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Article

The improvement of anti-HER2 scFv soluble expression in *Escherichia coli*

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The relationship between the expression of HER2 and malignity of breast tumors has led to the generation of antibodies targeting HER2$^+$ tumors. In addition, the expression of scFvs, as the smallest antigen-binding region of antibody containing two disulfide bonds in *Escherichia coli* often results in accumulating non-functional protein in the cytoplasm. A redox-modified strain of *E. coli* such as Origami (DE3) may facilitate the formation of proper disulfide bond in cytoplasm. The present study aimed to optimize the expression of anti-HER2 scFv in Origami and evaluate the influence of induction temperature, and host strain on the solubility of the protein. To this aim, chemically synthesized anti-HER2 scFv of Trastuzumab was cloned in pET-22b (+). The results demonstrated that anti-HER2 scFv is expressed in Origami, purified by using Ni-NTA column, and detected by anti-His antibody in Western blot analysis. The highest anti-HER2 scFv expression in Origami was achieved 24 h after IPTG induction (1 mM) at 37 °C. Further, the total anti-HER2 scFv expression level was higher in BL21, compared to Origami strain. However, the ratio of soluble/insoluble forms of anti-HER2 scFv increased in Origami strain. Furthermore, higher soluble expression was achieved when the culture of recombinant Origami was conducted at lower temperature (25 °C).

Keyword: Breast cancer. HER2. scFv. Expression. Solubility.

INTRODUCTION

The overexpression of HER2 receptor has been usually observed in 20-30 % of breast cancers (Kiewe *et al.*, 2006). HER2, a 185-kDa transmembrane protein (Rubin, Yarden, 2001), belongs to "human epidermal growth factor receptor" family including HER1 (ErbB1), HER3 (ErbB3) and HER4 (ErbB4) (Yarden, Sliwkowski, 2001). HER2 has no direct ligand and acts as a heterodimerization partner with other members of HER family, which plays a critical role in both development and cancer (Burgess *et al*., 2003). Therefore, monoclonal antibodies interfering with receptor dimerization of HER2 is considered as an attractive target for antibody-based therapeutic strategies in breast cancer therapy (Kiewe *et al*., 2006). Trastuzumab (Herceptin®) is a blocking antibody for HER2 signaling (i.e. ras- Raf-MAPK and PI3K/Akt) (Vrbic *et al.*, 2013) and plays a major role on treating HER2$^+$ metastatic breast cancer (Tan, Swain, 2003).

Single chain fragment variable (scFv), as the most widely used antibody fragment, includes the variable heavy (V_H) and variable light (V_L) domains of an antibody which linked together by a flexible polypeptide linker (Ahmad *et al*., 2012). scFv antibody demonstrates faster pharmacokinetics and potentially more homogenous tumor penetration related to large IgG molecules due to the reduced size and lack of Fc domain (Kelly *et al*., 2008). scFvs have been used in different therapeutic such as immunoliposomes (Park *et al.*, 2002), immunotoxins and radioimmunotherapy (Cao *et al.*, 2012; Cao *et al.*, 2009; Kelly *et al.*, 2008), as well as diagnostic strategies like radioimmunodetection (Kelly *et al.*, 2008; Pucca *et al*, 2011).

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Due to the lack of glycosylation in scFv fragment (Guglielmi, Martineau, 2009), a large number of studies focused on *E. coli* as a proper system for scFv production (Guglielmi, Martineau, 2009; Leong, Chen, 2008; Verma, Boleti, George, 1998). The overexpression of scFv proteins with two disulfide-bond in *E. coli* cytoplasm results in losing correct conformation and accumulation into insoluble aggregates and non-functional proteins (Guglielmi, Martineau, 2009). In this regard, many strategies consider the expression of proteins containing disulfide bonds in the *E. coli* cytoplasm because of these limitations. Further, adjusting the reduction-oxidation pathway in *E. coli* can significantly improve the effective expression of bioactive disulfide-containing proteins (De Marco, 2009) such as antibody fragments in *E. coli* cytoplasm (Guglielmi, Martineau, 2009).

