Production of bioactive trastuzumab and chimeric anti-VEGF antibody in the cytoplasm of *Escherichia coli*

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Abstract. Monoclonal antibody (mAb) is a very useful tool in not only medical field but also in research field. However, the current cost of its production is very high as most mAbs are produced in mammalian cell system. *Escherichia coli* is a good alternative but has many bottlenecks. Recently, it was demonstrated that biological active full-length Immunoglobulin G (IgG) could be produced in the reductive cytoplasm of an engineered *E. coli* strain called SHuffle. In this study, we investigated the effect of temperature and induction level on the production of full-length humanized anti-Her2 and chimeric anti-VEGF IgGs expressed as cyclonal. The solubility as well as the assembly of the IgG molecules was examined by western blot analysis. It was found that expression at 30 °C with 0.1 mM IPTG induction was the most suitable for trastuzumab. In contrast, the full-length expression of chimeric anti-VEGF required induction with 1 mM IPTG at 30 °C., however, 1 mM IPTG induction resulted in more full-length IgG. In conclusion, the present study indicated that E coli could be successfully used for the full-length expression of IgG by suitably modifying the expression conditions.

1. Introduction

Currently, there are more than 300 biopharmaceutical products in the market with combined market share of more than USD 100 billion [1]. Therapeutic monoclonal antibody (mAb) represents a large

class of biopharmaceutical products. They are used for many indications majority of them are used in various types of cancer and autoimmune disease. As new products are being developed and many are already in clinical trial, unsurprisingly, their revenue is continuing to grow and the combined global market is projected to be nearly USD 125 billion by 2020 [2]. In fact, the use of mAbs is not limited to therapeutic purposes but also as diagnostic and research tools due to their ability to recognize and bind specifically with respective antigens.

Currently, the most popular method for production of mAbs involves hybridoma cell line that expresses mAb in a bioreactor. It allows asparagine-linked (N-linked) glycosylation, which is important for antibody function, high level mAb production and high stability [3]. However, the major drawbacks of mammalian expression system for recombinant protein production include low productivity, low growth rate, potential human pathogenic viral contamination and the use of serum in the cell culture [4]. Moreover, in terms of bioprocessing and scaling up, long production cycle due to slow growth rate, high capital investment as well as expensive medium and serum consequently result in much higher production cost of mAbs [5-6]. Therefore, microbial system such as *E. coli* is a good alternative. Previous attempts were based on expression of mAbs in the naturally oxidative periplasmic compartment of *E. coli*, where it has similar conditions to the endoplasmic reticulum of mammalian cells, allowing for correct disulfide bond formation which is crucial to antibody's function. Nonetheless, periplasmic mAb production has limitations such as small periplasmic volume when compared to cytoplasmic volume leading to lower protein yield, lack of adenosine triphosphate (ATP)-dependent molecular chaperones that aid in antibody folding and efficiency of IgG heavy and light chain secretion through the tightly sealed cytoplasmic membrane [7].

Recently, Successful expression of bioactive full-length IgG was recently reported by using an engineered oxidative cytoplasm of an E coli strain called SHuffle T7 express [8]. They proved that the yield of the cytoplasmic IgG, termed 'cyclonal' was significantly more than the yield of typical IgG produced in the periplasm of *E. coli*. Furthermore, they also showed that the produced cyclonal could be redirected to newer antigens by swapping variable regions of the scaffold cyclonal with the new variable heavy chain (VH) and variable light chain (VL) domains with specificity for certain antigens [7]. These findings showed potentials in creating on-demand IgGs because of the ease with which new genes can be designed, cloned and expressed, in *E. coli*. This technique is a powerful tool for designing and producing humanized antibodies to reduce innate human immunogenic response against the conventional to animal-derived antibodies.

