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INVESTIGATION OF THE EFFECT OF ARGININE AND GLUTATHIONE ON RECOVERY OF A SINGLE DOMAIN ANTIBODY PRODUCED IN BACTERIA IN INCLUSION BODIES

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Abstract: Single domain antibodies (nanobodies) are the antigen binding domain of heavy chain only antibodies derived from camelids and sharks. Standard antibodies have heavy and light chains, and the antigen binding region is formed by the combination of variable heavy and variable light chain domains. Single domain antibodies are as good binders to their respective antigens as the combination of variable heavy and light chains. Due to their chemical and thermal stability, small size, and economic benefits, there is increasing interest in nanobodies for research use and from industry. In this article, we investigated the effect of arginine and a mixture of oxidized and reduced glutathione for recovering a nanobody produced in inclusion bodies in *E. coli*. Nanobody protein is solubilized in 6M urea buffer from the cell lysate. After a Ni-NTA chromatography, nanobody containing fractions were first diluted in different concentrations of arginine and/or reduced and oxidized glutathione containing buffers, followed by dialysis against a buffer to fold the protein. The best recovery yield was obtained in the presence of 400mM arginine. Nanobodies are important molecules in biotechnology and medicine, and, this study investigated ways to improve their production yield.

Keywords: Protein recovery, Single domain antibody, Arginine, Glutathione

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1. Introduction

Proteins are important biomacromolecules and are being heavily utilized in biotechnology and medicine. Immunoglobulins (antibodies) are one of the most important groups of proteins, generated by the immune system to help fight infections and foreign molecules within the body. Antibodies are indispensable components of serological diagnostic tests for detecting antigens. Therapeutic antibodies are becoming more promising in treating cancer, autoimmune diseases, and infections. Over the last three decades, research on antibodies, antibody fragments, and antibody engineering has expanded tremendously with the developments in recombinant DNA technology. In 2024, the FDA approved 50 novel drug molecules, 16 of which were antibody therapies, highlighting their significant role in treating various diseases, including cancers and autoimmune disorders (Martins et al., 2025).

Figure 1 has a schematic representation of an IgG (immunoglobulin G) type antibody and antibody fragments. Standard IgG type antibodies are composed of two identical heavy and two identical light chains. Its molecular weight is around 150 kDa. The tip of the antibody composed of variable heavy (represented in light blue) and variable light (represented in light purple) domains recognize and bind two identical antigen molecules. Dark colored constant chains both help stabilize the protein and are essential for effector function of antibodies i.e. alerting and activating the immune system (Rees, 2021). There are many examples of IgG type antibodies in clinical use and in serological diagnostic tests (The Antibody Society, 2025).

Depending on the application, it is also possible to work with antibody fragments. Antigen binding fragment (Fab) is a stable four domain piece derived from the whole antibody which includes the variable heavy, first constant domain of heavy chain, and the light chain (Figure 1). When two variable domains of heavy and light chains are connected through a linker, single chain variable fragment (scFv) is formed. Natural or synthetic libraries of scFvs are generated to discover and create certain antigen specific antibodies (Gezehagn and Tessema, 2024). Finally, in camelids and sharks heavy chain only antibodies are discovered which are smaller in size

(around 90 kDa) and have a single domain for antigen binding (Hamers-Casterman et al., 1993). This single antigen binding domain is isolated, and it is called single domain antibody or nanobody (Figure 1). Figure 1B shows the ribbon diagram of a nanobody which has an immunoglobulin fold. Nanobodies are particularly advantageous for both therapeutic and diagnostic applications because of their small size, high chemical and thermal stability, easy and affordable production in bacterial systems (Muyldermans, 2021). Their small size allows them to penetrate tissues more effectively and target antigens that are otherwise difficult to reach with conventional antibodies. However, antibody fragments (scFvs and nanobodies) are mostly produced in the insoluble form in bacterial production hosts. This limitation poses challenges and there are efforts to overcome solubility problems (Birnboim-Perach et al., 2019; Dingus et al., 2022; Hennigan et al., 2024).

