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Research Article

Cytoplasmic Chaperones Enhance Soluble Expression of Anti-EGFR huscFv in *Escherichia coli*

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Background: Overexpression of EGFR is associated with carcinogenesis in more than 70% of head and neck cancers. Anti-EGFR monoclonal antibodies bind to the extracellular domain of EGFR and block the EGFR downstream signaling pathway, which results in the suppression of the growth of the tumor cells. *Escherichia coli* is the preferred system for expressing various recombinant proteins, including single chain antibodies, but the formation of inclusion bodies negatively affects the efficacy of this system. Several strategies have been suggested to solve this problem, notably the utilization of molecular chaperones.
Objectives: In this study, we attempted to increase the soluble expression of huscfv antibody via co-expression with the cytoplasmic chaperones.
Materials and Methods: To achieve this purpose, chaperones plasmids pG-KJE8, pGro7, pKjE7, pTf16 and pG-Tf2 encoding cytoplasmic chaperones were co-expressed with the humanized anti-EGFR scFv construct in *E. coli*. Different temperatures, incubations times, and concentrations of IPTG were used to produce an active antibody with the highest solubility. Results were analyzed by SDS-PAGE. Soluble huscFv was purified by Ni-NTA column and the biologic activity of the recombinant protein was determined by ELISA.
Result: The results indicated that the highest concentrations of humanized anti-EGFR scFv were obtained by co-expression of huscFv via chaperone plasmid pG-KJE8 with 0.2 mM concentration of inducer (IPTG), culture temperature of 25 °C, and 4 h incubation time after induction.
Conclusion: In conclusion, co-expression with chaperones could be used as an efficient strategy to produce soluble active ScFvs in *E. coli.*
Keywords: EGFR; Inclusion body; Molecular chaperones; scFv; Soluble Expression

1. Background

Epidermal growth factor receptor (EGFR) is a member of the human epidermal growth factor receptor (HER) family receptor kinases (ErbBs). EGFR plays essential roles in regulating cell survival, proliferation, differentiation, and migration (1). Overexpression or increased activity of this receptor underlies the initiation and the progression of various types of cancers, especially colorectal, pancreas, prostate, lung, breast, and head and neck cancers (2). Thus, inhibiting the EGFR signaling pathway is an obvious strategy for targeted therapy (3, 4). One of the most effective approaches for EGFR-based targeted therapy is monoclonal antibodies (Mabs) (5). Mabs bind to the extracellular domain of EGFR and block autophosphorylation and the intracellular signaling pathway. Cetuximab is an anti-EGFR Mab used for colorectal and head and neck cancers (6). Despite the benefits of this Mab, it has some limitations (7), such as inducing human antimurine antibody (HAMA) response, that restrict multiple uses of the antibody. On the other hand, the large size of this full-length Mab reduces its penetration into the tumor tissues (8-10) (11).

Single chain antibodies (ScFv) are truncated recombinant antibodies that are composed of variable heavy (VH) and light (VL) chains that are joined together by a flexible peptide linker. Humanized ScFvs maintain antigen binding sites and overcome the limitations related to the immunogenicity and the large size of full-length antibodies (12, 13). Moreover, ScFvs could be expressed in *Escherichia coli;* an organism recognized as a fast and economic system for large-scale production of recombinant proteins (14-16). Despite several advantages of this system, production of biologically active

