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Original Article

Enhanced Solubility of Anti-HER2 scFv Using Bacterial Pelb Leader Sequence

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Abstract

Single chain Fragment variable (scFv) is an antibody fragment consisting variable regions of heavy and light chains. scFvs enhance their penetrability into tissues while maintaining specific affinity and having low immunogenicity. Insoluble inclusion bodies are formed when scFvs are expressed in reducing bacterial cytoplasm. One strategy for obtaining functionally active scFv is to translocate the scFv into the oxidized environment of the periplasm where the possibility for disulfide bond formation is increased. This can be achieved by cloning the gene in a vector containing N-terminal pelB leader peptide that exports foreign proteins to the periplasmic space. The aim of this study is to evaluate the influence of periplasmic localization using pelB leader peptide on the solubility of anti-HER2 scFv. Herein, anti-HER2 scFv gene was cloned between *Nco*I and *Xho*I sites of pET-22b (+) containing pelB leader peptide and in the same sites of pET-28b (+) (without pelB). The expression in BL21 (DE3) was induced using IPTG and was analyzed using SDS-PAGE and Western blot experiment. Then, the solubility of anti-HER2 scFv in BL21 (DE3) containing both pET-22 and pET-28 (anti-HER2 scFv) was determined. The results of the present study demonstrated that anti-HER2 scFv was expressed by both pET-22b (+) (+) and pET-28b (+) vectors in BL21 (DE3). The proper expression of anti-HER2 scFv was confirmed by appearance of a ~ 28 kDa band in Western blot analysis. The most anti-HER2 scFv expression from BL21 containing pET-28 (anti-HER2 scFv) was achieved when it was induced by 0.25 mM IPTG at 37 ˚C, 24 h post-induction. The ratio of soluble/insoluble anti-HER2 scFv was significantly higher in BL21 containing pET22-(anti-HER2 scFv) than in that containing pET-28 (anti-HER2 scFv). Totally, the fusion of pelB signal sequence to anti-HER2 scFv resulted in solubility enhancement. Therefore, the production of functional anti-HER2 scFv with proper disulfide bond can be achieved by directing the recombinant protein to periplasmic space using pelB signal peptide in pET-22b (+) vector.

Keywords: Disulfide bonds, HER2, pelB, signal peptide, scFv, solubility

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1. Introduction

Full-length antibodies are able to efficiently target antigen-expressing cells; however, due to their large size their penetration into tumor tissues are limited [1]. Therefore, small recombinant antibody fragments are being applied as a substitute for these whole size monoclonal antibodies (mAbs) in treatment and diagnosis of human disease [2]. Single-chain fragment variable (scFv) in which variable regions of heavy and light chains are fused together by a linker sequence, is one of the most common antibody fragments [3]. scFv is the smallest functional region of mAbs that penetrate more easily into tumors and has low immunogenicity while maintaining its affinity to specific antigen [4].

Many different hosts are used for the production of recombinant scFv; however, the best option is *Escherichia coli* due to its easy culturing and genetically manipulation and also having a very short life cycle [5]. High level expression of recombinant proteins in *E. coli* frequently results in the formation of insoluble and/or non-functional proteins accumulation in the cytoplasm as inclusion bodies [3]. It is highly required to refold the inclusion bodies into their proper structure that takes too much time and is an expensive and also difficult process [6].

There are a number of different strategies applied for enhancing the solubility of over-expressed protein [7]. One strategy is to express scFv into the periplasmic space which locates between inner and outer membranes of gram-negative bacteria [8]. Unlike cytoplasm, the periplasmic space is an oxidizing compartment and owns proteins including chaperones and disulfide isomerases that help the expressed proteins to fold correctly [9]. A signal peptide such as pelB is used to guide secretion of the recombinant protein into the periplasmic space. Lack of N-terminal signal peptide for periplasmic secretion leads to accumulation of the expressed recombinant proteins in the bacterial cytoplasm. The unfolded precursors can translocate into the periplasmic space by secretion system (Sec or SRP) through fusion of the protein gene to suitable leader peptides [10]. When the proteins that are attached to signal peptide at the N-terminus direct to periplasmic space, their signal peptide are sliced off allowing proper folding and assembling of the chains and subsequently formation of both the intra- and interdomain sulfide. So far, various specific scFv antibodies have been produced by periplasmic expression [4, 11].

The aim of this study is to evaluate the influence of periplasmic localization using pelB leader peptide on the solubility of anti- human epidermal growth factor receptor-2 (HER2) scFv that was selected as a model recombinant protein containing disulfide bonds. According to the relationship between overexpression of

HER2 which regulates cell growth and differentiation through specific signal transduction pathways [12] and malignity of breast tumors, many monoclonal antibodies and antibody fragments targeting $HER2^+$ tumors have been developed [13, 14]. The fusion of antibody fragment targeting HER2 to different toxins and the application of resultant immunotoxins as cancer therapeutics were reported [15, 16]. Furthermore, the tumor-targeting of anti-HER2 antibodies was combined to the ability of liposomes to deliver cytotoxic drug to construct Anti-HER2 immunoliposomes with increased antitumor efficacy [17, 18]. Therefore, production of high amount of functional anti-HER2 scFv with correctly formed disulfide bridges is highly required. In this regards, herein the effect of different parameters including induction temperatures, isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations and induction periods on anti-HER2 scFvexpression were also investigated.

