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*Original Article*

# Improvement of solubility and refolding of an anti-human epidermal growth factor receptor 2 single-chain antibody fragment inclusion bodies

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***Abstract***

Single chain variable fragment antibodies (scFvs) have attracted many attentions due to their small size, faster bio-distribution and better penetration in to the target tissues, and ease of expression in *Escherichia coli*. Although, scFv expression in *E. coli* usually leads to formation of inclusion bodies (IBs). The aim of this research was to improve solubilizing and refolding conditions for IBs of scFv version of pertuzumab (anti-human epidermal growth factor receptor 2 (HER2) antibody). After protein overexpression in *E. coli* BL21 (DE3), bacterial cells were lysed and IBs were extracted via repeated washing and centrifugation. The effect of different types, concentrations, pHs, and additive of denaturing agents on IBs solubility were evaluated. More than 40 refolding additives were screened and combinations of 10 of the best additives were check out using Plackett-Burman design to choose three refolding additives with the most positive effect on refolding of the scFv. Response surface methodology (RSM) was used to optimize the concentration of adopted additives. The most efficient buffer to solubilize IBs was a buffer containing 6 M urea with 6 mM beta mercaptoethanol, pH 11. The optimum concentration of three buffer additives for refolding of the scFv was 23 mM tricine, 0.55 mM arginine, and 14.3 mM imidazole. The bioactivity of the refolded scFv was confirmed by immunohistochemical staining of breast cancer tissue, a specific binding based method. The systematic optimization of refolding buffer developed in the present work will contribute to improve the refolding of other scFv fragments.

***Keywords:*** HER2; Inclusion bodies; Refolding additive; Response surface methodology; Single-chain antibodies; Solubilizing.

## INTRODUCTION

Today, recombinant small antibody fragments are used for many diagnostic and therapeutic applications instead of intact antibodies (1). Single chain variable fragment antibody (scFv) has faster distribution and better penetration into target tissue compared with other antibody fragments (2). As scFvs are small and non-glycosylated proteins, they can be easily overexpressed in eukaryotic hosts such as *Escherichia coli (E. coli)*. In comparison with mammalian cells, antibody production in large quantities is easier and faster in *E. coli* cells (3). Even production yield up to 2 g/L has been reported for expression of recombinant antibodies in *E. coli* (4). However, highly expressed scFvs usually accumulate as unfolded protein aggregates, which called inclusion bodies (IB) (5).

Inclusion bodies are lack of biological activity and insoluble molecules. Despite their problems, IBs formation can be considered as an advantage for separation and purification of expressed proteins (6). Inclusion bodies with more than 60% purity can be easily isolated from other component of the solution by high-speed centrifugation (5,000 - 22,000 *g*). Even more resuspension and centrifugation of IBs can slightly increase its purity (7). Sometimes, IBs are the only way to express proteins, which are toxic in their active form. Formation of IBs also increases resistance of protein to proteolytic degradation compared with soluble protein (8).

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There are four common steps to obtain active protein from IBs; separation IBs from other component of bacteria, solubilizing of IBs, refolding of soluble protein, and finally purification of refolded protein (9). High concentration of denaturing agents like urea and guanidine hydrochloride (GdnHCl) can be used for solubilization of IBs (10). For proteins with free cysteine, beta mercaptoethanol (BME) and dithiothreitol (DTT) can be added to solubilize agent resulting in more correct disulfide bonds formation. For protein refolding, solubilizing agents should be gradually eliminated. Dilution of solubilized protein in refolding buffer is a common way to recover protein to active form (9). Chemical agents like polyethylene glycol, L-arginin, cyclodextrins, proline, and polyols can be also added to refolding buffer (11). Different parameters may influence the efficiency of a refolding procedure including solubilizing or denaturing agents, methods which used for its elimination, and chemical additives which used to assist refolding (11).

Refolding of protein is a difficult task and condition unique to each protein must be experimentally obtained for a successful refolding process. Some studies have previously reported optimization of IBs refolding methods. For example, effect of different denaturing buffers with and without reducing agents on solubilizing of recombinant human growth hormone IBs was evaluated. It was observed that low concentrations of denaturing agents resulted in higher yield of IBs solubilizing in comparison to high concentrations (12). In another study, more than 200 different buffers were systematically evaluated to optimize refolding parameters for falcipain-2. The optimal refolding buffer was an alkaline buffer containing sucrose or glycerol and glutathione (reduced to oxide ratio 1:1) (13).

