Comparison of Gene Expression between Two Types of Anti-EGFRvIII ScFv Antibodies Having Different Variable Domain Orders In *Escherichia coli*

Kartika Sari Dewi* and Asrul Muhamad Fuad

Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Jalan Raya Bogor Km 46, 16911, Cibinong, Bogor, Indonesia.

Abstract

Several studies reported that the expression of various kinds of single-chain variable fragment (scFv) antibodies in *Escherichia coli* are significantly influenced by the order of their variable domains. To date, the effect of the order of variable domains in the expression of scFv antibodies against epidermal growth factor receptor variant III (EGFRvIII) has not been reported. This study aimed to compare the expression between V_H-linker-V_L and V_L-linker-V_H domain orders of the anti-EGFRvIII scFv antibodies in *E. coli* expression system. Recombinant plasmids inserted with DNA encoding scFv proteins were transformed into *E. coli* NiCo21 (DE3) competent cells and characterized by colony PCR. The expression of scFv proteins was done by using optimum concentration of inducer. Total proteins, soluble periplasmic and cytoplasmic proteins, also extracellular proteins were isolated, subsequently characterized by SDS-PAGE, Slot Blot, and ImageJ software analyses. The antigen-binding activity of both scFvs proteins against EGFRvIII was observed. The results showed that the relative percentage of scFv expression with V_H-linker-V_L domain order is higher than that of V_L-linker-V_H in each compartment. Moreover, both of scFvs proteins have antigen-binding activity against EGFRvIII.

Keywords: EGFRvIII, scFv, variable domain order, antibody, *Escherichia coli*

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*Corresponding author:
Cibinong Science Center, Jl. Raya Bogor Km. 46, Cibinong 16911, Indonesia
Tel. +62-21-8754587, Fax. +62-21-87754588
E-mail: kart008@lipi.go.id

Introduction

The type III epidermal growth factor receptor (EGFRvIII) is a mutant variant of EGFR that has in-frame deletion of 2-7 exons in mRNA, resulting in the deletion of amino acids 6-273 in the extracellular domain and the formation of a new Glycine in the junction of exon 1 and 8 (Wikstrand *et al*., 1998). EGFRvIII is not found in normal cells, but is the most common mutation observed in a number of human carcinomas, including gliomas, ovarian carcinomas, non-small-cell lung carcinomas, prostate carcinomas, and breast carcinomas (Gan *et al*., 2013). This biological characteristic of EGFRvIII makes it a highly promising therapeutic target for immunology-based cancer therapy (Greenall & Johns, 2016). Furthermore, single-chain variable fragment (scFv) antibodies are powerful tools for such targeted therapy.

ScFv is a minimized antibody consisting of heavy-chain (VH) and light-chain (VL) variable domains that are connected by a short peptide linker to stabilize the molecule. It has better pharmacokinetic properties comparing to its parental monoclonal antibody including better tumor penetration, lower retention time in non-target tissue, and lower immunogenicity. ScFv represents the smallest functional V_H-V_L of an antibody which is necessary for high-affinity binding of antigen. Because of its small size (~30 kDa), scFv can be easily expressed in a functional form in the bacterial system, offering the protein engineering to improve their properties such as increase of antigen-binding activity and specificity (Ahmad *et al*., 2012). However, scFv is known to have two disulfide bonds which contribute to their antigen-binding activity and stability.

Good expression levels for scFv in *E. coli* expression system can be achieved via soluble

production, secreting scFv into the oxidizing environment of the *E. coli* periplasm (Popplewell *et al*., 2005). In order to reach the periplasm, scFv must be fused with a signal peptide. Kipriyanov *et al*., (1997) reported that PelB signal peptide from *Erwinia carotovora* could lead the scFv proteins into the periplasmic space of *E. coli*. Therefore, in the previous study we fused PelB signal peptide onto N-terminal of the anti-EGFRvIII scFv antibody fragments either the V_H-linker-V_L (scFv) or V_L-linker-V_H domain orders (rscFv) (Dewi *et al*., 2015; Dewi *et al*., 2016).