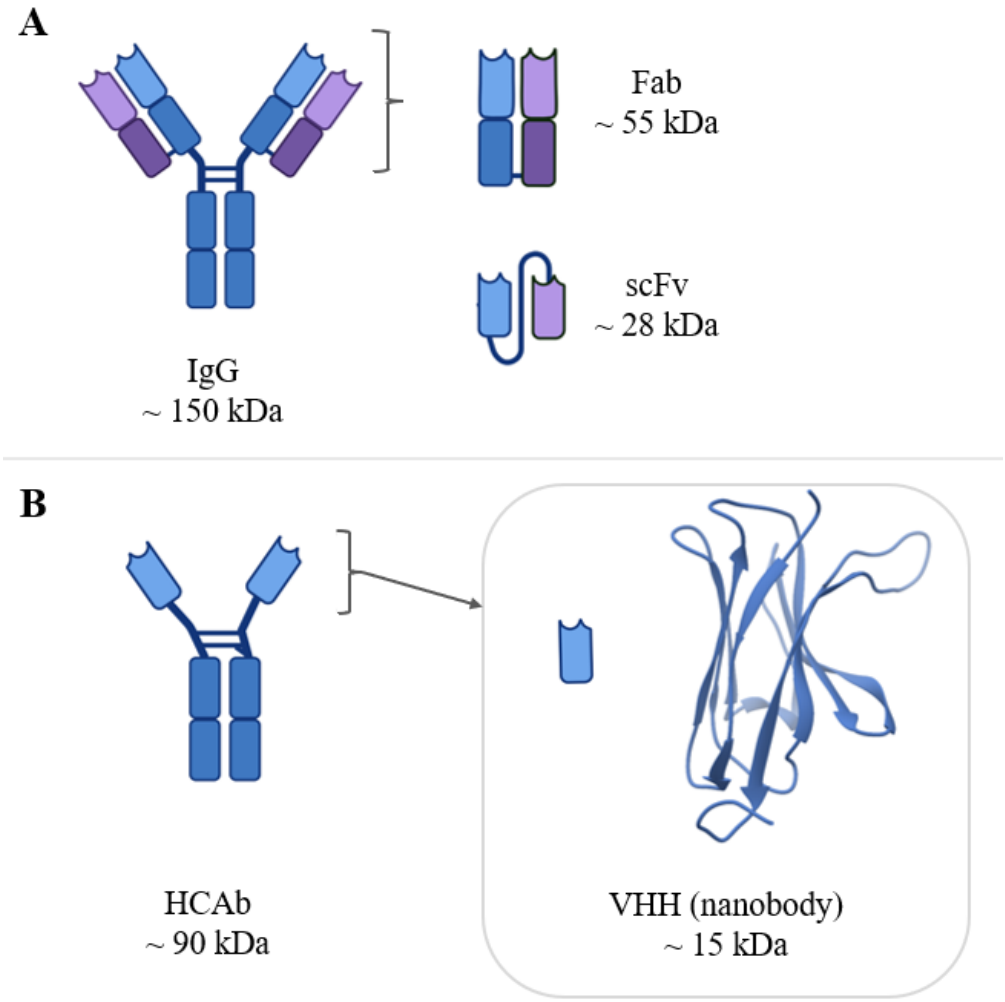

Figure 1. Schematic representation of an IgG antibody, heavy-chain only antibody, antibody fragments, and their approximate molecular weights. A) A standard IgG antibody (~150 kDa) consists of two identical heavy (blue) and two identical light (purple) chains. Smaller fragments such as the antigen binding fragment (Fab, ~55 kDa) or single-chain variable fragment (scFv, ~28 kDa) can be used in different applications. B) Heavy chain only antibody (HCAb, ~90 kDa) that is naturally missing light chains. Its single antigen binding domain (VHH or nanobody, ~15 kDa) that retains HCAb's antigen binding capacity. Ribbon (right) representation of VHH, created by UCSF Chimera.

The most preferred bacterial host for producing recombinant proteins is *Escherichia coli*. However, during overexpression of protein in *E. coli*, high transcription/translation rates and high plasmid copy number induce stress and protein misfolding, leading to formation of inclusion bodies (IBs). This problem is more prominent during cytoplasmic expression because the reducing environment of the cytoplasm prevents the formation of disulfide bonds needed for structural stability of proteins. In addition to environmental factors, structural characteristics also influence the formation of IBs. For instance, multi-domain proteins have a higher propensity to aggregate than single-domain proteins due to the presence of misfolded intermediate forms (Bhatwa et al., 2021).

In this study, we worked on a nanobody that was reported to bind HIV-1 capsid protein with picomolar affinity in the literature (Helma et al., 2012). The nanobody protein is expressed and sent to the periplasm of *E. coli*. However, the protein was insoluble. After

extracting the protein with 6M urea, we tested the effects of arginine and glutathione during refolding to identify the optimal environment that would allow for proper protein refolding and the highest soluble, functional protein recovery. Ultimately, diluting protein solution in 400mM arginine buffer and refolding maximized the yield of correctly folded nanobody suitable for further studies.

2. Materials and Methods

2.1. Protein Production

HIV-1 capsid protein binding nanobody protein gene was cloned into pET22b plasmid. Protein was expressed in *E. coli* BL21 (DE3) containing pET22b-Nb plasmid with a periplasmic PelB tag at the N-terminus and a His-tag at the C-terminus. 500mL LB culture started from overnight growth. Culture was incubated at 37°C, 220–rpm. When OD at 600 nm reached 0.8-1, 0.5mM IPTG was added, the temperature was reduced to 30°C, and expression continued overnight. Cultures were centrifuged at 2,370 g for 30 minutes. Cell pellet was stored at -20°C until purification.

2.2. Purification

2.2.1. Denatured Ni-NTA chromatography

All the purification steps are conducted at 4°C, and protein fractions are kept on ice at all times. Around 1.9-2.1 g of bacterial cell pellet from 500mL culture was resuspended in around 40-42 mL 6M urea, 20mM sodium phosphate pH 7.4, 0.4M NaCl, 10mM imidazole, 10mM 2-mercaptoethanol (2-ME) buffer and cells were disrupted by sonication. Soluble fraction, the supernatant, was separated from the cellular debris with centrifugation at 37,900 g. The supernatant was filtered with 0.45µm hydrophilic PES filter and applied to Ni-NTA column. C-terminally his-tagged denatured nanobody protein bound to the column, and after multiple washes with low concentration imidazole-containing buffers, nanobody was eluted with 4M urea, 300mM imidazole, 20mM sodium phosphate pH 7.4, 400mM NaCl, 5mM 2-ME buffer. The unfolded Ni-NTA column fractions were analyzed in 15% polyacrylamide SDS-PAGE gel. The 14 kDa band represents the nanobody protein. Unfolded Ni-NTA chromatography removed most bacterial proteins from the supernatant, and elution fractions (E1-E4) contain mostly nanobody protein on the gel (Figure 2B).

2.2.2. Nanobody folding

After the Ni-NTA column chromatography, protein was diluted three fold into buffers with various refolding reagents at different concentrations and ratios (Table 1). Proteins prepared with different concentration of folding additive were analyzed by SDS-PAGE.

