Original Article

Co-Expression of Chaperones for Improvement of Soluble Expression and Purification of An Anti-HER2 scFv in *Escherichia Coli*

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Abstract

Background: Single-chain fragment variable (scFv) is one of the most commonly used antibody fragments. They offer some advantages over full-length antibodies, including better penetration to target tissues. However, their functional production has been a challenge for manufacturers due to the potential misfolding and formation of inclusion bodies. Here we evaluated the soluble expression and purification of molecular chaperone co-expression.

Materials and Methods: *E. coli* BL21(DE3) cells were co-transformed with the mixture of plasmids pKJE7 and pET22b-scFv by the electroporation method. First, L-arabinose was added to induce the expression of molecular chaperones, and then IPTG was used as an inducer to start the expression of anti-HER2 scFv. The effect of cultivation temperature and IPTG concentration on soluble expression of the protein with or without chaperones was evaluated. The soluble expressed protein was subjected to native purification using the Ni-NTA affinity column.

Results: SDS-PAGE analysis confirmed the successful co-expression of anti-HER2-scFv and DnaK/DnaJ/GrpE chaperones. Co-expression with chaperones and low-temperature cultivation synergistically improved the soluble expression of anti-HER2 scFv. Co-expression with chaperone also exhibited an approximately four-fold increase in the final yield of purified soluble protein.

Conclusion: The combination of co-expression with chaperones and low temperature presented in this work may be useful for the improvement of commercial production of other scFvs in *E. coli* as functionally bioactive and soluble form.

Keywords: Co-expression, DnaK/DnaJ/GrpE, molecular chaperone, single-chain variable fragment, temperature

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Introduction

Antibody fragments have been widely evaluated as suitable alternatives to full-length antibodies for therapeutic and diagnostic applications.[1] Single-chain fragment variable (scFv) is one of the most commonly used antibody fragments that comprises the variable heavy and light chain domains (VH and VL) of an antibody linked through a flexible linker (*e.g.*, $(G_4S)_3$). scFvs offer some advantages over full-length antibodies, including better penetration to target tissues (*e.g.*, tumor), intracellular targeting, improved access to cryptic epitopes, and lower immunogenicity.[2] Furthermore, due to their small size (*i.e.,* 27 kDa) and non-glycosylated structure, scFvs can be easily overexpressed in bacterial host cells, including *Escherichia coli,* which results in a decrease in the time and cost of production processes.[3]

scFvs are mainly expressed either in the periplasm or cytoplasm of *E. coli*. The first approach allows for better formation of disulfide bridges and proper folding of scFvs.[4]

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However, the periplasmic expression of proteins usually leads to lower yields due to inefficient translocation and secretion of target protein into the periplasmic space.[5] The second method results in higher yields of proteins which mostly accumulates as insoluble aggregates (*i.e*., inclusion bodies) due to the reducing conditions of the cytoplasm.[6,7] To recover biological activity, inclusion bodies need to be solubilized and refolded, which is usually a time-consuming, complex, and costly procedure.[8] Alternatively, protein can be co-expressed with molecular chaperones facilitating proper protein folding.[9]

Chaperone proteins bind to nascent polypeptides, avoid harmful hydrophobic interactions, and assist them in correct folding and reaching the native structure.[10] *E. coli* uses two main sets of molecular chaperones, the GroES(HSP60)-GroEL system and the DnaK(HSP70)/DnaJ/GrpE system.[11] There are some reports on the improvement of soluble production of different proteins, including scFvs in *E. coli* by co-expression with molecular chaperones. For example, Hu *et al*.[12] found that co-expression of anti-domoic acid scFv with DnaK/DnaJ/GrpE resulted in a significant improvement of soluble protein production (up to 100 fold enhancement). We have previously developed an anti-HER2 scFv which was successfully expressed in the cytoplasm of *E. coli*.[13] However, most of the protein (more than 70%) was produced as inclusion bodies requiring *in vitro* refolding, which resulted in the loss of protein. Although the periplasmic expression of anti-HER2 scFv led to the improvement of its solubility and bioactivity, the majority of protein could not be secreted into the periplasm.[14] In the present work, we evaluate the effects of the molecular chaperones DnaK/DnaJ/GrpE on soluble expression and purification of anti-HER2 scFv. The effect of temperature and inducer concentration on the level of soluble production of proteins was also determined.