Several studies reported that the expression of scFv proteins is dependent on the order of their variable domain (Luo *et al*., 1995; Kim *et al*., 2008). However, the result does not always correspond to their antigen-binding activity (Desplancq, 1994). To date, no study has been reported regarding the difference between the expression of V_H-linker-V_L and V_L-linker-V_H domain orders of the anti-EGFRvIII scFv antibodies in *E. coli*. Thus, in this study we expressed the two types of anti-EGFRvIII scFv antibodies having different variable domain orders in *E. coli* NiCo21 (DE3) and analyzed the soluble proteins in each compartment. Besides, we also tested their antigen-binding activity against a recombinant EGFRvIII antigen that is conjugated with blue fluorescent proteins (BFP).

Materials and Methods

Materials and Microorganisms.

Recombinant plasmids pJ414_scFv and transformants of *E.coli* NiCo21(DE3) harboring pJ414_rscFv plasmids were obtained from previous research (Dewi *et al*., 2015; Dewi *et al*., 2016). *E. coli* NiCo21(DE3) host cells and EGFRvIII::BFP proteins were available in our laboratory. The primers used for PCR colony analysis were: scFv-F primer (5'-GCCATGGCTCAAGTTCAATTGGTTG-AGTCAGG-3'), scFv-R primer (5'-CGCCAT-GGCTCGAGTGATTAACAATGATGATGG-TGG-3'). All primers were purchased from Integrated DNA Technologies (IDT, USA).

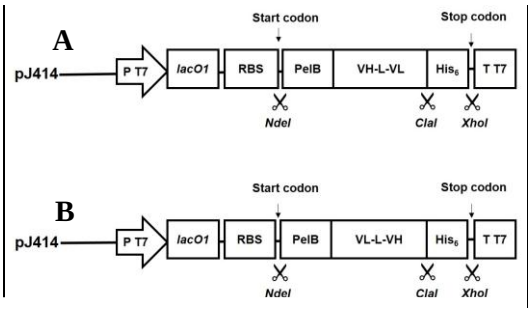


Figure 1. The expression cassettes of: **(A)** pJ414_scFv and **(B)** pJ414_rscFv.

Transformation of pJ414_scFv into *E. coli* NiCo21(DE3).

About 50 ng pJ414_scFv plasmids were transformed into 200 µL of *E. coli* NiCo21-(DE3) competent cells using one-step transformation and stock solution (TSS) method (Chung *et al*., 1989). After that, eight colonies of *E. coli* transformants were picked and cultured in Luria Bertani medium containing 100 µg/mL ampicillin (LBamp). These cultures were used as the template for colony PCR characterization.

Colony PCR was performed as follows: initial denaturation at 95°C for 1 m; 30 cycles of denaturation at 95°C for 1 m, annealing at 60°C for 30 s and extension at 72°C for 1 m, then final extension at 72°C for 5 m. The amplified fragments were then analyzed using 1% agarose gel electrophoresis.

Screening and IPTG Optimization of scFv Proteins Expression.

Every positive colony from colony PCR characterization was grown overnight in LBamp medium at 25°C. This overnight culture was diluted 1:50 with LBamp medium and grown at 25°C. After the cell concentration had reached OD_{600} = 0.8, IPTG was added and growth was continued at 25°C for 16-18 h. Screening of scFv expression was done by adding 1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG). Optimization of IPTG concentration was carried out by varying IPTG concentration from 0.1 to 1 mM.

Isolation of scFv and rscFv Proteins.