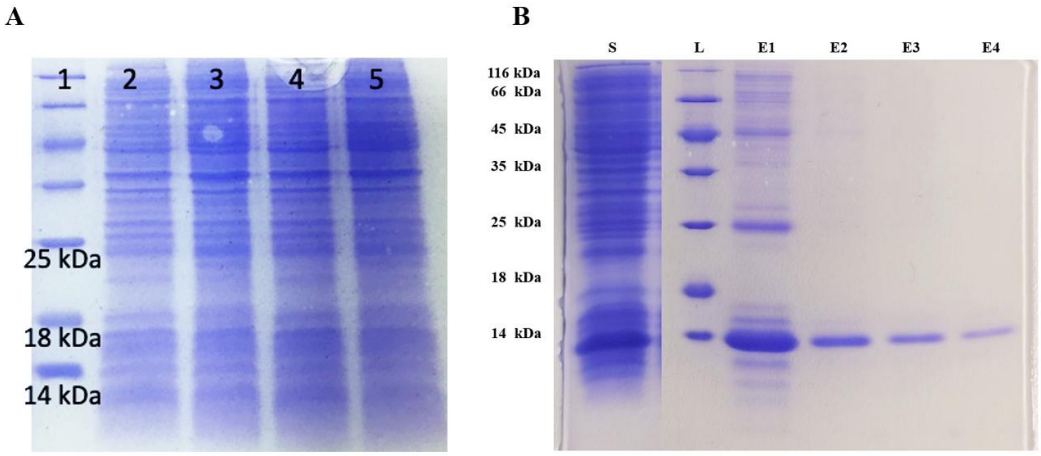

Figure 2. SDS-PAGE image of nanobody protein production in BL21 (DE3) cells, in pET21a (A) plasmid in the cytoplasm and pET22b (B) plasmid in the periplasm. A) First lane is showing the ladder with indicated marker band sizes (Thermo Scientific™ Pierce™ Unstained Protein MW Marker #26610). Second and fourth lanes have cell lysate before induction of two different cultures. Third and fifth lanes have the cell lysate after induction. Nanobody protein size is around 14 kDa. There are no protein bands around 14 kDa in the induced lanes (third and fifth lanes). B) Different fractions from purification of nanobody protein produced in the periplasm. SDS-PAGE gel image indicates supernatant (S), ladder (L) (Thermo Scientific™ Pierce™ Unstained Protein MW Marker #26610), and four elution fractions (E1-E4) of Ni-NTA chromatography, respectively. The 14 kDa band represents the nanobody protein.

The experimental setup is designed as follows. Nanobody protein-containing fractions from unfolded Ni-NTA chromatography are pooled. This nanobody solution is diluted three fold (with or without the indicated additives). The final buffer compositions after dilution are given in Table 1. After dilution, 20 µL sample (labeled as before dialysis) is saved for SDS-PAGE. Diluted protein solution is dialyzed against 1L solution. After dialysis, recovered protein volume was measured and it was the same as the volume before dialysis. After dialysis, recovered protein solution is labeled as after dialysis sample for analyzing in SDS-PAGE gel. Three replicates were done for every condition, and the results were analyzed in the SDS-PAGE gels. The 14 kDa band represents the nanobody protein.

2.2.2.1. Folding additive: Arginine (200 mM and 400 mM)

Fractions containing denatured nanobody proteins were pooled (total 20 mL) according to the SDS-PAGE gel results and diluted three fold with dilution buffer (400mM NaCl, 20mM sodium phosphate buffer 7.4, 1M urea) containing final concentrations of 200mM or 400mM arginine as folding additives. Approximately 60 ml protein solution (buffer composition is given in Table 1) was dialyzed against 1 L dialysis buffer (20mM sodium phosphate buffer pH 7.4, 300mM NaCl, 10mM 2-ME) overnight. Experiments were conducted in triplicates for each folding additive.

2.2.2.2. Folding additive: Oxidized Glutathione: Reduced Glutathione (GSH:GSSG) (1:1, 3:1, and 30:9 ratio)

Fractions containing denatured nanobody proteins were pooled (total 20mL) according to the SDS-PAGE gel results and were diluted three fold with dilution buffer (400mM NaCl, 20mM sodium phosphate buffer 7.4, 1M urea) containing different ratios of GSH (reduced glutathione) and GSSG (oxidized glutathione) as given in Table 1. Approximately 60 ml protein solution (buffer composition is given in Table 1) was dialyzed against 1 L dialysis buffer (20mM sodium phosphate buffer pH 7.4, 300mM NaCl, 10mM 2-ME) overnight. Experiments were conducted in triplicates for each folding additive.

Table 1. Final buffer composition for three fold diluted protein before dialysis

Tested refolding reagent	Final buffer composition for three fold diluted protein before dialysis
No additives	2M urea, 100mM imidazole, 20mM sodium phosphate, pH 7.4, 400mM NaCl, 2mM 2-ME*
Arginine (200 mM)	200mM Arginine, 2M urea, 100mM imidazole, 20mM sodium phosphate, pH 7.4, 400mM NaCl, 2mM 2-ME
Arginine (400 mM)	400mM Arginine, 2M urea, 100mM imidazole, 20mM sodium phosphate, pH 7.4, 400mM NaCl, 2mM 2-ME
GSH*: GSSG* (1:1)	1mM GSH: 1mM GSSG, 2M urea, 100mM imidazole, 20mM sodium phosphate, pH 7.4, 400mM NaCl, 2mM 2-ME
GSH: GSSG (3:1)	3mM GSH: 1mM GSSG, 2M urea, 100mM imidazole, 20mM sodium phosphate, pH 7.4, 400mM NaCl, 2mM 2-ME
GSH: GSSG (30:9)	30mM GSH: 9mM GSSG, 2M urea, 100mM imidazole, 20mM sodium phosphate, pH 7.4, 400mM NaCl, 2mM 2-ME
Arginine (400 mM), GSH: GSSG (1:1)	400mM Arginine, 1mM GSH: 1mM GSSG, 2M urea, 100mM imidazole, 20mM sodium phosphate, pH 7.4, 400mM NaCl, 2mM 2-ME

2-ME= 2-mercaptoethanol, GSH= reduced glutathione, GSSG= oxidized glutathione.

2.3. VersaDoc and ImageJ analysis

15% Tris-Glycine SDS-PAGE analysis was performed to assess the protein profiles of the samples for both before and after dialysis. 20uL of sample was loaded to wells. The gel images were acquired using the digital imaging system, VersaDoc MP 4000 Imaging System (Bio-Rad, USA). Band intensities around 14 kDa are determined and analyzed using ImageJ software. Ratio of the after and before dialysis band intensities gives the percentage of soluble recovered protein from dialysis (Protein refolding yield) (equation 1):

$$Protein\ refolding\ yield\ (\%) = \frac{Band\ intensity\ after\ dialysis}{Band\ intensity\ before\ dialysis} \times 100 \quad (1)$$

2.4. Statistical Analysis

All statistical analyses were performed using R software (version 4.3.2). The normality of the data was assessed using the Shapiro-Wilk test. It was confirmed to be a normal distribution. ANOVA was conducted to compare the differences among the seven groups. Subsequently, t-test was performed to identify the statistically significant differences between each group and the control. P-values < 0.05 were accepted as statistically significant.

