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CO-EXPRESSION OF RECOMBINANT SINGLE CHAIN VARIABLE FRAGMENT RECOGNIZING BLOOD ANTIGEN FUSED WITH SUMO AND CHAPERONES IN *Escherichia coli*

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ABSRACT

Single chain variable fragments (scFv) have widely been used in research, diagnosis and treatment, but the scFv is considered as difficult protein for expression in *Escherichia coli* (*E. coli*). In previous studies, we expressed a construction of recombinant single chain variable fragments again antigen specific for blood type A (antiA-scFv) individually or fused with Trx or SUMO. However, soluble fraction was low abandant and only approximately 40% when fused with Trx, the other cases were expressed in form of inclusion body. Therefore, it was difficult for purification, refolding and activity assessment. In this paper, we demonstrated a suitable construction for soluble production of antiA-scFv fused with SUMO (SUMO/antiA-scFv) in presence of chaparones. Under fermentation with 0.1 mM IPTG at 20^{o}C, the SUMO/antiA-scFv was entirely expressed in soluble form. Importantly, after cleavage from SUMO with SUMO protease, antiA-scFv was still maintained in the supernatant fraction. Therefore, it can help ensure bioactivity and is useful for purification process. To the best of our knowledge, this is the first report showing soluble recombinant scFv fused with SUMO in presence of chaperone for determination of blood group antigens. Thus, this result facilitates the optimal study of soluble expression, purification and bioactivity determination of the antiA-scFv recombinant antibody.

Keywords: *Escherichia coli*, antiA-scFv, chaperones, co-expression, soluble protein, SUMO.

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INTRODUCTION

Escherichia coli expression system is the most host of choice for producing heterologous protein because of high recombinant product level (Ni & Chen, 2009; Schmidt, 2004; Spadiut et al., 2014; Terpe, 2006). Besides, genetic properties of *E. coli* have been well known. However, *E. coli* has some disadvantages, such as limitation of the recombinant protein secretion and formation of inclusion body leading to lost biological activity.

For expression of antibody fragments, protein is produced in oxidized periplasmic space for correct disulfide bond formation (Skerra & Plückthun, 1988). Synthesis of the protein in reduced environment usually causes protein aggregate without activity (Wörn et al., 2000). Refolding of antibody fragment from the inclusion body form is generally ineffective. Therefore, mutation of genes encoding glutathione and thioredoxin reductase in host strains, co-expression of chaperones such as GroEL/ES, DnaK/J, DsbC, Skp, GroES/L as well as other proteins have been investigated to enhance production of active recombinant proteins (Bothmann & Plückthun, 2000; de Marco, 2009; Friedrich et al., 2010; Golchin et al., 2012; Sonoda et al., 2011; Yuan et al., 2013).

SUMO based protein expression system produces high level of soluble recombinant protein in *E. coli*, yeast, mammal. When fused with SUMO, soluble accumulation of heterologous proteins are significantly enhanced (Butt et al., 2005, Marblestone et al. 2006, Panavas et al., 2009). In addition, SUMO fused system is also more priority because enzyme SUMO protease has ability recognizing tertiary structure of SUMO and cleave generation of the recombinant protein with the desired N-terminus without addition of amino acid residues. Some findings showing that SUMO fused scFv for VEGF (Ye et al., 2008) and FGFR3 (Liu et al., 2015) was expressed in soluble form with bioactivity.

In this study, we showed that the single chain variable fragment recognizing blood antigen (antiA-scFv) when fused with SUMO in present of chaperone in *E. coli* was efficiently expressed. The recombinant protein was almost soluble product.

MATERIALS AND METHODS

E. coli DH10b (Invitrogen, USA) was used for cloning genes. *E. coli* JM109, BL21 (DE3) and Rosseta 2 (Invitrogen, USA) were used for gene expression. Plasmid pET22b+/antiA-scFv (GEL, IBT) was used as template for amplifying *antiA-scFv*. Plasmid pSUMOpro3 for gene expression in *E. coli* was purchased from LifeSensors, USA. Other chemicals, enzymes, antibodies were used in this study including: monoclonal antibody against C-myc produced from mouse and anti-mouse IgG-peroxidase secondary antibody (Sigma, USA), APS, TEMED, Chloroform, Ethidium brobmide, Glucose, Glycerol, Glycine, Isoamyl-alcohol, Ethanol, Methanol, Peptone, Yeast Extract, SDS, Tris, Acrylamid, Bis Acrylamide, Agar, Agarose, Coomassie (Merck, Germany), KIT DNA GFX^{TM} (code 28-9034-70, GE Healthcare Life Science, Englands), dNTP, Taq DNA polymerase, Dnase I, T4 DNA-ligase, restriction enzymes (Fermentas, USA), skim milk (Difco, USA), Ampiciline, TMB (Sigma, USA).

