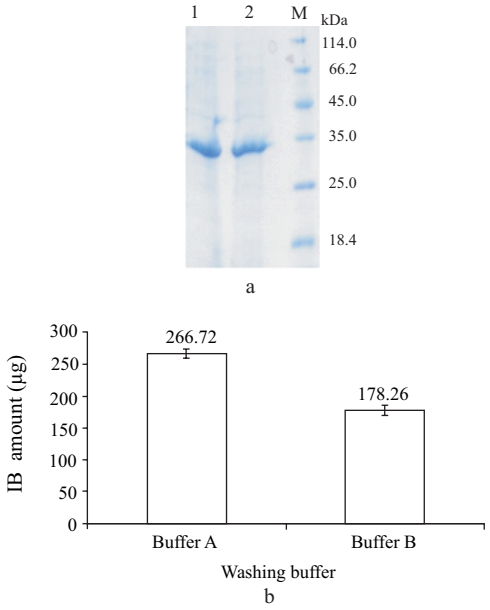

Fig 3 The effect of washing buffer on human interferon 2b (hIFN2b) inclusion bodies (IB) yield. a, Lane 1: IB washed with buffer A (50 mM Tris Cl pH 8 containing 1% Triton X-100); lane 2: IB washed with buffer B (50 mM NaCl and 1 mM EDTA containing 0.5% Triton X-100 pH 7.2). Pellet weight was 0.1 g and loaded volume was 5 µL. b, Amount of IB measured using Bradford method in different buffer.

protein (Fig 5 lane 1 and 3) and lower band had the same size with unreduced soluble protein (Fig 5 lane 4). This means that hIFNα2b from IB was not totally

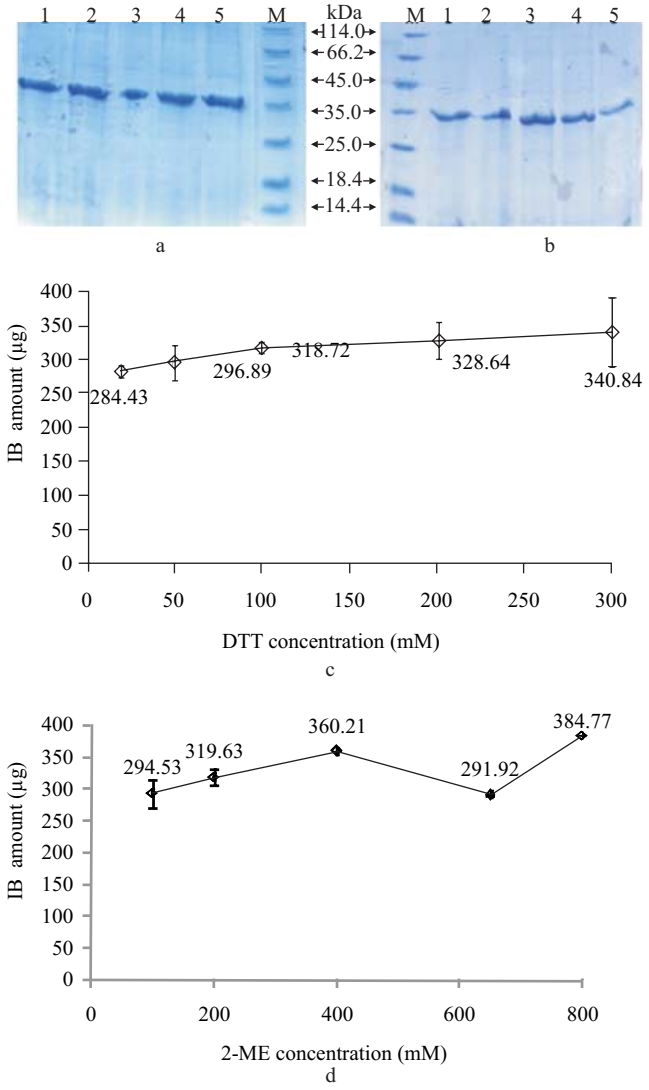

Fig 4 The effect of dithiotreitol and 2-merchapethanol concentrations on the solubilization of human interferon 2b (hIFN2b) inclusion bodies (IB) yield. a, Lanes 1-5: solubilized IB with GdnHCl containing 20, 50, 100, 200, and 300 mM DTT. b, The effect of 2-merchapethanol on the solubilization of hIFN2b IB. Lanes 1-5: solubilized IB with GdnHCl containing 100, 200, 400, 650, and 800 mM 2-merchapethanol (2-ME). The pellet weight was 0.1 g and sample volume loaded was 5 µL. c, IB amount measured by Bradford method in solubilizing buffer containing various concentration of DTT. d, IB amount measured by Bradford method in solubilizing buffer containing various concentration of 2-ME.

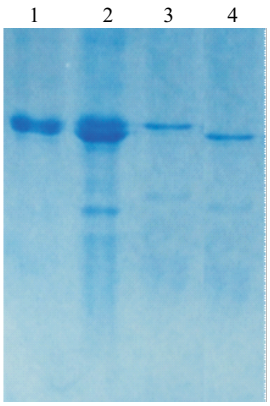

Fig 5 Non-reducing SDS-PAGE of refolded human interferon 2b (hIFN2b). Lane 1: solubilized inclusion bodies; 2: refolded hIFN2b ; 3: reduced soluble hIFN2b; 4: unreduced soluble hIFN2b.

refolded. Only about 50% of hIFNα2b was refolded and formed disulfide bridges.