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recombinant antibodies by this bacterium is limited due to the formation of insoluble inclusion bodies (IBs). IBs are aggregates of over-expressed misfolded proteins that lack correct structure and hence are biologically inactive (17). To overcome this problem, several strategies have been developed, including periplasmic expression by the aid of signal peptide, expression in low temperature/low inducer concentration, and fusion with solubility-enhancing tags and co-expression with molecular chaperones. It has shown that the co-expression of ScFv with molecular chaperones facilitates the correct folding of the recombinant antibodies (18). The classes of cytoplasmic chaperones that have an important role in protein folding are GroEL, DnaK, GroES, Tig, DnaJ, and GrpE. It has been shown that the co-expression of ScFvs and the chaperone plasmids PG-tf2 (GroES- GroEL- tig), ptf16 (tig), pGro7 (GroES- GroEL), pKJE7 (grp E- dnaJ- dnaK), and pG-KJE8 (groES- groEL- dnaK- dnaJ - grpE) leads to a noticeable increase in the expression of soluble proteins (19, 20). These chaperone plasmids contain pACYC- the origin of replication and carry a chloramphenicol-resistance gene (*Cmr*), *araB* and/ or *Tet* promoters, which can be induced by L-Arabinose and Tetracycline. The aim of this study was to evaluate the effects of cytoplasmic chaperones on the expression of soluble humanized anti-EGFR scFv in *E. coli* (21). In addition, we evaluated the effects of culture and induction condition on the solubility and the activity of the recombinant scFv co-expressed with selected chaperones.

2. Objectives

The aim of this study was to increase the expression of soluble humanized anti-EGFR scFv by co-expression with cytoplasmic chaperones under different conditions and to evaluate its activity on the EGFR-overexpressing tumor cell line A431.

3. Materials and Methods

3.1. Cell lines, ScFv Construct, Chaperone Plasmids

The *E. coli* strains, including BL21 (DE3), SHuffle® T7 Express Competent (NEB), BL21 Rosetta DE3, Inv, and the expression vector pET22b (+) were purchased from Novagen and New England Biolabs, respectively. The chaperone plasmid set was purchased from Takara Bio Inc. (**Table 1**). pET22b - humanized anti-EGFR ScFv construct was designed by Veisi K *et al*.(22) (**Fig. 1**).

Table 1. List of the molecular chaperones used in this study.

Plasmid	Proteins	Resistance marker	Promoter	Inducer
pG-KJE8	dnaK-dnaJ-grpE-groES-groEL	Cm	*araB* *Pzt1*	L-Arabinose Tetracycline
pGro7	groES-groEL	Cm	*araB*	L-Arabinose
pKjE7	DnaK-dnaJ-grpE	Cm	*araB*	L-Arabinose
pTf16	groES-groEL-tig	Cm	*Pzt1*	Tetracycline
pG-Tf2	tig	Cm	*araB*	L-Arabinose

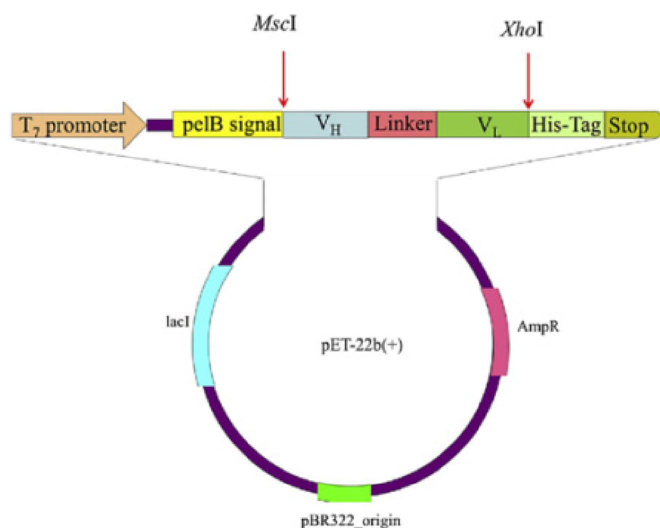

Figure 1. The schematic diagram of the recombinant pET22b-ScFv expression vector. The gene encoding anti-EGFR scFv was inserted into the pET22b vector under the control of the T7 lac promoter, in frame with a c-terminal histidine (6×His) tag.

The expression construct was transformed into *E. coli* BL21 (DE3) and the transformation was confirmed by PCR using designated primers (Forward: 5'-TGGCCATGCAAGTGCAGCTG-3' and reverse 5'- CTCGAGTTTGATTTC CAGTTTGGTG-3'). The conditions for PCR were as follow: initial denaturation at 94 °C for 3 min, cyclic denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min, and a final extension cycle at 72 °C for 5 min.