3. Results

3.1. Nanobody Expression and Purification

Initially, we tested HIV-1 capsid binding nanobody protein expression in BL21 (DE3) *E. coli* cells in the cytoplasm (in pET21a plasmid). However, we did not observe any protein expression in the cytoplasm (Fig. 2A). The same nanobody protein is expressed in the pET22b plasmid in BL21 (DE3) *E. coli* cells. After purification, pure protein yield was less than 1mg per liter of culture, indicating that purification optimization is necessary to improve overall yield. The low yield highlights the need for further exploration of alternative expression systems or modifications to the existing protocols that could enhance solubility and facilitate higher production levels of the nanobody protein.

In our hands, HIV-1 capsid binding nanobody expression in the periplasm did not produce any nanobody protein in the soluble fraction from the osmotic shock protocol for easy extraction of proteins present in the periplasm (unpublished results). Thus, the protein was extracted under denaturing conditions. It is not recommended to expose proteins to sudden environmental changes (buffer conditions, pH, or temperature), or they might aggregate and/or precipitate. Dilution and dialysis under

low temperature conditions over four or more hours is the gentlest form of buffer exchange for folding proteins (Rudolph and Lilie, 1996). The effect of arginine and glutathione in the refolding buffer was evaluated for nanobody protein recovery and yield.

3.2. Additives to Fold Proteins in High Yield

When the protein of interest is produced in inclusion bodies, various additives reported in the literature can be used to help fold and recover the protein in high yield with the refolding dialysis step. Literature on recombinant production of cysteine-rich antibody fragments recommends testing various ratio of reduced and oxidized glutathione (1:1, 2:1, 1:2, 3:1, 1:3, 5:1, 1:5, 10:1 etc) as redox pair (Bao et al., 2016; Ban et al., 2020). Arginine is another highly utilized additive to increase solubility and suppress protein aggregation during refolding (Yasuda et al., 1998; Yamaguchi et al., 2013). These two additives were reported to improve nanobody recovery from refolding experiments in the literature (Bao et al., 2016; Maggi and Scotti, 2017).

Before testing any additives for refolding nanobody protein, we dialyzed the protein directly into no additive buffer (Table 1) which caused most of the protein to aggregate and precipitate. Figure 3 shows the SDS-PAGE gel image of the nanobody protein after three-fold dilution and compares the results before and after refolding dialysis experiments without any refolding additives. Here, from three different replicates, 38% (±4.7) protein recovery was achieved in the absence of any refolding reagent.

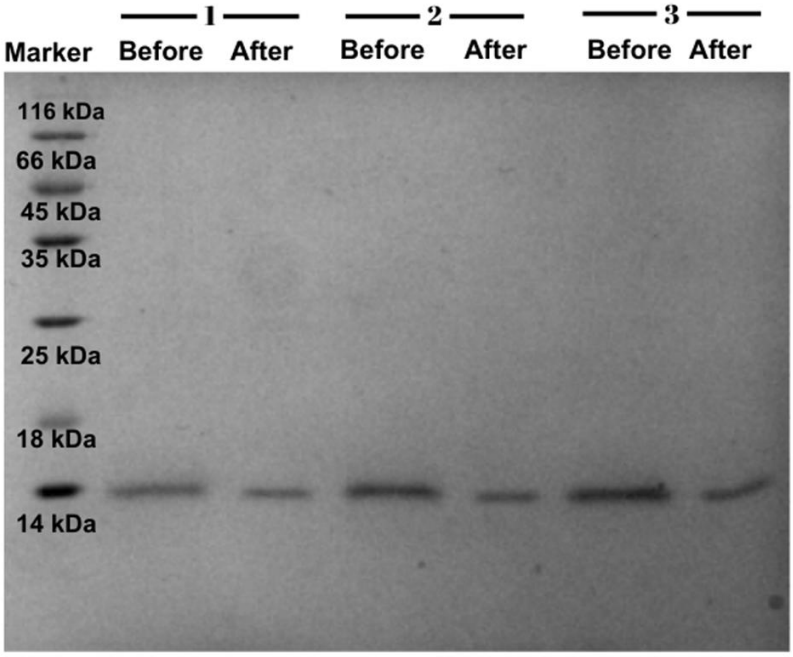

Figure 3. Refolding efficiency of the nanobody without any chemical additives (arginine or reduced glutathione (GSH): oxidized glutathione (GSSG)). SDS-PAGE analysis represents nanobody samples before and after refolding. 1, 2, and 3 indicates replicates. The target protein band appears at ~14 kDa.

Arginine and a mixture of oxidized and reduced glutathione were the two additives chosen to observe their effect on HIV-1 capsid binding nanobody refolding. We diluted the nanobody protein in the presence of varying concentrations of arginine (200mM and 400mM) or a mixture of reduced and oxidized glutathione at various ratios (1:1, 3:1, and 30:9) separately, followed by refolding dialysis. Later, the highest yielding arginine concentration and reduced (GSH) and oxidized (GSSG) glutathione ratio were combined to investigate the additive effect of these two protein folding chemicals on nanobody recovery.

Figure 4 shows SDS-PAGE gel images of nanobody protein before and after dilution refolding experiments in the presence of 200mM (Fig. 4A) and 400mM (Fig. 4B) arginine. Figure 4C has the bar graph representation of average protein recovery percentages from three replicates in 200mM and 400mM arginine, respectively. In the presence of 200mM arginine, the protein recovery is 46% (±0.6) which is slightly better than the effect of no refolding additives in Figure 3. In contrast, 82% (±6.6) nanobody protein is recovered in the presence of 400mM arginine from three replicates, which is a decent increase in protein refolding yield.