2. Materials and Methods

2.1 Materials

Escherichia coli Top 10 and BL21 (DE3) (Kindly gifted by Dr. Nematollahi, Pasteur Institute of Iran) were used for molecular cloning and expression of anti-HER2 scFv, respectively. The pET-22b (+) and pET-28b (+) vectors (Novagen USA, Kindly gifted by Dr. Nematollahi, Pasteur Institute of Iran) were used to clone the anti-HER2 scFv gene in downstream of the T7 promoter.

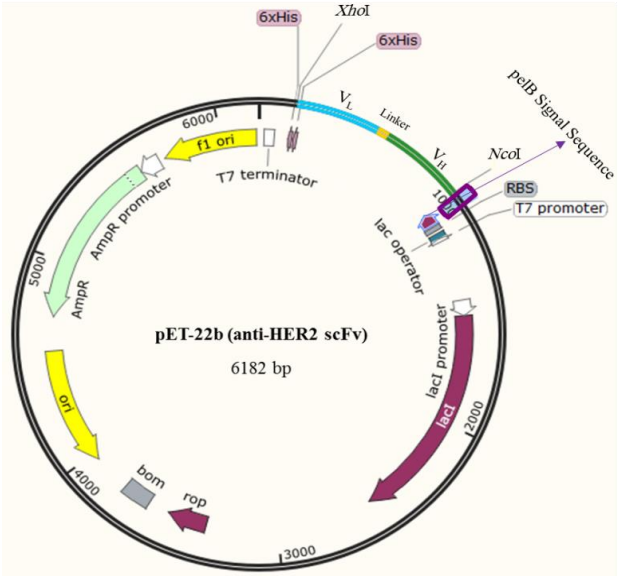
IPTG, prestained protein ladder, and DNA polymerase were obtained from Sinaclon, Iran. DNA ligase, unstained protein marker, DNA ladder, *Xho*I, and *Nco*I restriction enzymes were obtained from Fermentas, Germany. The chemicals and reagents used in the present study were of analytical grade and purchased from Merck, unless otherwise specified.

2.2. Methods

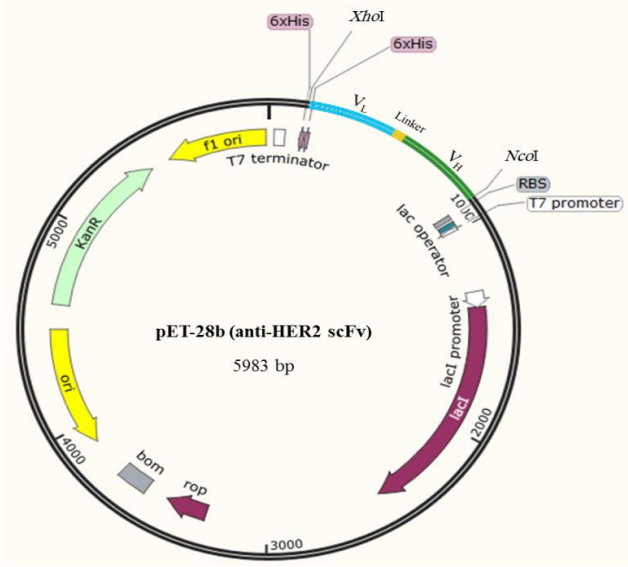
2.2.1. Construction of the Expression Vectors

The sequences of variable heavy (V_H) and light (V_L) chains of Trastuzumab (drug bank number DB00072) was codon optimized for expression in *E. coli*. Then, the pGH vector containing the chemically synthesized anti-HER2 scFv gene (Generay biotech, China) was digested with *Xho*I and *Nco*I restriction enzymes gel-extracted (MEGA quick gel extraction kit, iNtRON biotechnology), and subsequently ligated into digested pET-22b (+) (+) (Figure 1A) and pET-28b (+) (Figure 1B) vectors. Then, the ligation mixture was transformed into *E. coli* TOP10 strain. In the first step, the recombinant clones were confirmed by *Xho*I/*Nco*I double digestion and polymerase chain reaction (PCR) reaction. To perform PCR reaction for confirming the recombinant clones, the anti-HER2 scFv-expressing recombinant vectors were used as the template for PCR. PCR was done using the specific primer pair of 5′-GGGCGAAGTGCAGTTAGTTGA-3′ (forward) and 5′-GGTTGGAGGTGTTGTATAATGCTGCTG-3′ (reverse) that were designed by Oligo7 software (Molecular Biology Insights, Inc) and

in a thermal cycler (Analytikjena) with the temperature profile including (1) initial DNA denaturation at 94 °C for 5 min, (2) 30 PCR cycles of 94 °C for 1 min, 58.5 °C for 2 min, and 72 °C for 2 min, (3) extension period at 72 °C for 15 min. Finally, to ensure the correct frame for expression, the purified recombinant plasmids were sequenced by Bioneer Company (Korea).



A)



B)

Figure 1. Schematic diagram of the A) pET-22 (anti-HER2 scFv) and B) pET-28 (anti-HER2 scFv) expression vector.

