Research in Pharmaceutical Sciences, June 2026; 21(3): 323-332
Received: 11-01-2024
Peer Reviewed: 29-02-2024
Revised: 03-05-2025
Accepted: 09-05-2025
Published: 10-05-2026

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Enhancing soluble expression and purification of a bispecific scFv antibody in *Escherichia coli* using chaperones

Zahra Alidousti-Shahraki[1], Afrooz Amini-Dehkordi [2], Nafiseh Esmaeil[3], and Vajihe Akbari[2,*]

[1]Student Research Committee, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran. [2]Department of Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran. [3]Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

Abstract

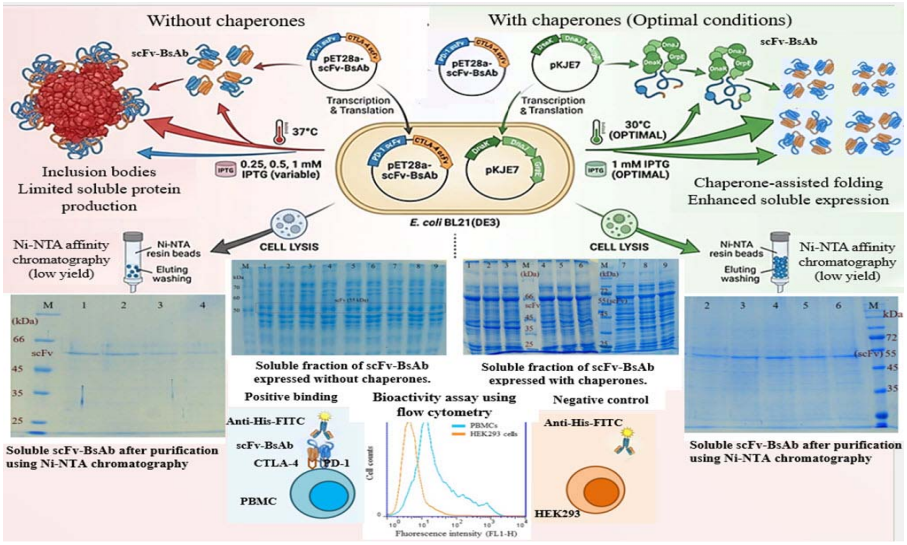
Background and purpose: The use of a bispecific antibody engineered as a tandem single-chain fragment variable antibody (scFv-BsAb) offers several advantages over full-length antibodies, including enhanced tissue penetration and the potential for production in bacterial cells. However, production of them in bacteria usually results in the formation of inclusion bodies. The current work aimed to facilitate proper folding and soluble expression of a recombinant scFv-BsAb targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) by co-expression with molecular chaperones.

Experimental approach: *Escherichia coli* BL21(DE3) cells harboring pET28a-scFv-BsAb and pKJE7 plasmids were used as host cells for protein expression. The effect of different temperatures and IPTG concentrations on the expression of soluble protein with or without expression of chaperones was investigated by SDS-PAGE and densitometry analysis. Subsequently, the expressed protein was subjected to native purification using a Ni-NTA affinity column. Then, the binding ability of the scFv-BsAb to the receptors on peripheral blood mononuclear cells (PBMCs) was evaluated by flow cytometry.

Findings/Results: Co-expression with chaperones slightly enhanced the expression of soluble scFv-BsAb. The optimal condition for soluble expression of protein is at a temperature of 30 °C and an IPTG concentration of 1 mM when co-expressed with chaperones. The final yield of protein in co-expression with chaperones was 2.9 mg protein per 1 L of culture.

Conclusion and implications: Optimization of culture conditions and co-expression with molecular chaperones may be useful for the production of soluble and functionally bioactive scFv antibodies in *E. coli*.

Keywords: Bispecific antibody; Immune checkpoint inhibitors; Molecular chaperones; Single-chain antibodies; Soluble expression.



*Corresponding author: V. Akbari
Tel: +98-3137927060, Fax: +98-3136680011
Email: v_akbari@pharm.mui.ac.ir

Access this article online

Website: http://rps.mui.ac.ir

DOI: 10.4103/RPS.RPS_10_24

INTRODUCTION

Many researchers have developed different formats of recombinant bispecific antibodies (BsAbs) that simultaneously block two different inhibitory immune checkpoints (1-4). One of these formats is tandem single-chain fragment variable (scFv) antibody (5).

Tandem scFv is one of the most commonly used BsAbs, which consists of two different scFvs linked by a flexible spacer. Tandem scFvs offer several advantages over full-length BsAbs, including better penetration into the target tissue and lower immunogenicity (5,6). Furthermore, due to their smaller size (*e.g.*, 55 kDa) and non-glycosylated structure, they can be easily overexpressed in bacterial host cells, including *E. coli*, leading to a gradual reduction in production costs (7,8).

Tandem scFvs are mainly expressed either in the periplasm or cytoplasm of *E. coli* cells. Periplasmic expression allows for better disulfide bond formation and proper folding of recombinant proteins (9). However, this approach usually results in lower yields of protein due to inefficient translocation and secretion of the target protein into the periplasmic space (10). The second method results in higher protein yields, which usually accumulate as insoluble aggregates (*i.e.,* inclusion bodies). To restore biological activity, inclusion bodies must be solubilized and refolded, which is usually a time-consuming, complex, and costly procedure (11-13). Alternatively, proteins can be co-expressed with molecular chaperones, which facilitate proper protein folding (14,15).