Pertuzumab is a humanized monoclonal antibody and the first member of drugs that inhibit dimerization of human epidermal growth factor receptor 2 (HER2). Because of specific mechanism of pertuzuamb, hindering sterically receptor dimerization, existence of Fc region is not essential for action of the antibody. It has been demonstrated that antibody fragments of pertuzumab can also inhibit growth of tumor cells (14). In our previous study, scFv version of pertuzumab was successfully expressed in *E. coli*. Despite of high expression of protein, most of the scFv (approximately 70%) were produced as insoluble protein. Refolding of insoluble protein through dialysis against a simple buffer resulted in loss of 25% of protein (15). In the present study, we aimed to improve recovery of anti-HER2 scFv from IBs by optimizing solubilizing and refolding agents.

## MATERIALS AND METHODS

### *Solubilizing of anti-HER2 scFv IBs*

Anti-HER2 scFv was overexpressed using *E. coli* BL21 (DE3) as previously reported (15). The culture was centrifuged at 7,500 *g* for 10 min and the pellet was used for IBs extraction (16). Isolated anti-HER2 scFv IBs were dissolved in different concentrations (2, 4, 6, and 8 M) of urea or GdnHCl and also their combinations (17). Inclusion bodies were also dissolved in solubilizing agent at different pH (5, 7, 9, and 11) containing 6 M urea (17, 18). Other solubilizing agent at pH 11 containing urea 6 M with reducing agent (DTT (4 mM) or BME (2, 4, 6, and 8 mM)) or 5% n-propanol were also used to solubilize anti-HER2 scFv IBs. Same amount of IBs pellet was dissolved in each solubilizing agent and solubilization was conducted by shaking the sample at 180 rpm for 1 h at room temperature. Then, the insoluble particles were removed by centrifugation at 13,000 rpm for 10 min. The total protein concentration for all supernatants (solubilized IBs) was measured by the Bradford assay (19) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16).

### *Refolding of anti-HER2 scFv IBs*

More than 40 additives were used for refolding of anti-HER2 scFv IBs. Refolding of IBs was performed by rapid dilution method. Twenty µL of solubilized protein was diluted in 180 µL of each additive and incubated for 24 h. Then, change in the turbidity (due to precipitation and aggregation of the protein) of

each sample was monitored by determination of optical density at 600 nm. Insoluble particles (misfolded protein) were removed by centrifugation at 13,000 rpm for 10 min and the concentration of protein in soluble fraction (refolded protein) was determined by Bradford assay and SDS-PAGE.

After the first screening of the best refolding additives (having the highest protein concentration and the lowest turbidity change), a Plackett-Burman design (Table 1) with 11 factors (10 additive and temperature) was used for selection of the three most important factors. Fifteen experimental runs (Table 2) were constructed by Box-Behnken model using a three-level three-factor design. The optimized refolding buffer composition was predicted by Design Expert software (ver 7.0.0.) and this condition was used for large scale refolding of anti-HER2 scFv.

### *Bioactivity of refolded anti-HER2 scFv*

The biological activity of anti-HER2 scFv refolded with optimum buffer composition, its ability to bind to HER2, was evaluated by immunohistochemistry staining. Breast cancer specimens, paraffin embedded sections, were incubated with refolded anti-HER2 scFv as the primary antibody. Monoclonal anti-polyhistidine-peroxidase antibody (Sigma, USA) and 3,3´diaminobenzidine (Sigma, USA) were used as the secondary antibody and the chromogen substrate, respectively. The sections were counterstained with hematoxilin.