Methods

Amplification of antiA-scFv gene from pET22b+/antiA-scFv

Gene antiA-scFv was amplified from pET22b+/antiA-scFv by PCR with following components: 18µl dH_2O, 2.5µl buffer 10X, 2.5µl dNTP 2 mM, 0.5µl F - BsaI 10 µM (5'-TAGGTCTCTAGGTCAGGTCCAAGTGCA GC-3'), 0.5µl R - XbaI 10 µM (5'-TGTCTAGATTACAGGTCTTCTTCGC-3'), 0.5µl pET22b+/antiA-scFv, 0.5µl Taq polymerase.

PCR programmes: initial denaturation at 95°C for 3 mins; 30 cycles of 3 steps: denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 60 sec; final extension at 72°C for 10 mins.

Construction of expression vector pSUMO/antiA-scFv

Amplified *antiA-scFv* gene was digested with two restriction enzymes *Bsa*I and *Xba*I. Besides, vector pSUMOpro3 was also cleaved by *Bsa*I. The gene fragment and vector products were purified using DNA Extraction Kit. After that, the gene and vector fragments were ligated using enzyme T_4 ligase to creat recombinant expression vector pSUMO/antiA-scFv. Subsequently, the ligate solution was transformed into *E. coli* DH10b by heat shock method. The positive colonies were selected on LB plates supplemented with 100 µg/ml ampicillin (LBA) (Sambrook & W Russell 2001). Plasmids isolated from selected transformants were checked for harboring insert of antiA-scFv gene using restriction enzyme *Sac*I. Finally, the constructed expression vector pSUMO/antiA-scFv was transformed into expression strains.

Expression of antiA-scFv

E. coli expression strains of BL21 (DE3), JM109 and Rosetta harboring expression vector pSUMO/antiA-scFv were inoculated into LBAmp with shaking 200 rpm at 37°C overnight. After that, the overnight culture was inoculated into fresh LBA medium at OD about 0.1 and continually incubated at 37°C with shaking 200 rpm to reach OD about 0.3 – 0.5. The culture was induced with 0.1 mM isopropyl β- D- thiogalactopyranoside (IPTG) (Studier et al., 1990) and fermented at 20^{o}C with shaking 200 rpm for 16 hours. After fermentation, the cells were harvested by centrifugation at 5000 rpm for 5 mins and resuspended in buffer 20 mM Tris-HCl, pH = 8 to final OD_{600} = 10.

Co-expression of chaperones and antiA-scFv in E. coli

The *E. coli* recombinant strain harboring vectors expressing chaperone pG-KJE8 and antiA-scFv gene (pSUMO/antiA-scFv) was inoculated into LB medium with 100 µg/ml Amp and 20 µg/ml Cm (Chloramphenicol) with shaking at 200 rpm, 37°C for overnight. The overnight culture was inoculated into fresh LB medium (or PE) with Amp and Cm added chaperone-inducers including 0.5 mg/ml L-arabinose and 5 µg/ml Tetracyline at OD = 0.1. The preculture was incubated at 26°C with shaking at 200 rpm until OD_{600} about 0.3–0.5. The culture was induced with 0.1 mM IPTG and fermented at 20°C with shaking 200 rpm for 16 hours. After fermentation, the cells were harvested by centrifugation of 5000 rpm for 5 mins and resuspended in buffer 20 mM Tris-HCl, pH = 8 to final OD_{600} = 10.

