Construction and Expression of Immunotoxin Anti EGFRvIII scFv-HPR Conjugate in *Pichia pastoris* as A Targeted Drug Candidate for Cancer Therapy

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Abstract

Epidermal growth factor receptor variant III (EGFRvIII) is a mutant of EGFR lacking 267 amino acids (exon-2 through 7) within its extracellular domain, resulting in the formation of a new epitope as a tumor-specific target. EGFRvIII is commonly found in GBM, breast, ovarian, prostate, and lung carcinomas. Antibodies or part of antibodies (e.g. single chain variable fragment or scFv) with specific activity against this receptor have been developed. These antibodies are internalized into the cell after receptor binding. Ribonucleases can be cytotoxic due to their inherent ability to degrade RNA, therefore causing cell death via inhibition of protein synthesis. The aim of this research is to construct, clone and express an immunotoxin-based conjugate combining an anti-EGFRvIII scFv with a HPRmut (human pancreatic ribonuclease mutant variant) in *Pichia pastoris*. The anti-EGFRvIII scFv gene was cloned into yeast expression vector pPICZαA and fused with EGFP gene as a marker. The HPRmut gene was subsequently cloned at the C-terminal of the scFv::EGFP fusion, resulting in the scFv::EGFP::HPR fusion construct. The fusion construct was successfully obtained and nucleotide sequence of plasmid was verified. This construct was used to transform *Pichia pastoris* SMD 1168H. The gene fusion was successfully transformed and expressed in *P. pastoris* with a transformation efficiency of 10^2 cfu/μg DNA. The transformed yeasts were screened on agar media containing up to 1000 μg/ml zeocin. Yeast transformants showed green fluorescent due to the expression of EGFP gene. The recombinant proteins have been expressed and secreted from *P. pastoris* as showed by immunoblotting and SDS-PAGE analyses, then purified by affinity chromatography method. The selected yeast transformant produced at least 15.85 mg of purified protein per liter of yeast culture.

Keywords: EGFRvIII, HPR (*human pancreatic ribonuclease*), immunotoxin conjugate, anti-EGFRvIII scFv, *Pichia pastoris*.

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Introduction

The association of *epidermal growth factor receptor* (EFGR) with cancer cells had been studied in the past. Overexpression of EGFR is often found on the surface of many cancer cells and is known to play a major role in the development of cancer cells. Therefore, EGFR or its various mutated forms become subject for molecular target in cancer therapy. EGFR was found on the cell surface and binds its natural ligands including growth factors such as EGF or TGFα that cause cells to divide. In some types of cancer cells, EGFR molecules are found in excessive quantities compared to normal cells. Abundance of EGFR molecules on cell surface makes cancer cells divide more rapidly than normal cells (Arteaga, 2002).

Natural EGFR is also present in normal tissue. However, it was found that EGFR has various forms of mutations which might be specific for each cancer type. Studies revealed that EGFR on cancer cells underwent mutations and many different variants of EGFR mutants were found in various types of cancer cells (Kuan *et al.*, 2001 and Frederick *et al.*, 2000). Epidermal growth factor receptor variant III (EGFRvIII) is the most common

mutant variant found in a number of solid tumors, including glioblastoma multiforme (GBM), breast cancer, brain cancer (medulloblastoma), and cervical cancer. EGFRvIII is rarely found in normal tissues. It is a mutant variant of EGFR which has deletions in exon-2 through exon-7. Most of the extracellular domain is lost and a new arrangement of amino acid at its N-terminal of the receptor becomes a unique new epitope. The amino acid glycine is formed at the junction of fusion between exon-1 and exon-8. Fusion between exons-1 and 8 as well as the formation of glycine create a new antigen-specific variant of EGFR mutant. This mutant variant is known as EGFRvIII variant (Gupta *et al.*, 2010).

Single chain antibody fragment (scFv) is part of the antibody molecule comprising a variable light-chain (VL) and variable heavy-chain domain (VH) connected by a peptide linker. scFv molecule is much smaller (30 kD) compared to an intact antibody molecule (e.g. IgG 150 kD). Therefore, it is expected that scFv molecules can penetrate tissue of tumor cells better (Sun *et al.*, 2003). Alternative strategy for targeted drug delivery can be done by means of an immunotoxin, which combines an antibody fragment (such as scFv) with a toxin molecule in a conjugated form of molecule. Immunotoxin-based immunotherapy has been developed in the last decade, such as 'ImunoRNase' (IR) using ribonuclease (RNase) molecule as active compounds (toxins) (Lorenzo *et al.*, 2004). One type of RNase that will be used in this study is human pancreatic RNase (HP-RNase or HPR). RNase is not cytotoxic on normal cells, but it becomes toxic when it enters cancer cells (Rybak & Newton, 1999).