3.2. Selection of E. coli Host for Expression of Humanized Single Chain Antibody

Four Different strains of *E. coli,* including SHuffle® T7 Express Competent (NEB), BL21 (DE3), BL21 Rosetta DE3, and BL21 Inv cells were competent. The expression vector pET-22b carrying the humanized anti-EGFR single chain antibody (huscFv) was separately transformed into the competent cells. A single colony of each transformed strains was inoculated into the LB medium containing 100 µg.mL^{-1} of ampicillin and was incubated for 24 h at 37 °C on a shaker (140 x g) until the cultures reached the optical density ~ 0.7 at 600 nm (OD600). The cultures were then induced by adding IPTG (1 mM) and each sample was evaluated after 4h and 24h. To analyze expression, the cells were harvested (10,000 x g, for 15 min) and analyzed by sodium dodecyl sulfate-polyacrylamide gel (12%) electrophoresis (SDS-PAGE).

For analyzing the solubility of the expressed scFv, the cells were dissolved in 10 mL of lysis buffer (50 mM NaH_2PO_4, 100 mM NaCl, pH-8) and lysed by sonication (ten 30 s pulses interrupted by 30 s on ice). The lysate was then centrifuged at 10,000 x g for 10 min at 4 °C and the soluble and the insoluble fractions were separated and analyzed by SDS-PAGE.

3.3. Co-Expression of Anti-EGFR-huscFv with Molecular Chaperones

E. coli BL21 (DE3) cells containing pET22b- huscFv were separately transformed with five chaperone plasmids (pG-KJE8, pGro7, pKjE7, pG-Tf2, and pTf16). The cells were cultured in LB medium containing 100 mL LB medium with 20 µg.mL^{-1} chloramphenicol and 50 µg.mL^{-1} ampicillin. Induction of cells carrying chaperones pGro7, pKjE7, and pG-Tf2 was done by 0.6 mM L-arabinose, pG-KJE8 by 0.6 mM L-arabinose and 10 ng.mL^{-1} tetracycline, and pTf16 by 10 ng.mL^{-1} tetracycline. For better results, plasmids were induced at the beginning of culturing. After OD (595 nm) reached 0.6, different IPTG concentrations (0.05, 0.1, 0.2, 0.4, and 1 mM) and different incubation times were investigated at various temperatures. Then, the cells were harvested by centrifugation at 10,000 g for 10 min, resuspended in lysis buffer and sonicated. The lysate was centrifuged and the soluble and the pellet fractions were tested by 12% SDS-PAGE. The SDS-PAGE images were scanned and the density of bands was analyzed with ImageJ 1.42.

3.4. Purification of Soluble huscFv Fragments

After 4 h of culturing cells at optimal condition (0.2 mM IPTG, 25 °C) in 200 mL of LB medium, cells were harvested and sonicated. The resultant supernatant was loaded on Ni-NTA column equilibrated with 2 mL of binding buffer (NaH_2Po_4 (100 mM), NaCl (300 mM) at pH 8). Then the column was washed with 25 mM imidazole to remove nonspecific proteins and the bound protein was released by adding the elution buffer (250 mM imidazole). Finally, the purified huscFv-anti-EGFR was dialyzed against PBS (pH 7.4) to eliminate imidazole. The quality of purified huscFv was assessed by 12 % SDS–PAGE. Based on the size of the coding sequence for *huscFv,* the predicted molecular weight of recombinant antibody was 28kDa.

3.5. Assessment of Antigen-Binding Activity of huscFv by ELISA

The binding specificity of the humanized single chain antibody against EGFR was examined by enzyme-linked immunosorbent assay (ELISA) test. The A431 cells (10^6 cells/well) were coated overnight at 4 °C on ELISA plates. Afterward, the plates were washed and blocked with PBS containing 2% bovine serum albumin (BSA), 300 µL per well, for 1 h at room temperature. Next, 100 µL of various dilutions of soluble purified huscFv and cetuximab – as the positive control - were added to the plate and incubated for 1 h at 4 °C. The wells were washed with PBS, 3 times and after that incubated with the HRP-conjugated Protein L (1:2000 dilutions in 1% BSA PBS) (for scFv) and the HRP-conjugated anti-human IgG (for cetuximab) for 1 h. Finally, the reaction was stopped using TMB substrate and the absorbance values were determined by spectrophotometer at 450 nm.

