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Improved Production of Single Domain Antibodies with Two Disulfide Bonds by Co-Expression of Chaperone Proteins in the *Escherichia coli* Periplasm

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Running Title: Plasmid pTUM4 Improves Production of SdAb with two DSB

Abstract

Single domain antibodies are recombinantly expressed variable domains derived from camelid heavy chain antibodies. Natural single domain antibodies can have noncanonical disulfide bonds between their complementarity-determining regions that help position the binding site. In addition, engineering a second disulfide bond serves to tie together β -sheets thereby inhibiting unfolding. Unfortunately, the additional disulfide bond often significantly decreases yield, presumably due to formation of incorrect disulfide bonds during the folding process. Here, we demonstrate that inclusion of the helper plasmid pTUM4, which results in the expression of four chaperones, DsbA, DsbC, FkpA, and SurA, increased yield on average 3.5-fold for the nine multi-disulfide bond single domain antibodies evaluated. No increase in production was observed for single domain antibodies containing only the canonical disulfide bond.

Keywords: Single domain antibody/thermal stability/helper plasmid/disulfide bond/chaperone

1.0 Introduction

Single domain antibodies (sdAb), derived from the variable domain of camelid heavy chain antibodies, are attractive alternatives to conventional immunoreagents as they are small, highly soluble, and able to refold following denaturation, yet they still possess high affinity for antigen (Muyldermans, 2013). In most instances sdAb produce well in the oxidizing environment of the *E. coli* periplasm, which facilitates the formation of its single highly conserved disulfide bond (DSB). However, a substantial fraction of sdAb, particularly those obtained from camels, possess non-canonical DSBs that generally link complementarity-determining regions, CDR1 to CDR3, which help to properly orient the binding site (Govaert et al., 2012). Naturally occurring additional DSBs in the variable domains of the heavy chain only antibodies have also been observed that link CDR3 to framework (FW) 2, and CDR2 (Govaert et al., 2012; Muyldermans, 2013). In addition, a proven technique to enhance the thermal stability of single domain antibodies is to insert an additional DSB, commonly linking FW2 and FW3 (Hagihara and Saerens, 2014). Placement of a similar additional DSB was also shown to provide increased protease stability (Hagihara and Saerens, 2014). In earlier work other groups as well as our own examined alternate positions for placement of the additional DSB and found that many locations could accommodate the extra DSB and result in an increased melting temperature (Hagihara and Saerens, 2014; Zabetakis et al., 2014). The primary limitation to thermal stabilization by DSB addition is the loss in protein production.

While multi-DSB sdAb may have the binding and/or stability desired, their production is often significantly lower than those with only the conserved DSB. The co-expression of four folding chaperones, the thiol-disulfide oxidoreductases DsbA and DsbC that catalyze the formation and isomerization of disulfide bridges and the peptidyl-prolyl cis/trans-isomerases with chaperone activity, FkpA and SurA combined on single plasmid, pTUM4, has been demonstrated to improve the yield of a number of recombinant proteins (Schlapschy et al., 2006). In this work, we performed an in depth examination on the utility of pTUM4 to improve the production of sdAb with more than one DSB.

2.0 Materials and Methods

2.1 Reagent and buffers

The wild type sequences for all but two of the sdAb prepared here have been previously published in other works (Conway et al., 2010; Richard et al., 2013; Vance et al., 2013; Liu et al., 2015; Turner et al., 2015). The sequences as produced for this work with the location of the added cysteines that formed the additional disulfide bond marked are shown in Fig. 1. The helper plasmid pTUM4 was prepared as described previously (Schlapschy et al., 2006; Schlapschy and Skerra, 2011), and provided to us as a kind

gift by Dr Skerra. Enzymes used for cloning were from New England Biolabs. Gene synthesis and DNA sequencing services were provided by Eurofins Genomics. Oligos were also purchased from Eurofins Genomics. Sequence alignments were performed using MultAlin (Corpet, 1988). Unless otherwise specified, chemical reagents were from Sigma Aldrich, Fisher Scientific, or VWR International.

2.2 Recombinant protein production and purification

The sdAb were produced as previously described (Turner et al., 2015). Briefly, the PET22b plasmid carrying the sdAb gene and the pTUM4 plasmid were transformed into Tuner (DE3) for expression and grown on agar plates containing both ampicillin and chloramphenicol (100 µg/mL and 34 µg/mL respectively). Single colonies were transferred to overnight cultures containing both antibiotics (50 mL in Terrific Broth [TB] grown at 25°C). The 50 mL cultures were used to inoculate 0.5 L of TB (with appropriate antibiotics) which was grown for 2 hours at 25°C and then induced with 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown a further 2 hours. Cultures were centrifuged and the cell pellets subject to an osmotic shock protocol (Turner et al., 2015). Protein was purified by Ni-NTA followed by size exclusion chromatography and was quantified by absorbance at 280 nm. Each sdAb was also produced without co-transforming with the pTUM4 in media containing only ampicillin.