Cysteines in the *E. coli* cytoplasm are retained reduced by thioredoxin reductase and glutaredoxin pathways (Faulkner *et al.*, 2008). The *trxB* and *gor* negative strains of *E. coli* alone or in combination with the expression of foldases/chaperones are commonly accepted and effectively exploited (De Marco, 2009). Among these strains, Origami from Novagen with mutation in trxB and gor genes can improve the folding of recombinant proteins containing disulfide bond in the *E. coli* cytoplasm (Salinas *et al.*, 2011; Sørensen, Mortensen, 2005).

It is known that different parameters such as the expression strain, IPTG concentration, the duration and temperature of induction (San-Miguel, Perez-Bermudez, Gavidia, 2013) can affect the yield and solubility of the expressed proteins (Tolia, Joshua-Tor, 2006). A large number of studies investigated the optimum expression condition to improve the yield of soluble and active protein without any need for refolding (San-Miguel *et al.*, 2013).

The current study aimed to express soluble anti-HER2 scFv version of trastuzumab in the cytoplasm of *E. coli*. Additionally, the influence of isopropyl-beta-D-thiogalactopyranoside (IPTG) concentrations, duration and temperature of induction, as well as strain type on the expression of anti-HER2 scFv was evaluated in this study.

MATERIAL AND METHODS

Bacterial strains, plasmid and reagents

E. coli strain Origami (DE3) and the protein expression vector pET-22b (+) were kindly gifted from Dr. Nematollahi and Dr. Behddani, respectively (Pasteur Institute of Iran). Then, *E. coli* strain was grown in Luria–Bertani (LB) medium (Floka). The growth medium was supplemented with the ampicillin (100 µg/mL) and kanamycin (biobasic) at the final concentration of 50 µg/mL, when required. All of the used chemicals and reagents were provided from standard commercial sources such as Merck.

Construction of anti-HER2 scFv expression vector

The anti-HER2 scFv gene fragment was cloned into pET-22b (+). Briefly, the gene encoding anti-HER2 scFv based on V_L and V_H sequences of Trastuzumab (drug bank number DB00072) was synthesized in which the codon usage was optimized for high expression in *E. coli.* Then, the *Nco*I-anti-HER2 scFv-*Xho*I digested fragment was cloned into pET-22b (+).

Expression of anti-HER2 scFv protein

A single colony of the pET-22b (anti-HER2 scFv)-transformed Origami (DE3) strain was used to inoculate pre-culture LB broth medium containing 100 µg/mL of ampicillin. Then, the culture were grown at 37 °C to an OD_{600} of 0.5-0.6. Then, the protein expression was induced at 37 °C with 1 mM IPTG and the cells were harvested within desired intervals. After induction, the bacterial biomass was collected by centrifugation (10,000 × g for 5 min). Then, the bacterial pellet was resuspended in sample buffer 2X (4% SDS, 20% glycerol, 2% 2-mercaptoethanol (2- ME), 0.01% bromophenol blue, 500 mM Tris-HCl, pH 6.5) and protein expression was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. Further, the stained SDS-PAGE gels were recorded and the percentage of anti-HER2 scFv band was evaluated by using the Total Lab TL 120 software. In addition, the total protein concentration of each sample was determined by BCA assay (Parstous Company) using the bovine serum albumin (BSA) as the protein standard. Finally, the concentration of anti-HER2 scFv protein was determined by multiplying the band percentage by the total protein concentration of the sample.

Determination of the optimized expression condition

The expression of anti-HER2 scFv protein was optimized by changing various parameters including induction temperature, concentration of inducer IPTG, and harvesting time following IPTG induction. In order to optimize culture temperature for anti-HER2 scFv expression in Origami (DE3), the cells were grown at 37 °C to reach the optimal density. Then, 1 mM IPTG was added, the expression was carried out at different temperatures

(25, 30 or 37 °C), and the samples were collected 2, 4, 6 and 24 h after induction. In the next procedure, the optimal IPTG concentration was determined. For this purpose, different concentrations of inducer (0.25, 0.5, 1 or 2 mM) were added and cultured at optimum cultivation temperature after reaching the desired OD_{600} of 0.6 at 37 °C. Finally, the samples were collected at the optimum harvesting time after induction.