4. Results

4.1. Transformation and Expression of the Humanized Single Chain Antibody

The pET-22b–huscFv construct encoding humanized anti-EGFR scFv with an in-frame C-terminal 6 His-tag was transformed into *E. coli* BL21 (DE3) and the transformation was confirmed by polymerase chain reaction (PCR) (**Fig. 2**).

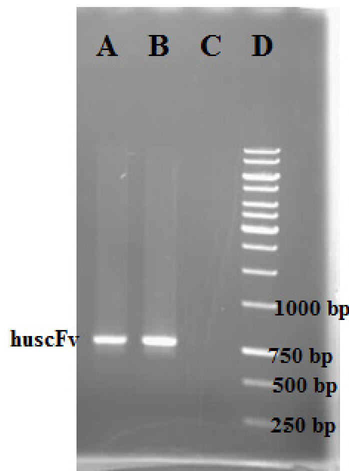

Figure 2. PCR confirmation of the pET-22b-huscFv constructs transformation into the *E. coli* BL21 (DE3). Lane A –B) indicates the pET-22b-huscFv clones; Lane C) indicates the negative control; Lane D) indicates the DNA size marker.

To attain an appropriate level of expression, the humanized anti- EGFR ScFv was expressed in various strains of *E. coli* (SHuffle® T7 Express Competent (NEB), BL21 (DE3), BL21 Rosetta DE3, and Inv). The cells containing ET22b-huscFv were cultured and induced by 1 mM IPTG for 4 h at 37 °C. SDS-PAGE analysis showed the highest expression of humanized anti- EGFR ScFv in *E. coli* BL21 (#c2527) with the molecular weight of 28 kDa, in concert with the computed molecular weight of huscFv (**Fig. 3**).

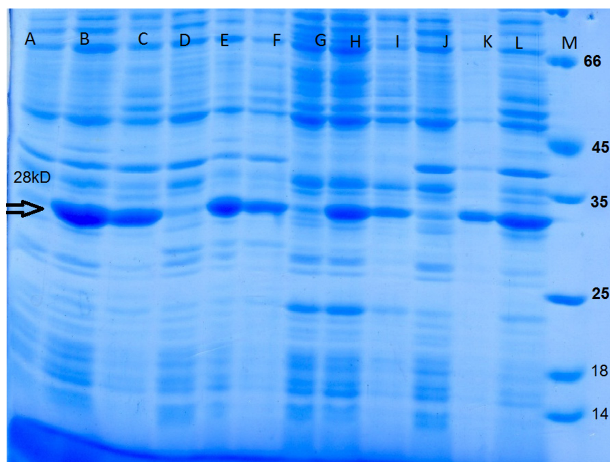

Figure 3. SDS-PAGE analysis of the expressions of huscFv in different strains of *E. coli*. Lane A) BL21 (DE3) before inductions; Lane B) BL21 (DE3) after 4h induction; Lane C) BL21 (DE3) after 24h induction; Lane D) Rosetta (DE3) before induction; Lane E) Rosetta (DE3) after 4h induction; Lane F) Rosetta (DE3) after 24h induction; Lane G) SHuffle® T7 strain before inductions; Lane H) SHuffle® T7 after 4h induction; Lane I) SHuffle® T7 after 24h induction; Lane J) BL21 (Inv) before induction; Lane K) BL21 (Inv) after 4h induction; Lane L) BL21 (Inv) after 4h induction and Lane M) indicates the protein marker.

Analysis for the solubility of the expressed huscFv in BL21 (#c2527 by SDS-PAGE indicated that the majority of expressed huscFv appeared as IBs (**Fig. 4**).

