Periplasmic expression of EGFRvIII extracellular domain as a fusion protein in *Escherichia coli* NiCo21(DE3)

K S Dewi*, Aminah, and A M Fuad

1Research Centre for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong Sciences Centre, Jalan Raya Bogor km. 46, Cibinong 16911, Bogor, Indonesia

*Email: kart008@lipi.go.id

Abstract. Epidermal growth factor receptor variant III is a mutant variant of EGFR that has a deletion on its DNA encoding extracellular ligand-binding domain. EGFRvIII is only found in cancer cells and not in normal cells, making it an ideal target for antibody-based cancer therapy. This study performed the expression and characterization of EGFRvIII extracellular domain conjugated with a blue fluorescent protein (BFP) as a fusion protein in *Escherichia coli* periplasmic space. Endoxylanase signal peptide was employed to guide the recombinant protein through the membrane. IPTG as inducer was added into expression medium with various concentrations of 0; 0.1; 0.25; 0.5; 1 mM, followed by periplasmic extraction using the hypertonic solution. Total proteins and periplasmic proteins were characterized using SDS-PAGE and slot blot analyses. Extracellular domain of EGFRvIII-BFP fusion protein was detected using confocal fluorescence microscopy. This study showed that the ~43 kDa target protein was successfully expressed on *E. coli* NiCo21(DE3) periplasmic space with optimum IPTG concentration of 0.1 mM and showed a blue fluorescence color under the microscope.

1. Introduction

Epidermal growth factor receptor variant III (EGFRvIII) is a mutant variant of normal EGFR, which is frequently found on the surface of malignant cells and detected as an oncoprotein. Several cancer cells that express EGFRvIII on their surface are breast cancer, prostate cancer, lung cancer, and glioblastoma multiforme. EGFRvIII has deletions in the DNA encoding extracellular ligand-binding domain. The deletion resulting in the lack of amino acids at position 6-273, and formed a new immunogenic glycine residue at position 6 between amino acids 5 and 274. Its characteristic of being expressed only on cancer cells makes EGFRvIII ideal as a molecular target in antibody-based cancer therapy [1-3]. The EGFRvIII as an antigen can be developed as a reporter molecule in selecting a specific antibody against EGFRvIII more useful if it is conjugated with a fluorescent protein.

Fluorescent protein (FP) is a protein that produces chromophores with visible wavelength. The fluorescence comes from 3 adjacent amino acids at positions 65, 66, and 67. This unique characteristic can be utilized as a powerful tool for protein structure and functional study [4]. FP can be fused into target protein as a protein reporter to form internal chromophores without additional cofactors, enzymes, or substrates besides molecular oxygen [5].

For producing a simple recombinant protein, *E. coli* is one of the favorable hosts. It offers many advantages, including easy growth on inexpensive and simple medium, easy to transform with exogenous DNA, and high protein yield [6]. Moreover, the production of recombinant proteins in the
E. coli periplasm has many benefits, including facilitating the purification stage, avoiding protease attacks and the addition of N-terminal methionine, and there is a better chance for proper protein folding [7]. Therefore, the recombinant protein produced will be more identical to the original protein.

In this study, the EGFRvIII extracellular domain was fused with Blue Fluorescent Protein (BFP) and expressed in the E. coli periplasmic space. Endoxyylanase signal peptide was fused in the N-terminal of the fusion protein to help translocation of target protein into the periplasmic space. Recombinant protein characterization was performed using SDS-PAGE and slot blot analyses. Moreover, the EGFRvIII-BFP protein was also visualized under confocal fluorescence microscopy.

2. Material and Methods

2.1. Microbial strain
E. coli NiCo21(DE3) transformant harboring pJ404_EGFRvIII_BFP was employed to produce EGFRvIII-BFP recombinant proteins [8,9].

2.2. Culture condition for EGFRvIII-BFP expression
About 20 µL glycerol stock of E. coli NiCo21(DE3) transformant harboring pJ404_EGFRvIII_BFP were added into 2 mL Lysogeny Broth medium containing 100 µg/mL ampicillin (LB-amp) and grown overnight at 25°C, 150 rpm. Pre-culture was diluted into ratio 1:50 with LB-amp medium and grown at 25°C with shaking until the optical density at λ=600 (OD600) reached 0.8. After that, IPTG was added with various concentration of 0; 0.1; 0.25; 0.5; 1 mM, then incubated overnight. For negative control, E. coli NiCo21(DE3) non-transformant was given the same treatment.