Chaperone proteins bind to nascent polypeptides, avoid deleterious hydrophobic interactions, and assist them in folding correctly and achieving native structure (16). *E. coli* uses two main sets of molecular chaperones, the GroEL/GroES (Hsp60/Hsp10) system and the DnaK/DnaJ/GrpE system (17-19). There are some reports on the enhancement of soluble production of various proteins, including scFvs in *E. coli* cells, by co-expression with molecular chaperones (20,21). For example, Mirzapour et al. found that co-expression of anti-HER2 scFv with DnaK/DnaJ/GrpE resulted in significant improvement in soluble protein purification and expression (up to 4-fold increase) (20).

Previously, we reported the expression of a bispecific tandem scFv against cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) in the periplasmic space of *E. coli* (9). To achieve a more biologically active bispecific antibody, in this study, we aimed to produce scFv-BsAb by cytoplasmic expression in an optimized condition and by co-expression with the molecular chaperones DnaK/DnaJ/GrpE. The effect of temperature and inducer concentration on the level of soluble protein production was also determined.

MATERIALS AND METHODS

Bacterial strain and plasmids

Protein expression was performed in *E. coli* BL21(DE3) bacteria (Novagen, USA), chemically competent *E. coli* cells. The gene of scFv-BsAb was chemically synthesized (Biomatik, Canada) and sub-cloned into the *Nco*I and *Xho*I restriction sites of the pET28a plasmid (Novagen, USA) (Fig. 1A) (22,23). In addition, the pKJE7 plasmid (carrying genes of dnaK, dnaJ, and grpE chaperones) was obtained from Takara (Shiga, Japan). The bacteria were transformed with the pET28a-scFv-BsAb plasmid or co-transformed with the pET28a-scFv-BsAb and pKJE7 plasmids by the heat shock method. Subsequently, positive colonies were spread on Luria-Bertani (LB) agar plates supplemented with 30 µg/mL kanamycin and 20 µg/mL chloramphenicol.

Effect of IPTG concentration and temperature on expression of scFv-BsAb

A single positive colony containing the pET28a-scFv-BsAb plasmid was added to LB medium containing 30 µg/mL kanamycin and cultured overnight in a shaker incubator at 37 °C and 180 rpm. After 10-fold dilution with LB medium containing 30 µg/mL kanamycin, the cells were again incubated in a shaker incubator at 37 °C and 180 rpm until the logarithmic phase was reached (OD_{600} = 0.5). Then, 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to the medium, and the incubation continued under the same conditions for a further 4 h to express the protein. For protein

co-expression with chaperones, a single colony containing both pET28a-scFv-BsAb and pKJE7 plasmids was inoculated in LB medium containing 30 μg/mL kanamycin and 20 μg/mL chloramphenicol, and after overnight cultivation and dilution, the medium was incubated in a shaker incubator at 37 °C and 180 rpm until the logarithmic phase was reached (OD_{600} = 0.5). To induce chaperone expression, 0.5 mg/mL L-arabinose was first added, and the culture was incubated for 30 min under the same conditions. To induce expression of the target protein, 0.5 mM IPTG was then added, and the incubation continued for an additional 4 h.

To examine the impact of IPTG concentration on the expression of the target protein, varying concentrations of IPTG (0.25, 0.5, or 1 mM) were utilized. Additionally, to assess the effect of temperature on the soluble expression of the target protein, bacterial cultures after addition of IPTG were incubated in a shaker incubator at different temperatures (25, 30, and 37 °C) (Table 1).