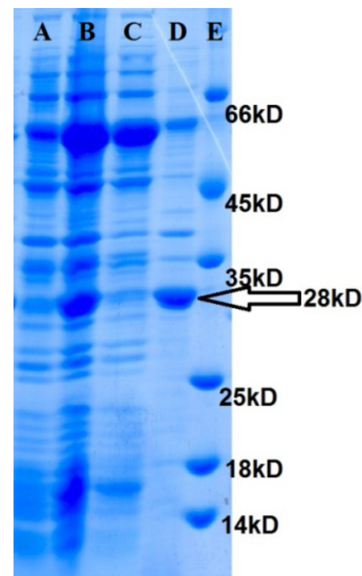

Figure 4. SDS-PAGE analysis of the solubility of huscFv expressed in *E. coli*. Lane A) before induction; Lane B) after induction; Lane C) the soluble fraction; Lane D) the insoluble fraction and Lane E) indicates the protein size marker.

4.2. Soluble Expression of huscFv

To increase the solubility of the anti-EGFR huscFv in *E. coli*, different chaperones plasmids (pGro7, pG-KJE8, pTf16, pKjE7, and pG-Tf2) were separately co-expressed with the humanized anti- EGFR ScFv. To optimize the expression of soluble proteins, different IPTG concentration (0.05, 0.1, 0.2, 0.4, and 1 mM) different incubation times and various temperatures were tested. The results indicated that at lower temperatures, low IPTG concentration, and incubation time up to 4h the co-expression of the humanized anti-EGFR ScFv with chaperones significantly increased the solubility of huscFv.

Comparing the effects of various chaperones by the ELISA test revealed that the co-expression with chaperone plasmids pG-KJE8 and pGro7 increased the solubility more than other chaperones evaluated (**Table 2**).

To optimize the soluble expression, co-expression of humanized anti- EGFR ScFv with pG-KJE8 and pGro7 chaperone were performed and different IPTG concentrations (0.05, 0.1, 0.2, 0.4, 1 mM) at 25 °C for 4 h were analyzed by SDS–PAGE. Then images of SDS–PAGEs were scanned and the densitometry of each band was analyzed with Image J software. The results indicated that co-expression of huscFv with chaperone plasmid pG-KJE8 by 0.2 IPTG at 25° C for 4h, extremely improved soluble of huscFv antibody (**Table 3**).

Table 2. The Comparison of the reactivity of soluble huscFv obtained by co-expression with different chaperones in ELISA.

Agent	OD (450)
Humanized scFv	0.674
Humanized scFv + pG-KJE8	2. 793
Humanized scFv + pGro7	2.180
Humanized scFv + pKjE7	1.434
Humanized scFv +pTf16	1.870
Humanized scFv + pG-Tf2	1.107
Control +	2.864

Table 3. Densitometric analysis of SDS-Page results using Image J software.

Chaperone	IPTG	Time	Supernatant	pellet
pGro7				
	0.05	4h	36%	63%
	0.1	4h	44%	56%
	0.2	4h	46%	53%
	0.4	4h	42%	58%
pG-KJE8				
	0.05	4h	37%	63%
	0.1	4h	39%	61%
	0.2	4h	54%	46%
	0.4	4h	42%	57%
Without chaperone				
	0.4	4h	26%	73%

4.3. Purification of Soluble Humanized Anti- EGFR ScFv

The BL21 (DE3) cells containing pG-KJE8 and pET22b-huscFv in frame with the C-terminal 6 His-tag were co-expressed and induced by 0.6 mM L-arabinose, 10 ng.mL^{-1} tetracycline and 0.2 mM IPTG at 25 °C for 4 h. After sonication, the supernatant was collected and purified by Ni–NTA column and results were checked by SDS–PAGE.

In addition, the BL21 (DE3) cells without plasmids and the BL21 (DE3) cells containing huscFv plasmid but lacking chaperone plasmid were used as the controls (**Fig. 5**).

