Rostinawati T et al /J. Pharm. Sci. & Res. Vol. 9(11), 2017, 2106-2113

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Construction and Expression of Synthetic-gene encoding anti-HER2 scFv Fused with pelB in Escherichia coli BL21 (DE3)

Rostinawati T[1*], Kusuma SAF[1], Yusuf M[2], Gaffar S[2] and Subroto, T[2]

[1]*Departemen of Pharmaceutical Biology, Faculty of Pharmacy, Padjadajaran University, Sumedang, West Java, 45363, Indonesia*

[2]*Departemen of Chemistry, Faculty of Mathematics and Natural Sciences, Padjadajaran University, Sumedang, West Java, 45363, Indonesia*

Abstract

Breast cancer is a malignant tumor located in breast cells that can grow and develop around the breast tissue. In breast cancer there are much more copies of HER2 (Human Epidermal Growth Factor 2) gene so as to induce dimerisation and induce the occurrence of migration and cancer cell metastasis. The amount of HER2 in the cell surface can be an indicator of the severity of this breast cancer and can be detected through an immunoassay test based on the antigen-antibody binding reaction. Anti-HER2 scFv (single chain fragment variable) is an antibody to HER2 that is small but able to maintain binding to antigen. The purpose of this study was to express the modified *anti-HER2 scFv* using an extracellular expression system with *pelB* signal peptide via sec-dependent pathway. In this research used Escherichia coli strain BL21 (DE3) as host cell to be transformed with pD861 plasmid which already contain *anti-HER2 scFv* synthetic gene fused with *pelB*. Then expressed by induction using L-Rhamnose. Further Anti-HER2 scFv protein was secreted via extracellular with PelB signal peptide and characterized using SDS PAGE electrophoresis. The result of this study showed that Anti-HER2 scFv can be extracellularly expressed through sec-dependent pathway characterized by the size of ± 27 kDa band on SDS-PAGE elekforegram fraction of protein in the cytoplasm, periplasm and extracellular medium.

Keywords: *anti-HER2 scFv*, *pelB*, *Escherichia coli* BL21(DE3), expression

INTRODUCTION

Breast cancer is one type of cancer that affects many women. About 1.7 million women are diagnosed with breast cancer that causes of death in 2013 (https://www.iarc.fr/). In Indonesia, breast cancer patients reach 25,208 per 100,000 people and as many as 43% of patients are die [1]. A total of 30% of breast cancer cases is caused by Human Epidermal Receptor-2 (HER-2) overexpression [2].

HER2 is one of the receptor families of tyrosine kinase which includes HER1 (EGFR), HER3 and HER4. These receptors mediate cell differentiation and proliferation processes in both embryonic and adult cells [3].The excessive expression of HER-2 receptors is capable of inducing spontaneous dimerization and autophosphorylation and triggers the activation of focal adhesion kinase (FAK) so as to induce the migratory process and cancer cell metastasis [4].

Therefore, it is important to be able to detect HER2 concentrations in cells in the human body as early detection of breast cancer. Today, recombinant antibody technology has evolved toward antibody fragments as molecules used for diagnosis and therapy. A number of models of recombinant antibodies have been engineered for use as a diagnostic tool, one of which the most famous is single chain fragment variable / scFV [5]. ScFv is an antibody fragment containing varying regions in the heavy (V_H) and light chain (V_L) chains of immunoglobulins linked by varying peptide connections. scFv has a different structure depending on the molecule bound on it . The advantage of scFv is more economical than other immunoglobulin antibodies because scFv has a smaller molecular weight [6].

A sensitive detection of breast cancer can be easily made economically by using an identifiable molecule such as scFv. Therefore, antibodies to HER2 called anti HER2-scFv are required to detect HER2 on the surface of breast cells. Anti-HER2 scFv will bind specifically to HER2 through the introduction of antibodies. Specific protein identification can be performed through specific protein reactions with appropriate antigens, as is usually done using ELISA (Enzyme-Linked Immunosorbent Assay), but this method requires expensive and time-consuming chemicals. Hence, the anti-HER2 scFv interaction test against HER2 can be developed through a simpler and more economical immunoassay imaging test by conjugating anti-HER2 scFv to nanoparticles of gold (AuNP) is further characterizedd by UV-Vis Spectrophotometry [7].

Many scFv have been expressed in various expression systems, among which are popular bacterial expression systems for the production of high levels of non-glycosylated antibody fragments. However, it is not uncommon for recombinant proteins to be expressed to fail to achieve precise conformation and to undergo proteolytic degradation or interaction of these protein each other resulting in insoluble aggregates or nonnative proteins known as inclusion bodies [8].

One strategy to overcome these problems is by secreting extracellular proteins, in which proteins that have been

synthesized in the cytoplasm will be secreted to the periplasm or extracellular medium. The production of recombinant extracellular protein secretion has several advantages over intracellular production, such as overcoming inclusion bodies by increasing protein folding, reducing proteolytic degradation and facilitating better purification [9]. The Anti-HER2 scFv secretion process will be performed by the PelB signal peptide diffused with Anti-HER2 protein scFv to form premature protein. The PelB signal peptide will aid the secretion of Anti-HER2 protein scFv via a sec-dependent pathway. This pathway is supported by several proteins that will carry proteins across the inner membranes to the periplasm and then proceed with the translocation process to the extracellular medium [10]. This study aims to construct the synthetic gene encoding *anti-Her2 scFv* and express recombinant protein using the expression system in *E. coli* BL21 (DE3). the recombinant Anti-HER2 scFv will be translated in a fused form together with the PelB signal peptide in the cytoplasm and then transocated to the periplasm and the extracellular medium.