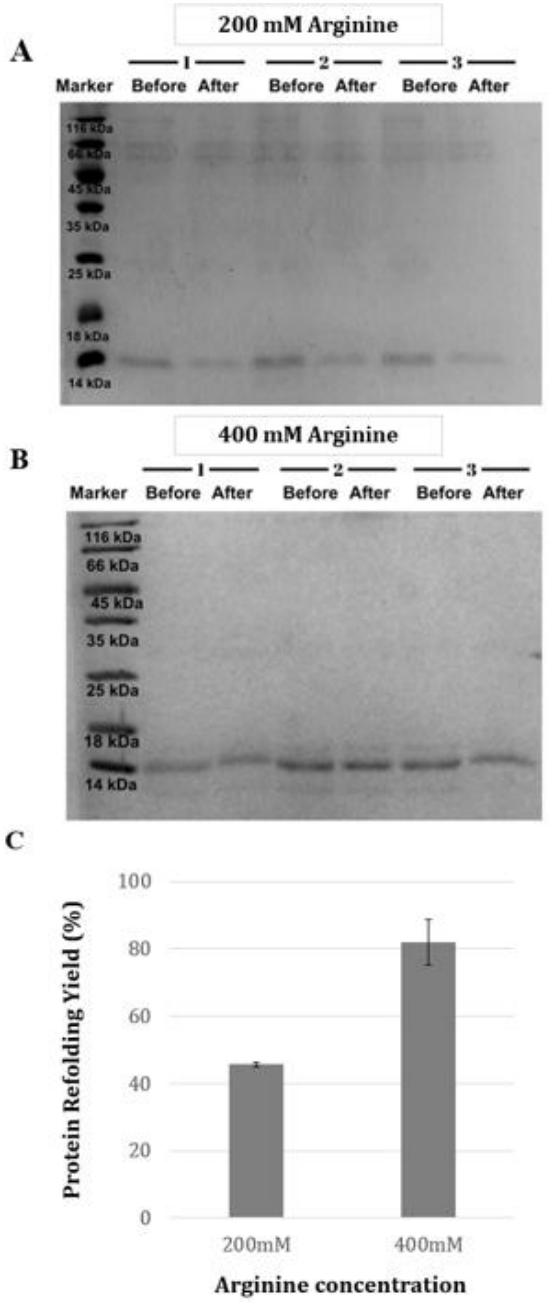

Figure 4. Refolding efficiency of the nanobody with different concentrations of arginine. SDS-PAGE analyses represent the nanobody samples before and after refolding with 200mM (A) or 400mM (B) arginine. The target protein band appears at ~14 kDa. 1, 2, and 3 indicates replicates. C) The percentage of nanobody refolding yields with 200mM or 400mM arginine, respectively.

In our initial preliminary experimental optimization, we used (30:9) ratio of reduced and oxidized glutathione and observed an improvement in recovered nanobody protein yield. Thus, in this study in addition to (1:1) and (3:1) GSH:GSSG ratios, we investigated (30:9) ratio. Figure 5 shows the SDS-PAGE gel images of nanobody protein before and after dilution refolding experiments in the presence of 1mM:1mM (Fig. 5A), 3mM:1mM (Fig. 5B), and 30mM:9mM (Fig. 5C) ratios of GSH:GSSG. Figures 5D is the bar graph representation of protein recovery from the mentioned GSH:GSSG ratios, respectively. For nanobody folded in 1mM:1mM GSH:GSSG, 62% (±9.0) of protein was recovered after dialysis. In the presence of 3mM:1mM GSH:GSSG, 47% (±7.0) protein was recovered. Finally, 47% (±6.0) protein recovery was achieved in the presence of 30mM:9mM GSH:GSSG. Best protein recovery yield was obtained with GSH:GSSG ratio of 1mM:1mM. This is similar to GSH:GSSG concentration and ratio in the eukaryotic cells' ER where protein folding takes place. GSH:GSSG ratio in the ER of eukaryotic cells range between 1:1 - 3:1 (Okumura et al., 2011; Bocedi, 2019).

Finally, we tested the effect of 400mM arginine and 1mM:1mM GSH:GSSG together on nanobody dilution refolding yield and the results are given in Figure 6. 63% (±15.5) protein recovery was achieved in the presence of 400mM arginine and 1mM:1mM GSH:GSSG. These recovery yields are less than the protein recovered in the presence of only 400mM arginine. All the refolding dialysis buffers contain 10mM 2-mercaptoethanol (2-ME), a reducing agent. We conclude that the presence of 2-ME keeps cysteine amino acids of the protein in the reduced form for all refolding conditions. Thus, reduced and oxidized glutathione do not contribute to recover more nanobody protein. Nanobodies are known to have at least one disulfide bond. The nanobody used in this study has two pairs of cysteine amino acids, possibly forming two disulfide bonds. Thus, despite the appearance of no additional advantage of using GSH and GSSG, it is useful to include a chemical additive specifically for the correct disulfide bond formation for cysteine containing proteins.

Figure 7 summarizes the effect of refolding additives on nanobody refolding yield via box plots. ANOVA revealed statistically significant differences among the seven groups ($P<0.01$). Control group (without any additives), had the lowest recovery with yields of roughly 38%. Addition of 200mM arginine into the refolding buffer slightly improved the protein recovery yield ($P>0.05$). However, increasing the arginine concentration to 400mM resulted in a statistically significant enhancement of protein recovery compared to the control ($P<0.001$). Among the redox conditions, only 1mM:1mM GSH:GSSG buffer resulted a statistically significant improvement in nanobody recovery yield ($P<0.01$). On the other hand, no statistical differences were found between different GSH:GSSG ratios in terms of their varied concentration. The final assessment was performed via the combination of best results in arginine and GSH:GSSG additives. Although combining different kinds of additives resulted in a statistically significant improvement in protein recovery ($P<0.01$) compared to no additive condition, it did not surpass refolding yield obtained with 400 mM arginine alone. These results imply that the contribution of GSG:GSSG on protein recovery may be masked in the presence of 2-ME.