**Table 1**. Plackett-Burman experimental design of 11 factors at 2 levels and effect of these factors on refolding of anti-HER2 scFv.

| Term | Tricin (mM) | Arginin (M) | SDS (%) | Glucose (M) | Citric acid (mM) | Tween 20 (%) | Betaiene (M) | Imidazole (mM) | DTT (mM) | Temperature (°C) | Glycerol (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Run 1 | 0.00 | 0.125 | 0.00 | 1.50 | 0.0 | 1.00 | 0.50 | 0.00 | 10 | 22 | 20 |
| Run 2 | 0.00 | 0.125 | 0.10 | 0.50 | 50 | 1.00 | 0.00 | 100 | 10 | 22 | 5.0 |
| Run 3 | 500 | 0.125 | 0.10 | 1.50 | 0.0 | 1.00 | 0.50 | 100 | 1.0 | 4.0 | 5.0 |
| Run 4 | 0.00 | 0.500 | 0.10 | 1.50 | 0.0 | 0.10 | 0.00 | 100 | 1.0 | 22 | 20 |
| Run 5 | 500 | 0.500 | 0.00 | 1.50 | 50 | 1.00 | 0.00 | 0.00 | 1.0 | 22 | 5.0 |
| Run 6 | 0.00 | 0.500 | 0.00 | 1.50 | 50 | 0.10 | 0.50 | 100 | 10 | 4.0 | 5.0 |
| Run 7 | 500 | 0.125 | 0.00 | 0.50 | 50 | 0.10 | 0.50 | 100 | 1.0 | 22 | 20 |
| Run 8 | 0.00 | 0.125 | 0.00 | 0.50 | 0.0 | 0.10 | 0.00 | 0.00 | 1.0 | 4.0 | 5.0 |
| Run 9 | 500 | 0.500 | 0.00 | 0.50 | 0.0 | 1.00 | 0.00 | 100 | 10 | 4.0 | 20 |
| Run 10 | 500 | 0.500 | 0.10 | 0.50 | 0.0 | 0.10 | 0.50 | 0.00 | 10 | 22 | 5.0 |
| Run 11 | 0.00 | 0.500 | 0.10 | 0.50 | 50 | 1.00 | 0.50 | 0.00 | 1.0 | 4.0 | 20 |
| Run 12 | 500 | 0.125 | 0.10 | 1.50 | 50 | 0.10 | 0.00 | 0.00 | 10 | 4.0 | 20 |
| Effect | 448.42 | 683.18 | -41.9733 | -401.70 | -905.27 | 199.94 | -387.57 | 451.48 | 193.96 | -263.85 | 322.82 |
| SumSqr | 603233 | 1400220 | 5285.28 | 484105 | 2458540 | 119928 | 450639 | 611521 | 112861 | 208856 | 312645 |
| Contribution (%) | 8.91 | 20.68 | 0.078 | 7.15 | 36.32 | 1.77 | 6.65 | 9.03 | 1.66 | 3.08 | 4.61 |
| Significance | NS | S | NS | NS | S | NS | NS | NS | NS | NS | NS |

S, significant; NS, non-significant.

**Table 2.** Box-Behnken experimental design of 3 factors (refolding additive) at 3 levels (concentration).

| Run | Tricine (mM) | Arginine mM) | Imidazole (mM) | Concentration of refolded anti-HER2 scFV[1] (µg/mL) |
|---|---|---|---|---|
| 1 | 20 | 250 | 200 | 29.01 |
| 2 | 35 | 0.00 | 0.00 | 144.5 |
| 3 | 35 | 500 | 200 | 30.42 |
| 4 | 20 | 500 | 100 | 28.47 |
| 5 | 35 | 250 | 100 | 29.74 |
| 6 | 35 | 0.00 | 200 | 134.7 |
| 7 | 35 | 250 | 100 | 27.45 |
| 8 | 35 | 250 | 100 | 28.47 |
| 9 | 50 | 250 | 0.00 | 29.41 |
| 10 | 20 | 250 | 0.00 | 29.69 |
| 11 | 50 | 0.00 | 100 | 87.33 |
| 12 | 35 | 500 | 0.00 | 31.91 |
| 13 | 20 | 0.00 | 100 | 136.7 |
| 14 | 50 | 250 | 200 | 26.03 |
| 15 | 50 | 500 | 100 | 27.12 |

[1]Anti-human epidermal growth factor receptor 2 single chain variable fragment antibody inclusion bodies.