MATERIALS AND METHODS

The main Materials used in this research were pD861-*pelB-anti-HER2 scFv* recombinat (synthesized by DNA 2.0, California, USA) and *E. coli* BL21 (DE3).

Optimization, Design, and Synthesis of *anti-HER2 scFv* gene

The sequence of human *anti-HER2 scFv* gene was obtained from Protein Data Bank (www.pdb.org) with acces code i.e 4LLU. Gene of *anti-Her2 scFv* was added a modified linker with the addition of cysteine. Then, sequence of *anti-HER2 scFv* gene was inserted into the web server of Graphical Codon Usage Analyzer (GCUA) (http://gcua.schoedl.de/) and produced graph with varying degrees of codon adaptability. The codon optimization was done by replacing the rare codon with the preferred optimum codon on *E. coli*. In this synthetic gene was also inserted *leader* sequence (*pelB*), start codon, restriction sites at 5' *sap*I and at 3' *eco*RI, *his* tag and stop codon. This gene was ligated with pD861 vector having the *kanamycin resistant* gene.

Transformation of *pD861-pelB-anti-HER2 ScFv* into *E. coli* BL21 (DE3)

The first, *E. coli* BL21 (DE3) cell was made into competent cell by using $CaCl_2$. A number of 5 µL *pD861-pelB-anti-HER2 scFv* 1ng/µL was added to a micro tube containing 50 µL competent cells. The mixture of recombinant plasmid and the competent cells was resuspended and incubated into the ice for 30 min. After that, micro tube was removed and placed at 42°C waterbath for 50 sec and reincubated in ice for 2 min. Then 100 µL of Lurea Bertani (LB) broth was added into the microtube and resuspended and incubated in shaker for 1 h at 37°C with 150 rpm. A total of 100 µL suspension was cultured on LB agar medium containing kanamycin 25 µg/mL at 37°C for 16-18 h [11].

3.2.7 Isolation and characterization of *pD861-pelB-anti-HER2 scFv* recombinat

One of transformant colonies was cultured in 5 ml of LB broth containing kanamycin for 18 h at 37°C. The cell pellet was collected by centrifugation at 12,000 rpm for 1 min. Then, recombinant plasmid was isolated using plasmid isolation kit (TIANprep Rapid Mini Plasmid Kit). The isolated plasmid recombinant was further characterized using *Sap*I restriction enzyme (Thermo Fisher Scientific). 5 µL of recombinant plasmid was placed into a micro tube and added 1 µL of buffer cut, 2 µL of nuclease free water and 1 µL of *Sap*I, then incubated for 2 h at 37°C. The results of the restriction were characterized by electrophoresis agarose 1% (w/v) containing 1% of Ethidium Bromida (EtBr). Electrophoresis was carried out with 80 V for 60 min using TAE 1x buffer as a current delivery medium. DNA bands were seen using ultra violet light at λ312 nm.

Determination of *anti-HER2 scFv* gene sequence

Sequence of gene encoding *anti-HER2 scFv* was sequenced using primer R1 5'-ttcagcaaaaaaccccctcaa-3'. Sequencing process was carried out in Macrogen, South Korea using isolated recombinant plasmid as sample. Then, sequencing result of anti-HER2 scFV gene sequence was aligned with synthetic-gene having anti-HER2 scFv.

Expression of gene encoding *anti-HER2 scFv*

Gene expression was performed as represented in Figure 1.

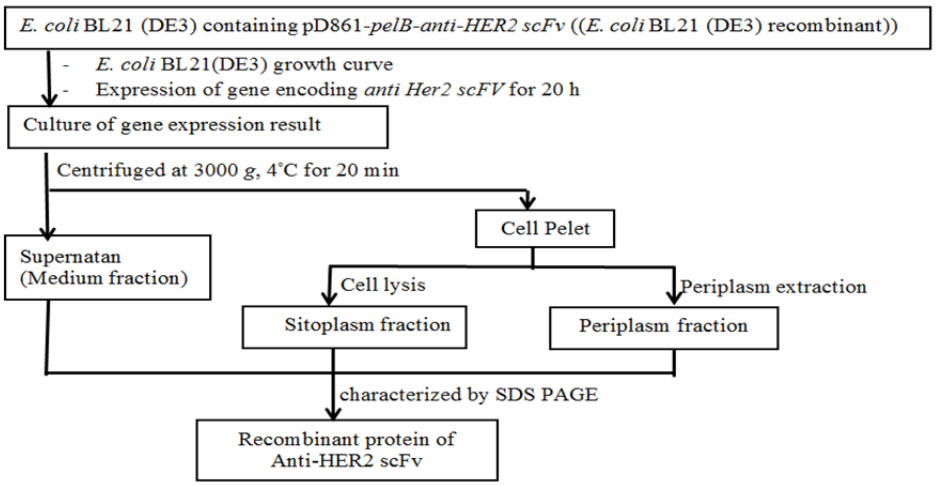

Figure 1. Stage of expression of gene encoding *anti Her2 scFv*

Growth curve determination of *E. coli* BL21 (DE3) having *pD861-pelB-anti-HER2 scFv*

Colony of *E. coli* BL21 (DE3) having *pD861-pelB-anti-HER2 scFv* was cultured in 50 mL of LB broth added kanamycin at 37°C for 20 h using shaker at 150 rpm. Every 1 hour, 1 mL of culture was taken from medium then measured Optical Density at 600 nm wavelength (OD_{600}). The growth curve was determined by linking the effect of growth time on the X-axis to its absorption on the Y-axis [12].