2.3. Fluorescence based melting assays

The melting temperature of each sdAb was measured by a fluorescent dye-based assay. This technique relies on the fluorescence enhancement of Sypro Orange (Thermo Fisher), as it interacts with the hydrophobic amino acids on a protein, which become accessible upon thermal unfolding. Each sdAb was diluted to a concentration of 500 µg/mL in a final volume of 20 µL PBS. The Sypro Orange dye (Thermo Fisher) was added to each sample at a dilution of 1:1000. Samples were measured in triplicate using a StepOne Real-Time PCR machine (Applied Biosystems). The heating program was run in continuous mode from 25°C – 99°C at a heating rate of 1% (~2°C per minute). Data was recorded using the ROX filter and the melting point was determined to be the peak of the first derivative of the fluorescence intensity. All three replicates gave essentially identical values for the melting temperature.

3.0 Results and Discussion

Our research has focused on the development of robust immunoreagents with the long term goal of increasing the shelf-life of immunoassays, both to decrease logistical costs and to improve the ability of those immunoassays to retain their activity during long term storage under the harsh conditions that occur when operating in austere locations.

SdAb, also known as nanobodies, have been our molecules of choice for the development of robust recognition reagents. Due to their small size, most sdAb start with many attractive attributes, such as the ability to rapidly refold following thermal denaturation. However, we have found that this attribute is a function of intrinsic as well as extrinsic properties. The intrinsic properties include their net charge, which impacts their propensity to aggregate while denatured, while the extrinsic properties include the temperature to which they are exposed, the duration of that exposure, their concentration, and the buffer conditions. This is a complex set of parameters that can be tweaked in attempts to facilitate protein refolding following thermal denaturation. However, the more direct approach is to simply prevent the unfolding in the first place by increasing the melting temperature of the sdAb.

There are a number of approaches that can be taken to improve the thermal stability of sdAb, such as: CDR grafting onto stable frameworks, consensus sequence matching, and random mutation followed by stringent selection, as well as addition of disulfide bonds. We and others have looked extensively at all of these techniques (Hagihara and Saelens, 2014; Zabetakis et al., 2014; Turner et al., 2015). Of these methods, the most straight forward and reliable tactic is the addition of a disulfide bond to increase the melting temperature (T_m). In addition, it can easily be applied after any of the other approaches, if the T_m of the sdAb is still below the desired temperature.

While affinity can be negatively impacted by disulfide bond addition to the sdAb (Hagihara and Saelens, 2014; Turner et al., 2015), a common limitation has been that the protein production frequently takes a substantial hit. While the decrease is of little concern in the research arena, it can make the production of sdAb prohibitively expensive for the world of commerce. For one particular sdAb with an extra DSB, we successfully recovered much of the production of the parental sdAb by decreasing the proteins net charge (Liu et al., 2015). Unfortunately, while decreasing the net charge appears to improve both an sdAb's solubility and refoldability (Turner et al., 2015), it did not prove to be a general method for improving production of sdAb with an extra DSB (Liu et al., 2015).

To overcome the production problems of sdAb with extra DSBs, we turned to the proven method of co-expression of chaperones. The pTUM4 plasmid that expresses four folding chaperones that accumulate in the periplasm was particularly attractive as it fit well with our standard production protocol and was readily available. To thoroughly evaluate pTUM4's ability to improve the production of sdAb with extra DSBs, we started by measuring the production of four sdAb, three with an extra DSB added to the FW (ACVE+, A3neg+, C10neg+) and one with a naturally occurring non-canonical DSB between CDR1 and CDR3 (F5). Each of these sdAb was produced three times both with and without the helper plasmid pTUM4. The sdAb produced with or without the pTUM4 eluted at the same volume from the SEC70 gel filtration column, however the increased monomer yields with the inclusion of pTUM4 also seem to correlate with increases in partially aggregated material (Fig. 2); the final purity of the purified

sdAb was comparable ($\geq 95\%$ as determined using an Experion automated electrophoresis system). Our results were most promising, with increases of ~3 to 10-fold due to inclusion of pTUM4, with the sdAb that produced most poorly gaining the most, thus the final yield for all the sdAb approached or was greater than 10 mg/L, Table I. This value compares well with literature results for the shake flask production of sdAb.