Determination of anti-HER2 scFv protein solubility

Following the induction step, the harvested cells were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM Imidazole, pH 8). Then, the bacterial suspension was incubated on ice for 30 min in the presence of lysozyme (1 mg/mL), DNase (Sigma, 100 µg/mL), and $MgSO_4$ (100 mM), and disrupted by sonication (300 W of 7 s separated by 8 s intervals for total of 30 min, Topsonics, Iran). Then, the suspension of disrupted cells was centrifuged at 10,000 × g for 20 min at 4 °C in order to separate soluble and insoluble fractions. Finally, the fractions were resuspended in sample buffer 6X (10% SDS, 30% glycerol, 6.8% 2-mercaptoethanol (2- ME), 0.01% bromophenol blue, 500 mM Tris-HCl, pH 6.5) and then analyzed by 12% SDS-PAGE gel, followed by coomassie blue staining. Finally, the SDS-PAGE gel was analyzed by using the Total Lab TL 120 software and the density of bands related to soluble and insoluble fragments of the lysate was determined.

Anti-HER2 scFv purification

First, the purification of recombinant anti-HER2 scFv was conducted by prepack Ni-NTA affinity chromatography column (Qiagen), based on the manufacturer's instructions for native condition. Briefly, the induced bacterial cells were resuspended in lysis buffer, incubated in the presence of lysozyme, disrupted by sonication, and finally centrifuged as described earlier. In the next step, the presence of anti-HER2 scFv in supernatant (the soluble fraction) was verified by SDS-PAGE analysis and then loaded onto Ni-NTA affinity chromatography column. Then, the column was washed by washing buffer (50 mM NaH_2PO_4, 300 mM NaCl and 20 mM Imidazole, pH 8). Finally, anti-HER2 scFv recombinant protein was eluted by elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM Imidazole, pH 8).

Western blot analysis

The equivalent amounts of samples were resolved on a 12% SDS-PAGE and the separated bands were transferred to a polyvinylidene difluoride (PVDF) membrane (Roche). Further, nonspecific binding was blocked using 2.5 % bovine serum albumin (BSA, Atocell) in tris bufferd saline with tween 20 (TBS-T) (Tris-HCl 10 mM pH 7.4, NaCl 150 mM ,and 0.1% tween 20) overnight at 4 °C. The membrane was incubated with mouse monoclonal anti-poly Histidine antibody (Sigma Aldrich) (diluted 1:10000 in blocking buffer) for 90 min at room temperature after washing with wash buffer (TBS-T) for three times. Furthermore, after another washing procedure, the membrane was incubated with goat peroxidase-conjugated anti-mouse IgG antibody (Sigma Aldrich) (diluted 1:5000) as a secondary antibody for 60 min at room temperature. Finally, the membrane was visualized by 0.6 mg/mL 3,3'-Diaminobenzidine (DAB, Sigma) in 0.12 % H_2O_2 and 1M Tris-HCl.

Statistical analysis

The data were analyzed by using GraphPad Prism software (version 6, USA). One-way ANOVA, followed by Tukey post-test, was used to compare multiple groups. $p<0.05$ was considered as statistically significant different.

RESULTS

Expression, purification and Western blot analysis of the recombinant anti-HER2 scFv in Origami (DE3)

The expression of the recombinant anti-HER2 scFv in Origami (DE3) host was induced by 1 mM IPTG at 37 °C. Then, the samples were collected by centrifugation 2, 4, 6 and 24 h after induction. The pET-22b without insert was used as negative control for expression. Compared to pET-22b without insert as negative control, the over-expressed protein band of approximately 28 kDa was observed in induced samples. The recombinant protein was highly expressed 24 h after IPTG induction (Figure 1A). In addition, the presence of an immunoreactive band of 28 kDa in Western blot analysis confirmed the expressed protein (Figure 1B). Further, anti-HER2 scFv was purified by using Ni-NTA affinity chromatography under native condition. Based on the results, a single band of approximately 28 kDa on coomasie blue stained-(SDS-PAGE) demonstrated the presence of highly purified protein (Figure 1C).