Determination of Expression of gene encoding *anti Her2 scFv*

Colony of *E. coli* BL21 recombinant was cultured in 5 mL of LB broth containing 25 µg/mL of kanamycin at 37°C for 18 h with speed shaker at 180 rpm. 1 mL of culture was taken and recultured in 100 mL of LB broth at 37°C for 20 h. Furthermore, the induction process was performed by adding L-Rhamnose 4 mM as an inducer into the expression medium at the exponential phase. The time to add the inducer can be determined based on the growth curve of *E. coli* BL21(DE3) recombinant. Prior an inducer was added in the medium, as much as 1 mL of medium was taken to be defined as (t_0) protein fraction. Furthermore, 2 mL of medium was taken every hour after the addition of the inducer. 1 mL of medium was used to determine the dissolved protein in the cytoplasm and the other of medium to determine the soluble protein in the periplasm and extracellular medium [13].

Qualitatif determination of recombinat protein in the cytoplasm, periplasm and extracellular media

1 mL of medium taken from induced media was centrifuged at a rate of 3000 *g*, 4°C for 20 min. The cell pellets were lysed to know protein present in the cytoplasm. Cell pellets were added 100 µL of lysis buffer and Phenylmethylsulfonyl fluoride (PMSF) 0.1 mM final concentration then homogenized by vortex. The cell lysis process was done by the sonicator with procedur i.e 2 seconds ON, 2 seconds OFF for 2 min then 4 min rest for three times cycle [13]. The lysed cells were centrifuged at 10,000 *g*, 4°C for 10 minutes. Then, supernatant was separated and placed into the new sterile eppendorf tube as the fraction of dissolved proteins in the cytoplasm. The protein was characterized with using SDS PAGE 12% .

Qualitatif determination of recombinant protein in periplasm

The other 1 mL of medium was also centrifuged at a rate of 3000 *g*, 4°C for 20 min. The supernatant was separated for subsequent characterization with using SDS PAGE 12 % to be known protein present in medium fraction. While cell pellets was added TSE buffer pH 8 (Tris 0.2 M, sukrosa 0.5 M dan EDTA 0.001 M) and 0.1 mM PMSF final concentration. The cell pellets were incubated in ice for 30 min then centrifuged at a rate of 20,000 *g*, 4°C for 30 min. Supernatan was separated and placed into new steril eppendorf tube as periplasm protein fraction. The protein was characterized with using SDS PAGE 12% [14].

4.6 Calculation of anti-HER2 scFV Molecular Weights

Calculation of AntiHER2scFv can be performed by using linear equations of standard protein curve [15].The standard protein curve was obtained from the comparison of the relative mobility value (Rf) marker protein used. The Rf value was obtained from the result of comparison of migration of marker protein to the migration distance of SDS PAGE solution. Furthermore the molecular weight of the recombinant protein was obtained from the Rf value of this protein was fed into the linear regression equation.

RESULT

Construction of synthetic-gene encoding *antiHER2 scFv*

anti-HER2 scFv gene was consisted of sequence of heavy chain (V_H) and light chain (V_L) scFv. Sequence of V_H and V_L of scFV was connected by linker SCGGGSGGGGSGGGGS i.e V_H-S**C**GGGSGGGGSGGGGS-V_L. Hence, a whole sequence of Anti-HER2 scFv protein with a total of 242 amino acid in the following order was obtained in Figure 2. While the sequence of gene encoding *anti-Her2 scFV* that has been optimized as follows in Figure 3.

From synthetic gene design result was obtained recombinant expression vector of *pD861-pelB-anti Her2 scFv* with size 3029 bp in Figure 4. This recombinant plasmid consisted of sequences of *kanamycin resistant* gene, rhaBAD promoter, strong RBS, gene encoding signal peptide *pelB*, Ori, *anti HER2 scFv* and *his* tag.

EVQLVESGGGLVQPGGSLRLSCAASGFTFTDYTMDWVRQAPGKG
LEWVADVNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAE
DTAVYYCARNLGPSFYFDYWGQGTLVTVSSSCGGGSGGGGSGGG
GSDIQMTQSPSSLSASVGDRVTITCKASQDVSIGVAWYQQKPGKA
PKLLIYSASYRYTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQ
YYIYPYTFGQGTKVEIK