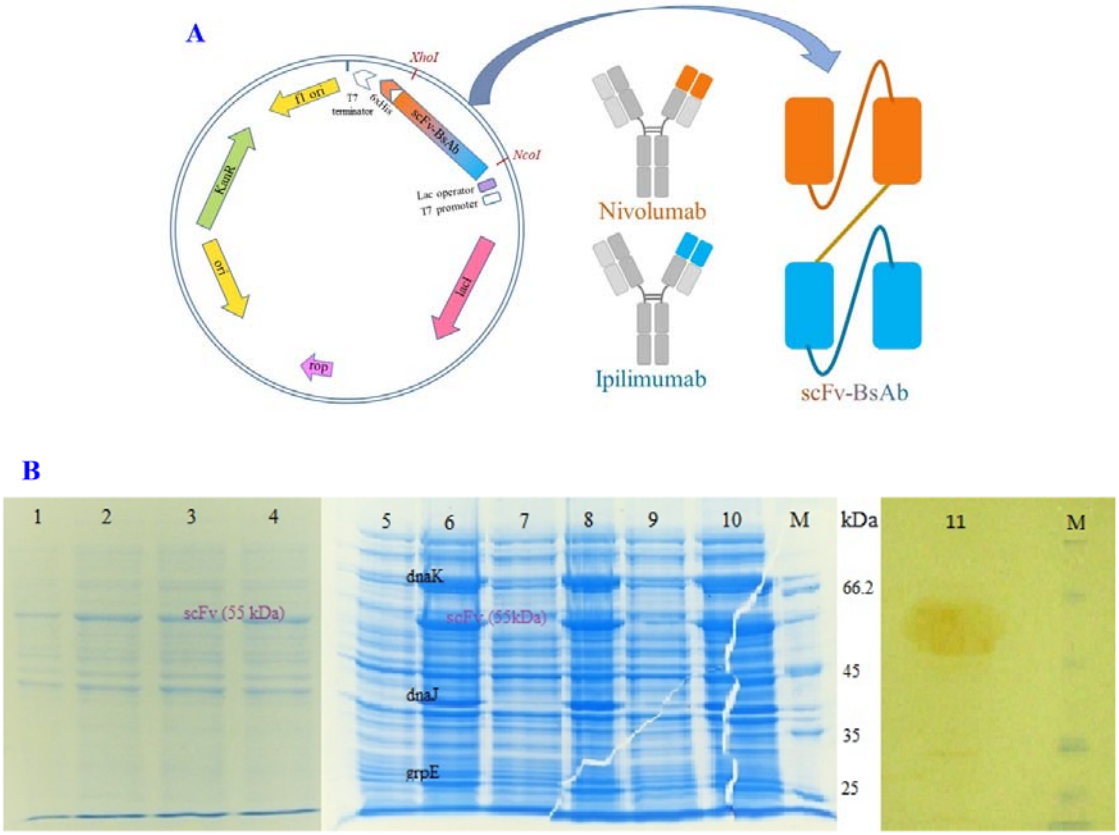


Fig. 1. (A) Schematic presentation of the pET28a-scFv-BsAb expression vector; (B) SDS-PAGE and western blot analysis of bacterial expression of histidine-tagged scFv-BsAb with or without chaperones. Lane 1: without chaperone before induction; lanes 2-4: without chaperone after induction by IPTG (0.25, 0.5, and 1 mM, respectively); lanes 5, 7, and 9: co-expression with chaperones before induction; lanes 6, 8, and 10: co-expression with chaperones after induction by IPTG (0.25, 0.5, and 1 mM, respectively); lane M: Thermofisher protein molecular weight marker 26610; Lane 11: western blot analysis using anti-His-HRP antibody. scFv, Single-chain variable fragment; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 1. Soluble protein expression under various conditions.

Condition	Inducer (IPTG) concentration (mM)	Temperature (°C)	Presence of chaperones	Total protein expression (mg/L)	Soluble protein (mg/L)	Soluble protein (%)
1	0.25	25	No	5.33	1.05	19.69
2	0.25	30	No	4.86	1.53	31.48
3	0.25	37	No	6.25	1.44	23.04
4	0.5	25	No	N/A	1.47	N/A
5	0.5	30	No	N/A	1.4	N/A
6	0.5	37	No	N/A	0.97	N/A
7	1	25	No	N/A	1.05	N/A
8	1	30	No	N/A	1.29	N/A
9	1	37	No	N/A	1.06	N/A
10	0.25	25	Yes	5.64	2.59	45.92
11	0.25	30	Yes	3.84	1.72	44.79
12	0.25	37	Yes	N/A	2.13	N/A
13	0.50	25	Yes	5.73	2.6	45.37
14	0.50	30	Yes	N/A	2.67	N/A
15	0.50	37	Yes	4.21	1.79	42.51
16	1	25	Yes	N/A	1.8	N/A
17	1	30	Yes	5.82	2.9	49.82
18	1	37	Yes	N/A	1.84	N/A

"N/A" explains the unavailability of data on total protein expression for those specific conditions.

Cell lysis, separation of soluble fraction, and purification of soluble scFv-BsAb

As the structure of this recombinant protein contains a polyhistidine sequence (6xHis, Fig. 1A), protein purification was performed using a nickel-nitriloacetic acid (Ni-NTA) column (Invitrogen, USA). Following protein expression, cells were collected through centrifugation at 5000 rpm for 5 min. The bacterial pellet was subsequently dispersed in native binding buffer (50 mM NaH_2PO_4, 0.5 M NaCl, 10 mM imidazole), and lysozyme (1 mg/mL) was added before the cells were incubated on ice for 30 min. To prepare the cell lysate, bacterial cells were then subjected to cell disruption by ultrasonication on ice at moderate intensity for 5 cycles of 20 s with 40 s rest intervals. To reduce the sample viscosity, RNase A (10 µg/mL) and DNase I (10 µg/mL) were added. After incubating the sample on ice for 15 min, centrifugation was performed at 3,000 *g* for 15 min at 4 °C to separate the soluble and insoluble fractions. The supernatant (soluble fraction) was then subjected to sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. For purification, the supernatant was loaded onto a Ni-NTA column. Four washes were performed with native wash buffer (50 mM NaH_2PO_4, 0.5 M NaCl, and 20 mM imidazole, pH 8.0), and then the target protein was eluted with native elution buffer (50 mM NaH_2PO_4, 0.5 M NaCl, and 250 mM imidazole, pH 8.0). The elution extracts were collected in 1 mL fractions, and each fraction was subjected to SDS-PAGE analysis separately.

Analytical methods

To confirm the protein expression and compare the soluble expression level in each condition, the samples were loaded on 12% sodium dodecyl sulfate-polyacrylamide gels, and electrophoresis was performed (SDS-PAGE) with a constant voltage of 150 V. Also, to confirm the expression of the His-tagged scFv-BsAb, the whole protein sample, which was first analyzed by SDS-PAGE, was transferred to polyvinylidene difluoride (PVDF) membranes, and western blotting was done using anti-His (C-term)-horseradish peroxidase (HRP) antibody (Invitrogen, USA). For the quantification of each lane on the gels, densitometry analysis was performed using ImageJ software. The band intensities of expressed scFv-BsAb were estimated and normalized to the protein molecular weight marker (SMBIO1500) (24). Furthermore, the concentration of soluble protein was measured using the Bradford assay both before and after protein purification.