2.2.2. Expression of the Anti-HER2 scFv Solubility Testing

The successfully constructed plasmids were extracted from TOP10 and transformed into competent BL21 (DE3). To express anti-HER2

scFv, a positive clone was incubated into 5 mL LB broth (Luria bertani) containing ampicillin (100 µg/mL) for pET-22 (anti-HER2 scFv) and kanamycin (25 µg/mL, Biobasic, Canada) for pET-28 (anti-HER2 scFv). Then, the overnight cultures were inoculated into 50 mL LB broth. The cultures were shaken at 37 °C and 180 rpm until the OD_{600} of the cells reached 0.6 - 0.7. Then, the expression was induced by addition of 1 mM IPTG and the induced cultures were shaken for another 2 h under identical conditions. The bacterial biomass from BL21 containing pET-22 and pET-28 (anti-HER2 scFv) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% gel followed by staining with Coomassie brilliant blue G-250.

2.2.3. Determining Anti-HER2 scFv Concentration

The total protein concentration was determined calorimetrically by BCA assay according to the manufacture's microplate protocol (Parstous Company, Iran) using bovine serum albumin (BSA, Atocell, Austria) as standard protein. Densitometry analysis of the stained SDS-PAGE gels by Total Lab TL120 software (Nonlinear Inc, Durham NC, USA) was performed to define the percentage of anti-HER2 scFv band in the stained SDS-PAGE gels. The band percentage was multiplied by total protein concentration of the sample to calculate the concentration of anti-HER2 scFv protein.

2.2.4. Expression of Anti-HER2 scFv in E. coli BL21 under Different Induction Conditions

The effects of three factors including temperature, duration of induction, and concentration of inducer (IPTG) were examined on the expression of anti-HER2 scFv from BL21 containing pET-28 (anti-HER2 scFv). First, to study the effect of the duration of induction, the expression of anti-HER2 scFv was induced by addition of 1 mM IPTG, and the induced culture was shaken at 37 °C and then to collect the bacterial biomass, the samples were centrifuged (4 °C, 10,000 × g, 5 min) 2, 4, 6, and 24 h after induction. In the next step, the influence of temperature on anti-HER2 scFv expression was considered. Accordingly, recombinant BL21 (DE3) cells was grown at 37 ° C until the cells reached OD_{600} of 0.6 - 0.7 (mid log growth phase). Afterward, the protein expression was induced with 1 mM IPTG and the growth temperature was changed to 25, 30, and 37 °C. Finally, to specify the optimum concentration of inducer, the anti-HER2 scFv expression was induced by adding different concentrations of IPTG (0.25, 0.5, 1, and 2 mM) and the bacterial incubation was performed at the optimum temperature.

2.2.5. Western Blot

Total proteins from BL21 containing pET-22 (anti-HER2 scFv) and pET-28 (anti-HER2 scFv) were analyzed by SDS-PAGE and then blotted onto a PVDF membrane (Roche). Next, the protein bands were appeared by Ponceau S stain and then the membrane was blocked by using 2.5% BSA in tris bufferd saline with tween 20 (TBS-T, Tris-HCl 10 mM, and NaCl 150 mM

containing 0.1% tween 20) overnight at 4°C. Subsequently, after 3 time washing with wash buffer (TBS-T), the addition of monoclonal anti-poly Histidine antibody produced in mouse (Sigma Aldrich) (diluted 1:10000 in blocking buffer (TBS-T/2.5% BSA)) was performed and incubated for 90 min at room temperature. After 3 washes, the membrane was incubated for 60 min at room temperature in the presence of secondary antibody, anti mouse IgG (FC specific) peroxidase antibody in goat (Sigma Aldrich, 1:5000). Finally, the reaction detection was performed by using 0.6 mg/mL 3,3'-Diaminobenzidine (DAB; Sigma, USA) in 0.12 % H_2O_2 and 1M. Tris-HCl which yields an insoluble brown product.

2.2.6. Comparison of pET-22b (+) and pET-28b (+) Vectors for Soluble Protein Expression

50 mL of LB broth was cultured and induced under the same condition by 1 mM IPTG at 37 °C for 24 h for both BL21 containing pET-22 (anti-HER2 scFv) and pET-28 (anti-HER2 scFv). To make cell suspension, lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl and 10 mM Imidazole, pH 8) was added to the harvested cells. Afterwards, lysozyme (1 mg/mL), DNase (Sigma, 100 µg/mL) and $MgSO_4$ (100 mM) were added to the bacterial suspension and incubated on ice for 30 min. Next, the cell suspension was disrupted by sonication (300 W of 7 s separated by 8 s intervals for total of 30 min, Topsonics, Iran). Then, centrifuge (10,000 × g at 4 °C for 20 min) was applied to separate soluble and insoluble fractions of the disrupted cells. Finally, 10 µL of each fraction was analyzed on 12% SDS-PAGE gel. Afterwards, the stained SDS-PAGE gel was analyzed using the Total Lab TL 120 software and the percentage of anti-HER2 scFv band in soluble and insoluble fraction of the lysate was determined.