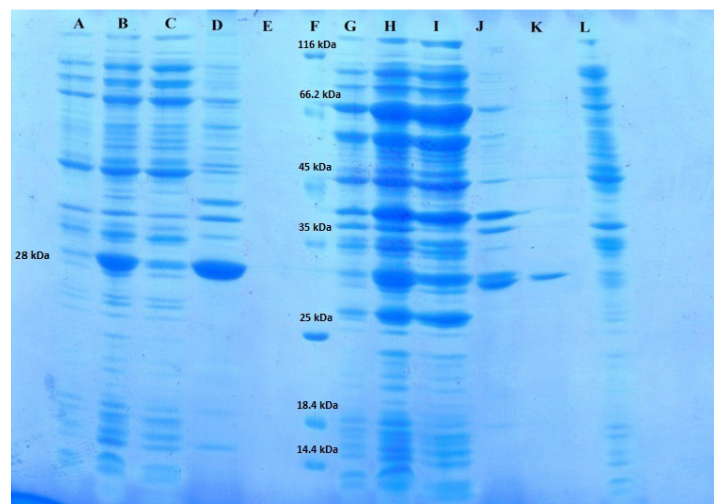

Figure 5. Analyses of huscFv expression by BL21 (DE3). Lane A) pET22b-huscFv before inductions; Lane B) pET22b-huscFv after 4h of induction; Lane C) pET22b-huscFv, sonication supernatant fraction; Lane D) pET22b-huscFv sonication pellet fraction; Lane E) pET22b-huscFv, purified huscFv; Lane F) protein marker; Lane G) pET22b-huscFv + pG-KJE8 chaperone, before inductions; Lane H) pET22b-huscFv + pG-KJE8 chaperone, after 4h induction; Lane I) pET22b-huscFv + pG-KJE8 chaperone, sonication supernatant fraction; Lane J) pET22b-huscFv + pG-KJE8 chaperone, sonication pellet fraction; Lane K) pET22b-huscFv + pG-KJE8 chaperone , purified huscFv and Lane L) indicates BL21 (DE3) without plasmid, 4h after induction (control).

4.4. ELISA Test for Reactivity Analysis
The reactivity of the purified soluble huscFv co-expressed with pG-KJE8 chaperon plasmid, with EGFR on A431 carcinoma cells was investigated using the ELISA test. The results indicated that huscFv co-expressed with chaperones were more reactive compared to huscFv expressed without the chaperone (**Fig. 6**) (OD value of 3.65), which indicates the correct folding of the purified protein.

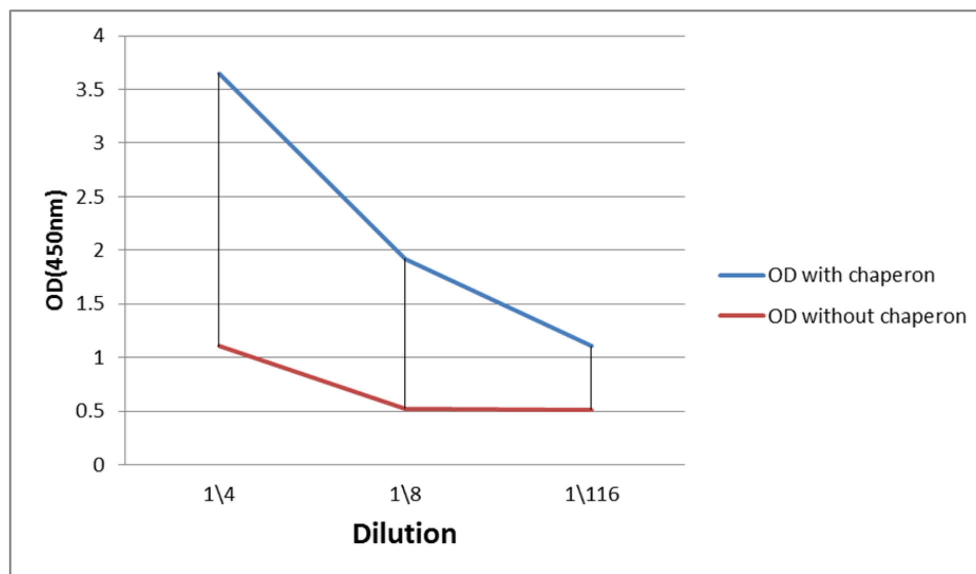

Figure 6. Analyses of the reactivity of the soluble anti-EGFR huscFv co-expressed with the chaperone plasmid pG-KJE8 and soluble anti-EGFR huscFv expressed without chaperones on A431 cells. Co-expressed huscFv showed higher reactivity in compare to that expressed without chaperone.