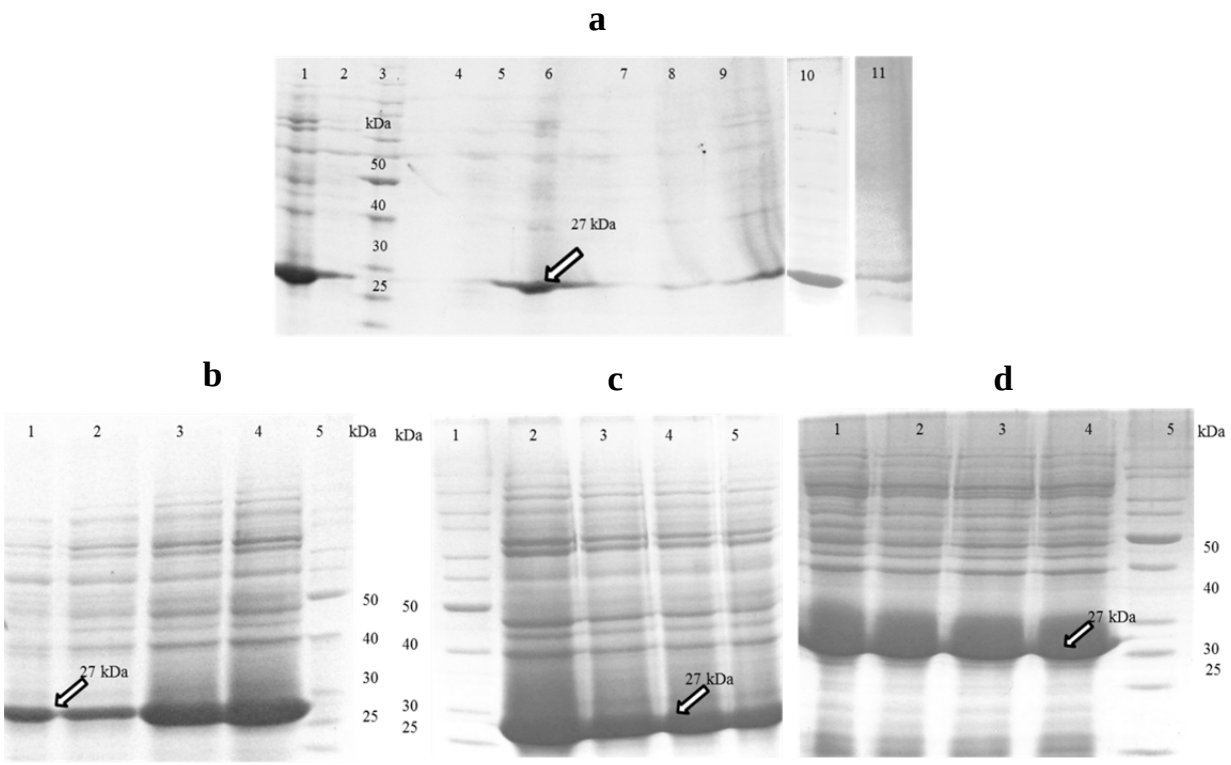


**Fig. 1.** Solubilizing of anti-human epidermal growth factor receptor 2 single chain variable fragment antibody inclusion bodies (HER2 scFv IBs) using (a**)** different concentrations of urea, guanidine hydrochloride (GdnHCl), and their combinations. Lane 1, urea 6 M; Lane 2, urea 4 M; Lane 4, urea 2 M; Lane 5, GdnHCl 2 M and urea 2 M; Lane 6, GdnHCl 4 M and urea 4 M; Lane 7, GdnHCl 2 M; lane 8, GdnHCl 4 M; Lane 9, GdnHCl 6 M; Lane 10, urea 8 M; and Lane 11, GdnHCl 8 M; (b) Urea 6 M at different pH. Lane 1-4, pH 5, pH 7, pH 9, and pH 11, (C) urea 6 M at pH 11 supplemented with different additives. Lane 2, beta mercaptoethanol (BME) 4 mM; Lane 3, n-Propanol 5%; Lane 4, dithiothreitol (DTT) 4 mM; and Lane 5, no additive; (d) Urea (6 M) at pH 11 supplemented with different concentrations of BME. Lane 1: BME 2 mM; Lane 2: BME 4 mM; Lane 3: BME 6 mM and Lane 4: BME 8 mM. Lanes 3a, 5b, 1c, and 5d are protein marker.