Extraction of recombinant protein from *E. coli*

The recombinant cells harvested from fermentation culture were resuspended in buffer 20 mM Tris HCl, pH=8 to an OD of 10. The cells were disrupted by sonication with Amplitude for 10 mins. After sonication, total soluble proteins were separated from pellet by centrifugation at 8000 *g* at 4°C for 15 mins. The pellet was resuspended in equivalent volume in 20 mM Tris HCl, pH=8 buffer. Proteins from soluble and insoluble fractions were checked by SDS-PAGE 12.6% (Laemmli 1970) .

Cleavage of SUMO/antiA-scFv with SUMO protease

One ml of the total soluble proteins was added with 5µl of enzyme SUMO protease 0.76 mg/ml (provided by Genetic engineering lab). The reaction was carried out in 20 mM Tris HCl, pH = 8 buffer, added with 2 mM DTT and incubated at 30°C for 3 hours.

Assessment of protein expression by SDS-PAGE and Western blot

SDS-PAGE (Laemmli 1970) and Western blot was carried out as described in Dang et al (Dang et al., 2017). Briefly, recombinant protein SUMO/antiA-scFv was detected by Western blotting using monoclonal antibody against C-myc. After SDS-PAGE, proteins were transferred from gel to PVDF membrane. Subsequently, the blot was incubated with blocking buffer, then antibody against C-myc, afterward antimouse IgG-peroxidase. Finally, the detection reaction was carried out in the TMB solution.

RESULTS AND DISCUSSION

In the previous paper, we reported the findings of expression of antiA-scFv in the construction with vector pET22b(+) in *E. coli* (Dang et al., 2017). Protein antiA-scFv was expressed in form of inclusion body. The antiA-scFv was purified in denaturation condition and refolding in some buffers. However, the refolded protein showed lost biological activity for red blood cell aggregation. Besides, the protein was also expressed with signal peptide pelB/antiA-scFv for secretion at periplasm, but the recombinant protein was produced at low abundant and insoluble (data not shown). Therefore, we shifted to express the recombinant protein fused with some proteins such as TRX, SUMO to enhance production of soluble recombinant protein. When fused with TRX, soluble fraction was only about 40% of total expressed recombinant protein (data submitted in Academia Journal of Biology). Following, we presented result on construction and expression of antiA-scFv fused with SUMO (SUMO/antiA-scFv) in presence of chaparones. SUMO/antiA-scFv was almost produced in soluble form.

Construction of expression vector pSUMO/antiA-scFv

An *antiA-scFv* gene was amplified from plasmid pET22b+/antiA-scFv using primer pairs F- *Bsa*I and R-*Xba*I. PCR product was a clear single DNA fragment of 900 bps as expected size of *antiA-scFv* gene (Fig. 1a).

In order to construct an expression vector pSUMO/antiA-scFv, firstly the PCR product was double digested with *Xba*I and *Bsa*I and the vector was treated with *Bsa*I to generate two compatible ends. Then, the cleavage products were purified and checked on agarose gel (Fig. 1b) afterward ligated together by T4 DNA ligase to generate pSUMO/antiA-scFv.

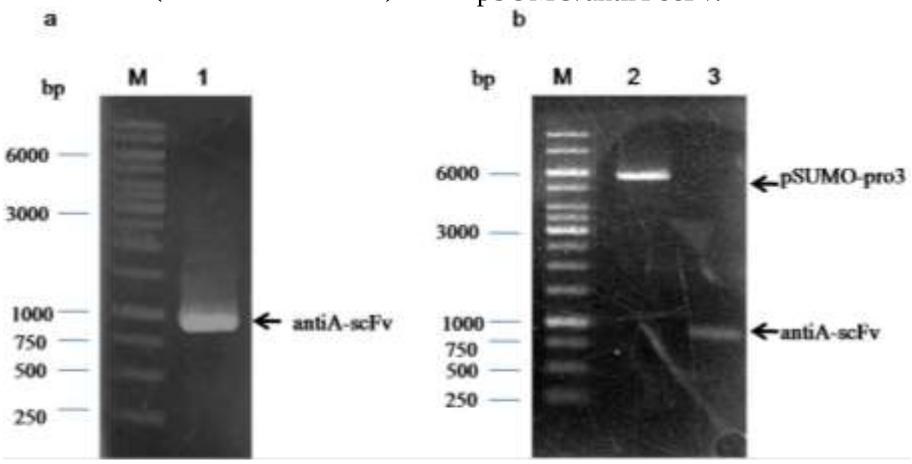

Figure 1. Assessment of antiA-scFv gene and vector pSUMOpro3

(a) PCR product amplifying antiA-scFv. (b) Cleavage products of antiA-scFv and vector pSUMOpro3 using restriction enzyme XbaI and BsaI. Lane M: DNA marker 1 kb (Fermentas). Lane 1: PCR product of antiA-scFv. Lane 2: Cleavage product of vector pSUMOpro3 using BsaI. Lane 3: Cleavage product of antiA-scFv using XbaI and BsaI