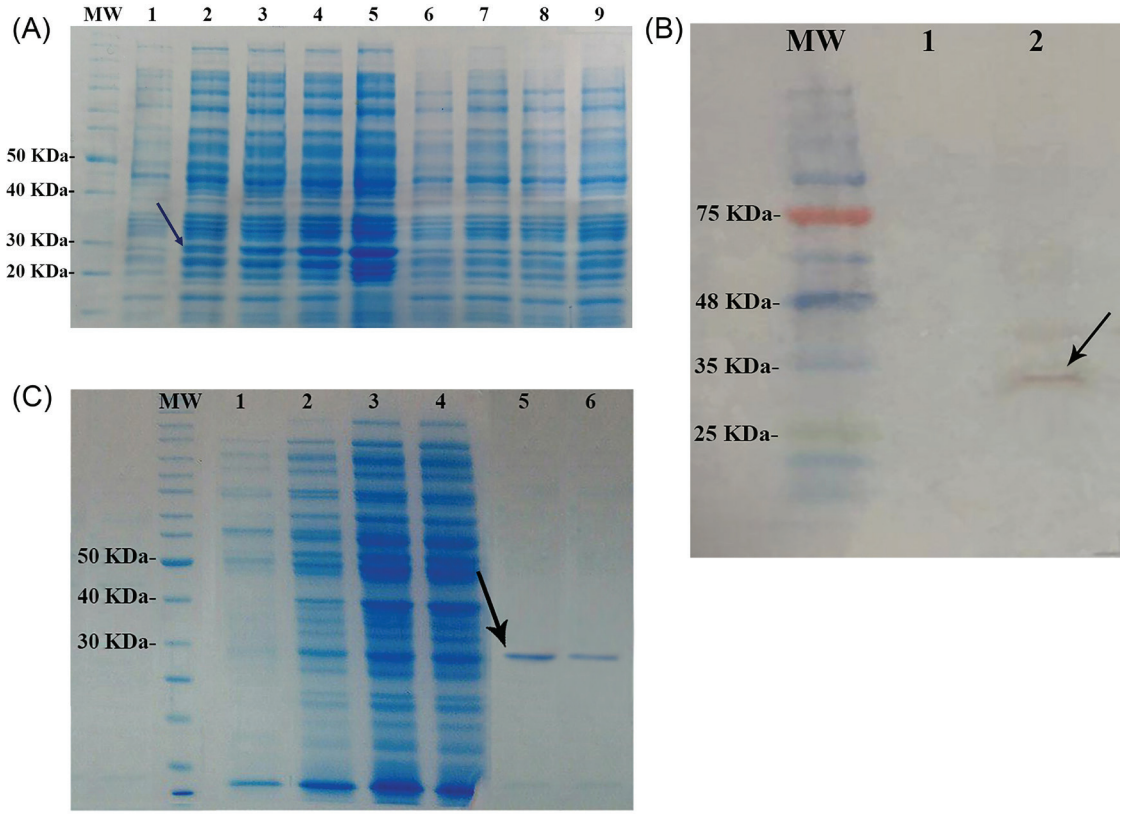

FIGURE 1 - Anti-HER2 scFv expression, purification and Western blot analysis. A) Analysis of anti-HER2 scFv expressed in Origami: Protein marker (Fermentas) (MW), total protein from Origami (DE3) containing pET-22b (anti-HER2 scFv) plasmid before induction (lane 1) after induction with 1 mM IPTG for 2 h (lane 2), 4 h (lane 3), 6 h (lane 4), 24 h (lane 5) at 37 °C, total protein from Origami (DE3) containing pET-22b (without insert) before induction (lane 6), after induction with 1 mM IPTG for 2 h (lane 7), 4h (lane 8), 6 h (lane 9) at 37 °C. B) Western blot analysis of the expressed anti-HER2 scFv using anti His tag antibody: Protein molecular size marker (Sinaclon) (MW), negative control (uninduced Origami (DE3) cell lysate) (lane 1), Origami cell lysate transformed by recombinant plasmid 24 h after induction with 1 mM IPTG (lane 2). C) anti-HER2 scFv purified using polyhistidine (His)-tag affinity column chromatography: Protein marker (Fermentas) (MW), Flowthrough (lane 1), wash 1-3 (lane 2-4), purified anti-HER2 scFv protein elution 1 (lane 5), and elution 2 (lane 6).