2.3. Statistical Analysis

GraphPad Prism software (version 6, USA) was used for statistical analysis. To compare the expression of anti-HER2 scFv in multiple groups, one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used. $p<0.05$ was considered as statistically significant different.

3. Results and Discussion

3.1. Construction of pET-22 and pET-28 (Anti-HER2 scFv)

Anti-HER2 scFv gene (756 bp) was successfully cloned between *Nco*I and *Xho*I sites of pET-22b (+) containing pelB leader peptide and in the same sites of pET-28b (+) (without pelB). These vectors include an inducible IPTG T7 promoter and a polyhistidine tail on scFv C-terminal sequence facilitating the purification [19].

The constructed plasmids were extracted from *E. coli* TOP10 cells and then the recombinant vectors were confirmed by enzyme digestion (Figure 2A). The 700 bp fragment of anti-HER2 scFv gene was successfully amplified using pET-22 and pET-28 (anti-HER2 scFv)as templates (Figure 2B). In addition, vector sequencing proved the authenticity of cloning procedure.

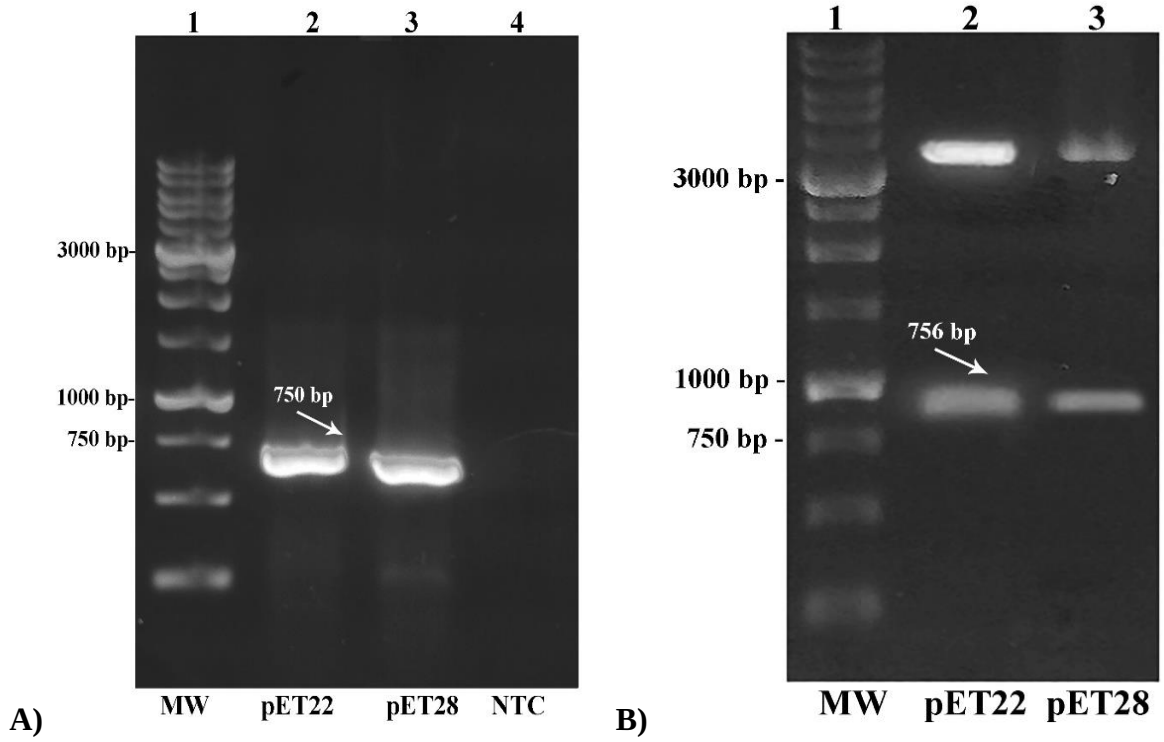


Figure 2. Confirmation of the recombinant plasmids by enzyme digestion and PCR.

A) Molecular weight marker 1 Kb (lane 1), pET-22 (anti-HER2 scFv) (lane 2) and pET-28 (anti-HER2 scFv) digested by NcoI/XhoI (lane 3). B) Molecular weight marker 1 Kb (lane 1), PCR products using pET-22 (anti-HER2 scFv) (lane 2) and pET-28 (anti-HER2 scFv) (lane 3) as template and no template control (NTC, lane 4).

3.2. Expression and Western Blot Analysis of BL21 (DE3) Containing pET-28 (anti-HER2 scFv)

The expression of the recombinant protein in BL21 (DE3) containing pET-28 (anti-HER2 scFv) was induced by 1 mM IPTG at 37 °C. The predicted molecular weight of anti-HER2 scFv(about 28 kDa), was confirmed by SDS-PAGE analysis (Figure 3A). As shown in Figure 3B, appearing ~ 28 kDa band in Western blot analysis using anti-His antibody confirmed the proper expression of anti-HER2 scFv in optimum condition

3.3. The Expression of Anti-HER2 scFv Under Different Induction Conditions

Expression conditions such as temperature of induction and concentration of inducer (IPTG) impact on the total yield of recombinant protein [20]. Therefore, we examined the effect of duration and temperature of induction and also IPTG concentration on anti-HER2 scFv expressed from BL21 (DE3) containing pET-28 (anti-HER2 scFv).