As much as 100 mL culture of scFv and rscFv proteins were prepared by the method described above, subsequently induced by optimized IPTG concentration. Pelleted cells from 100 mL culture were suspended in 5 mL

of hypertonic solution (20% sucrose, 30 mM Tris-Cl, 1 mM EDTA) and incubated on ice with gentle agitation for 1 h (Kipriyanov *et al*., 1997). After that, the cells were centrifuged at 10000 x *g* and 4°C for 15 m, leaving the supernatant as soluble periplasmic proteins. The remaining pellet was then resuspended in lysis buffer (50 mM Tris-Cl, 3 mM EDTA, 1 mM PMSF) and imposed to five freeze-thaw cycles by freezing at -20°C and thawing at room temperature. Thawed suspension was centrifuged at 10000 x *g* and 4°C for 15 m. Supernatant was collected as soluble cytoplasmic proteins (Johnson and Hecht, 1994).

Sample Preparation.

A sample of total proteins was prepared by suspending the pellet cells from 1 mL culture in 100 µL sample buffer and 100 µL denaturing lysis buffer (8 M urea, 10 mM Tris-Cl, 100 mM NaH_2PO_4) and then boiled for 15 m. About 5 µL of total proteins sample was used for analysis.

As much as 50 µL of soluble periplasmic proteins and also 900 µL of cell-free culture medium were precipitated using trichloroacetic acid (TCA) precipitation method (Link and Labaer, 2011). Then, the precipitated proteins were resuspended using 10 µL denaturing lysis buffer and 10 µL sample buffer. For soluble cytoplasmic proteins, about 20 µL sample was used for analysis.

SDS-PAGE Analysis.

The SDS-PAGE analysis was performed using 13% acrylamide gel. The proteins were separated in the electro-running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS) using Bio-Rad Mini-PROTEAN® at 90 V for 100 m. After that, the gel was immersed in Coomassie blue staining. The presences of the scFv and rscFv proteins in all compartments were analyzed using ImageJ software (Schneider *et al*., 2012).

Slot-Blot Analysis.

Nitrocellulose membrane and Whatman paper were soaked in Tris-buffered saline (TBS) pH 7.6 (50 mM Tris-Cl, 150 mM NaCl) and then placed in Slot Blot apparatus. Vacuum/atmosphere pressure was applied until the membrane dried and then samples were put into the well. Vacuum was continued until all samples were certainly absorbed.

The dried membrane containing samples was incubated with blocking buffer (5% milk in TBS pH 7.6) for 1 h at room temperature, and then washed with TBS-T (0.1% Tween-20 in TBS pH 7.6). After that, the membrane was incubated with His-Probe rabbit polyclonal IgG antibody (1:2000 dilution, Santa Cruz Biotechnology, Inc., USA) overnight at 16°C. The membrane was washed with TBS-T, subsequently incubated with AP-conjugated goat anti-rabbit IgG antibody (1:3500 dilution, Santa Cruz Biotechnology, Inc., USA) for 1 h at room temperature. After the membrane had been washed with TBS-T, the signals were then visualized with NBT-BCIP (Thermo Scientific, USA).

Antigen-binding Assay.

About 40 µL of HisPur™ Ni-NTA magnetic beads (Thermo Scientific, USA) were placed into a 1.5 mL microcentrifuge tube, and then washed 2 times with phosphate-buffered saline (PBS). The magnetic beads were then coated with 500 µL of soluble periplasmic scFv proteins at 4°C for 2 days. Coated magnetic beads were collected, subsequently washed with PBS. As much as 20 µL of EGFRvIII::BFP soluble proteins were added to magnetic beads and incubated for 1 h. The magnetic beads were washed again with PBS and then visualized using confocal microscopy.

Results

Transformation of pJ414_scFv into *E. coli* NiCo21(DE3) was carried out to obtain transformants which can produce scFv proteins. Growing transformants were subjected to colony PCR analysis. Specific scFv primers were used to determine the actual size of scFv gene, and pJ414_scFv plasmid was used as template for positive control. The amplified products were visualized on agarose gel as seen in Figure 2 and were approximately 0.75 kb in length which corresponds to the theoretical size of scFv gene.