## RESULTS

*Solubilizing of anti-HER2 scFv IBs*

Extracted anti-HER2 scFv IBs were solubilized in different concentration of urea, GdnHCl or their combinations. As shown in Fig. 1a, urea 6 M solubilized more amount of IBs compared with other solubilizing agents. The effect of pH on the yield of IBs solubilizing was also checked out and the optimum pH was 11 (Fig. 1b). Inclusion bodies were also dissolved with urea 6 M at pH 11 in the presence of DTT, BME or n-propanol and our results showed that addition of BME to solubilizing buffer resulted in improvement of IBs solubilization (Fig. 1c). Different concentrations of the reducing agent were also examined and most IBs were dissolved with urea 6 M at pH 11 containing 4 mM BME (Fig. 1d).

***Refolding of anti-HER2 scFv***

Different buffer additives (as a single agent) were used for refolding of anti-HER2 scFv using rapid micro-dilution. The samples were then subjected to SDS-PAGE and Bradford assay and the result obtained from Bradford assay were in agreement with SDS-PAGE results. Considering results of SDS-PAGE, Bradford, and turbidity assays, ten buffer additives were chosen and used in combination for Plackett-Burman design (Table 1). Six factors (tricine, arginine, glucose, citric acid, betaine, and imidazole) out of 11 factors had 88.8% contribution in refolding of anti-HER2 scFv. Tricin, arginine, and imidazole had positive effect while other buffer additive showed negative effect (Table 2). The optimum concentration of the three variables with positive effect was further evaluated by Box-Benhken design. The results of 15 runs

designed by Box-Benhken model is summarized in Table 3. Refolding of anti-HER2 scFv was carried out in triplicate under the central point conditions (*i.e*. tricine, 35 mM; Arginin, 250 mM; And imidazole, 100 mM) to confirm reproducibility of the model. The concentration of anti-HER2 scFv was 28.55 ± 1.15 (µg/mL) with the coefficient of variation being less than 4.1 %. The ANOVA of response surface quadratic model showed that the model was significant with *F*-value of 119 and a very low probability value ($P > F$) < 0.0001. Non-significant lack of fit *F*-value of 5.50 indicated the good fitness of the model. As presented in Table 3, the model terms, A, B, AB, and $B^2$ were significant ($P < 0.05$). The relationship between concentration of refolded anti-HER2 scFv and concentration of three buffer additives can be expressed in the following regression equation in terms of coded factors:

Log (refolded anti-HER2 concentration) = + 3.46 - 0.033A - 0.31B - 0.014C + 0.043AB - 0.011AC + 0.002369BC - $0.039A^2$ + $0.33B^2$ + $0.039C^2$

Based on the regression equation, the value of correlation coefficient ($R^2$) of the model was 0.9954. The "Pred R-squared" of 0.9326 is in high agreement with the "Adj R-squared" of 0.9870 indicating a high degree of model accuracy and high degree of relation between the actual and predicted value. The "Adeq precision" measuring the signal to noise ratio was 27.457 indicated an adequate signal.

Figure 2 exhibits 3 dimensional surface response plots of the interactions between two different buffer additives. As shown in Fig. 2a, concentration of refolded anti-HER2 scFv decreased significantly with an enhancment in arginine concentration, especially in the persence of low concentration of tricine ($P$ = 0.0486). At low concenration of arginine (25 mM) (Fig. 2b), higher concentration of tricine resulted in lower concentration of refolded anti-HER2 scFv in the higher concentration of imidazole. Negative effect of high cncentarion of arginine on refolding of anti-HER2 scFv was also observed in different range of immidazole concentration (Fig. 2c).

According to this response surface quadratic mode, the optimum concentrations of three buffer additives for refolding of anti-HER2 scFv were tricine, 23 mM; Arginin, 0.55 mM; And imidazole, 14.3 mM. This predicted condition was applied for large scale refolding of anti-HER2 scFv which yielded a concentration of 154 µg/mL. This expresimental result was in good agreemant with that perdicted by the model.

### *Bioactivity of anti-HER2 scFv*

The bioactivity of refolded anti-HER2 scFv under optimum condition was evaluated by immunohistochemical staining of breast cancer tissue. Figure 3 represents HER2-positive breast sections from the same sample, which were stained with refolded anti-HER2 scFv (Fig. 3a) and commercial anti-HER 2 antibody (Fig. 3b) where both antibody showed strong staining as compared with negative control (Fig. 3c).