Materials and Methods

Bacterial strain and plasmids

Expression experiments were performed in *E. coli* BL21 (DE3) (Novagen, USA).

The recombinant pET22b-anti-HER2-scFv plasmid was previously constructed in our laboratory, and pKJE7 plasmid (carrying the *dnaK*, *dnaJ*, and *grpE* gene) was provided from Takara (Shiga, Japan). pET22b-anti-HER2-scFv plasmid was transformed into chemically competent *E. coli* BL21 (DE3) by heat shock method and grown on Luria Bertani (LB) agar plates supplemented with 100 μg/ml ampicillin. pET22b-anti-HER2-scFv and pKJE7 plasmids were co-transformed into electrocompetent *E. coli* BL21 (DE3) by electroporation (Bio-Rad, Gene Pulser Xcell at 2500 V, 2000 Ω and 25 μF with 0.2 cm cuvette) and plated onto LB agar plates supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml).

General expression of anti-HER2 scFv

A single positive colony harboring pET22b-anti-HER2-scFv plasmid was cultured in LB broth containing 100 μg/ml ampicillin and incubated at 37°C and 180 rpm until reaching the logarithmic phase (OD_{600} = 0.5). The expression of the protein was induced by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and then the culture was further incubated at the same condition for 4 h.

For protein co-expression with chaperones, a single colony harboring both pET22b-anti-HER2-scFv and pKJE7 plasmids was inoculated into LB broth supplemented with appropriate antibiotics and grown until an OD_{600} ~ 0.5 was reached. Subsequently, to initiate the expression of molecular chaperones, 0.5 mg/ml L-arabinose was added, and the culture was incubated at 37°C and 180 rpm for 30 min.[15] Then, to induce the expression of target protein, 1 mM IPTG was added, and the culture was incubated with shaking at the same condition for an additional 4 h [Figure 1].

Effect of IPTG concentration and temperature on co-expression of anti-HER2-scFv

After reaching the exponential phase and adding L-arabinose, different IPTG concentrations (0.5, 1 or 1.5 mM) were used to induce the expression of the target protein.[13] Then, the cultures were incubated at 37°C and 180 rpm for 4 h. To evaluate the effect of temperature on soluble expression of anti-HER2-scFv, the logarithmic phase cultures were incubated at different temperatures (37, 30, and 23°C) for 30 min. Then, the expression of proteins was induced by thr addition of inducers (0.5 mg/ml L-arabinose and 0.5 mM IPTG), and the cultures were shaken for 4, 6, and 18 h at 37, 30, and 23°C, respectively.

Purification of soluble anti-HER2-scFv

At the end of the expression, bacterial cells were collected by centrifugation at 7,500 × *g* for 10 min. The pellets were subjected to cell disruption by ultra-sonication as described previously. Then, the samples were centrifuged at 11,000 × *g* for 20 min at 4°C to separate the soluble and insoluble fractions. The supernatant (the soluble fraction) was loaded into a Ni-NTA (Invitrogen, USA) column, and after four wash with the Native Wash Buffer (50 mM NaH_2PO_4, 0.5 M NaCl, and 20 mM imidazole, pH 8.0), the target protein was eluted using the Native Elution Buffer (50 mM NaH_2PO_4, 0.5 M NaCl and 250 mM imidazole, pH 8.0).

Analytical methods

The protein samples were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels and ran with a constant voltage of 150 V for 45 min (SDS-PAGE). The purified proteins were analyzed by SDS-PAGE and then were transferred to PVDF membranes before western blotting by anti-his(C-term)-HRP antibody (Invitrogen, USA) as previously explained.[14] The amount of soluble protein was determined by densitometry analysis of the corresponding band using TL120 software (USA). The concentration of purified anti-HER2-scFv was measured using the Bradford method.