Table I. Yield improvement due to pTUM4

Clone	replicates	Avg Yield no pTum4 (mg/L)	Avg Yield with pTum4 (mg/L)	Fold increase with pTum4
<u>+Noncononical DSB</u>				
ACVE+	3	4.2 $\pm$ 1.2	11.9 $\pm$ 1.9	2.8
A3neg+	3	2.1 $\pm$ 0.4	10.4 $\pm$ 2.2	5.0
C10neg+	3	8.9 $\pm$ 1.2	33.3 $\pm$ 16.5	3.7
F5	3	0.9 $\pm$ 0.2	8.9 $\pm$ 2.1	9.9
A8fneg+	2	2.8 $\pm$ 1.2	14.8 $\pm$ 3.9	5.3
F6m+	3	5.9 $\pm$ 0.4	11.1 $\pm$ 0.8	1.7
G2+	1	8	24	3.0
C20neg+	2	1.3 $\pm$ 0.1	3.1 $\pm$ 0.4	2.4
C2neg+	2	1.1 $\pm$ 0.1	4.1 $\pm$ 0.3	3.7
<u>Cononical DSB only</u>				
B2	2	9.6 $\pm$ 0.6	10.2 $\pm$ 1.6	1.1
A17	2	12.3 $\pm$ 1.8	14.6 $\pm$ 3.0	1.2
A18	2	14.9 $\pm$ 1.8	14.1 $\pm$ 1.4	0.9

We expanded the number of engineered sdAb tested for production with and without the pTUM4 helper plasmid, producing one clone in triplicate, three clones in duplicate and one just once. In each case, we saw similar results with increased production on co-transformation with pTUM4. While not always done with as many replicates, each sdAb with extra DSB showed improvements in yield consistent with the results of the first four evaluated. In addition, each of these clones was tested for binding activity by surface plasmon resonance (not shown) and all actively bound their target antigen with the little or no loss in affinity relative to the parental clone, indicating that each sdAb folded correctly making the proper disulfide bonds. For 75% of the sdAb with added DSBs, protein production with the inclusion of pTUM4 was equivalent to that of the parental sdAb without an engineered DSB; for the remaining 25% the yield was about half compared to the parental clone.

Each of the DSB additions led to an increased T_m as is seen in Table II. The most dramatic increase was seen for G2, which was nearly 20 °C. The smallest increase, 6 °C, was seen for clone ACVE, which already possessed a high melting temperature prior to addition of the extra DSB.

Due to the increased protein production with addition of pTUM4 in the sdAb that included an extra DSB, we also investigated the use of pTUM4 to increase production of sdAb containing only the

canonical DSB. We chose three sdAb which were expressed in duplicate with and without co-transformation with pTUM4. No increase in protein production was found for these clones, each of which produced ~ 10 mg/L.

Table II. Melting temperature (T_m) Increase due to additional DSB

Clone (+ds)	T _m of wt*	T _m °C of wt +ds	Net Increase
ACVE (+)	80	86	6
A3neg (+)	82	>90	>8
C10neg (+)	65	78	13
A8f (neg+)	60	79	19 [†]
F6m (+)	78	85	7
G2 (+)	62	80	18
C20neg (+)	66	75	9
C2neg (+)	74	86	12

*wt – represents the clone without the additional DSB

†Net increase of 19°C is for the A8fneg+ clone vs A8f, as the A8fneg was never made. The changes to decrease the clone's net charge can increase the T_m ~7 °C.

4.0 Conclusions

We demonstrated an increase in production by a factor of ~2 to 10-fold for multi-DSB containing sdAb by the addition of the helper plasmid pTUM4. The average yield increased from 3.9 ± 2.5 to 13.5 ± 7.0 mg/mL for the nine multi-DSB sdAb evaluated using pTUM4. No improvement for the production of sdAb with only the standard single DSB was obtained with pTUM4, which indicates that correcting improper DSB formation is the a critical limiting step when producing sdAb with multiple DSBs.

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Figure Legends

Figure 1. Protein sequences of the sdAb examined in this work. Sequences are aligned using MultAlin (Corpet, 1988). Stars mark the positions where cysteines were engineered in the “+” clones for the formation of a non-canonical DSB, position 54 and 78 using the IMGT numbering scheme. Clones B2, A17 and A18 are from Conway *et al.*, 2010; G2+ is described in Liu *et al.*, 2015; C10neg+ is from Turner *et al.*, 2015; A3neg+ is from Liu *et al.*, 2015; F5 is from Vance *et al.*, 2013. ACVE+ is derived from ACVE (Liu *et al.*, 2015); C2neg+ and C20neg+ are derived from C2 and C20 respectively (Richard *et al.*, 2013); F6m+ is derived from F6 (Vance *et al.*, 2016); A8fneg+ is unpublished.