**Table 3.** ANOVA for response surface quadratic model

| Source | Sum of squares | df | Mean square | F value | Prob > F | |
|---|---|---|---|---|---|---|
| Model | 1.20 | 9 | 0.13 | 119.00 | < 0.0001 | Significant |
| A-Tricin | 0.008917 | 1 | 0.008917 | 7.95 | 0.0371 | |
| B-Arginin | 0.78 | 1 | 0.78 | 691.85 | < 0.0001 | |
| C-Imidazole | 0.001629 | 1 | 0.001629 | 1.45 | 0.2821 | |
| AB | 0.007551 | 1 | 0.007551 | 6.73 | 0.0486 | |
| AC | 0.0004639 | 1 | 0.0004639 | 0.41 | 0.5485 | |
| BC | 0.00002246 | 1 | 0.00002246 | 0.020 | 0.8930 | |
| $A^2$ | 0.005692 | 1 | 0.005692 | 5.07 | 0.0740 | |
| $B^2$ | 0.39 | 1 | 0.39 | 347.80 | < 0.0001 | |
| $C^2$ | 0.005508 | 1 | 0.005508 | 4.91 | 0.0775 | |
| Residual | 0.005609 | 5 | 0.001122 | | | |
| Lack of fit | 0.005003 | 3 | 0.001668 | 5.50 | 0.1577 | Not significant |
| Pure Error | 0.0006066 | 2 | 0.0003033 | | | |
| Cor Total | 1.21 | 14 | | | | |