Ethical approval

Ethical approval The study was approved by the Ethics Committee of Isfahan University of Medical Sciences in 2017 (ethics approval code: IR.MUI.REC.1396.3.392).

Results

General expression of anti-HER2 scFv

First, the expression of anti-HER2 scFv with or without chaperones was performed under general induction conditions. SDS-PAGE analysis confirmed the successful co-expression of anti-HER2-scFv (27 KDa) and dnaK (69 KDa)/dnaJ (41 KDa)/grpE (24 KDa) chaperones and that the apparent molecular weight of each protein band on the gel exhibited consistently with its theoretical molecular weight [Figure 2]. It was observed that co-expression with chaperones improved soluble expression of the target protein [Figure 2].

Effect of IPTG concentration on co-expression of anti-HER2-scFv

The effect of three different IPTG concentrations (0.5, 1, and 1.5 mM) on co-expression of anti-HER2-scFv was evaluated. As SDS-PAGE analysis showed [Figure 3a], soluble expression of anti-HER2-scfv was remarkably decreased at higher concentrations of inducer (>1 mM). Our findings showed that approximately the same amount of soluble protein was obtained by induction with 0.5 and 1 mM IPTG. Thus, the following inductions were conducted with 0.5 mM IPTG.

Effect of cultivation temperature on co-expression of anti-HER2-scFv

Densitometry analysis of SDS-PAGE results revealed that the optimum temperature for co-expression of soluble anti-HER2-scFv was 23°C [Figure 3b]. Our results also showed that the reduction of cultivation temperature significantly improved soluble expression of anti-HER2-scFv with or without chaperones.

Purification of soluble anti-HER2-scFv

Our results showed that co-expression with chaperones

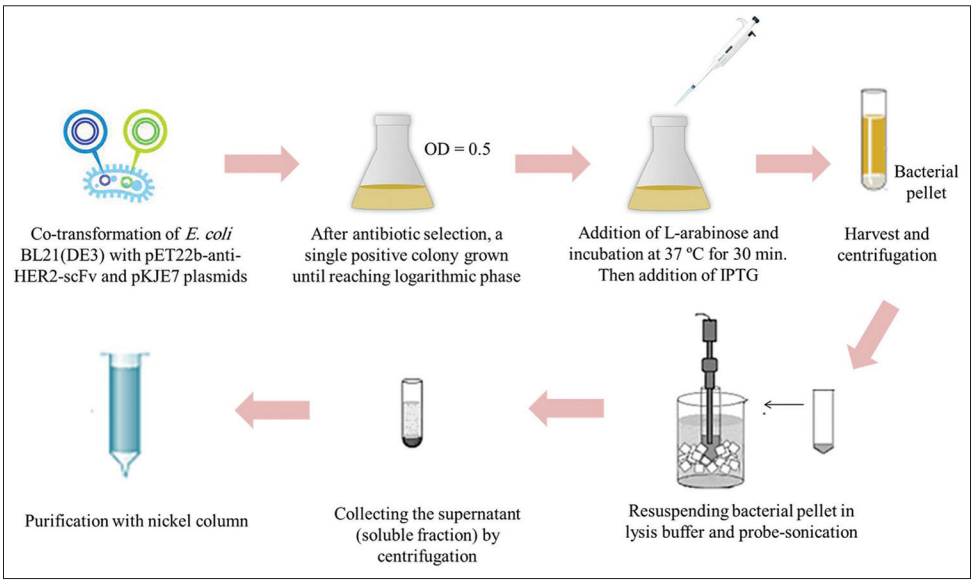

Figure 1: Schematic illustration of co-expression with chaperones and soluble purification approaches used in this work