2.3. Extraction of EGFRvIII-BFP from E. coli NiCo21(DE3) periplasmic space
About 15 mL cell cultures were harvested by centrifugation at 5,000 x g, 4°C for 15 minutes. Cell pellets were resuspended with 750 µL hypertonic solution consisting of 20% sucrose, 30 mM Tris-Cl and 1 mM EDTA pH 8. The cell suspension was incubated on ice for 1 hour with gentle agitation, centrifuged at 11,600 x g, and 4°C for 30 min. The supernatant containing periplasmic proteins were used for further analysis [10].

2.4. SDS-PAGE analysis
For total protein analysis, as much as 1 mL of cell culture was centrifuged at 6,000 x g, 4°C for 6 min. Pelleted cells were resuspended with 100 µL of 5x denaturing sample buffer and then heated for 5 min at 95°C. Subsequently, the sample was centrifuged at 12,000 x g for 15 min. About 5 µL of the sample was used for analysis using 15% SDS-PAGE for 90 min, 90 V according to the Laemli method [11]. For periplasmic protein analysis, about 15 µL of periplasmic protein extract was mixed with 5 µL of 5x denaturing sample buffer and then heated for 5 minutes at 95°C. Samples were analyzed using the same condition as for total protein.

2.5. Slot blot analysis
Whatman paper and nitrocellulose membrane 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 pressure was applied until the membrane dried, and then 50 µL samples were put into the well. A vacuum was continued until samples were surely absorbed [12].

The membrane containing samples was incubated in blocking buffer (5% milk in TBS pH 7.6) for 1h at room temperature, then washed with TBS-T (0.1% Tween-20 in TBS pH 7.6). The membrane was then incubated with anti-flag tag mouse monoclonal IgG antibody (1:2000 dilution, Santa Cruz Biotechnology, Inc., USA) overnight at 4°C. The membrane was washed with TBS-T, and then incubated with AP-conjugated goat anti-mouse IgG antibody (1:3500 dilution, Santa Cruz Biotechnology, Inc., USA) for 1h at room temperature. The membrane was again using TBS-T and finally, the signals were visualized with NBT-BCIP (Thermo Scientific, USA) [12].
2.6. Characterization of EGFRvIII-BFP recombinant proteins using confocal fluorescence microscopy

A total of 20 µL of HisPurTM Ni-NTA magnetic beads were put into a microtube, then washed twice with phosphate buffer saline (PBS) pH 7.4. Magnetic beads were mixed with 200 µL of periplasmic protein extract and then incubated for 1h at 4°C. After that, the magnetic beads were rewashed with PBS and visualized using confocal fluorescence microscopy.

3. Results and Discussion

3.1 Expression and characterization of EGFRvIII-BFP

SDS-PAGE analysis was performed to determine the profile of EGFRvIII-BFP proteins expressed by E. coli NiCo21 (DE3). In this study, the optimization of IPTG concentration as an inducer was carried out. Figure 1A showed that the total protein of EGFRvIII-BFP could be expressed at IPTG concentration of 0.1; 0.25; 0.5; 1 mM with a molecular size of approximately 43 kDa. Whereas Fig. 1B showed the profile of the protein expressed in periplasmic space. It has been demonstrated that the EGFRvIII-BFP periplasmic proteins were only expressed using IPTG induction with a concentration of 0.1-0.5 mM.

IPTG inducer was added with various concentrations to determine the optimum concentration inducers for expressing target protein. There was no significant difference in the amount of protein produced by IPTG induction of 0.1; 0.25; 0.5 mM from the analysis conducted. Therefore, it can be concluded that the induction of IPTG 0.1 mM is optimum in inducing the expression of EGFRvIII-BFP extracellular domain fusion protein in the periplasm of the E. coli NiCo21(DE3).

Theoretically, the EGFRvIII-BFP fusion protein has a molecular weight of 43 kDa. The result was consistent with Figure 1A (lane 3-6) which showed that the target protein band is experimentally sized below the 44.6 kDa or approximately 43 kDa. Figure 1, lanes 1 and 2, are the total protein profiles of non-transformant E. coli NiCo21(DE3) and non-induced E. coli NiCo21(DE3) transformant. The result showed that the target protein band only appeared when IPTG induced the transformant. The absence of a host cell protein band at the same size as the target protein proved that the EGFRvIII-BFP was successfully expressed in E. coli NiCo21(DE3) host cell and the LacI protein as a repressor was correctly worked [13].

Endoxylanase signal peptides were fused at the N-terminal position when plasmid construction was carried out. It was intended to assist the translocation of target proteins into the periplasmic space [14]. Signal peptide will be recognized by Sec translocase which then translocated the target protein into the periplasm. Eventually, the signal peptide will be cut off by a peptidase produced on the membrane and produce a recombinant protein that is identical to its natural protein [15]. The presence of EGFRvIII-
BFP proteins in periplasmic protein extract (Figure 1B) indicated that endoxylanase signal peptide was worked well.