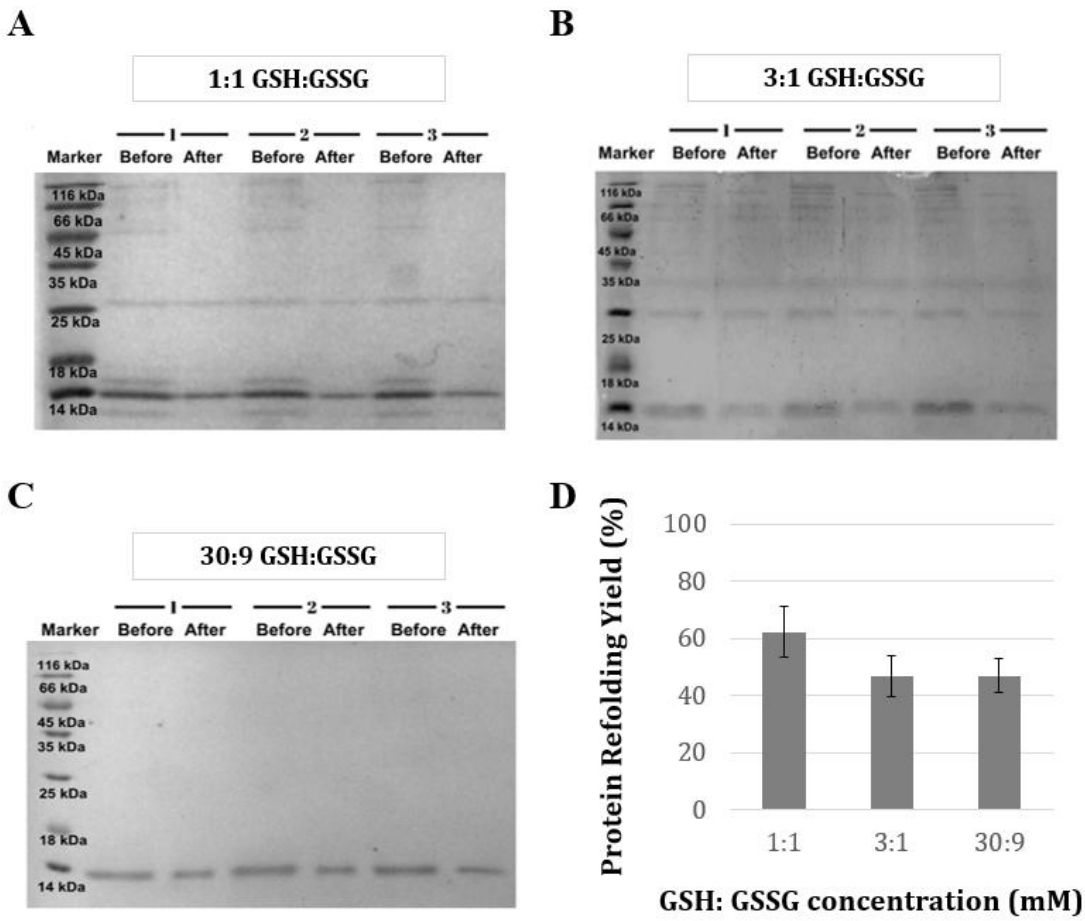

Figure 5. Refolding efficiency of the nanobody under different concentrations of GSH:GSSG. SDS-PAGE analyses represent the nanobody samples before and after refolding with 1:1 (A), 3:1 (B) or 30:9 (C) GSH:GSSG. The target protein band appears at ~14 kDa. 1, 2, and 3 indicates replicates. D) The percentage of nanobody refolding yields with 1:1, 3:1, 30:9 GSH:GSSG, respectively.

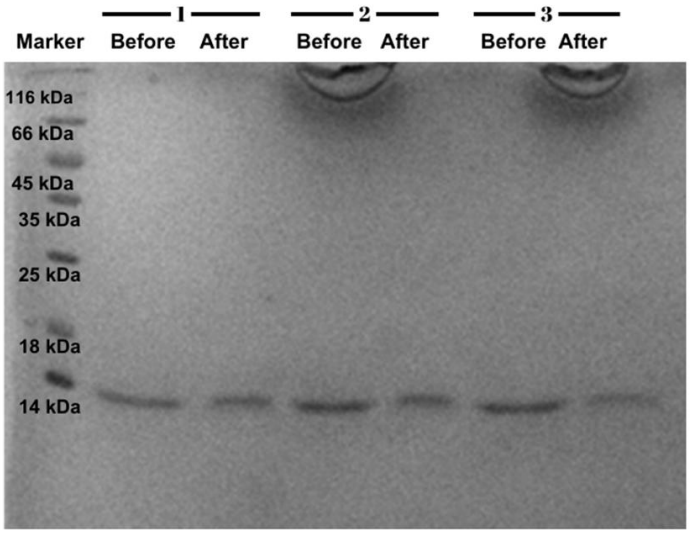

Figure 6. The combined effect of best arginine (Arg) and oxidizing and reducing agents (GSH:GSSG) on nanobody refolding efficiency. SDS-PAGE analysis indicates the nanobody samples before and after refolding with 400mM arginine and (1:1) GSH:GSSG. The target protein band appears at ~14 kDa. 1, 2, and 3 indicates replicates.