As demonstrated in Figure 3C, significant higher amount of anti-HER2 scFv was

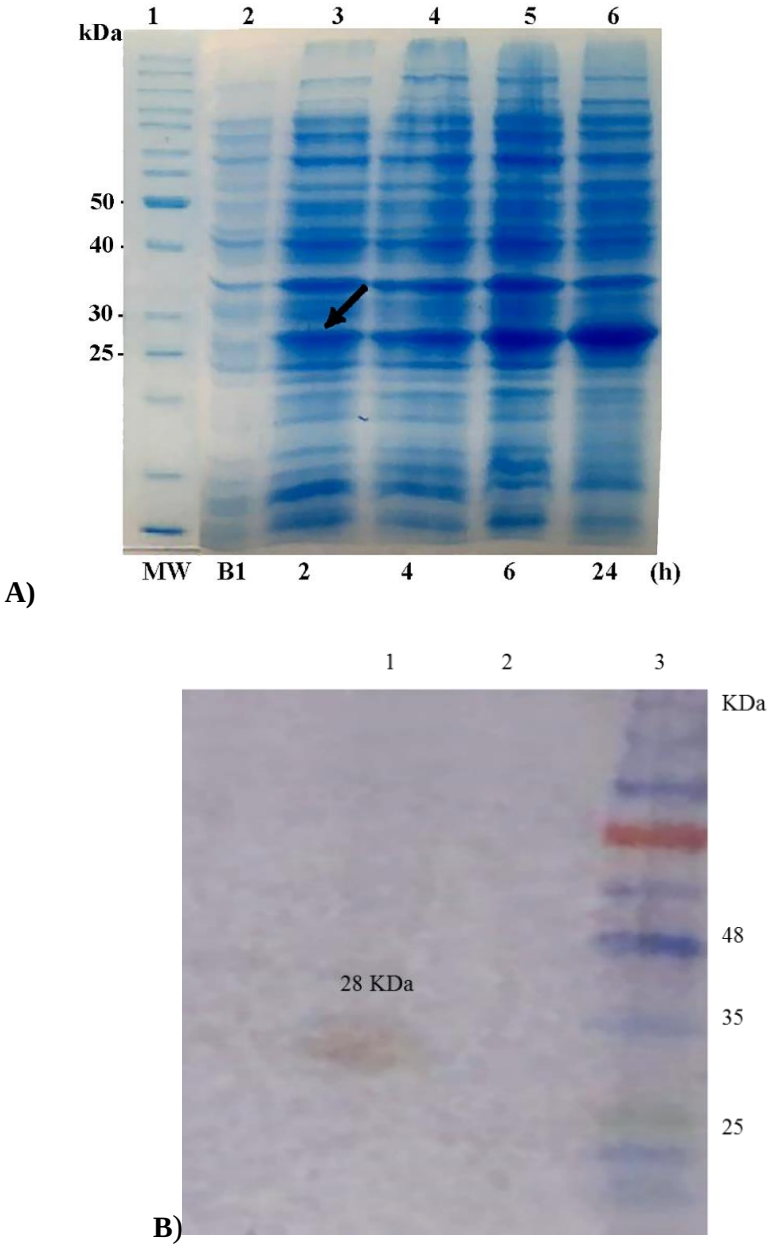
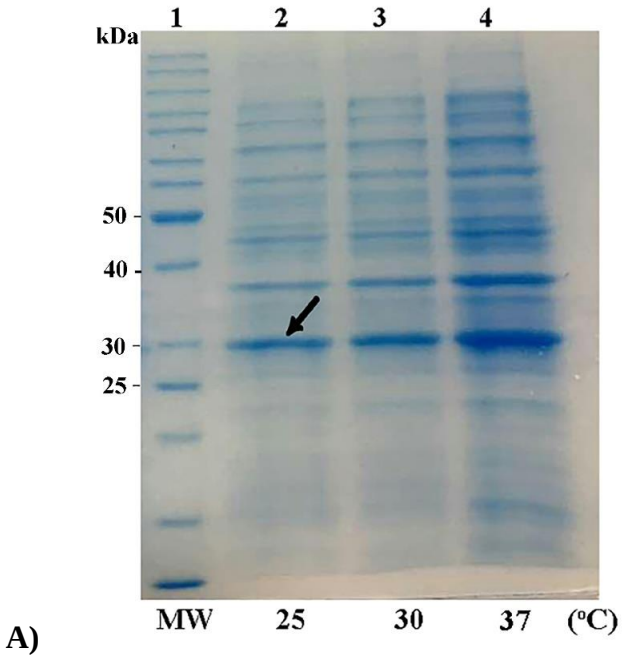


Figure 3. Anti-HER2 scFv expression and Western blot analysis.