3.2 Characterization of EGFRvIII-BFP proteins

Slot-blot analysis was performed to confirm the presence of soluble EGFRvIII-BFP proteins in periplasmic space. Unlike the Western blot technique, in slot blot analysis, the sample proteins were spotted directly onto the membrane and hybridized with antibody probes. Figure 2 showed that the colors were only seen in samples induced with IPTG concentration of 0.1; 0.25; 0.5 mM. This result was in line with SDS-PAGE characterization (Figure 1B) which showed that the protein bands of EGFRvIII-BFP in periplasmic space were only detected in 0.1; 0.25; 0.5 mM of IPTG induction cultures.

![Figure 2. Slot-blot analysis of soluble periplasmic proteins. Lane 1, E. coli NiCo21(DE3) non-transformant, Lane 2-6, E. coli NiCo21(DE3) transformant induced with various concentration of IPTG (0, 0.1, 0.25, 0.5, 1 mM respectively).](image1)

The ability of BFP to glow makes it useful for gene expression markers. BFP is known to retain its fluorescence in the context of genetically engineered fusion proteins without affecting the function or localization of its fusion partner within the cellular environment [4]. In this study, the EGFRvIII-BFP was conjugated with a 6x His-tag to facilitate the purification stage. Moreover, this provides advantages for detecting periplasmic protein expression using affinity between Ni-NTA magnetic beads and His-tag. EGFRvIII-BFP bound magnetic beads will glow when it was viewed under a confocal fluorescence microscopy. figure 3 showed blue fluorescence under the microscope, which indicated that the EGFRvIII-BFP was successfully expressed in the periplasmic space of E. coli NiCo21(DE3).

![Figure 3. Characterization of EGFRvIII-BFP extracellular domain using confocal fluorescence microscopy with 100x magnification.](image2)
4. Conclusion

EGFRvIII-BFP was successfully expressed in the periplasmic space of *E. coli* NiCo21(DE3) at a size of approximately 43 kDa using Endoxylanase signal peptide with optimum IPTG concentration of 0.1 mM. Moreover, it showed a blue fluorescence color under the confocal fluorescence microscopy. For future research, this fusion protein is potential as a reporter molecule in selecting anti-EGFRvIII monoclonal antibody as a drug delivery system to develop antibody-based targeted cancer therapy.

References
[1] Wikstrand C J, Reist C J, Archer G E, Zalutsky M R and Bigner D D 1998 *J. Neurovirol.* 4(2)148-58
[2] Lammering G, Hewit T H, Holmes M, Valerie K, Hawkins W, Lin P S, Mikkelsen R B and Schmidt-Ullrich R K 2004 *Clin. Cancer Res.* 10(19) 6732-43
[3] Pedersen M W, Meltorn M, Damstrup L and Poulsen HS 2001 *Ann. Oncol.* 12(6) 745-60
[4] Sample V, Newman R H and Zhang J 2009 *Chem. Soc. Rev.* 38(10) 2852-64
[5] Stepanenko O V, Verkhusha V V, Kuznetsova I M, Uversky V N and Turoverov K K 2008 *Curr. Protein. Pept. Sci.* 9(4) 338-69
[6] Rosano G L and Ceccarelli E A 2014 *Front. Microbiol.* 5 172
[7] Choi J H and Lee S 2004 *Microbiol. Biotechnol.* 64(5) 625-35
[8] Utami E R, Dewi K S, Abinawanto and Fuad AM 2020 *IOP Conference Series: E & ES.* 481(1) 012009
[9] Gardiani R W 2016 *Thesis* Konstruksi vector dan ekspresi protein human EGFRvIII domain ekstraseluler pada *Escherichia coli* Program Studi Bioteknologi, Sekolah Pascasarjana Institut Pertanian Bogor. Bogor.
[10] Dewi K S, Retnoningrum D S, Riani C and Fuad A M 2016 *Sci. Pharm.* 84(1) 141-52
[11] Laemmli U K 1970 *Nature* 227(5259) 680-5
[12] Dewi K S and Fuad A M 2017 *Ann. Bogor.* 21(1) 29-37
[13] Lewis M 2005 *C R. Biol.* 328(6) 521-48
[14] Singh P, Sharma L, Kulothungan S R, Adkar B V, Prajapati R S, Ali P S, Krishnan B and Varadarajan R 2013 *PloS one* 8(5) e63442
[15] Deb A, Johnson W A, Kline A P, Scott B J, Meador L R, Srinivas D, Martin-Garcia J M, Dörner K, Borges C R, Misra R and Hogue B G 2017 *PloS one* 12(2) e0172529