RNase is a small protein (10-28 kDa). The toxicity of RNase is due to its enzymatic activity that is able to cut phosphodiester bonds in RNA strands. RNAse binds to negatively charged cell membranes, enters the cell by endocytosis and translocates into the cytosol to avoid ribonuclease inhibitor (RI) protein that degrades RNase (Ardelt *et al.,* 2009). Gaur *et al.* (2001) stated that human pancreatic ribonuclease (HPR) is secreted in nature and has been considered as a counterpart of bovine pancreatic RNase A. Although HPR shares 70% homology with RNase A and possesses similar key structural and catalytic residues, it displays some unique features. Unlike bovine serum RNase and onconase which are toxic when they enter a human cell, human pancreatic RNase (HPR) is not so or far less toxic when it enters the cell. This is due to the fact that there is a tight interaction between this HPR and RNase inhibitor (RI) within the cytosol that neutralizes its hydrolytic activity. Leland *et al.* (2001) showed that affinity of a ribonuclease for RI plays an integral role in defining the potency of a cytotoxic ribonuclease. HPR holds tremendous promise as a therapeutic agent for humans. Compared to other RNases, it is likely to be less immunogenic and thus more efficacious (Gaur *et al.*, 2001; Lorenzo, *et al.*, 2002; Castro, *et al.*, 2011). Therefore, generating HPR variants which exhibit enhanced resistance toward inactivation against human RI (hRI) and more cytotoxic is very attracting. Gaur *et al.* (2001) have investigated HPR in its interaction with hRI. Contact residues were mutated either individually or in combination to generate mutants. Some mutants showed an ability to evade hRI more effective than wild type HPR. Leland *et al.* (2001) constructed a mutant variant ERDD-RNase1, which has conformational stability and ribonucleic activity similar to the wild-type enzyme, but exhibited less affinity for endogenous cytosolic hRI. Most significantly, ERDD RNase1 was found to be toxic to human leukemia cells (Leland et al., 2001)

Pichia pastoris was chosen for the production of this chimeric protein. *P. pastoris* is a methilotropic yeast that has been shown to be suitable to produce complex recombinant proteins at high yield and is able to grow in a simple, low-cost medium. Other advantages of the use of *P. pastoris* include very high intracellular or extracellular protein production, high cell density in fermentation, genetically stable cell-lines and production of large amount of heterologous protein for analytical studies (Hames & Higgins, 1999). This *Pichia* expression system offers ability to express therapeutic complex proteins that is free from animal viruses and endotoxin contamination, which can be applied not only for drugs but also for other biotechnology applications (Joosten *et al.*, 2003). Protein expression in *P. pastoris* is based on the use of tightly regulated *alcohol oxidase* (AOX1) promoter. The activity of this promoter is strictly regulated by the presence of methanol

as carbon source in the medium. The protein yield can be significantly enhanced by manipulating factors that influence gene expression and product stability.

In this study, we have cloned the open reading frame fusion of scFv::EGFP::HPRmut into pPICZα expression vector under the control of inducible AOX1 promoter (P_{AOX}) in attempt to express an immunotoxin conjugate molecule of anti-EGFRvIII scFv and HPR mutant variant as a toxic agent.

Several studies have reported construction of such immunotoxin molecules, including MR1 scFv, which was fused to a bacterial toxin (domains II and III of Pseudomonas exotoxin A) (Lorimer *et al*., 1996), gene fusion of recombinant human EGFR and HPR that showed therapeutic benefit (Psarras *et al*., 1998), and construction of an anti-CD30 directed immunotoxin fused to hpRNase (Braschoss *et al*., 2007). Thus, the aim of this research was to construct an immunotoxin conjugate of an anti-EFGRvIII antibody scFv fragment with a mutant variant of HPR (ERDD-HPR) and use of EGFP in the gene fusion as reporter gene in the *Pichia* expression system. Here we describe the construction of the fusion gene of scFv::EGFP::HPRmut by subsequent cloning of individual gene into the pPICZα vector, transformation of the recombinant plasmid into *P. pastoris* SMD1168H strain, selection of transformed cells, the chimeric protein overexpression, and their purification.

Materials and Methods

Microorganisms, plasmids, primers and genes. *Escherichia coli* TOP10F' and *Pichia pastoris* SMD 1168H were purchased from Invitrogen. *E. coli* DH5α was used for cloning purpose. Plasmids used were pPICZαA (Invitrogen), pJ201-scFv (DNA 2.0), pJ912-HPR (DNA2.0), pEGFP-1 (Clontech). All DNA primers used in this study were purchased from 1st Base. The anti-EGFRvIII scFv and HPRmut genes were ordered and made synthetically by DNA 2.0, cloned in pJ201-scFv and pJ912-HPR, respectively.