A) Protein marker (lane 1), Total protein from BL21 (DE3) containing pET-28 (anti-HER2 scFv) before induction (lane 2) and after induction with 1 mM IPTG at 37 °C for 2 (lane 3), 4 (lane 4), 6 (lane 5), 24 h (lane 6). B) Western analysis of total protein from BL21 (DE3) containing pET-28 (anti-HER2 scFv) after induction with 0.25 mM IPTG for 24 h at 37 °C (lane 1), before induction (lane 2), prestained protein ladder (lane 3). C) The mean ± SD of two independent experiments of anti-HER2 scFv induced in different induction durations was analyzed using ANOVA statistical experiment. Significant difference was demonstrated by $p<0.001$ (***) and $p<0.0001$ (****).

expressed 24 h after IPTG induction, compared to 2 and 4 h post-induction time ($p<0.0001$). It was demonstrated that due to active expression of many *E. coli* chaperones at low temperatures, the stability and folding of recombinant scFv is increased at lower temperature (25 °C) [14].

Therefore, a common strategy to decrease hydrophobic interactions and subsequently aggregation of recombinant proteins is to reduce the cultivation temperature [21]. In contrast, Zhu *et al*. reported that the contents of the inclusion body of anti-human ovarian carcinoma × anti-human CD3 single-chain bispecific antibody is increased when the temperature is enhanced from 20 to 37 °C [6]. However, the retardation of the cell growth rate also happened at low temperature [6] which led to decreased volumetric protein concentration



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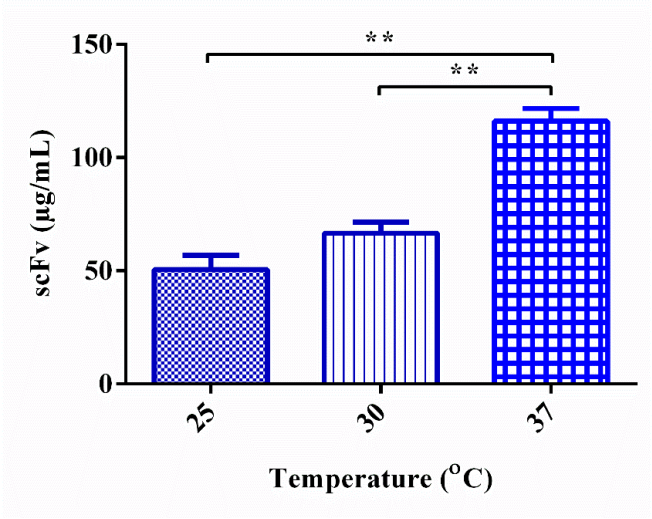
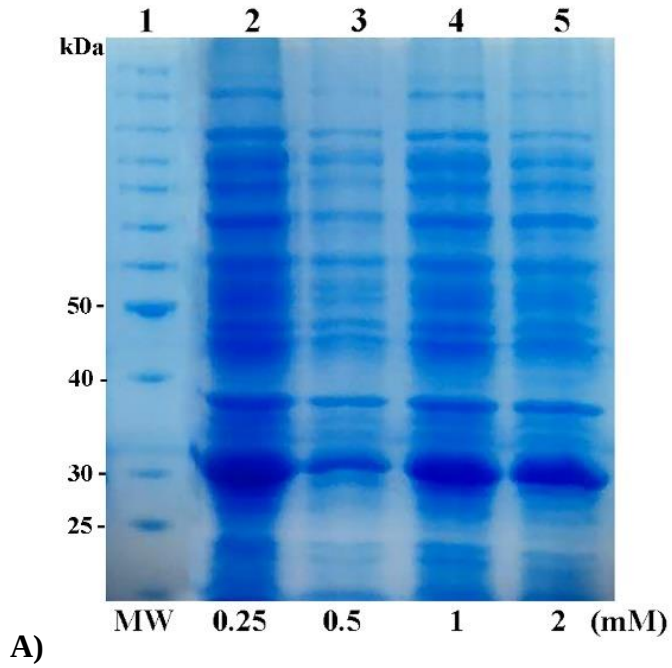


B)

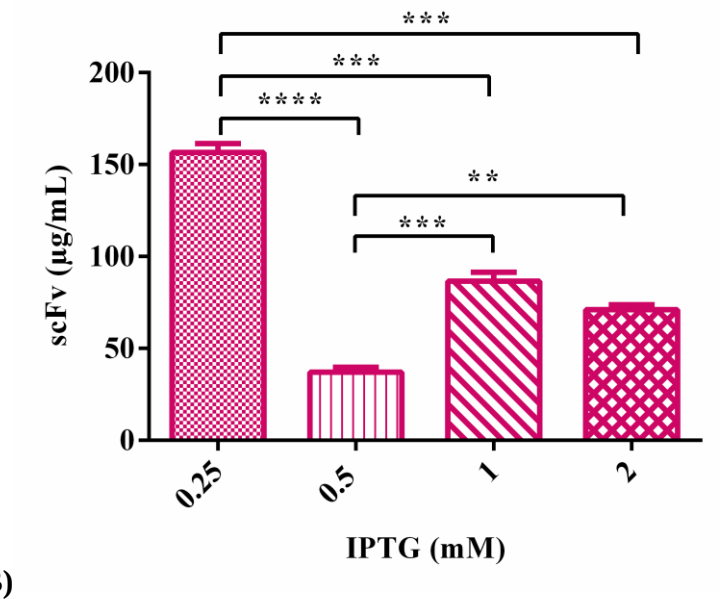
Figure 4. Effect of temperature on anti-HER2 scFv expression.