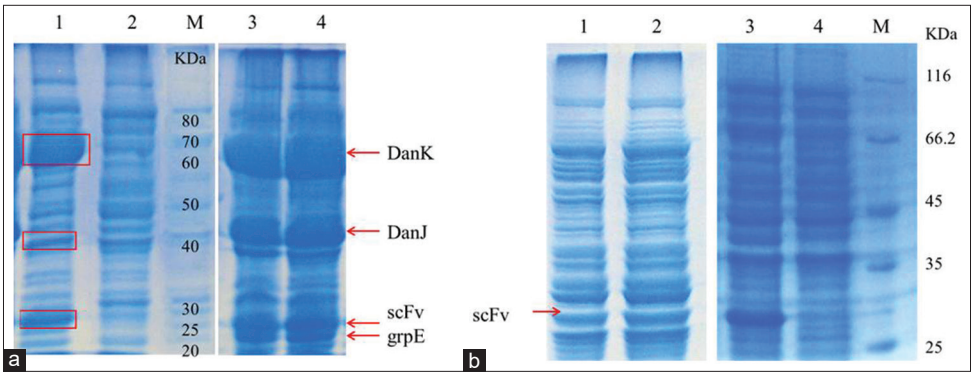

Figure 2: (a) SDS-PAGE analysis of expression of anti-HER2 scFv with chaperones. Lane 1: Total protein from *E. coli* BL21 (DE3) containing pET22b-scFv and pKJE7 plasmids after the induction with 1 mM IPTG and 0.5 mg/ml L-arabinose for 4 h at 37°C; Lane 2: Uninduced total bacterial protein; Lanes 3 and 4: Soluble fractions from *E. coli* BL21 (DE3) containing pET22b-scFv and pKJE7 plasmids after the induction with 1 mM IPTG and 0.5 mg/ml L-arabinose for 4 h at 37°C; M: Protein marker (Thermo Scientific 26619). (b) SDS-PAGE analysis of expression of anti-HER2-scFv without chaperones. Lanes 1 and 2: Soluble fractions from *E. coli* BL21 (DE3) containing pET22b-scFv plasmid after the induction with 1 mM IPTG for 4 h at 37°C; Lane 3: Total protein from *E. coli* BL21 (DE3) containing pET22b-scFv plasmid after the induction with 1 mM IPTG for 4 h at 37°C; Lane 4: Uninduced total bacterial protein; M: Protein marker (Thermo Scientific 26610)

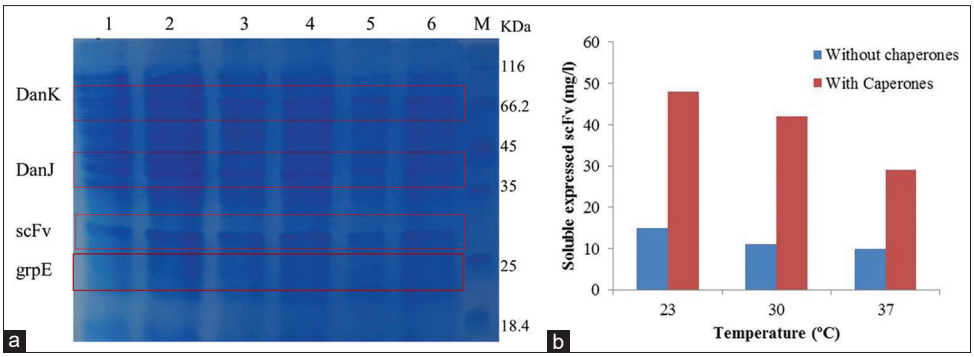

Figure 3: (a) The effects of the inducer concentration on soluble co-expression of scFv with chaperones at 37°C. Proteins were separated on a 12% SDS-PAGE gel and visualized by coomassie brilliant blue R250 staining. Lanes 1 and 2: Soluble fractions from recombinant *E. coli* BL21 (DE3) after the induction with 0.5 mM IPTG and 0.5 mg/ml L-arabinose for 2 and 4 h, respectively; Lanes 3 and 4: Soluble fractions from recombinant *E. coli* BL21 (DE3) after the induction with 1 mM IPTG and 0.5 mg/ml L-arabinose for 2 and 4 h, respectively; Lanes 5 and 6: Soluble fractions from recombinant *E. coli* BL21 (DE3) after the induction with 1.5 mM IPTG and 0.5 mg/ml L-arabinose for 2 and 4 h, respectively; M: Protein marker (Thermo Scientific 26610). (b) Comparison between the amounts of soluble anti-HER2-scFv expressed with or without chaperones after the induction with 0.5 mM IPTG and 0.5 mg/ml L-arabinose at 23, 30, or 37°C. The protein quantity was determined by densitometry analysis of the corresponding band using TL120 software