Figure 2. Amino acid sequence of anti-HER2 scFV protein connected by linker

GAAGTGCAGCTGGTGGAAAGCGGCGGCGGCCTGGTGCAGCCGGGCGGCAGCCTGCGCCTG
AGCTGCGCGGCGAGCGGCTTTACCTTTACCGATTATACCATGGATTGGGTGCGCCAGGCG
CCGGGCAAAGGCCTGGAATGGGTGGCGGATGTGAACCCGAACAGCGGCGGCAGCATTTAT
AACCAGCGCTTTAAAGGCCGCTTTACCCTGAGCGTGGATCGCAGCAAAAACACCCTGTAT
CTGCAGATGAACAGCCTGCGCGCGGAAGATACCGCGGTGTATTATTGCGCGCGCAACCTG
GGCCCGAGCTTTTATTTTGATTATTGGGGCCAGGGCACCCTGGTGACCGTGAGCAGCAGC
TGCGGCGGCGGCAGCGGCGGCGGCGGCAGCGGCGGCGGCGGCAGCGATATTCAGATGACC
CAGAGCCCGAGCAGCCTGAGCGCGAGCGTGGGCGATCGCGTGACCATTACCTGCAAAGCG
AGCCAGGATGTGAGCATTGGCGTGGCGTGGTATCAGCAGAAACCGGGCAAAGCGCCGAAA
CTGCTGATTTATAGCGCGAGCTATCGCTATACCGGCGTGCCGAGCCGCTTTAGCGGCAGC
GGCAGCGGCACCGATTTTACCCTGACCATTAGCAGCCTGCAGCCGGAAGATTTTGCGACC
TATTATTGCCAGCAGTATTATATTTATCCGTATACCTTTGGCCAGGGCACCAAAGTGGAA
ATTAAA

Figure 3. Sequence of *anti-Her2 scFv* synthetic gene

Characterization of *pD861-pelB-anti-HER2 scFv* recombinant plasmid

Furthermore, the presence of a recombinant *pD861-pelB-anti-HER2 scFv* in *E. coli* BL21(DE3) cell through a transformation process can be determined from the characterization of plasmid isolated. The result of recombinant *pD861-pelB-anti-HER2 scFv* characterization can be seen in Figure 5. The path 1 in Figure 5a showed some bands indicating that the DNA plasmid was circular and had some different conformations such as supercoiled monomer, supercoiled dimer and nicked circular (nc) [16]. Line 2 electroforegram in Figure 5b showed that *pD861-pelB anti HER2-scFv* t was successfully cut with a *Sap*I

restriction enzyme and produced only one DNA band. This was a linearly shaped pD861-pelB-ani Her2 scFV having a size of about 3029 bp.

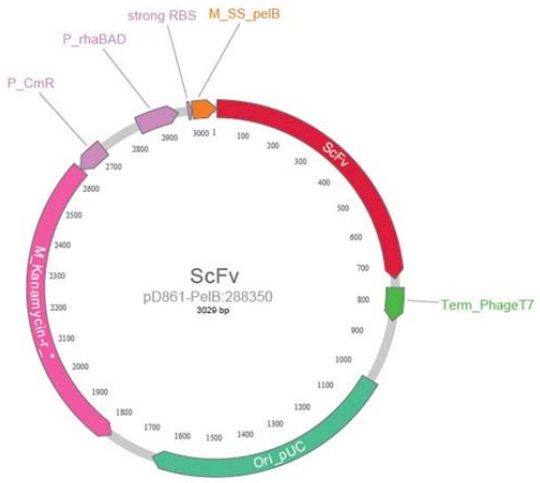

Figure 4. Map of recombinant plasmid construction of *pD861-pelB-anti-HER2 scFv*

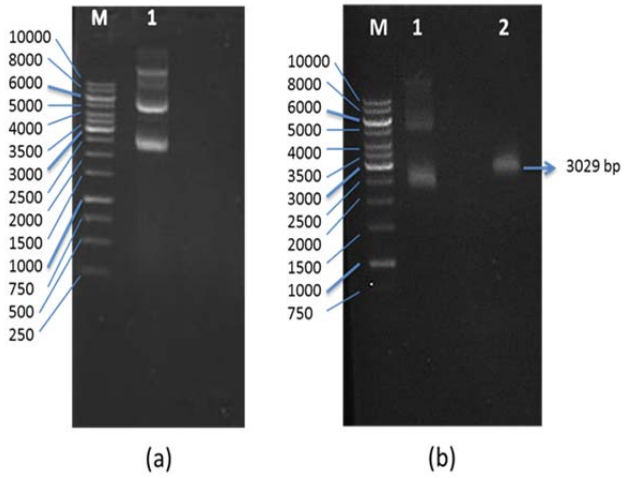

Figure 5. Characterization of pD861-pelB-anti-HER2 scFv recombinant plasmid. (a) Result of recombinant plasmid isolation and (b) Result of recombinant plasmid cut with SapI restriction enzyme

Figure 6. Result of sequence determination of *pD861-pelB-anti-HER2 scFv* recombinant plasmid

Result of sequencing determination of gene encoding *anti-HER2 scFv*

Result of sequence aligment between anti-HER2 scFV sequenced with anti-HER2 scFV synthetic gene revealed 100%. It means that *pD861-pelB-ani Her2 scFv* was successfully transformated in *E. coli* BL21 (DE3) and correctly encode *anti-HER2 scFv* gene.

The growth curve of E. coli BL21 (DE3)

The growth curve of *E. coli* BL21(DE3) *pD861-pelB-anti-HER2 scFv* has been determined and performed in the Figure 7. It was known that the growth of *E. coli* BL21(DE3) consisted of four phases i.e the lag phase, exponential phase or logarithmic phase, stationary phase and phase of death. The log phase occured 4 hours after the colony was cultured with OD value i.e 0.731. So, L-Rhamnose as an inducer was added after the colony was cultured at 37°C at 180 rpm.