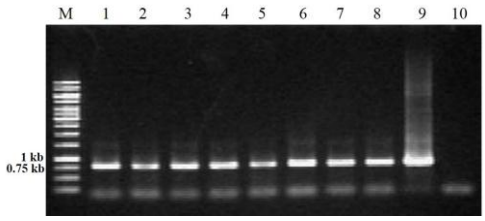


Figure 2. Colony PCR analysis of transformants *E. coli* NiCo21(DE3) using scFv-F and scFv-R primers. **Lane 1-8**, amplification fragment from colony number 1-8 (respectively); lane 9, positive control; lane 10, negative control.

To examine the expression of scFv proteins, each colony that gives a positive result in colony PCR characterization was cultured and induced with 1 mM IPTG. Screening is important because the colonies harboring the same recombinant plasmid might produce different expression level. The SDS-PAGE characterization of total proteins showed that high expression level obtained from colony number 1 and 7 (Fig. 3).

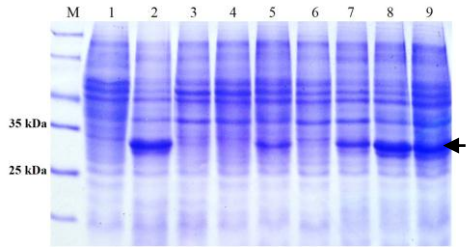


Figure 3. SDS-PAGE analysis of: **lane 1,** total proteins from untransformed *E. coli* NiCo21(DE3); **lane 2-9**, total proteins from colony transformant number 1-8 (respectively), induced with 1 mM IPTG. ScFv proteins is marked with an arrow.

To determine the optimal concentration of inducer, the culture was induced with various IPTG concentration. Result from SDS-PAGE analysis showed that there was no significant difference of scFv expression level in the range of 0.1-1 mM IPTG induction (Fig. 4). It can be concluded that low concentration level of IPTG was optimum for expressing scFv proteins.

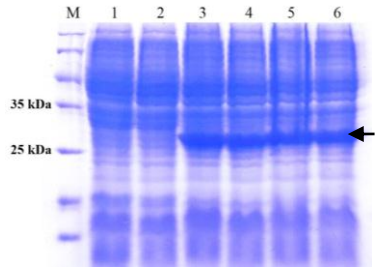
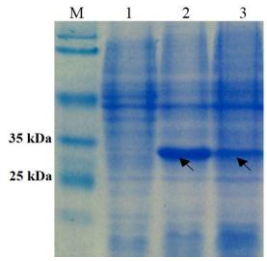
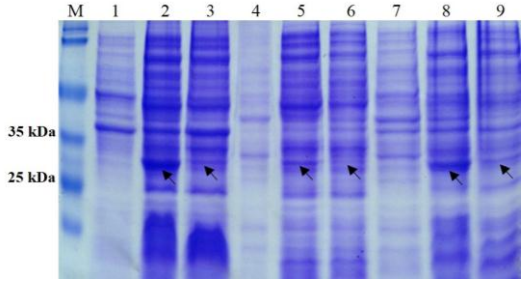


Figure 4. Total proteins of *E. coli* NiCo21(DE3) induced with various IPTG concentration; **lane 1**, untransformed *E. coli* NiCo21(DE3) induced with 0 mM IPTG; **lane 2-5**, Transformant *E. coli* NiCo21(DE3) from colony number 1 induced with 0, 0.1, 0.25, 0.5, and 1 mM IPTG (respectively). The scFv band is marked with an arrow.

Based on previous experience, low concentration of IPTG was also optimum for expressing rscFv proteins (Dewi *et al*., 2016). Thus, in this experiment 0.1 mM IPTG was used to induce rscFv proteins expression. Figure 5A showed that total scFv proteins have a thicker band compared to rscFv proteins. This phenomenon was also seen in soluble periplasmic proteins, soluble cytoplasmic proteins, and extracellular proteins (Fig. 5B). Using Photo-CaptMw program, scFv and rscFv proteins bands were deduced to have the actual size of 28.5 and 29 kDa respectively, which corresponds to the theoretical size of antibody in scFv format (Ahmad *et al*., 2012).