Optimization of anti-HER2 scFv expression

In the present study, different parameters such as the duration and temperature of induction, along with the concentration of inducer (IPTG), were investigated to optimize anti-HER2 scFv expression in Origami (DE3). Significant higher amount of anti-HER2 scFv was expressed 6 and 24 h following IPTG induction, compared to 2 h post induction time (Figure 2A). Further, anti-HER2 scFv expression was compared at three induction temperatures of 25, 30 and 37 °C. Based on the results, the highest anti-HER2 scFv level was expressed at 37 °C. Furthermore, the anti-HER2 scFv expressed at 37 °C was ~2 fold higher than that expressed at 25 and 30 °C. As demonstrated in Figure 2B, the amount of anti-HER2 scFv at 37 °C was significantly higher than that of 25 and 30 °C (p <0.01). In addition, the effect of different IPTG concentrations (0.25, 0.5, 1 or 2 mM) on anti-HER2 scFv expression level was examined in the present study. The expression of anti-HER2 scFv at 37 °C was not affected significantly by different concentrations of IPTG (Figure 2C).

Comparison of BL21 (DE3) and Origami *E. coli* strains for soluble protein expression

First, the pET-22b (anti-HER2 scFv) vector was separately transformed into *E. coli* strains BL21 (DE3) and Origami (DE3) *E. coli* strains. Then, the growth of both transformed strains was monitored by measuring the OD_{600} of the culture. The protein expression was induced in the same condition for both Origami (DE3) and BL21 (DE3) strains (1 mM IPTG at 37 °C for 24 h) when the bacterial cells reached the OD_{600} 0.6. As shown in Figure 3, BL21 (DE3) and Origami (DE3) reached their log phase (OD_{600} 0.6) 2 and 3 h after bacterial inoculation, respectively.