Figure 2. Representative elution profiles from the SEC70 gel filtration column. Production with the pTUM4 helper plasmid are shown as solid lines, and standard production (lacking the helper plasmid) are shown as dashed lines. Traces are shown for A3neg+ (left), C10neg+ (center), and F5 (right).

B2 QVKLQASGGGLVQAGGSLRLSCAASGRTFSTYALGHWFRQAPGKEREF^{*}AAAI^{*}S---RNGI^{*}IKDYAESVKGRFTISRDNAKNTIYLEMNSLKPEDTAVYYCAATGTYYDYMA--HALARSYEFWGQGTQVTVSS

A18 QVKLQESGGGLVQAGDSLRLSCAASRRTFSEYTLAWFRQAPGKEREFVTGIS---YNGVTTWYADSVKGRFTISRDNAKNTLYLQMNSLKREDTAVYYCAADPRTADYSG-----SYRYWGQGTQVTVSS

A17 QVKLQESGGGLVQAGGSLRLSCAASGRPFSTYYMGWFRQAPGKEREFIIGID---SNGGIT^{*}YARTSVKGRFTISRDNAKNTVYLQMNDLKPEDTAVYYCYADT--GRGAG--H-----WDSWGQGTQVTVSS

G2+ EVQLQASGGGLVQAGGSLRLSCTASGRAFTTNMGWFRQAPGKEREFVCRVN---WSGGVTSYADSVKGRFTCSRDN^{*}TKNTVYLQMDSLKPEDTAVYYCAGSTVWSTDRK--H-----YNYWGQGTQVTVSS

ACVE+ DVQLVESGGGLVQPGGSLRLTCAASGLIFGSYMGWFRQAPGKAREFVCAI---SWSGGDTYADSVKGRFTCSRDN^{*}AKNTVYLQMNSLEPEDTAVYYCSAAYGSKYYIS---KDA-KDYGYWGQGTQVTVSS

C2neg+ EVQLVESGGGLVLPGGSLRLSCAASGSISSIYAMGWYRQAPGKEREFVCFI---TNGNSPNYADSVKGRFTCSRDN^{*}AKNTVYLQMNSLKPEDTAVYYCNVEGVRY-----GDSWYDGDYWGQGTQVTVSS

20neg+ EVQLVESGGGLVQPGGSLRLSCYVGSIS^{*}SFNGMGWYRQAPGKEREFVCFI---SSGGRSKYTDSVKGRFTCSGDNAKSTVYLQMINKPEDTAVYYCNVGSVLSYVT---GNYYEPSDYWGQGTQVTVSS

8fneg+ EVQLVESGGGLVQAGDSLRLSCAAPGRPYRTYTMGWFRQAPGKEREFVCA-----NNMRGTRYAGSVRGRFSCSRDN^{*}SKNTVYLQMNSLKPEDTAVYYCASAVTADLLSGNYFRGDDYDYGQGTQVTVSS

F6n+ EVQLVESGGGLVQAGDSLRLSCATSGGTFSDYMGWFRQAPGKEREFVCAIRRNNGNGNGIEYADSVKGRFTCSRDN^{*}AKNTVYLQMNSLTPEDTAVYYCASISGY--A---YNTIERYNWGQGTQVTVSS

A3neg+ EVQLVESGGGLVQDGSRLRLSCTASGRTFSDAYMGWFRQAPGKEREFVCAISAPGTAYYAFYADSVRGRFSCSADSAKNTVYLQMNSLKPEDTAVYYCAADLKMQYAA---YMNQRSVDYWGQGTQVTVSS

10neg+ DVQLQASGGGLVQDGGSLRLCAHSGRE-GTTTVGWFRQAPGKEREFVCAIR---WTDGRISYADSVKGRFTCSDDNAKKT^{*}VYLQMNSLKPEDSALYYCAEDSWPYVTG-----TAYWGQGTQVTVSS

F5 QVQLVESGGGLVQPGGSLRLSCAASGFTLDYAI^{*}GWFRQVPGKEREGVACV-----KDGSTYYADSVKGRFTISRDN--GAVY^{*}LQMNSLKPEDTAVYYCASR^{*}PCFLGVP-----LIDFGSWGQGTQVTVSS

Figure 2.