A



B

Figure 5. SDS-PAGE analysis of: **(A) lane 1-3**, total proteins of untransformed *E. coli* NiCo21(DE3) (UE), transformant *E. coli* NiCo21(DE3) harboring pJ414_scFv (TS) and transformant *E. coli* NiCo21(DE3) harboring pJ414_rscFv (TR), respectively. **(B) Lane 1-3**, soluble periplasmic proteins from UE, TS and TR, respectively; **lane 4-6**, soluble cytoplasmic proteins from UE, TS, and TR respectively; **lane 7-9**, extracellular proteins from UE, TS, and TR respectively. ScFv and rscFv proteins are marked with arrows.

Slot-blot analysis was carried out to confirm the presence of soluble scFv and rscFv proteins in periplasmic space, cytoplasmic space, and culture medium. Protein detection using Slot blot methodology differs from Western blot techniques that request protein separation by using SDS-PAGE. Sample proteins are instead spotted onto membranes and hybridized with an antibody probe. Figure 6 showed that there is a difference in color intensity between samples and negative control. It can be concluded that soluble scFv and rscFv proteins were found in all fractions.

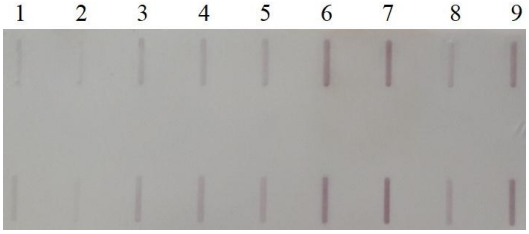


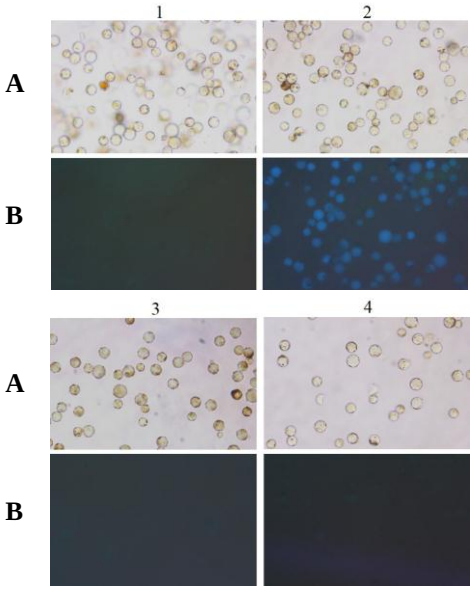
Figure 6. Slot-blot analysis of soluble periplasmic proteins (SPP), soluble cytoplasmic proteins (SCP), and Extracellular proteins (EP) of *E. coli* NiCo21(DE3) with duplication; **lane 1-3,** SPP, SCP, and EP from untransformed *E. coli* NiCo21(DE3); **lane 4-6,** SPP, SCP, and EP from transformant *E. coli* NiCo21(DE3) harboring pJ414_scFv; **lane 7-9,** SPP, SCP, and EP from transformant *E. coli* NiCo21(DE3) harboring pJ414_rscFv.

A semi-quantitative analysis using ImageJ software was done to find out the relative percentage of scFv and rscFv in total proteins and each compartment. Table 1 showed that the relative percentage of scFv and rscFv proteins in soluble form was much lower than in total proteins. These results indicate that the majority of scFv and rscFv proteins were found as inclusion bodies. The expression of soluble periplasmic scFv proteins was approximately eight times higher than that of rscFv proteins at the same expression condition (Table 1). The significant differences were also seen in total proteins, soluble cytoplasmic proteins, and extracellular proteins.

Table 1.The relative percentage of scFv and rscFv expression in *E. coli* NiCo21-(DE3) compartments.