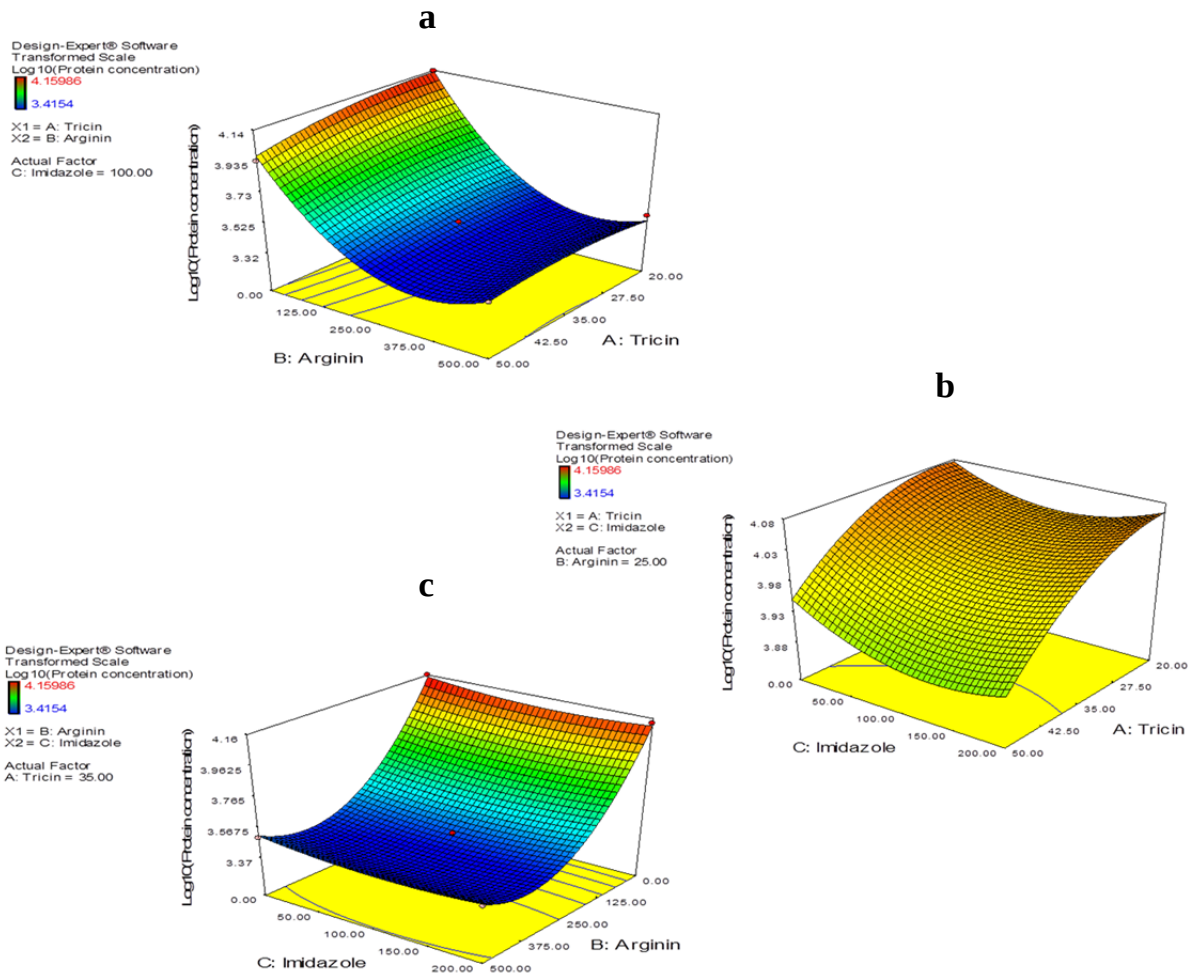


**Fig. 2**. Response surface of single chain variable fragment antibody (ScFv) refolding buffer represents the interaction between two additives in the concentration of ScFv (mg/L) by keeping other additives constant. (a) Interaction between tricine and arginine while the concentration of imidazole is 100 mM; (b) Interaction between tricine and imidazole while the concentration of arginine is 250 mM; (c) Interaction between tricine and imidazole while the concentration of tricine is 35 mM.

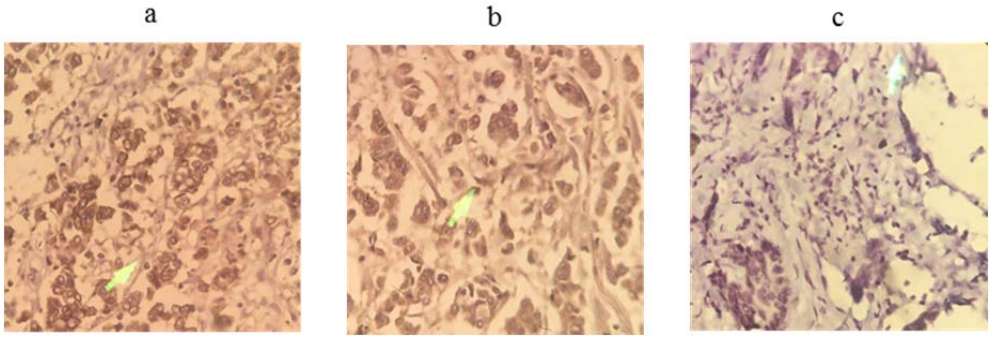


**Fig. 3.** Immunohistochemical staining of breast cancer tissue. (a) The sample was stained with refolded anti-human epidermal growth factor receptor 2 single chain variable fragment antibody inclusion bodies (HER2 scFv IBs), (b) the sample was stained with commercial anti-HER2 antibody, and (c) negative control.

## DISSCUSION

Single chain variable fragment antibodys are non-glycosylated proteins which can be easily overexpressed in *E. coli*. However, high level expression of proteins in cytoplasmic space of bacteria usually leads to formation of insoluble and inactive aggregates, IBs. Recovery of active protein from IBs is a challenging and difficult task, which required optimizing multitude processing including isolation, solubilization, and refolding. In the present study, we described an optimum method for high yield recovery of bioactive anti-HER2 scFv from IBs.

Solubilization of IBs is a critical step in its downstream processing which remarkably influences efficiency and yield of the next step, IBs refolding (20). In the present study, effect of two commonly used denaturants (urea and GdnHCl) at different concentrations (2-8 M) and combinations, addition of reducing agents (BME, DTT) and n-propanol, and pH on efficiency of anti-HER2 scFv IBs solubilization was evaluated. We observed that urea (6 M) containing BME (4 mM) at pH 11 dissolved IBs most efficiently compared with other solubilizing agents. In some reports, detergents, instead of denaturants, were used for solubilization of scFvs. Inclusion bodies of different scFvs were solublized by 2.5% lauroyl-glutamate, an amino acid-based detergent. It was proposed that some detergents could increase the solubility of protein and inhibit formation of aggregates and therefore facilitate the refolding (21).