Sub-cloning of scFv gene into pPICZαA vector. All procedures for plasmid isolation, gene cloning, ligation and transformation of recombinant plasmid into *E. coli* referred to Ausubel *et al.* (2002). All restriction enzymes used were purchased from Thermo Scientific. The scFv gene of anti-EGFRvIII was made synthetically at DNA2.0 based on previously published sequence (Weber *et al*., 2009), cloned in pJ201-scFv. The scFv gene was PCR-amplified by two-steps PCR method. First, the gene was amplified from pJ201-scFv using a pair of primers, VH101-F (5'-AAGTTCAATTGGTTGAGTCAGGAG-3') and VL101-R (5'-TTTGATTTCGACTTTA GTTCCTTGACCA-3'). Secondly, the gene was re-amplified using another pair of primers containing *XhoI* sites, VH-101-F-NdeXho (5'-CATATGCTC-GAGAAGAGGGAG-3') and VL101-R-NdeXho (5'-CATATGCTCGAGTC ATTAACAATGATG). Both gene and vector pPICZα were digested with *XhoI*, isolated and purified. The gene was cloned into the vector at *XhoI* site right after α-factor secretion signal, resulted in the recombinant plasmid pPICZα-scFv. The plasmid sequence was verified and the correct one was used for the next sub-cloning.

Sub-cloning of EGFP gene into pPICZα-scFv. EGFP gene was PCR-amplified from pEGFP-1 in two-step PCR. First, the gene was amplified using the following primers, EGFP-F (5'-CTTGTACAG CTCGTCCATGCCG- 3') and EGFP-R (5'-ATGGTGAGCAAGGGCGA GGAG-3'). Secondly, the gene was re-amplified using another pair of primers containing a short spacer (G4S) and *ClaI* sites, EGFP-F-G4S-ClaI (5'-GGGATCGATGGTGG CGGTTCTGGAATGGTGAGCAAGGGCGA GGAG-3') and EGFP-R-ClaI (5'-GGGGATA TCCTTGTACAGCTCGTCCATGCC-3').
Both gene and plasmid were ligated and transformed into *E. coli* TOP10F' according to the general protocol of molecular biology (Ausubel *et al*., 2002). *E. coli* transformants were selected on LS-LB agar medium (1%peptone, 0.05% NaCl, 0.5 % yeast extract, 1.7% agar) containing zeocin (25 ug/ml) and analyzed by colony PCR. Positive clones containing DNA inserts were analyzed further and sequence verified.

Sub-cloning of HPR gene into pPICZα-scFv-EGFP. The HPRmut gene (Leland *et al.*, 2001) was PCR-amplified from pJ912-HPR in two steps. First, the gene was amplified using the following primers, HPRmut-F (5'-AGTGCTATCCTCCACTGAAGC-3') and

HPRmut-R (5'-AAAGAGTCTTGTGCTAA AAAGTTTCAA-3'). Secondly, the gene was re-amplified using another pair of primers containing a short spacer (G4S) and *XbaI* sites, HPRmut-F-G4S-Xba (5'-GGGTCTAGAGGA GGTGGCGGTTCTAAAGAGTCTTGTGCT AAA-AAGTTTCAA-3') and HPRmut-R-Xba-mod (5'-CTTTCTAGAAGTGCTATCCTC CACTGAAGCCC-3'). Both gene and plasmid were cut with *XbaI*, isolated and purified. The gene was ligated and transformed into *E. coli* TOP10F' according to Ausubel (2002). *E. coli* transformants were selected on LS-LB zeocin agar medium. Transformant cells were analyzed by colony-PCR using primers AOX1-F (5'-GACTTGTTCCAATTGACAA GC-3') and AOX1-R (5'-GCAAATGGC ATTCTGACATCC-3'). Positive clones were further analyzed to determine gene orientation by PCR using primers HPRmut-F (5'-AGTGCTATCCTC-CACTGAAGC-3') and AOX1-R (5'-GCAAATGGCATTCTGACAT CC-3'). The recombinant plasmid was isolated from the correct clone and verified by sequencing.

***P. pastoris* transformation and selection of transformed cells.** The selected recombinant plasmid pPICZα-scFv-EGFP-HPR was linearized with *SacI*. Transformation of the plasmid into yeast cells was carried out by electroporation according to the protocol suggested by Invitrogen and Biorad. A total of 0.5 μg of linearized DNA was transferred into 60 μl of competent cells of *P. pastoris* SMD1168H strain in an electroporation cuvette (2mm gap, BioRad). The cells and DNA mixture in the cuvette was incubated on ice for 2 minutes, then electroporation was carried out using Gene Pulser II (Bio-Rad) with the following conditions: voltage (V) 2.5 kV, capacitor (C) 50 μF, resistance (R) 200 Ω, and 5 ms pulse. Transformed colonies were grown and selected on YPDS zeocin selection medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 1.7% agar) containing 100 μg/ml zeocin (Invitrogen).