The high cost of pharmaceuticals, has become an important issue for ever-increasing healthcare costs. Here, we demonstrated the effect of temperature and inducer concentration on the production of trastuzumab and chimeric anti-vascular endothelial growth factor (VEGF) in the cytoplasm of *E.coli* Shuffle T7 express strain. Trastuzumab is a humanized anti-human epidermal growth factor receptor 2 (HER2) mAb used to treat breast cancer. It is sold under the trade name Herceptin by Roche and was the world's eighth-largest-selling pharmaceutical product in 2015 (USD 6,603 million) [9]. While another product from Roche, humanized anti-VEGF or bevacizumab, sold under the trade name Avastin, was the world's seventh-largest-selling pharmaceutical product in 2015 (USD 6,751 million) [9]. Both of these mAbs are currently produced in mammalian cell system. In view of above, the current study might be helpful to understand factors that affect cytoplasmic IgG production in *E. coli*, which can be optimized further for large scale production. In the future, *E. coli* may replace the

current mammalian cell system and can reduce high cost of mAbs such that therapeutic antibodies can be more affordable for patients around the world.

2. Materials and methods

2.1. Bacterial strains and growth conditions
E. coli SHuffle T7 express strain (NEB) was used for all experiments expect for cloning, where DH5α strain was used instead. Typically, SHuffle T7 Express cells transformed with one of the pMAZ360–cIgG expression vectors were grown in Luria–Bertani (LB) medium, supplemented with 100 μg/ml ampicillin and 25 μg/ml spectinomycin, overnight at 37 ℃. The next day, cells were subcultured into fresh LB supplemented with antibiotics and grown at 23, 30 or 37 ℃ until the absorbance at 600 nm reached 0.7 then protein expression was induced by addition of 0.01, 0.1 or 1.0 mM isopropyl b-D-thiogalactopyranoside (IPTG), after which cells were incubated an additional 16 h at indicated temperatures.

2.2. Construction of recombinant plasmid
Chimeric anti-VEGF was created by replacing Fv of anti-HER2 (trastuzumab) with Fv of anti-VEGF antibody. To generate pMAZ360-anti-VEGF (mouse/human chimeric Ab), *E. coli* codon optimized V_H and V_L domains of anti-VEGF murine mAb (muMAb A.4.6.1) [10] were chemically synthesized by GenScript (USA) and cloned into pMAZ360-Herceptin. Briefly, murine anti-VEGF V_L domain was PCR amplified and religated into *Nde*I-*Not*I digested pMAZ360-Herceptin, thus replacing the original anti-HER2 V_L domain creating pMAZ360-anti-VEGF V_L/Herceptin plasmid.   To replace anti-HER2 V_H domain, murine anti-VEGF V_H domain was PCR amplified using primers that appended a 5' RBS sequence with *Hind* III cut site flanking on both ends and religated into *Hind*III treated pMAZ360-anti-VEGF V_L/Herceptin plasmid to obtain pMAZ360 anti-VEGF (mouse/human chimeric Ab). Sequencing analysis confirmed correct sequences and directions of both V_H and V_L.

2.3. Protein analysis
SHuffle cells expressing cyclonals were harvested 16 hours after induction by centrifugation at 5,000 rpm, at 4 ℃ for 10 min.   Cell pellets were resuspended in lysis buffer (1 mg/mL lysozyme, 50 mM Tris-HCl, 1 mM EDTA and 25 mM NaCl) and sonicated for 10 s 3 times at 40% amplitude on a Branson 150 sonifier (Branson Ultrasonics Co., Danbury, CT). The supernatants containing soluble cytoplasmic proteins were recovered as cell lysates containing soluble proteins. SDS-PAGE analysis was performed under non-reducing conditions. Samples were diluted 1:1 in 2 x Laemmli sample buffer without addition of 2-mercaptoethanol and heated at 100 ℃ for 10 min. All samples were normalized by total protein, as determined by the Bradford assay and loaded on 10% Tris-HCl gels. Western blotting was performed as previously described [7]. Herceptin or anti-VEGF (mouse/human chimeric Ab) were probed with 1:15,000-diluted anti-human IgG (H+L)-HRP conjugate (Abcam). The result was obtained using a Bio-Rad ChemiDoc™ MP Imaging System (USA).