Some transformants from ligation of pSUMOpro3 and antiA-scFv gene were selected to extract plasmids. The plasmids from transformants were higher than control plasmid of pSUMOpro3 (Fig. 2a). Result checking the plasmid using *Sac*I showed that the recombinant plasmid harboring *antiA-scFv* generated two DNA bands. Theoretically, enzyme *Sac*I has a sequence for recognizing in vector pSUMOpro3 and a site in *antiA-scFv* gene, therefore when the recombinant plasmid treated with *Sac*I to create the two bands of 400 bps and 6300 bps (Fig. 2b). Thus, we inserted *antiA-scFv* gene fragment into pSUMOpro3 (called pSUMO/antiA-scFv).

Expression of fusion protein SUMO/antiA-scFv

pSUMO/antiA-scFv was transformed into *E. coli* strains including BL21, JM109, Origami, Rosseta 1, Rosseta 2 and Soluble.

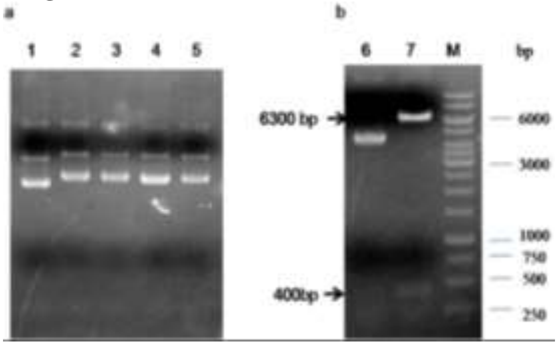

Figure 2. Creation of recombinant plasmid pSUMO/antiA-scFv

(a) Plasmids extracted from transformants of pSUMO/antiA-scFv. (b) Cleavage product of pSUMO/antiA-scFv using *Sac*I. Lane M: DNA marker 1 kb (Fermentas). Lane 1: vector pSUMOpro3. Lane 2, 3, 4, 5: plasmids from transformants pSUMO/antiA-scFv. Lane 6, 7: plasmid pSUMO/antiA-scFv before and after treated with *Sac*I

Recombinant strains were induced at OD = 0.6–0.8 with IPTG of 0.5 mM, incubation at 30°C for 6 hours. Harvesting OD was not significantly different between the strains. In which, Rosseta 1, Rosseta 2 and JM109 well produced the recombinant protein with molecular weight of approximately 47 kDa higher than those produced by Origami strain. In contrast, Soluble strain had no visible band of SUMO/antiA-scFv. Moreover, the assessment of soluble recombinant protein showed that SUMO/antiA-scFv was produced inclusion body. Besides, the strains were also tested by fermentation in different media at lower temperatures (16°C and 20°C) and lower concentrations of IPTG. However, SUMO/antiA-scFv was still produced insoluble form (Fig. 3). Therefore, we decided to co-expression with chaperones.

Co-expression of SUMO/antiA-scFv and chaparone

Recombinant proteins synthesized in *E. coli* were usually formed inclusion body due to process of folding correct structure as native protein. Accordingly, this is resulted the lost of biological activity. Although, we designed the construct in which the target gene was fused with a factor for enhancing solubility of recombinant protein such as SUMO. However, the enhancer was not always effect for every case. Thus, the requirement for optimum expression is to find the increasing amount of soluble target protein. In some situations, presence of some chaperones such as GroEL-GroES and DnaK-DnaJ-GrpE had facilitated the precise folding and reduced inclusion body (Wang et al. 2013, Young et al. 2004).

