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Optimal expression of a Fab-effector fusion protein in *Escherichia coli* by removing the cysteine residues responsible for an interchain disulfide bond of a Fab molecule

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ABSTRACT

Development of novel bi-functional or even tri-functional Fab-effector fusion proteins would have a great potential in the biomedical sciences. However, the expression of Fab-effector fusion proteins in *Escherichia coli* is problematic especially when a eukaryotic effector moiety is genetically linked to a Fab due to the lack of proper chaperone proteins and an inappropriate physicochemical environment intrinsic to the microbial hosts. We previously reported that a human Fab molecule, referred to as SL335, reactive to human serum albumin has a prolonged *in vivo* serum half-life in rats. We, herein, tested six discrete SL335-human growth hormone (hGH) fusion constructs as a model system to define an optimal Fab-effector fusion format for *E. coli* expression. We found that one variant, referred to as HserG/Lser, outperformed the others in terms of a soluble expression yield and functionality in that HserG/Lser has a functional hGH bioactivity and possesses an serum albumin-binding affinity comparable to SL335. Our results clearly demonstrated that the genetic linkage of an effector domain to the C-terminus of Fd ($V_H + C_{H1}$) and the removal of cysteine (Cys) residues responsible for an interchain disulfide bond (IDB) ina Fab molecule optimize the periplasmic expression of a Fab-effector fusion protein in *E. coli*. We believe that our approach can contribute the development of diverse bi-functional Fab-effector fusion proteins by providing a simple strategy that enables the reliable expression of a functional fusion proteins in *E. coli*.

1. Introduction

Producing functional antibody fragments such as Fab, scFv and Fv in the periplasm of *Escherichia coli* has been a major breakthrough in the field of antibody engineering, owing to its oxidizing environment and the presence of chaperone proteins enabling correct folding of expressed proteins [1,2]. These antibody fragments have been exploited to engineer "customized" therapeutics, with pharmacological properties optimized for specific applications. For example, the conjugation or genetic linkage of exogenous effector moieties such as cellular toxins, cytokines or enzymes to antibody fragments is an important approach where the antibody fragments provide means to deliver the functionality of therapeutic proteins more precisely and effectively to the desired targets [3–9].

Fab molecules are particularly attractive for therapeutic usage among antibody fragments, because of their excellent thermostability and the proven clinical safety in humans. Furthermore, unlike scFv, Fv or dsFv, Fab molecules can easily be produced up to 1–2 g/L as a soluble form in the *E. coli* periplasm [10–13], or even in *Pseudomonas fluorescens* [14]. In reality, however, reliable expression of soluble antibody fragments including Fab in *E. coli* is rather difficult and unpredictable. In addition, the references exploiting Fab-effector fusions are rare, especially when eukaryotic effector proteins are genetically linked to a Fab molecule, implying that the soluble expression of Fab-effector fusions in *E. coli* is more problematic. This is presumably due to the fact that the *E. Coli* host system

Abbreviation: Fab, fragment of antigen binding; IDS, interchain disulfide bond; hGH, human growth hormone; IPTG, isopropyl-β-D-1-thiogalactopyranoside; mAb, monoclonal antibody; pAb, polyclonal antibody.

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suffers from the lack of proper chaperone proteins and an inappropriate physicochemical environment for the efficient production of recombinant proteins of eukaryotic origin [15].

The Fc from IgG antibodies, along with albumin and albumin-binding moieties such as small peptides or domain antibodies (dAb), have been actively utilized to extend serum half-lives of therapeutic proteins throughthe neonatal Fc receptor (FcRn)-mediated recycling mechanism [16–19]. However, human anti-serum albumin Fab antibodies have not been applied for the development of long-acting protein therapeutics, perhaps because of the perception that the expression of a recombinant Fab-effector fusion protein in *E. coli* is suboptimal for industrial applications. We have previously reported the isolation of human anti-serum albumin Fab antibodies where one of them, referred to as SL335, displayed a prolonged serum half-life in rats [20]. It is reasonable to assume that an anti-serum albumin Fab will have unique advantages over the long-acting fusion technologies described above, owing to the thermostable nature of a Fab molecule as well as its feasibility in the microbial expression system. As long as the expression yield of SL335-effector fusion protein is satisfactory, one can assume that human growth hormone (hGH) would be an exemplary candidate as an effector fusion molecule because it is widely used to treat diseases linked to growth hormone deficiency in children and adults [21], yet its short half-life (>30 min) currently necessitate the development of a long-acting version. Since our initial trial to produce soluble SL335-hGH fusion protein in *E. coli* using a conventional method had not been quite satisfactory, we, herein, attempted to define an optimal SL335-hGH fusion format for the periplasmic expression in *E. coli* as a model Fab-fusion effector.

2. Materials and methods

2.1. Molecular cloning

DNA cloning experiments were performed according to the standard procedures [22]. Primers for polymerase chain reaction (PCR), codon-optimized SL335 Fd ($V_H + C_{H1}$), L ($V_L + C_L$) and hGH (27–191aa) genes were synthesized from Bioneer, Daejeon, South Korea. PCR reactions were carried out with Pyrobest or Ex-*Taq* DNA polymerase (Takara, Ōtsu, Japan). Reaction conditions were 25 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 10 min unless otherwise noted. Restriction endonucleases, shrimp alkaline phosphatase (SIP) and T4 DNA ligase were also purchased from Takara. *E. coli* MC1061 strain [*ara*D139 Del(*ara*A-*leu*)7697 Del(*lac*)X74 *gal*K16 *gal*E15(GalS) lambda- e14- *mcr*A0 *rel*A1 *rps*L150(*str*R) *spo*T1 *mcr*B1 *hsd*R2] was used for cloning, and *E. coli* SUPEX5 strain (AprilBio, Chuncheon, South Korea) was used as host cell to express SL335 and SL335-hGH fusion proteins. The SUPEX5 strain is a derivative of MC1061 strain generated by chemical mutagenesis that shows improved periplasmic expression of soluble antibody fragments (unpublished data). pHEKA (AprilBio, Chuncheon, South Korea), a derivative of pTrcHis (Life Technologies, CA, USA), which enables monocistronic expression of a Fab, was used as a periplasmic expression vector for SL335 and SL335-hGH fusion proteins [20].