DISCUSSION

Several approaches have been reported to improve the solubility of recombinant hIFN2b including using different expression systems (using *Streptococcus lividans, Pichia pastoris, Bacillus subtilis*, murine myeloma cell, and baculovirus) and different affinity tags (thioredoxin or gluthatione S transferase) (Fischer and Sumner 1993). The purification of soluble hIFN2b is more cost effective and less time consuming over refolding and purification from IB. Hence, optimizing production step of recombinant hIFN2b is the most reasonable alternative to reduce IB formation. Previously, we tried to improve hIFN2b solubility by using low copy number plasmid (pET32b) and constructing the hIFN2b fusion protein containing thioredoxin tag. We observed that environmental factors, i.e temperature and IPTG concentration, affected the yield. Current results confirmed that the best expression level was achieved at 25 °C with 0.5 mM IPTG. Nevertheless, at 37 °C, soluble protein was not obtained when induction was done using 0.25 and 0.5 mM IPTG. This might be due to high level of IB formation. Only when induction was performed using 1.0 mM IPTG soluble protein produced at low level. The result indicates that lowering temperature to 25 °C whilst still using 0.5 mM IPTG can decrease protein synthesis, therefore, increasing the formation of soluble hIFN2b. Our result is comparable with previous work showing the overproduction of gluthatione S transferase hIFN2b fusion protein at 25 °C with 0.5 mM IPTG and GFP CheY fusion protein at 25 °C with 8 µM IPTG (Rabhi-Essafi *et al.* 2007; Sevastsyanovich *et al.* 2009). However, Cirkovas and Serekaite (2010) reported that overproduction conducted under low temperature 20 °C improved the solubility of mink growth hormone. In this study, it was demonstrated that simultaneous decrease IPTG concentration into micromolar range did not reinforce the effect of temperature.

The centrifugation speeds at 5000 to 20 000 g commonly used to separate IB from homogenate (Fischer and Sumner 1993). Since the high speed can lead to co-sedimentation of *E. coli* proteins, hence increasing the impurities, we studied various centrifugation speeds (500 to 10 000 g) to obtain the highest hIFNα2b recovery with the lowest impurities. We observed that the impuritiy profiles from each speed did not demonstrate significantly different and

the highest amount of hIFNα2b from IB was obtained at 10 000 g. Lowering centrifugation speeds did not lead to high hIFNα2b recovery and did not have any effect on eliminating impurities. Valente *et al.* (2006) reported that at least 5000 g was needed to recover more than 93% of hIFNα2b from IB but increasing the speed tend to give higher impurities.

IB may contain protein impurities originated from *E. coli* such as RNA polymerase, outer membrane proteins, or enzymes. Membrane proteins or kanamycin phosphotransferase are soluble impurities. Other IB impurities were washed using buffer containing Triton X-100, sucrose or urea. Triton X-100 that is normally used at concentrations ranging from 0.5% to 5% (Fischer and Sumner1993). We observed that varying concentrations of Triton (0.5% to 1%) to remove the impurities did not affect hIFNα2b purity. The higher recovery achieved by buffer A might be due to higher pH (washing condition at pH 8.0 on buffer A comparing to at pH 7.2 on buffer B) that stabilized the hIFNα2b in washing step. Valente *et al.* (2006) compared two washing buffer, i.e. 20 mM Tris in 50 mM EDTA pH 8.0 and 50 mM Tris-Cl containing 1% Triton pH 8. Although the latter buffer resulted in higher purity of IFNα2b, it did not have significant effects on hIFNα2b recovery.

Generally, there are two denaturants used to break existing intramolecular and intermolecular disulfide bonds i.e. urea and GdnHCl. It has been proven that urea combined with DTT is not good for IFNα2b IB solubilization (Valente *et al.* 2006). We examined GdnHCl buffer containing various concentration of 2-ME or DTT as reducing agents. Higher amount of reducing agents reported might lead to co-solubilization of *E. coli* protein (Valente *et al.* 2006), however current results showed no difference in impurity profiles of each DTT and 2-ME concentration. Higher hIFNα2b recovery was achieved when the highest concentration (800 mM) of 2-ME was applied. So far, the highest concentration of 2-ME that ever used is 500 mM (Fischer and Sumner 1993). However, there is no good explanation of why using 650 mM of 2-ME resulted in lowest recovery. The total yield of hIFNα2b protein was 64%, which is higher than previous study reported by Srivasta et *al*. (2005) and Valente *et al.* (2006).

Renaturation step after IB solubilization is required to obtain native conformation and to catalyze disulfide bond formation. The denaturants must be removed and IB must be renaturated in oxidizing buffer (Fischer and Sumner 1993). In this study, we used buffer containing Tris and EDTA pH 8.0 to remove denaturant and oxidizing buffer containing urea, DTT, and EDTA pH 8.0. hIFNα2b protein was renatured in refolding buffer for 9 days based on previous publication report (Valente *et al*. 2006). The refolded hIFNα2b protein profile on native gel showed two different bands. The lower band that has same size with the non-reduced soluble protein should correspond to biologically active hIFNα2b. So it seems that only about 50% of hIFNα2b that could be successfully refolded, so further study is still needed to increase the refolding result. Valente *et al*. (2006) reported three different bands after 9 d renaturation. We suggest that thioredoxin tag in the hIFNα2b facilitates the disulfide bridge formation, although Sachdev and Chirgwin (1998) reported that the MBP and thioredoxin fusions of pepsinogen expressed in *E. coli* did not necessarily facilitate native refolding, but enhanced the recovery of soluble protein.

To conclude, the optimal condition to obtain hIFNα2b soluble protein and primary recovery of its inclusion bodies has been established yielding 8.9 mg protein of soluble protein with 14% recovery and 28.85 mg of IB with 50% recovery from 1 L culture.

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