2.4. Relative quantification of IgG fragments
The program on Bio-Rad ChemiDoc™ MP Imaging System (USA) was used for densitometric analysis of the protein bands. Solubility of each sample was calculated as total IgG of each sample by

addition of all the bands for each sample. On the other hand, full-length IgG yield was determined by considering only the band representing constructs with 2 heavy and 2 light chains. Relative solubility and relative full-length IgG yield were calculated by comparing data of each sample with the data of sample incubated at 30 ℃ with 1 mM IPTG induction. This condition was used as the reference because it was the condition reported previously [7]. % assembly efficiency was the ratio of full-length IgG to total IgG.

3. Results and discussion

3.1. Humanization of anti-VEGF using variable-domain grafting technique

For clinical use, animal-derived antibodies can cause immunogenicity in patients. With advances in recombinant DNA technology, therapeutic antibodies are now undergone 'humanization', which involves combining the antigen biding portions of a mouse antibody with the constant regions of a human antibody. In the current study, we tried to create a chimeric antibody by grafting murine anti-VEGF variable fragment (Fv) [11] on to the humanized anti-HER2 antibody or trastuzumab

scaffold [7], The result showed that we can create on-demand antibody that can be produced in *E. coli*, thus can reduce the production cost greatly compared to the mammalian cell systems used currently. It is noteworthy that this trastuzumab version contains E382V and M428I mutations, which allow aglycosylated IgG expressed from *E. coli* to bind effector FcγRI thus potentiating the killing of Her2 oeverexpressing tumor cells with dendritic cells as effecotrs [12]. Figure 1 shows the comparison between the amino acid sequences of Fv of anti-HER2 (trastuzumab) and the Fv of murine anti-VEGF named A.4.6.1, which was used to replace the Fv of trastuzumab and expressed as a mouse/human chimeric antibody.
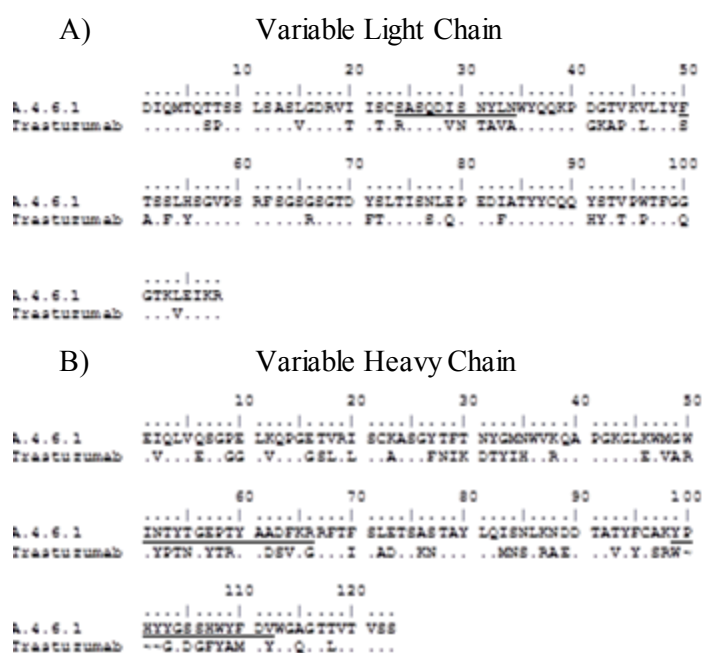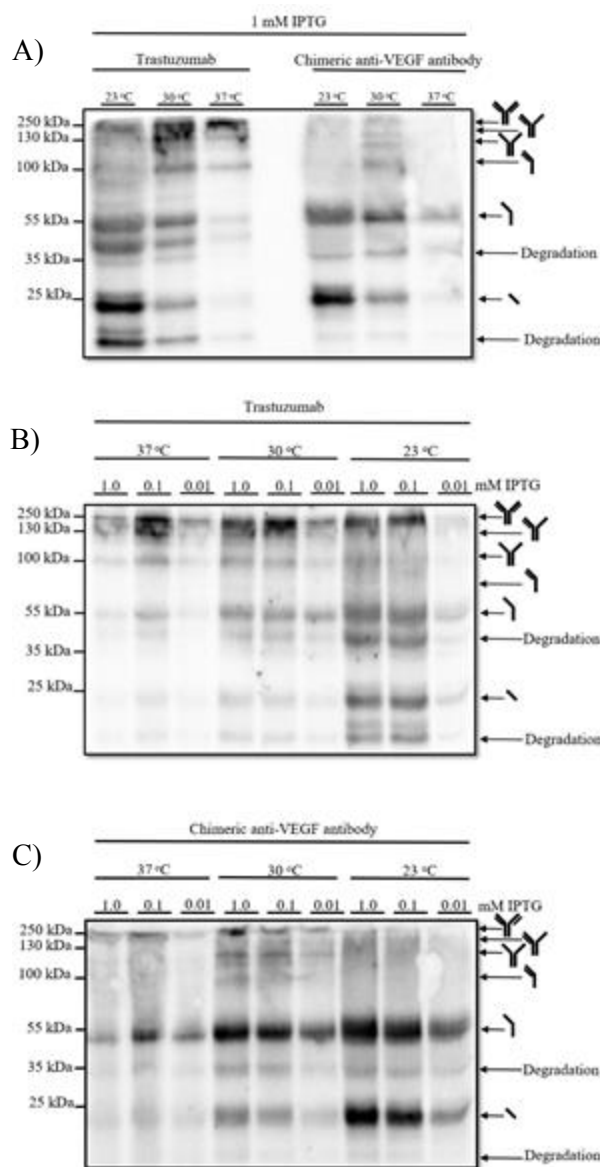