Flow cytometry analysis

Biological activity of the purified scFv-BsAb was evaluated by the determination of the binding ability of the protein. Briefly, peripheral blood mononuclear cells (PBMCs) (25) expressing PD-1 and CTLA-4 receptors on the cell surface, and human embryonic kidney 293 (HEK293) cells, as a negative control not expressing receptors, were incubated with scFv-BsAb. After the incubation period, cells were exposed to a secondary antibody (anti-His-FITC). Finally, after three washes, cells were analyzed using a flow cytometer (BD, USA).

Statistical analysis

The experiments were repeated three times, and the mean of the results from each set of three repetitions was calculated and used for analysis to ensure the reliability and accuracy of the results.

To compare the results of the expression in the two main groups (with and without chaperones), an independent samples t-test was used. To compare expression levels in nine groups with different expression conditions based on temperature and IPTG concentration (Table 1), a two-way ANOVA test was used, followed by the Tukey test. Moreover, the Pearson coefficient calculation was used to reveal any possible trend line between variables and expression levels.

RESULTS

General expression of scFv-BsAb

SDS-PAGE results showed successful expression of the target protein with and without chaperone under different conditions (37 °C temperature, 0.5 mg/mL L-arabinose, and 0.25, 0.5, or 1 mM IPTG). It was also observed that co-expression with chaperones results in higher amounts of protein (Fig. 1B). Furthermore, Western blot analysis using anti-His (C-term)-HRP antibody (Invitrogen, USA) confirmed the expression of the target protein (Fig. 1B).

Effect of IPTG concentration and temperature on expression of soluble scFv-BsAb without chaperone

The study investigated the effect of IPTG concentration (0.25, 0.5, and 1 mM) and expression temperature (25, 30, and 37 °C) on soluble protein expression. SDS-PAGE results and gel analysis using ImageJ software indicated that in protein expression without a chaperone, the highest amount of soluble protein was obtained at 30 °C and an IPTG concentration of 0.25 mM (Fig. 2A). As the IPTG concentration increased, a decrease in soluble protein and an increase in insoluble protein were observed. Moreover, at 25 °C, the expression of soluble protein decreased, and the expression of insoluble protein increased. At 37 °C, both soluble and insoluble expression decreased. Although the highest level of soluble protein expression was achieved at 30 °C and an IPTG concentration of 0.25 mM, IPTG concentrations of 0.25 and 0.5 mM did not lead to much difference in soluble protein expression at different temperatures. However, increasing the inducer concentration to 1 mM resulted in a decrease in the amount of soluble protein (Fig. 2A and Fig. 3A).

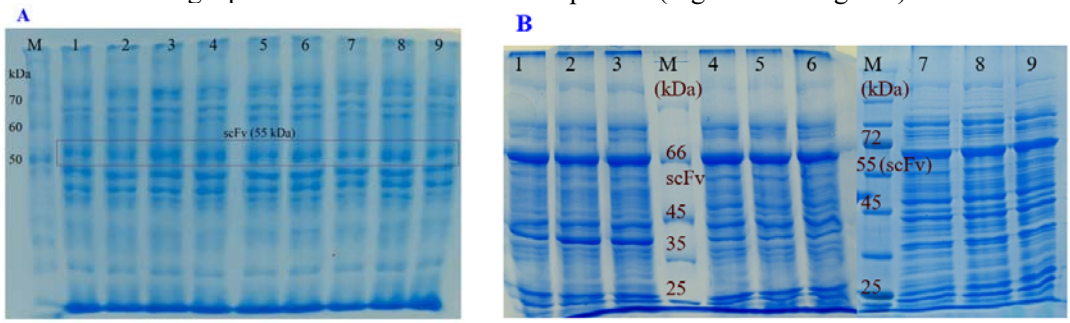


Fig. 2. SDS-PAGE analysis of soluble fraction of scFv-BsAb expressed in *Escherichia coli* BL21(DE3) cells. (A) Expression without chaperones. Lane M: Fisherbioreagents protein molecular weight marker BP3602; lanes 1-3: at 37 °C using 1, 0.5, and 0.25 mM IPTG, respectively; lanes 4-6: at 30 °C using 1, 0.5, and 0.25 mM IPTG, respectively; lanes 7-9: at 25 °C using 1, 0.5, and 0.25 mM IPTG, respectively. (B) Co-expression with chaperones using 1 mM L-arabinose. Lane M: Smobio PM1500 ExcelBand protein molecular weight marker; lanes 1-3: at 37 °C using 1, 0.5, and 0.25 mM IPTG, respectively; lanes 4-6: at 30 °C using 1, 0.5, and 0.25 mM IPTG, respectively; lanes 7-9: at 25 °C using 0.25, 0.5, and 1 mM IPTG, respectively. scFv, Single-chain variable fragment; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