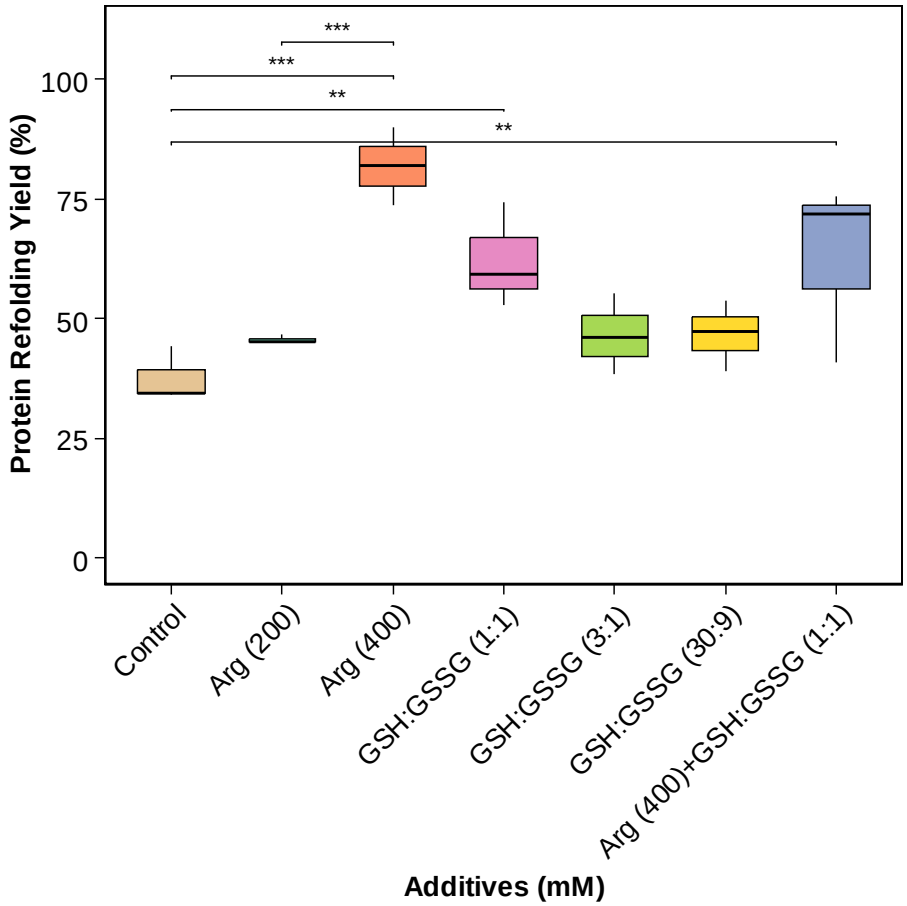

Figure 7. Box plot comparison of nanobody refolding yields under various chemical additive conditions. Control sample was refolded without any chemical additives. Arginine was tested at 200mM and 400mM, whereas reduced (GSH) and oxidized (GSSG) glutathione were assessed at molar ratios of 1:1, 3:1, and 30:9. In addition, a combination of 400mM arginine with GSH:GSSG (1mM:1mM), representing the best condition within their own classes, was evaluated. The horizontal line within the box indicates the median, and the whiskers correspond to variability outside the quartiles. Statistical significance is indicated as *P < 0.05, **P < 0.01, ***P < 0.001.

4. Discussion

Antibodies and antibody fragments are highly valuable products for the biotechnology industry. Antibody fragments can be produced in engineered bacterial host strains either in the cytoplasm or secreted into the periplasm. However, high-level expression frequently leads to the formation of insoluble aggregates known as inclusion bodies, particularly in the cytoplasm but sometimes also in the periplasm. For proteins containing disulfide bonds, such as antibody fragments, periplasmic expression is often preferred despite lower yields, due to the oxidizing environment that favors disulfide bond formation (Berkmen, 2012; Manta et al., 2019).

Proteins expressed in inclusion bodies can be recovered through solubilization and refolding, and protein recovery yields can be improved using various strategies, including chemical additives, molecular crowding agents, denaturants, redox reagents, detergents, on-column refolding, dilution, and dialysis-based refolding methods. Among these approaches, the use of refolding additives and controlled removal of denaturants are particularly important for minimizing aggregation and promoting correct folding (Arakawa et al., 2014).

In the present study, an HIV-1 capsid–binding nanobody was expressed in the periplasm of *E. coli* BL21 (DE3) cells but predominantly accumulated in inclusion bodies. In the absence of refolding additives, protein recovery was limited to 38% (±4.7). To improve refolding efficiency, the effects of arginine and a mixture with different ratios of reduced and oxidized glutathione (GSH:GSSG) were investigated. The nanobody contains two pairs of cysteine residues, making correct disulfide bond formation a critical factor for successful refolding. Antibodies and antibody fragments commonly contain cysteine residues, and the redox environment plays a critical role in the folding of disulfide-bonded proteins. The presence of reducing agents such as 2-mercaptoethanol (2-ME), dithiothreitol, or glutathione during the early stages of refolding is essential to prevent incorrect disulfide bond formation until the protein has adopted its native conformation (Ban et al., 2020).

Arginine has been widely used as an aggregation suppressor during recombinant protein refolding for several decades (Arakawa et al., 2007). Previous studies have reported that arginine concentrations around 400

mM can significantly improve the refolding yield of antibody fragments (Arakawa et al., 2014; Bao et al., 2016). Consistent with these reports, 200 mM arginine led to a modest increase in recovery yield (average 46%), while 400 mM arginine resulted in a substantial improvement, with an average recovery of 82%. These findings confirm the strong anti-aggregation effect of arginine during nanobody refolding.

In this study, stepwise reduction of denaturant concentration—from 6 M urea in the lysis buffer to 4M urea in elution buffer and to 2 M urea prior to dialysis—allowed gradual folding of the nanobody. Subsequent dilution of the protein solution reduced protein concentration, thereby minimizing intermolecular interactions and aggregation, which are known to be concentration dependent (Arakawa et al., 2014).

Dialysis against a large buffer volume enabled gentle removal of denaturants and refolding additives. All dialysis buffers contained 10 mM 2-ME, maintaining cysteine residues in a reduced state during folding. Under these conditions, the addition of GSH:GSSG alone resulted in moderate recovery yields (approximately 62% (±9.0) for a 1mM:1mM ratio). However, combining GSH:GSSG (1mM:1mM) with 400 mM arginine did not further enhance recovery (63% (±15.5)) beyond that achieved with 400mM arginine alone (82% (±6.6)). The lack of synergistic effect between arginine and GSH:GSSG is likely due to the presence of 10 mM 2-ME in all refolding buffers, which maintained cysteine residues in a reduced state and reduced the requirement for an additional glutathione redox system.