Figure 1. Amino acid sequences of variable light and heavy domains of murine anti-VEGF monoclonal antibody (A.4.6.1) and anti-HER2 scaffold. CDRs are underlined.

3.2. Comparison of cytoplasmic IgG expressions at various temperatures

We first investigated the effect of temperature on the cytoplasmic IgG expression of trastuzumab and the chimeric anti-VEGF using the same condition previously reported for trastuzumab which included induction with 1 mM IPTG for 16 h of protein expression [7]. In addition, the expression was carried out at 23, 30 and 37 ℃. In both cases, it was found that the higher the temperature the less amount of soluble IgG fragments were observed (Figure 2A). It is clear that chimeric anti-VEGF can be

expressed but, unfortunately, the amount of the soluble anti-VEGF is less than the trastuzumab used as scaffold for chimeric antibody grafting. Moreover, full-length IgG assembly was observed in the case of trastuzumab expressed at all temperatures investigated.

Only small amount of full-length anti-VEGF was observed when expressed at 30 ℃ (Figure 2A). This is not surprising because it has been reported that the yield of anti-PA-63, an antibody against anthrax protective antigen, when expressed in the cytoplasm of *E. coli* as heterotetrameric IgG in mFab/hFc format, where the whole Fab is replaced, is about 15 fold higher than of the chimeric format, where only Fv region was replaced (17.0 mg/L vs. 1.1 mg/L) [7]. In order to obtain the highest yield of full-length IgG, it is crucial to balance the expression conditions for high soluble protein expression and high assembly efficiency.

Figure 2. Expressions of trastuzumab and chimeric anti-VEGF antibodies in SHuffle T7 cells. Representative non-reducing western blot of A) both IgGs expressed at 23 °C, 30 °C, and 37 °C with 1 mM IPTG after 16 h of induction. B) Trastuzumab and C) chimeric anti-VEGF antibody with varying amount of inducers in SHufffle cells are shown here.