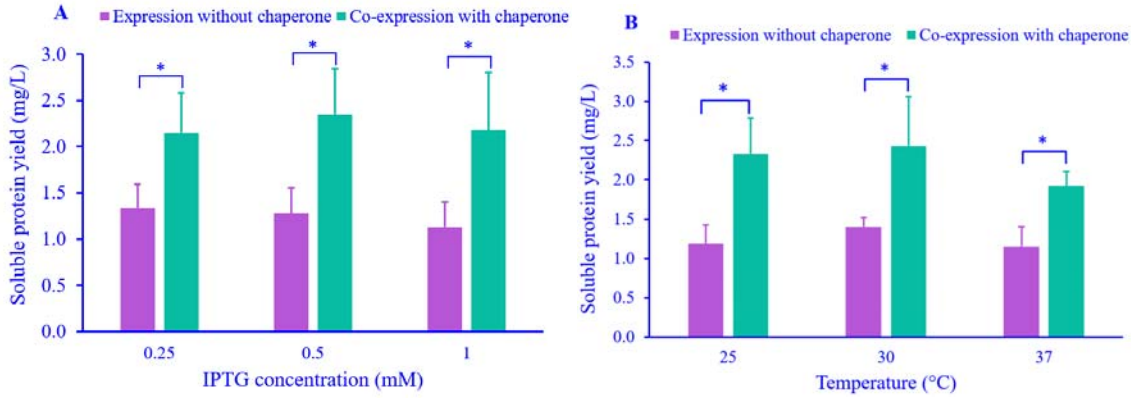


Fig. 3. Densitometry analysis of SDS-PAGE results using ImageJ software. Soluble protein expression with and without chaperones in different (A) IPTG concentrations and (B) temperatures. $P < 0.05$ indicates significant differences between the designated groups. IPTG, isopropyl-β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Effect of IPTG concentration and temperature on co-expression of scFv-BsAb with chaperones

According to SDS-PAGE results, in co-expression of protein with chaperones, the highest amount of soluble protein was obtained at 30 °C and 1 mM IPTG concentration (Fig. 2B). Densitometry analysis of SDS-PAGE results also revealed that, as the temperature increases to 37 °C, the expression of soluble protein decreased and insoluble protein increased. Also, at 25 °C, the amount of soluble protein slightly decreases compared to 30 °C. In terms of IPTG concentration, although on average at different temperatures, 0.5 mM IPTG results in higher amounts of soluble protein, at the temperature of 30 °C, increasing the amount of IPTG to 1 mM has a positive effect on the expression of soluble protein (Fig. 2B and Fig. 3B), which may indicate the synergistic effect of these two factors (temperature and inducer concentration).

Comparing soluble protein expression and purification with and without chaperones

To facilitate comparison and highlight the best conditions for soluble protein expression, the percentage of soluble protein and the total amount of protein expression in soluble and non-soluble forms under different conditions (inducer concentration, temperature, and presence or absence of chaperone) are presented in Table 1. For the comparison of expression levels across nine groups with varied expression conditions based on temperature and IPTG concentration, a two-way ANOVA test was employed. The obtained *P*-values for each condition factor were all above 0.05. This suggests that there was no significant difference in expression levels among the different conditions (*i.e,* IPTG concentration and temperature).

However, according to SDS-PAGE results and densitometry analysis, the amount of soluble protein is approximately 1.7-fold higher when co-expressed with chaperones than when expressed without chaperones. (Figs. 2 and 3). Furthermore, after protein purification, it was also observed that the purified soluble protein yield is 1.6 times higher in co-expression with chaperones (Fig. 4A and B). This result, which was based on the densitometry analysis of SDS-PAGE, was also confirmed by measuring purified protein concentration using the Bradford assay. The final yield of protein was 2.9 mg and 1.5 mg protein per 1 L of culture with and without chaperone expression, respectively.

Binding activity of scFv-BsAb

Flow cytometry analysis revealed a marked difference in fluorescence intensity between test and control cells. Lymphocytes gated within PBMCs, which express PD-1/CTLA-4 receptors on their surface, exhibited significantly higher fluorescence compared to HEK293 cells used as negative controls (Fig. 5).

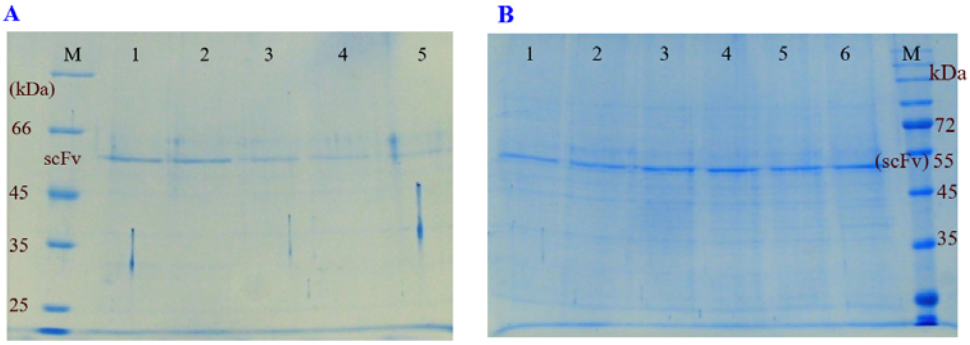


Fig. 4. SDS-PAGE analysis of purified soluble scFv-BsAb after purification using Ni-NTA chromatography. (A) protein expression at 30 °C using 0.5 mM IPTG. Lane M: Thermofisher protein molecular weight marker 26610; lanes 1-5: 1 mL elution fractions 5-1, respectively. (B) Co-expression of protein with chaperones at 30 °C using 0.5 mM IPTG. Lane M: Smobio PM1500 ExcelBand protein molecular weight marker; lanes 1-6: 1 mL elution fractions 6-1, respectively. scFv, Single-chain variable fragment; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Ni-NTA, nickel-nitriloacetic acid.