Compartment	scFv (%)	rscFv (%)
Total proteins	47.6	25.94
Periplasm	8.41	0.96
Cytoplasm	3.75	1.09
Extracellular	17.22	6.47

The qualitative study of the antigen-binding activity of scFv antibody against EGFRvIII antigen was worked on this experiment. The EGFRvIII antigen had been already conjugated with blue fluorescent proteins. Therefore, even though the antigen-binding activity of scFv and rscFv proteins could not quantitatively comparable, it useful to visualize their antigen-binding activity trough the fluorescence produced by BFP. Figure 7 showed that both of scFv and rscFv proteins generated fluorescence after incubated with EGFRvIII:BFP antigen. The result indicated that both of scFv and rscFv proteins have antigen-binding activity against EGFRvIII antigen.



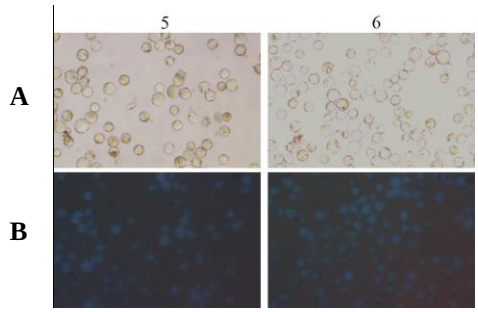


Figure 7. Antigen-binding assay of scFv and rscFv proteins against EGFRvIII::BFP observed under confocal microscopy. **Lane 1-6**, uncoated beads, EGFRvIII::BFP-coated beads, scFv-coated beads, rscFv-coated beads, scFv-coated beads subsequently incubated with EGFRvIII::BFP, rscFv-coated beads subsequently incubated with EGFRvIII::BFP, respectively. **A**, contrast phase; **B,** fluorescence phase.

Discussion

Owing to their extreme specificity, monoclonal antibodies (mAbs) have been used as important tools for research, therapy, and diagnostic applications (Pucca *et al*., 2011). However, there are several undesirable effects present in mAbs applications including activation of Fc receptor-expressing cells leading to toxicity through cytokine release. In the imaging application, the long serum half-life could also be undesirable because faster clearance is required to minimize exposure to radionucleotide molecules. To overcome these problems, mAbs have been manipulated to improve their therapeutic application (Monnier *et al*., 2013).

Later, advances in antibody engineering have developed an efficient method to reduce mAbs to scFv antibodies. The Fv fragment is the smallest unit of an antibody molecule with function in antigen-binding activities. In comparison to the mAbs, scFv antibodies have several advantages including better tumor penetration, rapid blood clearance, and lower retention times in non-target tissue which may be beneficial in cancer therapy and diagnostic applications (Monnier *et al*., 2013; Ahmad *et al*., 2012). Moreover, scFvs are less immunogenic due to a lack of Fc domain (Liu, 2014).

Disulfide bonds are crucial for scFv proteins in order to preserve its antigen-binding affinity. Periplasmic space in *E. coli* has an oxidative environment that can facilitate disulfide bond formation. Therefore, at least some of secreted scFv proteins fold into its native state in the periplasm (Kipriyanov *et al*., 1997).

The secretion machinery in *E. coli* expression system directs the scFv protein that fused with signal peptide into the periplasmic space. In this study, PelB signal sequence guides the scFv proteins to the Sec-translocon, which mediates the translocation of scFv proteins into periplasm via post-translational SecB-targeting pathway (Randall *et al*., 2004). The chaperone protein (SecB) passes the unfolded scFv proteins into SecA (ATPase), and then the scFv proteins will be translocated into periplasm through the channel comprising three integral membrane proteins, SecY, SecE and SecG (Rusch and Kendall, 2007).

The cell-free culture supernatant was concentrated to detect the presence of scFv and rscFv proteins in the culture medium. Figure 5B showed that some of scFv and rscFv proteins were released into the medium. In general, the leakage of scFv proteins into the medium is caused by the leaky outer membrane cell due to the accumulation of either scFv or rscFv proteins in the periplasmic space (King, 1998). This result was also proved by Kipriyanov *et al*., (1997) which found the scFv proteins in the culture medium.