3.3. Investigation of the effect of temperature and induction level

Temperature and induction level are crucial parameters that can greatly effect a solubility of recombinant proteins expressing in *E. coli* system as they can affect translation rate. Typically, proteins of mammalian origin are expressed as soluble form when the translation rate is low, which could be easily achieved by growing the cells at lower temperature or inducing with lower IPTG levels [13]. Therefore, a full factorial experiment was performed in order to study the interaction effect of both factors. Three temperatures, i.e. 23 , 30 and 37 ℃, and three induction levels, i.e. 0.01, 0.1 and 1 mM IPTG, were investigated. Figure 2B and 2C show the expression of trastuzumab and chimeric anti-VEGF analyzed by immunoblotting. IgGs were detected using polyclonal anti-human antibodies that detect both heavy and light chains such that various types of assembly can be detected. Bio-Rad ChemiDoc[TM] MP Imaging

System was used to quantify the results. It was confirmed that low temperature was better forexpression of the IgGs since, the highest yield of soluble IgG was obtain when cells were grown at 23 ℃. as quantified by band intensity (Figure 3A). Among the various IPTG levels tested, induction with 0.01 mM IPTG induction was too low for all temperatures while 0.1 and 1 mM IPTG seemed to have similar yield of IgG fragments at 23 ℃ and 30 ℃ while at 37 ℃ induction with 1 mM IPTG seemed to be too high resulting in less soluble IgG. The possible explanation might be that at high induction level, the rate of translation is too high resulting in partially folded protein having hydrophobic regions that can interact and form large aggregates. Eventhough the amount of full-length trastuzumab was similar when expressed at 23 ℃ and 30 ℃, the assembly efficiency was better at 30 ℃, which would ease purification process of full-length antibody in industrial scale. Currently, the majority of mAb recovery process employs Protein A chromatography following harvest [14]. Protein A binds strongly to Fc region of the heavy chain. If the assembly efficiency is low, it can result in high amount of unwanted product variant species. Moreover, as induction with 0.1 or 1 mM IPTG gave rise to similar amount of soluble IgG, it would be more economical to use lower IPTG concentration in industrial scale. Similar to trastuzumab, low temperature was better for expression of the chimeric anti-VEGF as more amount of soluble IgG was observed (Figure 3D). However, as % assembly of chimeric anti-VEGF at 23 ℃ was too low, 30 ℃ is the most suitable expression temperature for this IgG. Chimeric anti-VEGF showed highest relative full-lengh IgG yield following induction with 1 mM IPTG (Figure 3F) but the amount is still quite low ; therefore, further optimization maybe required.
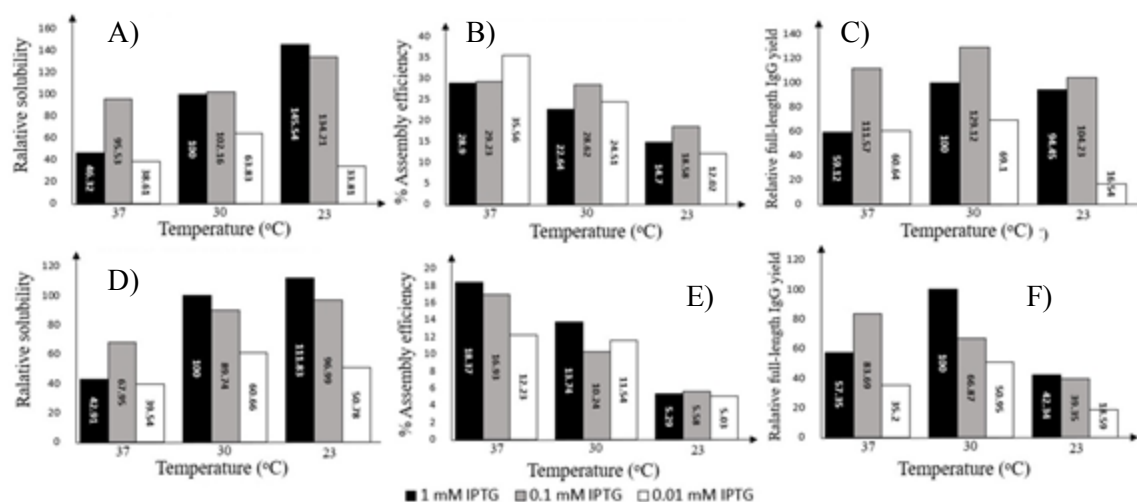
Figure 3. Relative quantification of IgG expressions. Band intensities were quantified using Bio-Rad ChemiDoc^{TM} MP Imaging System to calculate A) and D) relative solubility, B) and E) % assembly efficiency, and C) and F) relative full-length IgG yield for trastuzumab and chimeric anti-VEGF, respectively.