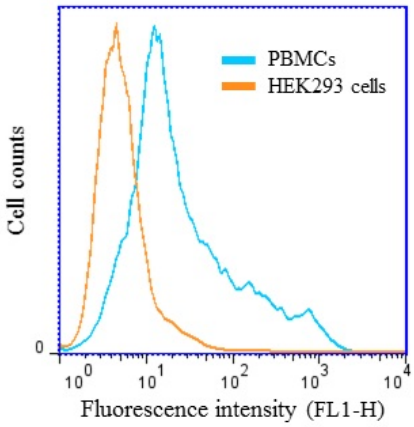


Fig. 5. Fluorescence intensity histogram based on the results of flow cytometry of PBMCs and HEK293 cells after incubation with scFv-BsAb and a secondary antibody (anti-His-FITC). The lymphocyte population in PBMCs was gated based on forward scatter and side scatter. scFv, Single-chain variable fragment.

Further analysis of antibody binding to lymphocyte surfaces indicated a concentration-dependent relationship, characterized by exponential growth in binding affinity. In contrast, binding to HEK293 cells showed a marginal increase with antibody concentration, significantly lower than that observed with PBMCs.

Comparison of antibody binding to receptor sites on PBMCs from healthy volunteers and HEK293 cells highlighted substantial differences. At approximately 800 nm concentration, the antibody bound to over 30% of lymphocyte receptors, whereas it bound to only about 2% of HEK293 cell receptors under the same conditions.

DISCUSSION

Antibody fragments, especially scFvs, have been widely used as diagnostic and therapeutic agents in cancer therapy (26). However, their functional production has been a challenge for manufacturers due to misfolding potential and the formation of inclusion bodies. Various strategies have been employed to improve the expression of soluble scFvs. One of these approaches is co-expression with chaperones to improve the folding and solubility of recombinant proteins (14,27). Here, we used co-expression with chaperons along with optimization of culture conditions to improve soluble expression of anti-PD-1/CTLA-4 scFv in the cytoplasm of *E. coli*.

DnaK, DnaJ, and GrpE are cytoplasmic chaperones that play an important role in the correct folding of proteins. Yousefi *et al.* evaluated the level of soluble anti-CD20 scFv when co-expressed with different cytoplasmic chaperones, including GroEL, DnaK, GroES, Tig, DnaJ, and GrpE. They found that among the investigated chaperones, DnaJ, GrpE, and DnaK chaperones significantly improved soluble expression of the scFv (28). In this study, it was observed that protein co-expression with the same cytoplasmic chaperones increased the amount of soluble protein by 60% (1.6 times; Fig. 4). Other researchers also used periplasmic

chaperons, DsbA and DsbC, to improve soluble expression of a scFv against type 1 insulin-like growth factor receptor. They reported 1.5 times enhancement in the recovery of soluble protein using co-expression with DsbC and up to 80% soluble expression when using co-expression with DsbA and DsbC (29). Furthermore, in other studies, co-expression with chaperones elevated the amount of soluble protein from 1.1 times up to 264 times in comparison with protein expression without chaperones (27,30,31). According to these results, it seems that the capability of chaperones in the improvement of correct protein folding is also dependent on the intrinsic and structural characteristics of the recombinant protein, and maybe other factors that need to be investigated.

On the other hand, optimization of temperature and inducer concentration can also play a role in increasing the soluble protein expression (32,33). The results of this study showed that, in scFv expression without a chaperone, the IPTG concentrations of both 0.5 and 0.25 mM lead to higher soluble expression, but with increasing the concentration to 1 mM, the soluble expression decreases. It may indicate that increasing the amount of inducer increases the rate of protein expression in bacteria and does not provide enough time and space for proper protein folding. Notably, in this work, co-expression with chaperones could reduce the negative effect of the inducer concentration on soluble expression of the scFv, and soluble expression was also observed at higher concentrations of IPTG (0.5 mM). In other words, changes in the concentration of IPTG in co-expression with chaperones do not have a great effect on the soluble protein expression, and chaperones can be effective on correct protein folding even at higher concentrations of the inducer and higher rates of protein expression.

In this study, we observed that co-expression with chaperones significantly increases the soluble protein expression under various conditions. Table 1 summarizes the soluble protein expression data and highlights that the highest soluble protein yield was achieved at 30 °C with 0.5 mM IPTG in the presence of chaperones.

While the table provides a comprehensive comparison, it is important to note some limitations. For certain conditions, data on total protein expression were not available, resulting in entries marked as "N/A". These missing data points could limit the ability to fully assess the relative efficiency of different conditions. Despite this, the trends observed are consistent with previous studies and suggest that the use of chaperones, along with optimization of temperature and inducer concentration, can significantly enhance soluble protein yield.

Regarding temperature, in expression without chaperones, the temperature of 30 °C was optimal, and in co-expression with chaperones, temperatures of 30 and 25 °C had almost similar results and showed high soluble expression, but at 37 °C, soluble expression decreased significantly. This result is consistent with other studies, because in most of the studies conducted on co-expression with chaperones, the optimal temperature was less than 30 °C and often around 25 °C (31), which may indicate that at 37 °C, chaperones cannot perform as well as at lower temperatures.

We used flow cytometric analysis to confirm the biological activity of the produced scFv-BsAb. Previous works found that stimulation of PBMC with ConA resulted in enhancement of CTLA-4 and PD-1 expression on the surface of cells (34). Our findings indicated that scFv-BsAb can bind more to the ConA-stimulated PBMCs (expressing PD-/CTLA-4) compared to HEK293. The final yield of protein in co-expression with chaperones was 2.9 mg protein per 1 L of culture, while the final yield of protein expression in the periplasmic space was 4.5 mg/L. Interestingly, the protein co-expressed with chaperones exhibited more binding ability compared with periplasmic-expressed scFv-BsAb (9). However, more experiments should still be performed to evaluate the binding activity of scFv-BsAb.