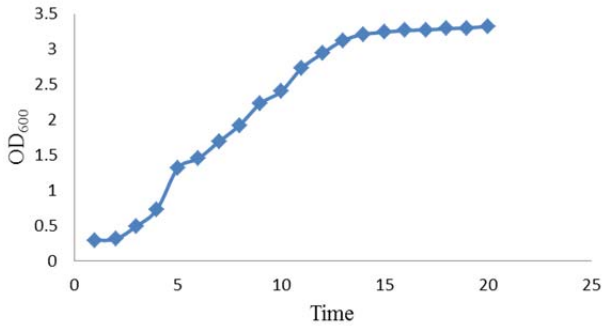

Figure 7. Growth curve of E.coli BL21(DE3) tranformated with pD861-pelB-anti-HER2 scFv

The expression of gene encoding pD861-pelB-anti-HER2 scFv in cytoplasm, periplasm and extracellular medium

The expression of gene encoding *pD861-pelB-anti-HER2 scFv* in cytoplasm has been determined. The dissolved proteins in the cytoplasm were shown in Figure 8. The SDS-PAGE results showed that the Anti-HER2 scFv protein was expressed in the cytoplasm with the presence of an Anti Her2 scFV protein band with the size ± 27 kDa. The resulting protein bands had a relatively constant thickness after 2 until 19 hours after induction.

Anti-HER2 scFv was expressed by fusion with a pelB peptide signal so that protein expressed in the cytoplasm will be translocated to periplasm. SDS-PAGE characterization of periplasmic protein fraction was revealed in Figure 9. After 4 hours of induction, protein had been was secreted in the periplasm but the appearance of protein band was thin. After induction for 6 hours, the number of proteins in the periplasm was more. Then, the amount of protein in the periplasm decreased after 12 hours of induction. Even at 17 hours after the induction, Anti-Her2 scFv protein was no longer present in the periplasm.

The Anti-HER2 scFv protein was also secreted up to the extracellular medium based on the characterization of SDS-PAG 12 % in Figure 10. At 16 hours after induction, anti-HER2 scFv protein was initially in extracellular media. The presence of this protein in extracellular media was increasing at 17, 18, 19 and 20 hours after induction.

Standard curve of marka protein molecular weight

Based on the linear line equation of molecular weight of the marker protein, the recombinant protein molecular weight with Rf = 0.675 wa 27.3 kDa. While the recombinant protein molecular weight was theoretically ± 27 kDa Figure 11.

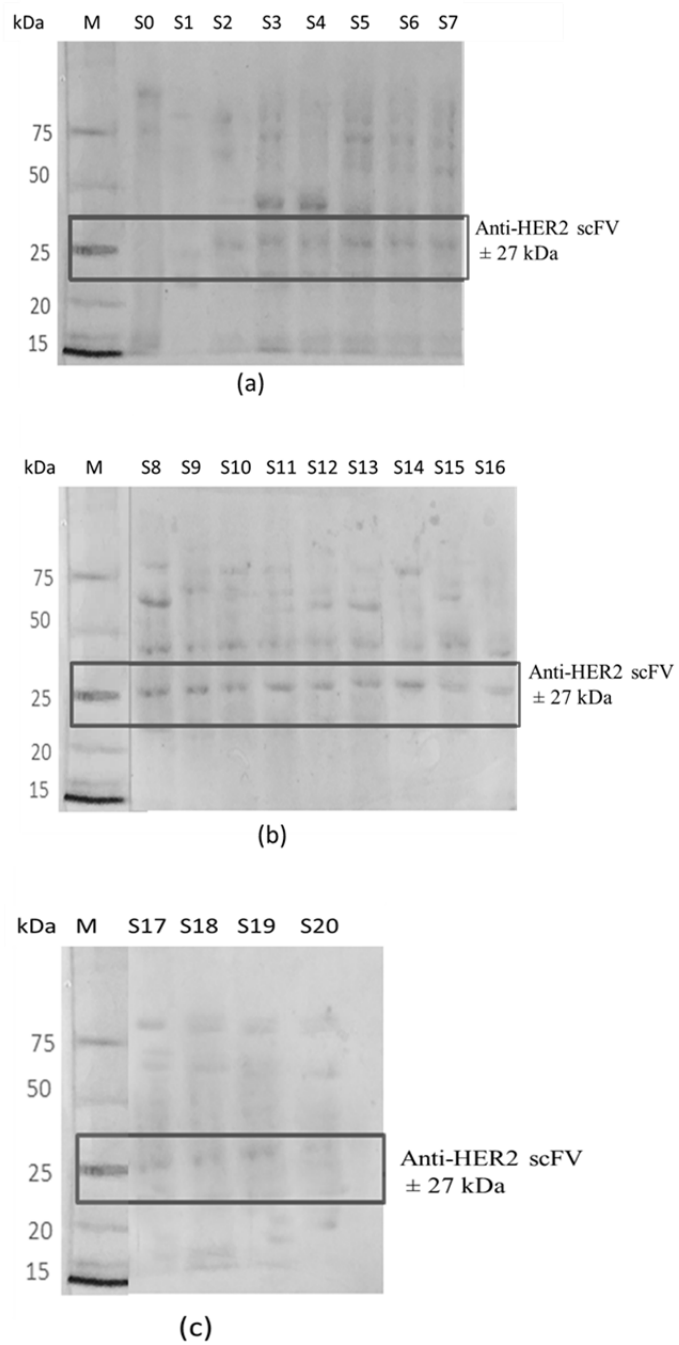

Figure 8. SDS-PAGE characterization of dissolved anti-HER2 scFV protein in cytoplasm with 4 mM L-ramnose induction. (M) protein markers; (S0) fraction before induction of L-Rhamnose; (Si) fraction after induction of L-ramnosa at i-hour.
(a) S0-S7 fraction, (b) S8-S16 fraction and (c) S17-S20 fraction