5. Discussion

Single-chain antibodies have several advantages and are considered a promising treatment in cancer targeted therapy (23, 24). The *E. coli* expression system is the most commonly used system to produce recombinant proteins. In spite of economical and biological benefits of *E. coli* expression system, it suffers from several noticeable shortcomings (15). One of its most serious limitations is the aggregation of misfolded recombinant proteins which leads to the formation of IBs (17, 25). Insoluble IBs are serious obstacles in producing biologically active recombinant proteins. Various approaches have been used to overcome these restrictions, including applying various molecular chaperones (26) and expression in the periplasmic space (27). In addition, reducing the inducer concentrations and the culture temperature decrease the speed and the level of expression, providing adequate time for proteins to fold correctly (28), (29).

The periplasmic expression has several benefits compared to the cytoplasmic expression as the more appropriate formation of disulfide bonds, the small number of proteases and the presence of fewer proteins in the periplasmic space lead to higher quality proteins (30, 31). Targeting recombinant proteins to the periplasmic space by using signal sequences (e.g., *PelB, ompA, PhoA* …) in a Sec-dependent system poses some difficulties, such as protein aggregation in the cytoplasm. Using low temperature and co-expression with secretion chaperones (e.g., DnaK/ DnaJ and GroEL-GroES) could overcome this obstacle (32). In this study, we applied various strategies to produce active huscFv in the *E. coli* expression system. Co-expression of different chaperone plasmids with huscFv under low temperature and low inducer concentration noticeably enhanced (up to 54%) the solubility of the recombinant scFv. Co-expressing pG-KJE8 chaperone plasmid carrying dnaK-dnaJ-grpE-groES-groEL cytoplasmic chaperone with huscFv resulted in highest solubility. This finding was in agreement with the report of Ha *et al.* (33) and showed that the co-expressing with pG-KJE8 chaperone plasmid had the highest effect on the solubility of anti-blood type A scFv and the target protein was expressed entirely in soluble form. In another study, Nishihara *et al*. showed that chaperone plasmids containing GroEL and GroES, alongside GrpE, DnaK, DnaJ, have synergic effects on the expression of insoluble proteins in *E. coli* (21). It has also been shown that the co-expression of Trigger factor (TF) with the recombinant human lysozyme

leads to higher expression of soluble proteins (20). The pGro7 chaperone plasmid, which contains groES-groEL, is effective in enhancing the solubility as well. Jin *et al.* showed that co-expression of 3-OST-1 with pGro7 chaperone significantly increased the solubility of target protein (34). In a similar study, Watanabe K showed that various chaperones have different effects on the soluble expression of human ST6Gal I (hST6Gal I), and chaperon pGro7 is the most efficient chaperon (35). Fang *et al.* reported that co-expression with GroEs-GroEL enhanced the solubility of chondroitinase AC II in *E. coli* (36). The higher efficiency of the pG-KJE8 may be ascribed to the presence of groES-groEL chaperones. Consequently, the pG-KJE8 may be considered a particularly suitable chaperone for huscFv. Despite the presence of groES-groEL, pGro7 plasmid was not as effective as pG-KJE8, indicating that groES-groEL functions better in the presence of dnaK-dnaJ-grpE. In our study, pG-Tf2, which contains the tig chaperone, was the least efficacious. Ultimately, comparing our results with previous works (37) illustrates that each specific chaperone has protein-specific effects on the enhancement of solubility. Furthermore, applying the optimal condition, such as lower IPTG concentration and reducing temperature down to 25 °C, could significantly affect the solubility.

6. Conclusion

The results of this study revealed that co-expression of the pG-KJE8 cytoplasmic chaperone plasmid containing groES- groEL- dnaK- dnaJ – grpE chaperone with humanized anti-EGFR scFv under special conditions could increase the amount of the active soluble scFv. In addition, testing the binding activity of huscFv on the EGFR-overexpressing A431 cell line demonstrated that the solubility was associated with the higher binding activity, indicating the correct folding of scFv antibody in soluble form.

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