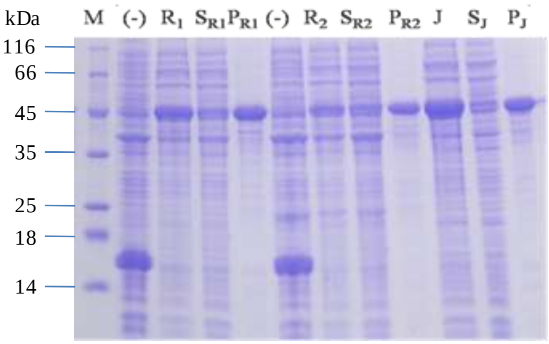

Figure 3. Evaluation of SUMO/antiA-scFv expression in recombinant strains at 16°C

R1. Rosseta 1, R2. Rosseta 2, J. JM109, M. protein marker, S, P. soluble and pellet fraction, respectively

We used plasmid pGKJE8 that can produce chaperones GroEL- GroES and DnaK-DnaJ-GrpE to co-express with SUMO/antiA-scFv in *E. coli* JM109. Protein chaperones were synthesized with estimated weight of 60, 10, 70, 40 and 22 kDa for GroEL, GroES, DnaK, DnaJ and GrpE, respectively (Fig. 4). In presence of the chaperones, SUMO/antiA-scFv was produced with molecular weight of 47 kDa. Especially, recombinant SUMO/antiA-scFv almost existed in a soluble form. Remarkably, after cleavage from SUMO, antiA-scFv with the weight of 33 kDa was still in soluble fraction (Figs 4 b, c). Thus, it was useful for recombinant protein purification. In contrast, absence of the chaperone, even at low temperature, SUMO/antiA-scFv was completely produced as inclusion body (Fig. 3). In addition, we also emphasized that SUMO also supported to the soluble recombinant protein. Because co-expression of these chaperones and individual antiA-scFv produced insoluble form (date not shown). Thus, this was demonstrated that chaperone molecular primarily contributed to structural formation during the folding of SUMO/antiA-scFv. In agreement, some findings showed that co-expression of chaperone enhances production of scFv recognizing TLH from a bacterium causing digestive diseases in human (Wang et al. 2013) and scFv for BNP protein (Maeng et al., 2011).

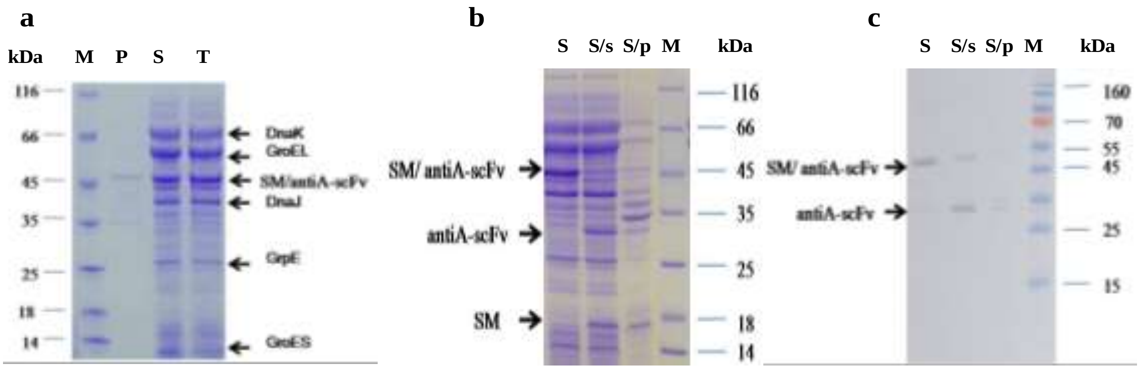

Figure 4. Analysis of SUMO/antiA-scFv co-expressed with chaperones
(a) Evaluation of soluble and insoluble of SUMO/antiA-scFv, (b) Cleavage of SUMO/antiA-scFv with SUMO protease, (c) Western blot. Lane M: Protein marker (Fermentas). Lane P, S, T: pellet, soluble, total fraction, respectively. Lane S/s, S/p: Soluble and pellet fractions of protein after treated with SUMO protease

CONCLUSION

Our data demonstrate that the expression of the antiA-scFv fused with SUMO in presence of chaperones was greatly increased soluble production. Thus, the results are useful basis for antiA-scFv purification process in *E. coli* for determination of blood types.

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