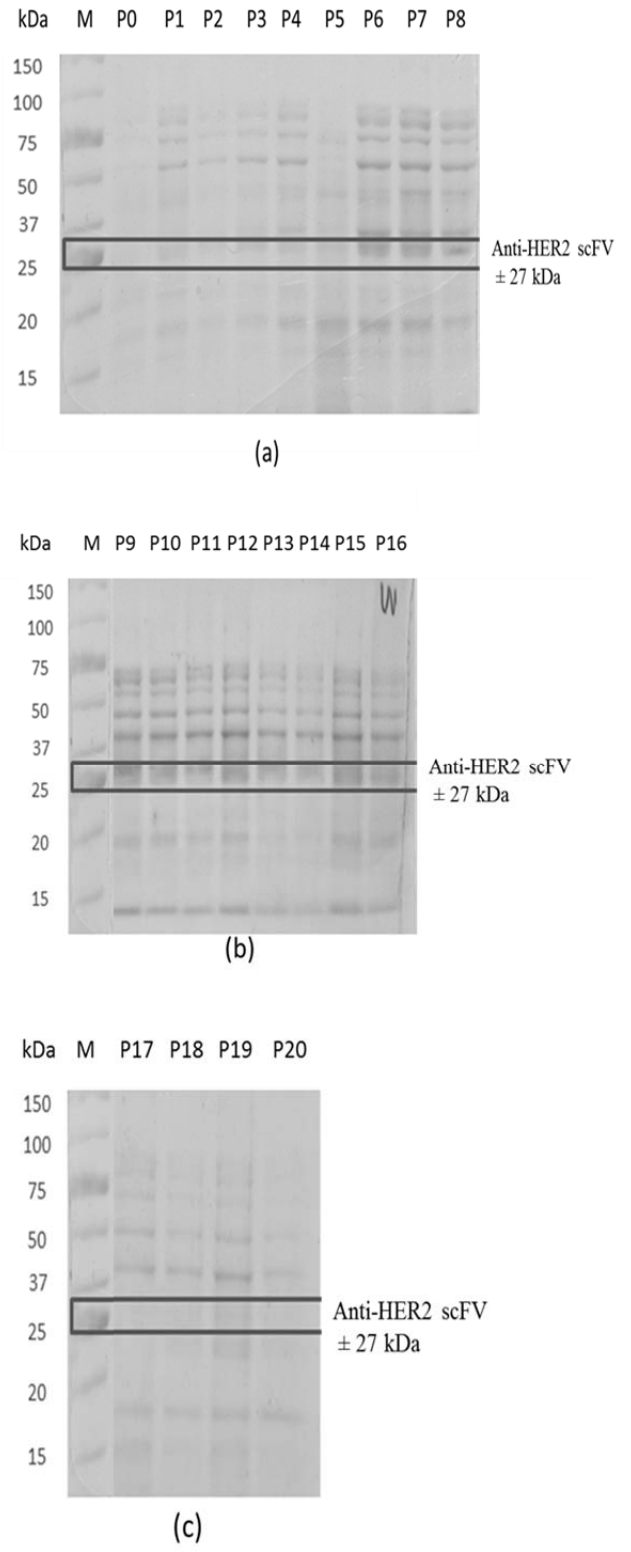

Figure 9. SDS-PAGE characterization of dissolved anti-HER2 scFv protein in periplasm with 4 mM L-ramnose induction. (M) protein markers; (P0) fraction before induction of L-Rhamnose; (Pi) fraction after induction of L-ramnosa at i- hour.
(a) P0-P8 fraction, (b) P9-P16 and (c) P17-P20)