CONCLUSION

According to the results of this study and other studies conducted on different proteins, optimizing culture temperature and inducer concentration, as well as co-expression with chaperones, can be effective in increasing soluble protein expression. In this study, the optimal conditions for the expression of

a scFv-BsAb were determined, and the soluble protein was purified using affinity chromatography in native conditions for use in subsequent studies and evaluation of the biological activity of the protein.

Acknowledgements

This work was financially supported by the Vice-Chancellery of Research of the Isfahan University of Medical Sciences through Grant Nos. 396392 and 340214. We also thank Mrs. Fatemeh Moazen for her excellent technical assistance.

Conflict of interest statement

All authors declared no conflict of interest for this study.

Authors' contributions

Z. Alidousti-Shahraki contributed to the concept and design of the study, definition of intellectual content, literature search, experimental studies, data acquisition, analysis, and interpretation of data, and manuscript preparation. A. Amini contributed to the literature search, experimental studies, and acquisition of data, manuscript review and editing, and final approval of the manuscript to be published. N. Esmaeil contributed to the design of the study, interpretation of data, and manuscript preparation. V. Akbari contributed to the concept and design of the study, definition of intellectual content, literature search, analysis and interpretation of data, and revision of the manuscript. All authors have read and approved the finalized article. Each author has fulfilled the authorship criteria and affirmed that this article represents honest and original work.

Ethical approval

This study was approved by the Research Ethics Committee of the Vice-Chancellor's in Research Affairs of Isfahan University of Medical Sciences. (Ethical approval code: IR.MUI.RESEARCH.REC.1398.577)

AI declaration

The authors did not use any AI-assisted technologies in the preparation of this manuscript.

REFERENCES

1. Zahavi D, Weiner L. Monoclonal antibodies in cancer therapy. Antibodies (Basel). 2020;9(3):34,1-20. DOI: 10.3390/antib9030034.
2. Kumar AR, Devan AR, Nair B, Vinod BS, Nath LR. Harnessing the immune system against cancer: current immunotherapy approaches and therapeutic targets. Mol Biol Rep. 2021;48(12):8075-8095. DOI: 10.1007/s11033-021-06752-9.
3. Korman AJ, Peggs KS, Allison JP. Checkpoint blockade in cancer immunotherapy. Adv Immunol. 2006;90:297-339. DOI: 10.1016/S0065-2776(06)90008-X.
4. Li X, Shao C, Shi Y, Han W. Lessons learned from the blockade of immune checkpoints in cancer immunotherapy. J Hematol Oncol. 2018;11(1):31,1-26. DOI: 10.1186/s13045-018-0578-4.
5. Jin S, Sun Y, Liang X, Gu X, Ning J, Xu Y, *et al*. Emerging new therapeutic antibody derivatives for cancer treatment. Signal Transduct Target Ther. 2022;7(1):39,1-28. DOI: 10.1038/s41392-021-00868-x.
6. Li H, Er Saw P, Song E. Challenges and strategies for next-generation bispecific antibody-based antitumor therapeutics. Cell Mol Immunol. 2020;17(5): 451-461. DOI: 10.1038/s41423-020-0417-8.
7. Nelson AL. Antibody fragments. mAbs. 2010;2(1):77-83. DOI: 10.4161/mabs.2.1.10786.
8. Paerhati P, Jakos T, Bai SY, Zhu JW, Yuan YS. Strategies and applications of antigen-binding fragment (Fab) production in *Escherichia coli*. Pharm Fronts. 2021;3(2):e39-e49. DOI: 10.1055/s-0041-1735145.
9. Rashti A, Akbari V. Construction and periplasmic expression of a bispecific tandem scFv for dual targeting of immune checkpoints. Adv Biomed Res. 2023;12:42,1-5. DOI: 10.4103/abr.abr_31_22.
10. Kipriyanov SM. High-level periplasmic expression and purification of scFvs. Methods Mol Biol. 2009:562:205-214. DOI: 10.1007/978-1-60327-302-2_16.
11. Tungekar AA, Ruddock LW. Design of an alternate antibody fragment format that can be produced in the cytoplasm of *Escherichia coli*. Sci Rep. 2023;13(1):14188,1-10. DOI: 10.1038/s41598-023-41525-3.
12. Gaciarz A, Veijola J, Uchida Y, Saaranen MJ, Wang C, Hörkkö S, *et al*. Systematic screening of soluble expression of antibody fragments in the cytoplasm of *E. coli*. Microb Cell Fact. 2016;15:22. DOI: 10.1186/s12934-016-0419-5.
13. Guglielmi L, Martineau P. Expression of single-chain Fv fragments in *E. coli* cytoplasm. Methods Mol Biol. 2009;562:215-224. DOI: 10.1007/978-1-60327-302-2_17.