A semi-quantitative analysis using ImageJ software (Table 1) showed that although cytoplasm has larger space than periplasm, only limited soluble cytoplasmic scFv and rscFv proteins have been found. This result indicates that both of scFvs proteins in the cytoplasm were accumulated as insoluble form. Theoretically, this situation occurs because disulfide bonds inside the proteins cannot be formed due to a reductive environment in the cytoplasm (Eser *et al*., 2008).

Use of high inducer concentration during protein expression, high temperature, and expression under strong promoter systems often results in expression of the desired protein at a high translational rate. However, these situations could lead the formation of inclusion bodies (Singh *et al*., 2015). Thus, in this experiment low concentration of IPTG and room temperature during induction were used

to slow down the expression rate. This is expected to provide an opportunity for bacterial systems to perform the correct folding.

Instead of lactose, which is eventually metabolized by the cells, IPTG was used as an inducer in this experiment. The advantage of IPTG is that because it could not be metabolized by the cells, its concentration remains constant and the cells growth rate will not variable during the protein expression stage. Therefore, the entire course of experiment could be controlled (Goulding, 1986).

As shown in Figure 5A and 5B, the expression of anti-EGFRvIII scFv antibody with V_H-linker-V_L scFv domain order is much higher than that of V_L-linker-V_H in each compartment. This phenomenon was also proved by imageJ analysis which the results are listed in Table 1. There are several possible causes for explaining this situation. Tsumoto *et al*. (1994) suggested the possibility of mistranslation or ribonucleic cleavage of the mRNA. They could detect the fragmentation in V_H-linker-V_L of anti-lysozyme scFv antibody when the target protein was expressed using the cell-free transcription/ translation system, which was not observed in V_L-linker-V_H scFv antibody. However, there was no fragmentation detected in V_H-linker-V_L and V_L-linker-V_H of anti-c-Met scFv antibody despite the expression of V_H-linker-V_L scFv antibody was much higher than that of V_L-linker-V_H (Kim *et al*., 2008). These experiences suggested that either *in vivo* or *in vitro* expression system could be involved in the different expression level of scFv caused by the order of its variable domain. Further studies should be performed in order to understand how far the exchange of variable domain orders influences the different expression level of anti-EGFRvIII scFv antibody, including transcription rates, translation rates, mRNA stabilities, and protein stabilities.

In this study, *E. coli* NiCo21(DE3) was used as an expression host. This cell was chosen because it has been engineered to minimize contamination from endogenous *E. coli* metal binding proteins (Robichon, 2011). Such proteins can compete with both of scFv and rscFv proteins to bind the magnetic beads. This situation is undesirable because it might interfere the result of antigen-binding assay.

A long-time incubation between scFv antibodies and magnetic beads was needed to get the fully-coated beads. Besides, it is important to ensure that the fluorescence generated was not from the direct binding between magnetic beads and EGFRvIII::BFP antigen. Under the fluorescence phase of confocal microscopy, both of scFv and rscFv proteins showed a fluorescence after reacted with EGFRvIII::BFP antigen. Even though the binding constant can not be determined, this result indicates that both proteins have antigen-binding activity. Advances research has to be worked out to determine the effect of variable domain orders into the scFv binding constant.

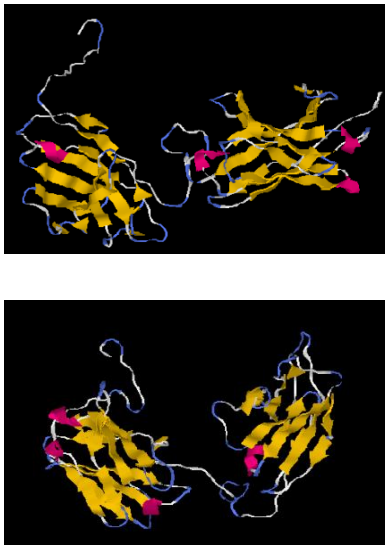


Figure 8. Three dimensional stucture prediction of: **(A)** scFv anti-EGFRvIII and **(B)** rscFv anti-EGFRvIII proteins. The protein structure prediction was done by I-TASSER (Iterative Threading ASSEmbly Refinement) on-line server.

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