2.2. Generation of SL335-hGH fusion constructs

To create $SL335_{\Delta ds}$, Hser (the Fd with $Cys^{233} \rightarrow Ser^{233}$substitution) was obtained by PCR amplification from the codon-optimized wild typeSL335 ($SL335_{wt}$) Fd chain gene (Hcys, the Fd with Cys^{233}) using PCR primers #1 and #2. The resulting ~750 bp PCR product was treated with *Eco*R I/*Hind* III and ligated with pHEKA. Lser (the L chain with $Cys^{214} \rightarrow Ser^{214}$substitution) was also obtained from the codon-optimized $SL335_{wt}$ L chain gene (Lcys, the L chain with Cys^{214}) by PCR amplification using PCR primers #3 and #4. Product was cut with *Bam*H I/*Xho* I and cloned into pHEKA containing Hser.

Cloning procedures for generating six fusion constructs (HcysG/Lcys, Hcys/LcysG, HserG/Lcys, Hcys/LserG, HserG/Lser and Hser/LserG) were as follows. 1) HcysG/Lcys: Hcys was PCR amplified from the SL335 Fd gene using PCR primer #1 and #5, and hGH cDNA containing a short peptide linker sequence was also PCR amplified from the codon-optimized hGH gene corresponding to 27–191 aa using PCR primers #6 and #7. Hcys and hGH genes were linked together to generate HcysG by assembly PCR using primers #1 and #7,cut with *Eco*R I/*Hind* III and subsequently cloned into pHEKA containing Lcys. 2) Hcys/LcysG construct: Lcys was PCR amplified from the SL335 L chain gene using primers #3 and #8. hGH cDNA containing a short peptide linker sequence was also PCR amplified from the hGH gene using primers #6 and #9. Lcys and hGH genes were linked to generate LcysG by assembly PCR using primers #2 and #9. Resulting product was cut with *Bam*H I/*Xho* I and cloned into pHEKA containing Hcys. 3) HserG/Lcys: HserG was PCR amplified using primers #1 and #10, and cloned into pHEKA containing Lcys. 4) Hcys/LserG: LserG was PCR amplified using primers #3 and #11, and cloned into pHEKA containing Hcys. 5) HserG/Lser: HserG was obtained by PCR using primers #1 and #10, and cloned into pHEKA containing Lser. 6) Hser/LserG: LserG was PCR amplified using primers #3 and #11, and cloned into pHEKA containing Hser.

2.3. Preparation of soluble Fab fragments and SL335-hGH fusion proteins

Soluble SL335 Fab fragments and SL335-hGH fusion proteins were produced by culturing *E. coli* MC1061 or SUPEX5 host cells in 10 ml or 1 l of 2 × YT medium containing 50 μg/ml kanamycin at 37 °C until an OD_{600nm} = 0.5 was reached, at which point 0.08 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, MI, USA) was added to the culture media. After 20 h of incubation at 20 °C, the culture supernatant and cell pellet were separated by centrifugation at 3300 × *g* for 20 min. Periplasmic extract was obtained from the cell pellet as described [20,23]. Purification of SL335 and SL335-hGH fusion proteins was carried out by affinity purification, followed by gel filtration. Briefly, CNBr-Sepharose 4 B resins (GE Healthcare Life Sciences, PA, USA) were conjugated with recombinant human serum albumin (HSA) (Sigma-Aldrich, MI, USA), and the *E. coli* culture supernatant and periplasmic extracts were mixed with the resin. After extensive washing of resins with phosphate-buffered saline (PBS), the bound SL335or SL335-hGH fusion molecules were eluted with elution buffer (0.1 M glycine, 10% glycerol, pH 3), followed by immediate neutralization using Tris buffer (0.5 M Tris HCl, 2 M NaCl, pH 9. 0). Gel filtration of HserG/Lser was also performed after affinity purification using AKTA FPLC (GE Healthcare Life Sciences, PA, USA). Briefly, Hiprep™ 16/60 Sephacryl ™ S-200 HR Prepacked Column (GE Healthcare Life Sciences, PA, USA)was equilibrated with equilibration buffer (20 mM HEPES, 150 mM NaCl, pH 7.4), and loaded with 5 ml of affinity-purified protein samples. Elution was performed with equilibration buffer at 0.35 Mpa alarm pressure and 0.5 ml/min running flow rate.

2.4. Differential scanning calorimeter (DSC) analysis

Heat stability analysis of purified $SL335_{wt}$ and $SL335_{\Delta ds}$ was carried out to determine protein-melting temperature (Tm) using Nano DSC (TA Instruments, DE, USA). Temperature increment step was 35 °C to 100 °C at 1 °C/min with 600 s of equilibration time, and 1 mg/ml of the sample proteins in the pre-formulation