14. Mamipour M, Yousefi M, Hasanzadeh M. An overview on molecular chaperones enhancing solubility of expressed recombinant proteins with correct folding. Int J Biol Macromol. 2017;102:367-375. DOI: 10.1016/j.ijbiomac.2017.04.025.
15. de Marco A. Strategies for successful recombinant expression of disulfide bond-dependent proteins in *Escherichia coli*. Microb Cell Fact. 2009;8:26,1-18. DOI: 10.1186/1475-2859-8-26.
16. Kravats AN, Wickner S, Camberg JL. Molecular Chaperones. Reference Module in Life Sciences: Elsevier; 2022.
17. Hayer-Hartl M, Bracher A, Hartl FU. The GroEL–GroES chaperonin machine:a nano-cage for protein folding. Trends Biochem Sci. 2016;41(1):62-76. DOI: 10.1016/j.tibs.2015.07.009.
18. Ziemienowicz A, Skowyra D, Zeilstra-Ryalls J, Fayet O, Georgopoulos C, Zylicz M. Both the *Escherichia coli* chaperone systems, GroEL/GroES and DnaK/DnaJ/GrpE, can reactivate heat-treated RNA polymerase. Different mechanisms for the same activity. J Biol Chem. 1993;268(34):25425-25431. PMID: 7902351.
19. Sandomenico A, Sivaccumar JP, Ruvo M. Evolution of *Escherichia coli* expression system in producing antibody recombinant fragments. Int J Mol Sci. 2020;21(17):6324,1-39. DOI: 10.3390/ijms21176324.
20. Mirzapour Estabragh A, Sadeghi HMM, Akbari V. Co-expression of chaperones for improvement of soluble expression and purification of an anti-HER2 scFv in *Escherichia coli*. Adv Biomed Res. 2022;11:117,1-6. DOI: 10.4103/abr.abr_351_21.
21. Saibil H. Chaperone machines for protein folding, unfolding and disaggregation. Nat Rev Mol Cell Biol. 2013;14(10):630-642. DOI: 10.1038/nrm3658.
22. Mierendorf R, Yeager K, Novy R. The pET system:your choice for expression. Innovations. 1994;1(1):1-3.
23. Soheili S, Jahanian-Najafabadi A, Akbari V. Evaluation of soluble expression of recombinant granulocyte macrophage stimulating factor (rGM-CSF) by three different *E. coli* strains. Res Pharm Sci. 2020;15(3):218-225. DOI: 10.4103/1735-5362.288424.
24. Akbari V, Sadeghi HM, Jafarian-Dehkordi A, Chou CP, Abedi D. Optimization of a single-chain antibody fragment overexpression in *Escherichia coli* using response surface methodology. Res Pharm Sci. 2015;10(1):75-83. PMID: 26430460.
25. Roufarshbaf M, Esmaeil N, Akbari V. Comparison of four methods of colon cancer cell lysates preparation for *ex vivo* maturation of dendritic cells. Res Pharm Sci. 2021;17(1):43-52. DOI: 10.4103/1735-5362.329925.
26. Muñoz-López P, Ribas-Aparicio RM, Becerra-Báez EI, Fraga-Pérez K, Flores-Martínez LF, Mateos-Chávez AA, *et al*. Single-chain fragment variable:recent progress in cancer diagnosis and therapy. Cancers (Basel). 2022;14(17): 4206,1-26. DOI: 10.3390/cancers14174206.
27. Bhatwa A, Wang W, Hassan YI, Abraham N, Li XZ, Zhou T. Challenges associated with the formation of recombinant protein inclusion bodies in *Escherichia coli* and strategies to address them for industrial applications. Front Bioeng Biotechnol. 2021;9:630551,1-18. DOI: 10.3389/fbioe. 2021.630551.
28. Yousefi M, Farajnia S, Mokhtarzadeh A, Akbari B, Ahdi Khosroshahi S, Mamipour M, *et al*. Soluble expression of humanized anti-CD20 single-chain antibody in *Escherichia coli* by cytoplasmic chaperones co-expression. Avicenna J Med Biotechnol. 2018;10(3):141-146. PMID: 30090206.
29. Sun XW, Wang XH, Yao YB. Co-expression of Dsb proteins enables soluble expression of a single-chain variable fragment (scFv) against human type 1 insulin-like growth factor receptor (IGF-1R) in *E. coli*. World J Microbiol Biotechnol. 2014;30(12):3221-3227. DOI: 10.1007/s11274-014-1749-2.
30. Utami DF, Azizah MI, Sriwidodo S, Haryanto RA, Pratiwi RD, Maksum IP. Effect of co-expression chaperones on the expression of intracellular recombinant proteins in *Escherichia coli*. Chim Nat Acta. 2023;11(2):25-33. DOI: 10.24198/cna.v11.n2.46480.
31. Fatima K, Naqvi F, Younas H. A review: molecular chaperone-mediated folding, unfolding and disaggregation of expressed recombinant proteins. Cell Biochem Biophys. 2021;79:153-174. DOI: 10.1007/s12013-021-00970-5.
32. Francis DM, Page R. Strategies to optimize protein expression in *E. coli*. Curr Protoc Protein Sci. 2010;5(1):5.24.1-5.24.29. DOI: 10.1002/0471140864.ps0524s61.
33. Shishparenok AN, Gladilina YA, Zhdanov DD. Engineering and expression strategies for optimization of L-asparaginase development and production. Int J Mol Sci. 2023;24(20): 15220,1-60. DOI: 10.3390/ijms242015220.
34. Judge SJ, Dunai C, Sturgill IR, Stoffel KM, Murphy WJ, Canter RJ. Assessment of PD-1 expression in human resting and activated natural killer cells and murine tumor models. J Clin Oncol. 2019;37(8 Suppl):36-36. DOI: 10.1200/JCO.2019.37.8_suppl.36.