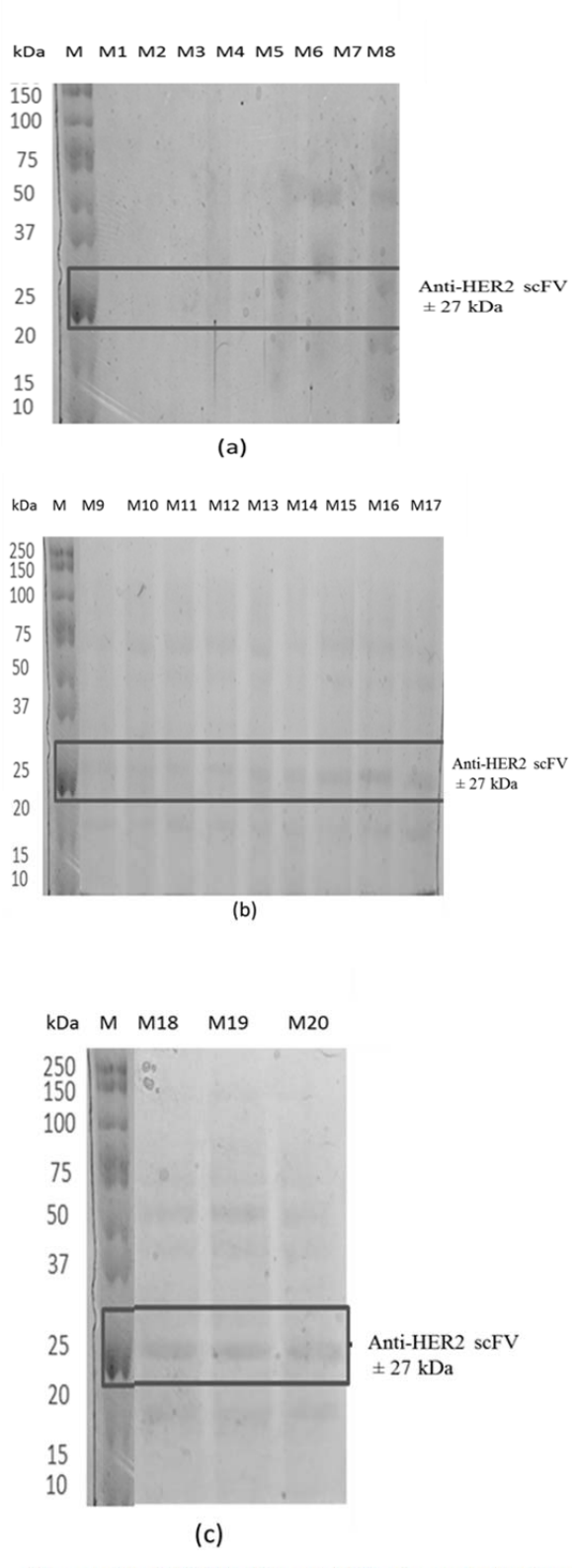

Figure 10. SDS-PAGE gel 12% characterization of dissolved anti-HER2 scFv protein in extracellular media with 4 mM L-ramnose induction. (M) protein markers; (M0) fraction before induction of L-Rhamnose; (Mi) fraction after induction of L-Rhamnose at i-hour.
(a) M0-M8 fraction, (b) M9-M17 fraction and (c) M18-M20 fraction.

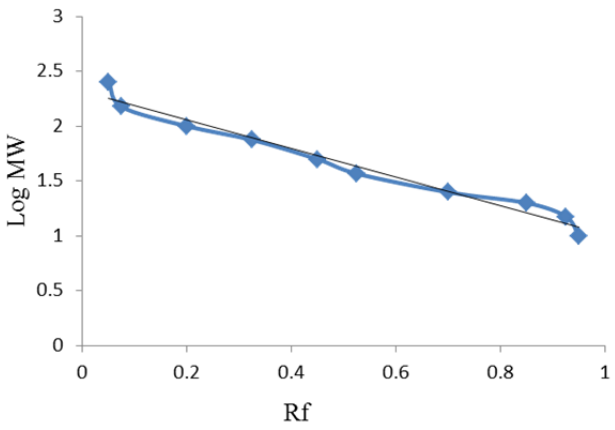

Figure 11. Linear regression curve of marka protein molecular weight with equation y= 1.3 x+2.3

DISCUSSION

The Anti Her2scFv protein had been designed as V_H-SCGGGSGGGGSGGGGS-V_L sequence. The linker sequence has been tested and proven to be used to connect V_H and V_L well on scFv. The addition of cysteine amino acids to the linker sequence can support the immunoassay test process [7].

Recombinant plasmid *pD861-pelB-anti-HER2 scFv* was successfully transformed into *E. coli* BL21 (DE3). This was characterized by the presence of linier recombinant plasmids cut by a *Sap*I with size ± 3000 pb. Sequencing result revealed that gene was the *anti-Her2 scFv* gene.

In construction of synthetic-gene encoding *anti-Her2 scFv* had selected pD861 with high expression specification as expression vector. The rhaBAD promoter in this vector has a high level of expression control and prevents leaky expression (recombinant protein expression before induction). The expression of the gene encoding *anti Her2 scFv* in recombinant plasmid *pD861-pelB-anti-HER2 scFv* was regulated by L-Rhamnose as inducer. The addition of L-Rhamnose into recombinant *E. coli* BL21 (DE3) culture was carried out at 4 h after colonies were grown on based of growth curve determination where the OD_{600} value was 0.731 and cells had reached exponential growth. Anti-Her2 scFv protein was not detected in cytoplasmic, periplasm and extracellular media fraction when L-Rhamnose has not been added to culture media. So, recombinant protein in construction with the pD861 vector had no leaky expression. After two hour administration of the inducer, the recombinant protein was in the cytoplasmic fraction and its existence for 17 hours. Recombinant proteins were produced by positive regulation performed by the rhaBAD promoter due to the presence of L-Ramnose as an inducer in the cytoplasm. After being in the cytoplasm for 2 hours, the AntiHer2 scFv protein began to migrate to the periplasm.

The Anti-HER2 scFv protein was successfully translocated into the periplasmic for approximately 12 hours. Anti-HER2 scFv was expressed in a fusion with a Pel B peptide signal that serves to secrete proteins from the cytoplasm into the periplasm space .Then, Anti-HER2 scFv that was present in periplasm was supported its folding by the DsbA, DsbB, DsbC and DsbD chaperones making formation of the disulphide bond. The highly oxidative periplasmic environment is optimum as a folding area of the protein so there is little chance of protein folding error [17]. Next, Anti Her2scFV was secretion into extracellular medium.