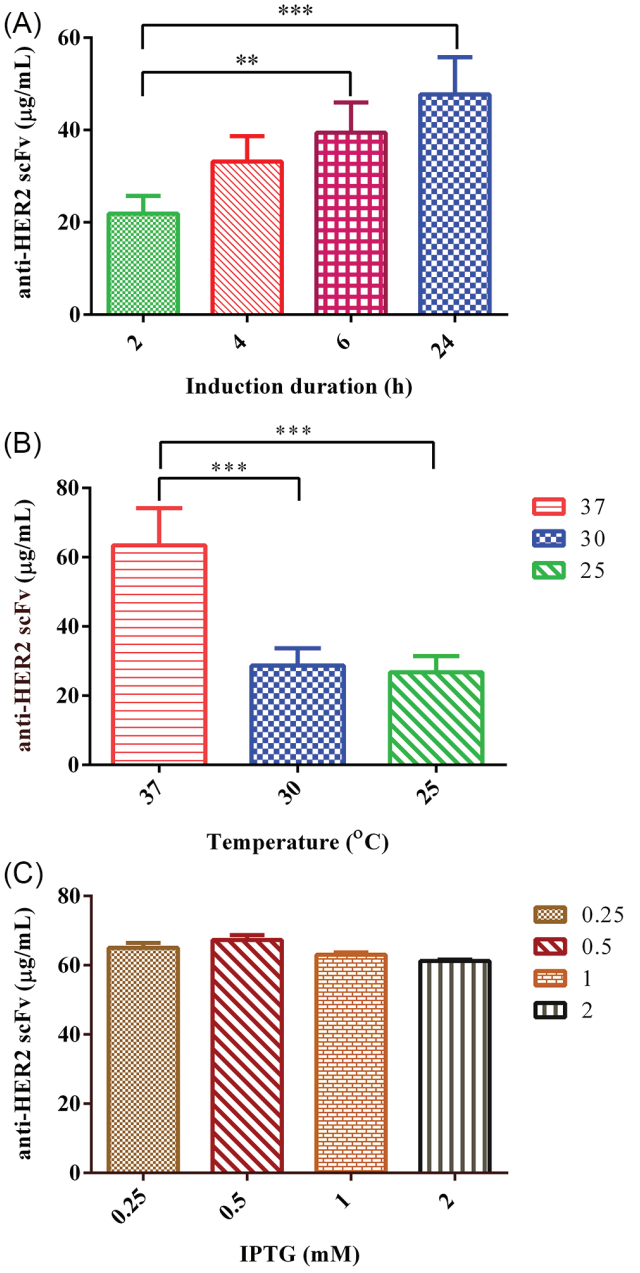

FIGURE 2 - Optimization of anti-HER2 scFv expression condition in Origami (DE3). A) The expression of anti-HER2 scFv protein at various harvesting times following IPTG induction (1 mM) at 37 °C. B) Anti-HER2 scFv expression at different temperatures induced by IPTG (1 mM) after 24 h. C) Induction of recombinant anti-HER2 scFv by different concentrations of IPTG at 37 °C for 24 h. Mean±SD of two independent experiments was analyzed by ANOVA method. $P<0.01$ (**) and $P<0.001$ (***) were considered as the significant difference.

Totally, the growth rate of Origami (DE3) was slower than that of BL21 (DE3).

The level of anti-HER2 scFv expression was 40% of the total protein in *E. coli* BL21 (DE3) and 16.2% in *E. coli* Origami (DE3) (Figure 4A). However, the soluble/insoluble ratio of anti-HER2 scFv was higher in Origami (DE3) than in BL21 (DE3) (Figure 4B).

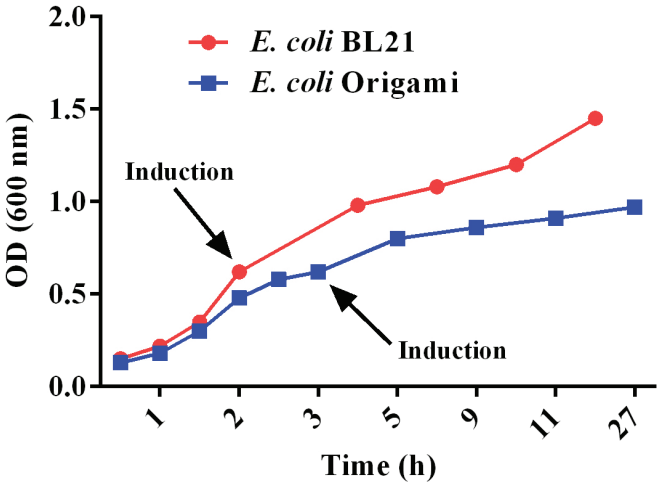

FIGURE 3 - Growth curves of BL21 (DE3) and Origami (DE3) harboring pET-22b (anti-HER2 scFv) plasmid. Recombinant BL21 (DE3) and Origami (DE3) were cultured at 37 °C and the $OD6_{00}$ value was determined at different time points.

Effect of the induction temperature on the solubility of anti-HER2 scFv

Lowering the cultivation temperature is regarded as a common approach to reduce the aggregation of recombinant proteins (Sørensen, Mortensen, 2005). To this end, the effect of induction temperature on the solubility of anti-HER2 scFv was analyzed. Based on the results, the soluble/insoluble ratio of anti-HER2 scFv increased ~20 fold at 25 °C, compared to the Origami (DE3) with induction temperature at 37 °C (Figure 5).

DISCUSSION

The expression of folded antibody fragments due to the formation of inclusion body and the need for refolding processes is difficult in *Escherichia coli* cytoplasm (Arakawa, Ejima, 2014). In addition, obtaining high level of soluble protein is often desirable (Fathi-Roudsari, Akhavian-Tehrani, Maghsoudi, 2016). Thus, the present study emphasized on the expression of anti-HER2 scFv protein in *E. coli* Origami (DE3) with deactivated thioredoxin reductase and glutathione reductase (Bessette *et al.*, 1999) which may lead to the formation of disulfide bonds in more oxidative cytoplasm.