BL21 containing pET-28 (anti-HER2 scFv) was induced by IPTG (1 mM), incubated at different induction temperatures and collected after 24 h. A) Protein marker (lane 1); total protein from recombinant BL21 (DE3) after 24 h induction at 25 (lane 2), 30 (lane 3), and 37 °C (lane 4). B) Data represented as mean ± SD of two independent experiments. $p<0.01$ (**) was considered as significant difference.

[14]. In the present study, three different induction temperatures (25, 30 and 37 °C) were compared for anti-HER2 scFv expression (Figure 4A). Induction at 37 °C led to significant higher amount of anti-HER2 scFv compared to induction at 25 and 30 °C (Figure, 4A and B, $p<0.01$). Consistent with the result of our study, several studies reported 37 °C as the optimum induction temperature for maximum protein production [22, 23]. Our results are also



A)



B)

Figure 5. Induction of recombinant anti-HER2 scFv expression by different concentrations of IPTG.

BL21 containing pET-28 (anti-HER2 scFv) was induced by different concentrations of IPTG at 37 °C for 24 h. A) Protein marker (lane 1); total protein from BL21 (DE3) containing pET-28 (anti-HER2 scFv) after 24 h induction with 0.25 (lane 2), 0.5 (lane 3), 1 (lane 4), and 2 (lane 5) mM IPTG at 37 °C. B) ANOVA analysis of the data (mean ± SD) related to two independent experiments was performed. (**), (***) and (****) represent for $p<0.01$, $p<0.001$ and $p<0.0001$, respectively.

in agreement with the previous studies which demonstrate lowering the cultivation temperature leading to decreasing the quantity of newly expressed protein [14, 21].

IPTG has the ability to inhibit cell growth and also has impact on protein expression [21]. The expression rate can be lowered by decreasing IPTG concentration and consequently intracellular folding efficiency can be enhanced [20]. Therefore, the optimization of IPTG concentration is highly required. Herein, the effect of IPTG concentration (0.25, 0.5, 1 or 2 mM) on total protein extraction was examined at optimum induction temperature (37 °C) after the optimum post-induction time (24 h) (Figure 5). The expression of anti-HER2 scFv with 0.25 mM IPTG was significantly enhanced compared to that with 0.5, 1 and 2 mM IPTG, (Induction with 0.25 vs. 0.5 IPTG mM, $p<0.0001$; Induction with 0.25 vs. 1 and 2 mM, mM, $p<0.001$; Figure 5 A and B). In another study by our group, the expression condition of anti-HER2 scFv from BL21 (DE3) containing pET-22 (anti-HER2 scFv) was optimized (data was not shown). In this study, IPTG concentration demonstrated significant effect on total protein expression. Our results are consistent with the study of Heo *et al.* which demonstrated that reducing the IPTG concentration from 1 to 0.05 mM results in approximately 1.6-fold increase in productivity of functional anti-c Met scFv [20]. However, the total protein expression did not alter by varying IPTG concentrations in the study of Napathorn and his colleagues. Although, lower transcription rate using lesser concentration of IPTG than standard (1 mM) resulted in proper folding and more soluble fragments [21]. Totally, the anti-HER2 scFv expressed in optimum condition from BL21 containing pET-28 (anti-HER2 scFv) was 45% of the total protein after 24 h induction.

3.4. Comparison of pET-22b (+) and pET-28b (+) Vectors for Soluble Protein Expression

The solubility of a protein demonstrates its correct folding [24]. Thus, we compared anti-HER2 scFv solubility related to each vector. In this study, the protein expression was induced in the same condition for both vectors (1 mM IPTG at 37 °C for 24 h). Then, the solubility of BL21 containing pET-22 (anti-HER2 scFv) and pET-28 (anti-HER2 scFv) were compared. In this expression condition, the level of anti-HER2 scFv expression was 40% of the total protein in BL21 (DE3) containing recombinant pET-22b (+) and was 33.2 % in this host containing pET-28 (anti-HER2 scFv) (Figure 6A). Furthermore, the soluble/insoluble ratio of anti-HER2 scFv was ~ 3.5 fold more in BL21 containing pET-22 (anti-HER2 scFv) compared to BL21 containing pET-28 (anti-HER2 scFv) (soluble/insoluble ratio of BL21 containing recombinant pET-22 vs. pET-28, $p<0.001$, Figure 6B). Similar results were previously reported for anti-idiotypic catalytic scFv with β-lactamase like activity expressed in BL21 (DE3) periplasm space using pelB leader peptide. The recombinant protein was soluble and retained the catalytic parameters for