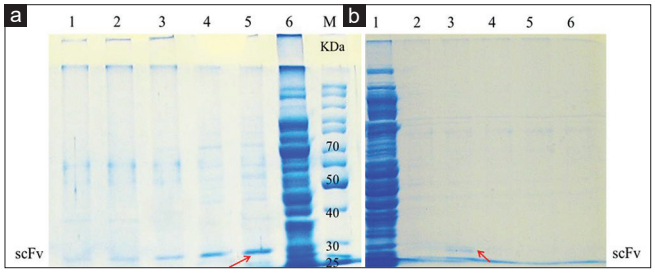

Figure 4: SDS-PAGE analysis of the purified scFv using native NIT purification procedure. (a) Purification of scFv co-expressed with chaperones. Lanes 1-5: Eluted fractions with 250 mM imidazole; Lane 6: Soluble fractions from *E. coli* BL21 (DE3) containing pET22b-scFv and pKJE7 plasmids after the induction with 0.5 mM IPTG and 0.5 mg/ml L-arabinose at 23°C; M: Protein marker (Thermo Scientific 26619). (b) Purification of scFv expressed without chaperones. Lane 1: Soluble fractions from *E. coli* BL21 (DE3) containing pET22b-scFv plasmid after the induction with 0.5 mM IPTG at 23°C Lanes 2-6: Eluted fractions with 250 mM imidazole

significantly improved the yield of purification of soluble anti-HER2-scFv [Figures 4 and 5]. The final yield for scFv co-expressed with chaperones was about 34.3% from the initial 3.2 mg of total protein, and the purity was 93% [Table 1].

Discussion

Antibody fragments, particularly scFvs, have been wildly used as diagnostic and therapeutic agents.[16] However, their functional production has been a challenge for manufacturers due to potential misfolding and the formation of inclusion bodies. Different strategies have been applied to improve the soluble expression of scFvs with reasonable costs.[17] Co-expression with chaperones has been applied to effectively improve the proper folding and solubility of recombinant proteins, including scFvs in *E. coli*. DnaK, DnaJ, and GrpE are members of the HSP70 family whose activity requires ATP hydrolysis.[18] They play an important role in the quality control of expressed proteins in *E. coli* and assist their correct folding. These molecular chaperones attach to the polypeptide chain released from the ribosome and maintain it in partially unfolded conformations, thus avoiding misfolding and aggregation of proteins. They also promote refolding of non-native proteins.[19]

In the present work, DnaK/DnaJ/GrpE and anti-HER2 scFv were independently co-expressed using different inducers (L-arabinose and IPTG), and the expression of molecular chaperones was induced before expression of scFv. Similar to our approach, another research group reported when the expression of chaperones under control of the *araB* promoter started before the expression of polyhydroxyalkanoate synthase, the yield of soluble and active enzymes enhanced more than 6-fold.[15,20]

To improve anti-HER2 scFv solubility, three different cultivation temperatures were evaluated. Our finding showed that the soluble expression of scFv, either with or without co-expression with molecular chaperones, remarkably increased at a low temperature (23°C). Co-expression with chaperones and low-temperature cultivation synergistically improved the soluble expression of anti-HER2 scFv. Similarly, Jhamb *et al*.[21] showed that co-expression with DnaK/DnaJ/GrpE chaperones and reducing cultivation temperature led to significant enhancement of the yield of soluble xylanases. Different mechanisms could explain this improvement. The low temperature might reduce hydrophobic interactions leading to the formation of inclusion bodies.[22] Furthermore, the low-temperature culture results in a reduced rate of protein expression and more efficient protein folding. Finally, the expression and activity of chaperones is temperature-dependent and improves at low temperatures.[23,24]