Anti-Her2 scFv protein was initially accumulated more obvious in the periplasm then secreted into extracellular medium characterized by a loss of protein at 17 hours after induction. Furthermore, recombinant protein was constantly seen in extracellular medium up to the 20th hour. Recombinant proteins can be translocated across membrane outer then are in the extracellular medium involving a particular protein known as secreton [17]. Even it is known that the protein must adopt a conformation that qualifies the secretion to proceed further, there is no secretion signal in the folded protein identified to make protein migrate to extracellular medium. The accumulation of recombinant proteins in periplasma can lead to the formation of osmotic pressure, which could be the driving force of transport across the outer membrane. The production of recombinant proteins may cause membrane disruption and increase selective permeability, which may support leakage. Periplasm secretion may also cause lysis, which results in the release of periplasmic content in older cultures condition [18,19,20].

CONCLUSION

This study revealed that construction of synthetic-gene encoding anti-HER2 scFv was successfully expressed. Then, Anti-HER2 scFv protein that was guided by PelB signal peptide to the periplasm and also excreted to extracellular medium had potensial good activity.

ACKNOWLEDGEMENT

This research was funded by Internal Grand of Padjadjaran University, Indonesia in 2017.

REFERENCES

1. Green M and Raina V. Epidemiology, screening and diagnosis of breast cancer in the Asia–Pacific region: Current perspectives and important considerations. Asia-Pacific Journal of Clinical Oncology 2008; 4:S5-S13
2. Gibbs, J. B. 2000. Mechanism-based target identification and drug discovery in cancer research. Science 2000; 287(5460):1969-73
3. Pohlmann PR, Mayer I A and Mernaugh R. 2009. Resistance to trastuzumab in breast cancer. Clinical Cancer Research 2009; 15(24): 7479–7491
4. Johnson E, Seachrist DD, DeLeon-Rodriguez CM, Lozada KL, Miedler J, Abdul-Karim FW and Keri RA. 'HER2/ErbB2-induced breast cancer cell migration and invasion require p120 catenin activation of Rac1 and Cdc42', Journal of Biological Chemistry 2010; 285(38): 29491–29501
5. Yin SLS. The Development of brain Chain Variable Fragment (scFv) Antibodies Against Toxoplasma Gondii By Phage-Display. Thesis. Universitas Malaya. Kuala Lumpur 2012.
6. Bach HY, Mazor S, Shaky A, Shoham-Lev Y, Berdichevsky DL, Gutnick and Benhar I. Escherichia coli maltose-binding protein as a molecular chaperone for recombinant intracellular cytoplasmic single-chain antibodies. J Mol Biol 2001; 312:79-93
7. Liu Y, Liu Y., Raymond L, Mernaugh and Zeng, X. Single chain fragment variable recombinant antibody functionalized gold nanoparticles for a highly sensitive colorimetric immunoassay. Biosensor and Bioelectronics 2009;24: 2853-2857
8. Baneyx F and Mujacic M. Recombinant protein folding and misfolding in E. coli. Nature Biotechnology 2004; 22(11):1399-1408

9. Choi JH and Lee SY. Secretory and Extraceluller Production of Recombinant Proteins Using Escherichia coli. Appl Microbiol Biotechnol 2004; 64: 625-635
10. Kudva R, Denks K, Kuhn P, Vogt A, Muller M and Koch HG. Translocation Across The Inner Membrane of Gram-Negatif Bacteria: The Sec and Tat Dependent Protein Transport Pathways. Research in Microbiology 2013;164:505-534
11. Sambrook J, Maniatis T, Fritsch EF. Molecular Cloning: a Laboratory Manual. cold Spring Harbor Laboratory Press. New York 2001;pp. 1.74-1.84
12. Kaitu, RAM, Sidharta BR and Atmodjo K. Aktivitas antibakteri fungi endofit jahe merah (Zingiber officinale var. rubrum) terhadap Escherichia coli dan Streptococcus pyogenes. http://e-journal.uajy.ac.id/3970/
13. Sriwidodo, Imam PM, Riswanto N, Rostinawati T and Subroto T. Extracellular secretion recombinant of human epidermal growth factor (hEGF) using pectate lyase B signal peptide in Escherichia coli BL 21(DE3). Int.J.Res.Pharm.Sci 2017; 8(1): 33-40
14. Quan S, Hiniker A, Collet JF, Bardwell JC. Isolation of bacteria envelope proteins.Quan Methods Mol Biol 2013; 966:359-66
15. Coligan, JE, Dunn BM, Ploegh HI, Speicher DW and Wingfield PA. Current protocols in protein science. John Willey and Sons, Inc., Washington 2006
16. Jazwinski SM and Edelman GM. Evidence for participation of a multiprotein complex in yeast DNA replication in vitro. J Biol Chem 1982;11:6852–6857
17. Mergulhao FJ, Summers DK, Monteiro GA. Recombinant protein secretion in Escherichia coli. Biotechnol Adv 2005; 23: 177-202
18. Sandkvist M. Biology of type II secretion. Mol Microbiol 2001;40:271–83.
19. Lory S. Secretion of proteins and assembly of bacterial surface organelles: shared pathways of extracellular protein targeting. Curr Opin Microbiol 1998;1:27–35.
20. Pugsley AP, Francetic O, Possot OM, Sauvonnet N and Hardie K. Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria—a review. Gene 1997;192:13–9