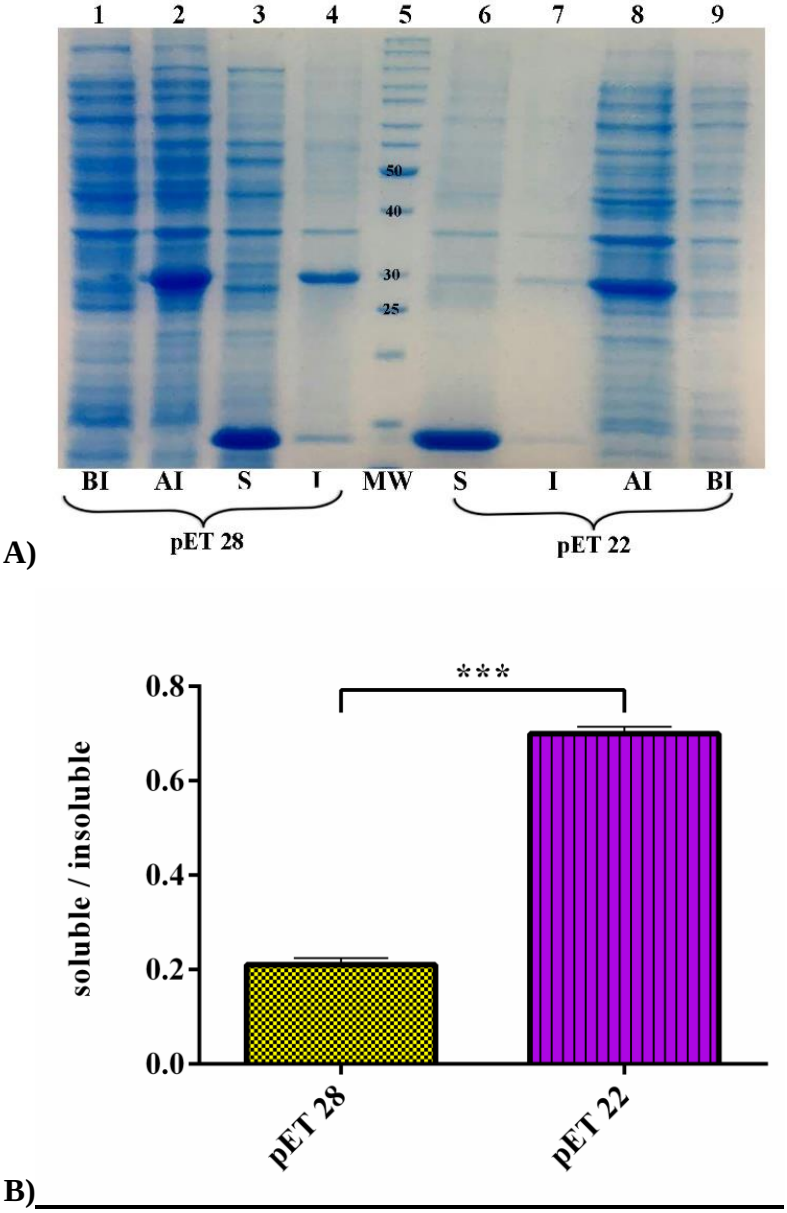


Figure 6. Comparison of pET-22b (+) and pET-28b (+) recombinant vectors for soluble anti-HER2 scFv expression.

A) Total protein from BL21 (DE3) containing pET-28 (anti-HER2 scFv) before induction (lane 1) and after induction with 1 mM IPTG for 24 h at 37 °C (lane 2); soluble (lane 3) and insoluble fractions (lane 4), protein marker (lane 5), soluble (lane 6) and insoluble (lane 7) fractions of BL21 containing pET-22 (anti-HER2 scFv) after induction with 1 mM IPTG for 24 h at 37 °C, total protein after (lane 8) and before induction (lane 9). B) Data represented as mean ± SD of two independent experiments. $P<0.001$ (***) was considered as significant difference.

hydrolysis of ampicillin [25]. In the study of Tiwari *et al.*, anti- Hepatitis B surface antigen scFv was cloned into pET-22b (+) (with pelB) and expressed in *E. coli*. Consistent with the result of our study, in their study the pelB leader sequence led to expression of humanized scFv in a secretory manner and subsequently resulted in enhancement in the recombinant protein solubility [26].

4. Conclusion

Breast cancer is the most frequent incident cancers in women worldwide [27, 28]. The relationship between overexpression of HER2 and malignity of breast tumors has been led to the production of monoclonal antibodies and antibody fragments targeting $HER2^{+}$ tumors [13]. In this study, anti-HER2 scFv was selected as the disulfide bond-containing protein. The insoluble inclusion bodies are produced when scFvs are expressed in reducing bacterial cytoplasm [29, 30]. One approach to achieve a functionally active scFv is to translocate the scFv into the oxidized environment of the periplasm where the formation of proper disulfide bond is possible. This can be achieved by cloning the gene in a vector containing N-terminal pelB leader peptide that exports foreign proteins to the periplasm [26]. In the present study, recombinant anti-HER2 scFv was successfully expressed and confirmed by Western blot analysis. The most expression level of anti-HER2 scFv by BL21 (DE3) containing pET-28 (anti-HER2 scFv) was observed 24 h after induction with 0.25 mM IPTG at 37 °C. Our results demonstrated that the total amount of scFv expression was higher in BL21 containing pET-22 (anti-HER2 scFv) in comparison with pET-28b (+). Additionally, the ratio of soluble to insoluble protein in this vector was significantly higher than BL21 containing pET-22 (anti-HER2 scFv). Therefore, it can be concluded that pelB leader sequence can lead to translocation of anti-HER2 scFv to oxidative environment of periplasmic space which provides appropriate folding.

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