Interestingly, co-expression with chaperone significantly improved anti-HER2 scFv purification under native conditions (approximately four-fold increase in the final yield of purified soluble protein). In agreement with our

Table 1: Summary of the yields of anti-HER2his-scFv protein expressed with or without chaperones during purification steps. After the addition of lysis buffer, the bacterial pellet was disrupted by probe-sonication (whole cell lysate). The soluble fraction (crude supernatant) was collected by centrifugation and then loaded onto a nickel column and purified (Native NIT purification). The yield of each step was calculated using the following equation: Amount of the target protein recovered after the purification step/Initial amount of the target protein in whole cell lysate ×100

Purification step	scFv (mg)		Yield (%)		Purity (%)	
	Without chaperones	With chaperones	Without chaperones	With chaperones	Without chaperones	With chaperones
Whole cell lysate[a]	2.9	3.2	100	100	29.4	21.5
Crude supernatant	0.8	2.4	27.6	75	9.2	6.1
Native NIT purification	0.28	1.1	9.7	34.3	91	93

[a]From pellet obtained from 50 ml cell culture

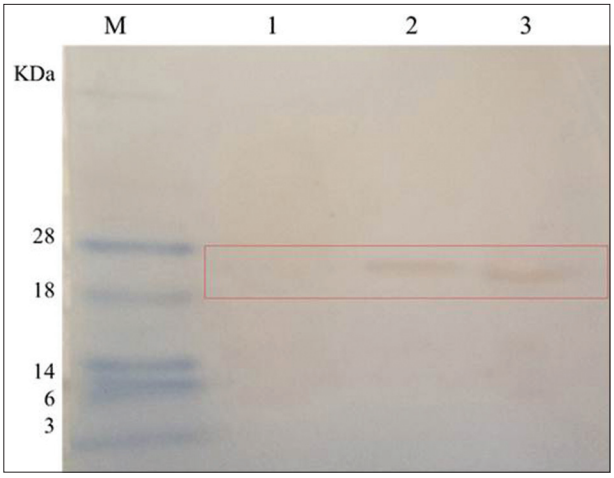

Figure 5: Western blot analysis of purified soluble scFv using native NIT purification procedure. Lane 1: Soluble purified scFv from *E. coli* BL21 (DE3) containing pET22b-scFv plasmid after the induction with 0.5 mM IPTG at 23°C; Lane 2: Soluble purified scFv from *E. coli* BL21 (DE3) containing pET22b-scFv and pKJE7 plasmids after the induction with 0.5 mM IPTG and 0.5 mg/ml L-arabinose at 23°C; Lane 3: Soluble purified scFv from *E. coli* BL21 (DE3) containing pET22b-scFv and pKJE7 plasmids after the induction with 0.5 mM IPTG and 0.5 mg/ml L-arabinose at 30°C; M: Protein marker (Thermo Scientific SeeBlue)

results, de Marco *et al*.[25] reported that chaperones enhanced yields (up to 5.5-fold) of purified soluble proteins expressed in *E. coli*. They evaluated the effect of four chaperone systems, including DnaK/DnaJ/GrpE, on the recovery rate of 50 target proteins during native purification and showed a remarkable improvement in the yield of purification for near 50% of the proteins examined.[25] These data, as well as ours, indicate that co-expression with chaperons not only leads to increased solubility of target proteins but also improves the folding of protein to the native state.

Conclusion

In this work, we reported the improvement of soluble anti-HER2 scFv expression in *E. coli* using co-expression with DnaK/DnaJ/GrpE chaperones and reducing cultivation temperature. The obtained soluble protein was efficiently purified using affinity chromatography under native conditions. The combination of co-expression with chaperones and low temperature presented in this work may be useful for the improvement of commercial production of other scFvs in *E. coli* as functionally bioactive and soluble form.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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