It was reported that the optimization of cultivation conditions is useful to enhance soluble protein expression (Mahgoub, 2012). In this study, the effect of duration and

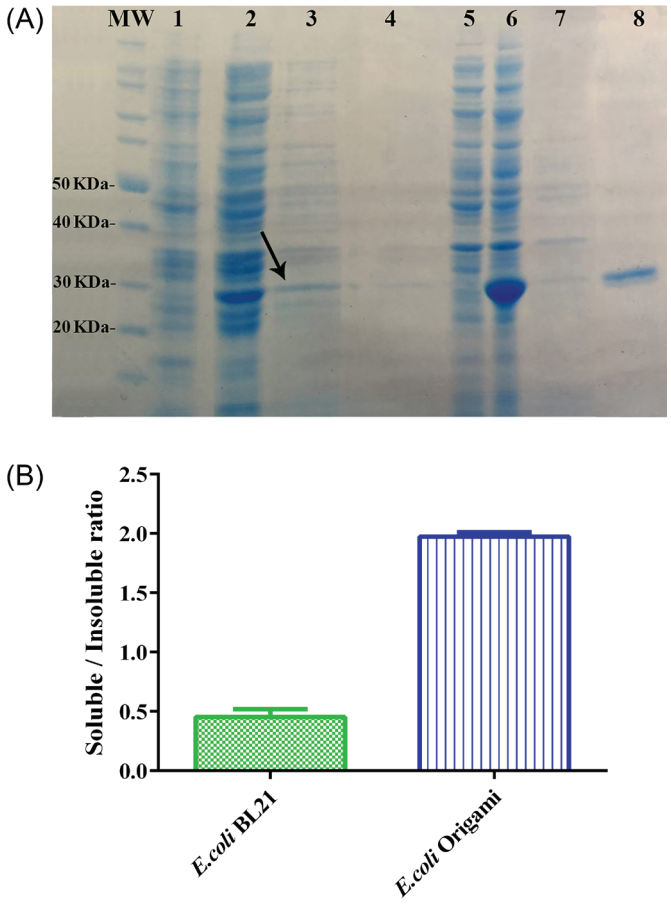

FIGURE 4 - Analysis of solubility of recombinant anti-HER2 scFv expressed in *E. coli* Origami (DE3) and BL21 (DE3). A) Proteins expression was induced for 24 h at 37 °C using 1 mM IPTG. (MW) protein marker (Fermentas), the total protein of *E. coli* Origami (DE3) containing pET-22b (anti-HER2 scFv) plasmid before induction (lane 1), after induction (lane 2), soluble (lane 3) and insoluble (lane 4) fractions of recombinant Origami (DE3) lysate, total protein of *E. coli* BL21 (DE3) containing pET-22b (anti-HER2 scFv) plasmid before induction (lane 5), after induction (lane 6), soluble fraction (lane 7) and insoluble fractions (lane 8) of recombinant BL21 (DE3) lysate. B) The soluble to insoluble ratio of anti-HER2 scFv expression in *E. coli* Origami (DE3) and BL21 (DE3) strain which were evaluated by using the Total Lab TL 120 software.

temperature of induction was evaluated along with the concentration of inducer (IPTG) on the total yield of anti-HER2 scFv. The results demonstrated that the most anti-HER2 scFv was expressed at 37 °C, which are consistent with those of some other studies which reported 37 °C as the best temperature for maximum protein production (Jaliani *et al*., 2014).

The reduction of temperature is considered as a strategy to obtain soluble expression of recombinant protein in *E. coli*. Cultivation at the reduced temperature increases expression and the activity of *E. coli* chaperones.