Low concentration of reducing agents can disrupt intra- and inter-chain disulfide bonds and prevents formation of incorrect disulfide bonds which increases unfolding and solubilizing of the protein and avoids its misfolding (22). We also found that addition of reducing agent to solubilizing buffer improves yield of IBs solubilization. However, effect of BME was more significant than DTT. Additionally, BME has lower cost and dose not interfere with protein purification (*e.g.*, immobilized metal affinity chromatography) (23). Furthermore, it was shown that alkaline pH significantly improved the efficiency of ani-HER2 scFv IBs solubilization. Khan *et al*. also reported that bovine growth hormone IBs could be successfully solubilized with high pH buffer containing low concentration of denaturing agent (urea, 2 M) (10). It was proposed that alkaline pH enhances IBs solubiliziation efficiency by disruption of interchain disulfide bonds and keeping the molecule in its reduced form (24).

Buffer additive is another important factor influencing the yield and efficiency of IBs refolding. In the present study, to increase the yield of IBs refolding different buffer additives including sugars, amino acids, alcohols, polyoles, polymers, detergents, chelating agents, thiol agents, and buffering agents were used. Among more than forty screened buffer additives, tricine, arginine, and imidazole exhibited more improvement in refolding yield of anti-HER2 scFv. In our previous study, refolding of denatured anti-HER2 scFv using stepwise dialysis against water resulted in 25% loss of protein (15). The optimum condition of buffer additives (*i.e.*, combination of tricine, arginine, and imidazole) exhibited a significant improvement of refolding yield in comparison with simple buffer (tris 10 mM, Triton X-100 0.1 %).

In agreement with our findings, Tsumoto *et al*. reported that addition of arginine into refolding buffer significantly increased the yield of refolded scFv (25). Arginine is an amino acid, which inhibits aggregate formation due to hydrophobic interaction. However, arginine did not improve the refolding of some proteins containing disulfide bonds. There are some reports that arginine could not suppress aggregation due to disulfide binding and even indirectly enhance formation of incorrect intermolecular disulfide bonds (26).

Tricine is a buffer additive, which is derived from tris and glycine. Based on our results, the most refolding yield of anti-HER2 scFv observed when tricine with concentration of 23 mM was used. Fischer *et al*., reported that tricine up to 4 mM has no significant impact on efficiency and speed of ribonuclease refolding (27). Buffers (*e.g.*, tricine) can significantly influence conformational stability of refolded protein. However, different kinds

of buffer exhibit different effects on refolding yield of protein even in the same pH. For example, Sakamoto *et al*., reported tricine exhibited more improvement in refolding of reduced lysozyme than Na-phosphate (28). Imidazole is an amino acid derivative, which can increase yield and speed of refolding and suppress formation of aggregates. Shi *et al.*, proposed that imidazole, via enhancing prolyl isomerization, a rate-limiting step, improves refolding yield of green florescent protein (29).

There are different structure-based and function-based methods for evaluation of the refolding efficiency (30, 31). However, functional assays like enzymatic activity and immunoassays or bioassays are simpler and provide very reliable information on protein folding. In the present study, immuno-histochemical staining, a specific binding based method was used to confirm refolding of the protein. It was found that under the optimum condition of buffer additives, anti-HER2 scFv was efficiently refolded with acceptable bioactivity similar to commercial anti-HER 2 antibody. In agreement with our study, Jain *et al*., (32) used immuno-histochemical staining for evaluation of biological activity of a novel monoclonal antibody against canine CD20. They reported that the developed antibody exhibited staining pattern similar to the antibody used for diagnostic applications.

## CONCLUSION

In the present study, we aimed to improve the recovery efficiency of IBs of anti-HER2 scFv by optimization of solubilizing and refolding agents. The most solubilizing yield was observed when IBs was dissolved at high concentration of alkaline urea containing low concentration of reducing agent. Our present research emphasized on screening and optimizing of refolding additives by (statistical) design of experiments (DOE). Our result showed that the statistically optimized refolding condition (tricine, 23 mM; Arginine, 0.55 mM; And imidazole, 14.3 mM) exhibited a significant improvement in the refolding yield of IBs. The refolded scFv had an acceptable bioactivity similar to the commercial anti-HER2 antibody which confirmed by immunohistochemical staining of breast cancer samples. The systematic optimization of refolding buffer developed in the present work will contribute to the improvement of the refolding of other single-chainsc Fv fragments.

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