5. Conclusion

In this study, we evaluated the effects of arginine (200 mM and 400 mM) and different GSH:GSSG ratios (1:1, 3:1, and 30:9) on the refolding and recovery yield of an HIV-1 capsid–binding nanobody produced as inclusion bodies in *E. coli* BL21 (DE3) cells. The highest recovery was achieved with 400 mM arginine, yielding an average of 82% (±6.6) folded protein. In contrast, GSH:GSSG alone resulted in moderate recovery, and its combination with arginine did not lead to further improvement. Overall, our results demonstrate that high-concentration of arginine is highly effective for improving nanobody recovery from inclusion bodies, while the presence of a reducing agent is sufficient to support proper folding of cysteine-containing antibody fragments.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	G.T.	İ.A.	E.A.	H.T.S.
C				100
D				100
S				100
DCP	40	30	30	
DAI	25	25	20	30
L	25	25	25	25
W	30	20	20	40
CR	40			60
SR				100
PM				100
FA			10	90

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because there was no study on animals or humans.

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References

Arakawa, T., & Ejima, D. (2014). Refolding technologies for antibody fragments. *Antibodies*, *3*(3), 232–241.

Arakawa, T., Ejima, D., Tsumoto, K., Obeyama, N., Tanaka, Y., Kita, Y., & Timasheff, S. N. (2007). Suppression of protein interactions by arginine: A proposed mechanism of the arginine effects. *Biophysical Chemistry*, *127*(1-2), 1–8.

Ban, B., Sharma, M., & Shetty, J. (2020). Optimization of methods for the production and refolding of biologically active disulfide bond-rich antibody fragments in microbial hosts. *Antibodies*, *9*(3), 39–57.

Bao, X., Xu, L., Lu, X., & Jia, L. (2016). Optimization of dilution refolding conditions for a camelid single domain antibody

against human beta-2-microglobulin. *Protein Expression and Purification*, *117*, 59–66.

Berkmen, M. (2012). Production of disulfide-bonded proteins in Escherichia coli. *Protein Expression and Purification*, *82*(1), 240–251.

Bhatwa, A., Wang, W., Hassan, Y. I., Abraham, N., Li, X.-Z., & Zhou, T. (2021). Challenges associated with the formation of recombinant protein inclusion bodies in Escherichia coli and strategies to address them for industrial applications. *Frontiers in Bioengineering and Biotechnology*, *9*, 630551.

Birnboim-Perach, R., Grinberg, Y., Vaks, L., Nahary, L., & Benhar, I. (2019). Production of stabilized antibody fragments in the E. coli bacterial cytoplasm and in transiently transfected mammalian cells. In *Human Monoclonal Antibodies: Methods and Protocols* (Vol. 1904, pp. 455–480). Springer.

Bocedi, A., Cattani, G., Gambardella, G., Ticconi, S., Cozzolino, F., Di Fusco, O., Pucci, P., & Ricci, G. (2019). Ultra-rapid glutathionylation of ribonuclease: Is this the real incipit of its oxidative folding? *International Journal of Molecular Sciences*, *20*(21), 5440.

Dingus, J. G., Tang, J. C. Y., Amamoto, R., Wallick, G. K., & Cepko, C. L. (2022). A general approach for stabilizing nanobodies for intracellular expression. *eLife*, *11*, e68253.

Gezehagn, K. G., & Tessema, T. S. (2024). The potential of single-chain variable fragment antibody: Role in future therapeutic and diagnostic biologics. *Journal of Immunology Research*, *2024*(1), 1804038.

Hamers-Casterman, C., Atarhouch, T., Muyledermans, S., Robinson, G., Hamers, C., Bajyana Songa, E., Bendarham, N., & Hamers, R. (1993). Naturally occurring antibodies devoid of light chains. *Nature*, *363*(6428), 446–448.

Helma, J., Schmidthals, K., Lux, V., Nüske, S., Scholz, A. M., Kräusslich, H. G., Rothbauer, U., & Leonhardt, H. (2012). Direct and dynamic detection of HIV-1 in living cells. *PLoS One*, *7*(11), e50026. https://doi.org/10.1371/journal.pone.0050026

Hennigan, J. N., Menacho-Melgar, R., Sarkar, P., Golovsky, M., & Lynch, M. D. (2024). Scalable, robust, high-throughput expression & purification of nanobodies enabled by 2-stage dynamic control. *Metabolic Engineering*, *85*, 116–130.

Maggi, M., & Scotti, C. (2017). Enhanced expression and purification of camelid single domain VHH antibodies from classical inclusion bodies. *Protein Expression and Purification*, *136*, 39–44.

Manta, B., Boyd, D., & Berkmen, M. (2019). Disulfide bond formation in the periplasm of Escherichia coli. *EcoSal Plus*, *8*(2). https://doi.org/10.1128/ecosalplus.ESP-0010-2018

Martins, A. C., Oshiro, Y. M., Schiavon, B. N., de Jesus, G. A., de la Torre, B. G., & Albericio, F. (2025). Monoclonal antibodies (mAbs) and proteins: The biologic drugs approved by the FDA in 2024. *Biomedicines*, *13*(8), 1962.

Muyldermans, S. (2021). Applications of nanobodies. *Annual Review of Animal Biosciences*, *9*(1), 401–421.

Okumura, M., Saiki, M., Yamaguchi, H., & Hidaka, Y. (2011). Acceleration of disulfide-coupled protein folding using glutathione derivatives. *FEBS Journal*, *278*(7), 1137–1144.

Rees, A. (2021). Antibodies: A history of their discovery and properties. In F. Rüker & G. Wozniak-Knopp (Eds.), *Introduction to Antibody Engineering* (1st ed., pp. 5–40). Springer Nature.

Rudolph, R., & Lilie, H. (1996). In vitro folding of inclusion body proteins. *The FASEB Journal*, *10*(1), 49–56.

The Antibody Society. (2025). *Therapeutic monoclonal antibodies approved or in review in the EU or US*. https://www.antibodysociety.org/resources/approved-antibodies.

Yamaguchi, S., Yamamoto, E., Mannen, T., & Nagamune, T. (2013). Protein refolding using chemical refolding additives. *Biotechnology Journal*, *8*(1), 17–31.

Yasuda, M., Murakami, Y., Sowa, A., Ogino, H., & Ishikawa, H. (1998). Effect of additives on refolding of a denatured protein. *Biotechnology Progress*, *14*(4), 601–606.