4. Conclusions

A method to produce mAb in the cytoplasm of *E. coli* has emerged in recent years which could revolutionize the industrial production of mAb as it will not only reduce the cost of production but

also allow for mAb engineering as genetic manipulation can be performed easily using *E. coli* as a host. In this study, we investigated the effect of temperature and induction level which affect the translation rate of the IgG. In conclusion, full-factorial experiment showed that expression at 30 ℃ with 0.1 mM IPTG induction was the most suitable for trastuzumab. Expression of chimeric anti-VEGF was also most suitable at 30 ℃, however, higher induction level resulted in more full-length IgG. Since high assembly efficiency of chimeric anti-VEGF was still not achieved, other factors or different grafting methods could be investigated in the future.

Acknowledgment

References

[1]   Nielsen J 2013 Production of biopharmaceutical proteins by yeast: advances through metabolic engineering *Bioengineered* **4** 207-11

[2]   Ecker D M, Jones S D and Levine H L 2015 The therapeutic monoclonal antibody market *mAbs* **7** 9-14

[3]   Li F, Vijayasankaran N, Shen A Y, Kiss R and Amanullah A 2010 Cell culture processes for monoclonal antibody production *mAbs* **2**, 466-79

[4]   Spadiut O, Capone S, Krainer F, Glieder A and Herwig C 2014 Microbials for the production of monoclonal antibodies and antibody fragments *Trends Biotechnol* **32**, 54-60

[5]   Chartrain M and Chu L 2008 Development and production of commercial therapeutic monoclonal antibodies in Mammalian cell expression systems: an overview of the current upstream technologies *Curr Pharm Biotechnol* **9**, 447-67

[6]   Farid S S 2007 Process economics of industrial monoclonal antibody manufacture *J Chromatogr B Analyt Technol Biomed Life Sci* **848**, 8-18

[7]   Robinson M P, Ke N, Lobstein J, Peterson C, Szkodny A, Mansell T J, Tuckey C, Riggs P D, Colussi P A, Noren C J, Taron C H, DeLisa M P and Berkmen M 2015 Efficient expression of full-length antibodies in the cytoplasm of engineered bacteria *Nat Commun* **6**, 8072

[8]   Lobstein J, Emrich C A, Jeans C, Faulkner M, Riggs P and Berkmen M 2012 SHuffle, a novel Escherichia coli protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm *Microb Cell Fact* **11** 56

[9]   Top drugs by sales revenue in 2015: Who sold the biggest blockbuster drugs? March 10, 2016 Retrieved April 18, 2018

[10]   Kim K J, Li B, Houck K, Winer J and Ferrara N 1992 The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies *Growth Factors* **7** 53-64

[11]   Presta L G, Chen H, O'Connor S J, Chisholm V, Meng Y G, Krummen L, Winkler M and Ferrara N 1997 Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders *Cancer Res.* **57** 4593-9

[12]   Jung S T, Reddy S T, Kang T H, Borrok M J, Sandlie I, Tucker P W and Georgiou G 2010 Aglycosylated IgG variants expressed in bacteria that selectively bind FcγRI potentiate tumor cell killing by monocyte-dendritic cells *PNAS* **107** 604-9

[13]   Dyson M R, Shadbolt S P, Vincent K J, Perera R and McCafferty J 2004 Production of soluble mammalian proteins in Escherichia coli: identification of protein features that correlate with successful expression *BMC Biotechnol* **4**:32

[14]   Liu H F, Ma J, Winter C and Bayer R 2010 Recovery and purification process development for monoclonal antibody production *mAbs* **2**:5 480-499