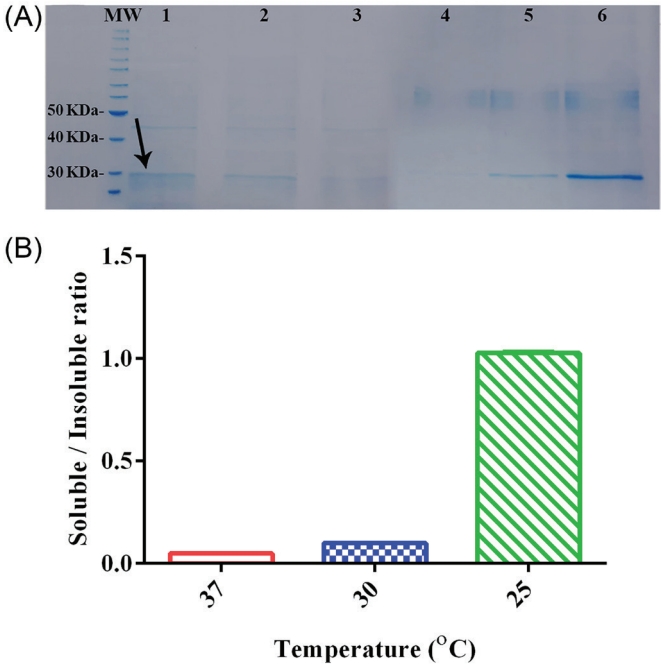

FIGURE 5 - The effect of induction temperature on solubility of anti-HER2 scFv expressed in Origami (DE3). A) The soluble fractions of cell lysates after induction with 1 mM IPTG at 25 (lane 1), 30 (lane 2) and 37 °C (lane 3) for 24 h. The insoluble fractions of cell lysates after induction with 1 mM IPTG at 25 (lane 4), 30 (lane 5) and 37 °C (lane 6) for 24 h. B) The soluble/insoluble ratio of anti-HER2 scFv expression in *E. coli* Origami (DE3) at different temperatures which were evaluated by using the Total Lab TL 120 software.

In addition, hydrophobic interactions which determine the aggregation reaction decrease at low temperature (Sørensen, Mortensen, 2005). Further, the reduction of induction temperature partly excludes the heat shock proteases which are induced under overexpression conditions (Chesshyre, Hipkiss, 1989). Therefore, the influence of induction temperature on the solubility of anti-HER2 scFv was evaluated in this study. Based on the results of this study, the ratio of soluble to insoluble anti-HER2 scFv protein increased when the expression temperature decreased from 37 to 25 °C. The positive effect of reducing the temperature of induction on the protein solubility was confirmed in some other studies. For example, Kim *et al.* reported that the soluble/insoluble ratio of His-tagged scFvMEL/TNF significantly influenced by induction temperature. Furthermore, the higher expression of soluble His-tagged scFv MEL/TNF was obtained with lactose induction at low temperature. The soluble/insoluble ratio of target protein at temperature below 15 °C was higher than that of other temperatures (Kim *et al.*, 2007). Additionally, according to Santala *et al.* (2004), the best yield of soluble biotinylated-scFv was

obtained at the lowest examined induction temperature, 24 °C (Santala, Lamminmäki, 2004).

In addition, the expression of anti-HER2 scFv was compared in BL21 (DE3) and Origami (DE3) *E. coli* strains. Our results demonstrated that the total expression level of anti-HER2 scFv in Origami (DE3) was 40.5% of the level in BL21 (DE3). However, the soluble fraction of anti-HER2 scFv produced in Origami (DE3) was 2-fold of its insoluble fraction. Further, the soluble fraction expressed in BL21 (DE3) cells was 50% of its insoluble fraction. In another study, Yang *et al.* (2009) reported that Origami (DE3) can express multifunctional scFv-H4 and less inclusion body, compared with BL21 (DE3). Similar to the results in the present study, induction temperature could influence more on the expression of scFv-H4 rather than IPTG concentration (Yang *et al.*, 2009). Furthermore, the results of this study are in line with those of Subedi *et al.* (2012) in which the solubility of scFv anti MLS128 fused to protein disulfide isomerase (PDI) increased in an Origami (DE3) bacterial strain, compared to BL21-CodonPlus (DE3). In addition, the results are in agreement with those of Xiong *et al.* (2005) in which higher total yield of the BbFGF proteins, as a model of simple proteins with a single disulfide bond and free cysteines, was achieved in BL21 (DE3), compared to Origami (DE3). Further, the total yield of the HBscFv as the model molecule of complex protein with 2 disulfide bonds in Origami (DE3) was 63-68% of that in M15 [pREP4]. However, they reported that the yield of soluble HBscFv in Origami (DE3) was 4.5-11.1% of the inclusion bodies (Xiong *et al*., 2005). The successful expression and purification of anti-HER2 scFv in Origami (DE3) were reported in the present study. Based on the results, the optimum condition for anti-HER2 scFv expression in Origami (DE3) was obtained 24 h after IPTG induction (1 mM) at 37 °C. Totally, the soluble fraction of recombinant anti-HER2 scFv protein increased in redox-modified host *E. coli* Origami (DE3). However, the growth rate and total expression yield decreased in this host. Finally, the culture related to recombinant Origami (DE3) strain at lower temperature (25 °C) resulted in higher soluble expression.

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