Selection of *P. pastoris* transformants. Selection of *P. pastoris* transformants were carried out according to procedures given by Invitrogen. All of transformant colonies that grew on the selection medium were transferred into a new fresh YPD zeocin medium containing 100 μg/ml zeocin. The plates were incubated at 30°C for 2 to 4 days. Stable transformants that grew on this medium were further transferred into YPD zeocin agar medium containing higher concentrations of zeocin (200, 500, and 1000 μg/ml, respectively) to acquire recombinant clones having multiple copies of target gene.

Protein expression of *P. pastoris* transformants. Ten individual transformant colonies were cultured to evaluate recombinant protein expression according to manual instructions of Pichia expression kit (Invitrogen). Transformed and non-transformed *P. pastoris* cultivated in 2 ml of YPD medium (with or without zeocin) were incubated at 30°C and 250 rpm for 24 hours. These cultures were used to inoculate 25 ml of BMGY production medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 1% glycerol). Cultures were incubated at 30°C and 250 rpm for 24 hours. The cells were harvested aseptically by centrifugation before they were transferred into 25 ml BMMY induction medium (1% yeast extract, 2% peptone, 100mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 0.5% methanol) with starting OD_{600}=1.0. Cultures were incubated at 30°C and 250 rpm for 3 days and methanol was added at 0.5% (v/v) into the culture every 24 hours. Cultures were harvested by centrifugation (10.000 rpm, 15 min, 4°C). Cell biomass was stored at -20°C and the cell-free culture medium (supernatant) was stored at 4°C for further protein analysis with SDS-PAGE and immunoblotting (slot blot).

Analysis of expressing cells with microscopy and immunoblotting. Due to fusion of EGFP within the antibody construct, transformed yeast cells could be observed for recombinant protein expression using fluorescence microscope. Yeast cell cultures were observed using ApoTome-2 fluorescence microscope (Carl Zeiss) with 40× magnification using FITC filter.

Recombinant protein (extracellular product) was analyzed using SDS-PAGE and immunoblotting (slot-blot) methods. Proteins were precipitated from cell-free supernatant samples by TCA solution (trichloro acetic acid) (Sanchez, 2001). Subsequently, protein samples were applied onto nitrocellulose

membranes (Biorad) by vacuum process. Membrane was blocked with 5% fat-free milk in PBS (Phosphate buffered saline)-Tween20 for 1 hour, then washed three times with PBS-Tween20 each for 5 minutes. Membrane was incubated with 1:2,500 dilution of anti-His antibody (Santa Cruz) in 10% non-fat milk in PBS-Tween20 for 1 hour, and washed three times with PBS-Tween20 each for 5 minutes. Membrane was incubated with the secondary anti-mouse IgG-alkaline phosphatase conjugate (Santa Cruz) in 10% non-fat milk for 1 hour and washed three times with PBS-Tween20 each for 5 minutes. Detection was carried out using WesternBlue stabilized substrate for alkaline phosphatase (Promega).

Protein purification with affinity chromatography. Protein sample in cell-free culture medium (supernatant) was concentrated using ammonium sulfate precipitation method according to Page and Thorpe (2009). An amount of 70 ml protein solution was precipitated with 80% saturation of ammonium sulfate. Precipitated proteins were dissolved in 1 ml of 50 mM phosphate buffer pH 7.4 and dialyzed against the same buffer using dialysis tubing with 3,500 Da cut-off (Spectra/Por® Dialysis Membrane) resulting in 2.93 ml of dialyzed protein. Protein purification was performed using 1 ml of dialyzed protein and 250 µl of Ni-NTA resin (Qiagen). 10× CV Binding buffer was added into protein sample (in 1:10 ratio) before it was applied into Ni-NTA column previously equilibrated with 5 column volumes (5×CV) of binding buffer. Protein sample was added to the matrix in a sealed tube and incubated overnight at 4°C with rotation. They were put back into the column, and rinsed for purification of proteins. Flow-through fraction (FT) was collected as well as washing fractions (W) (5×CV). The column was then eluted with elution buffer containing 100 mM imidazole and elution fractions (E) were collected (8×CV). Purified protein was analyzed by SDS-PAGE and protein concentration was measured using Qubits® Fluorometer (Invitrogen).

Results and Discussion

Construction of fusion protein scFv::EGFP

Plasmid pPICZαA containing the gene encoding anti-EGFRvIII scFv (pPICZαA-scFv) has been previously constructed (Hertati, 2012). The scFv gene was cloned at *XhoI* next after yeast secretion signal mating factor-α (MF-α) for secretion of the recombinant scFv fragment. The sequence of pPICZα-scFv plasmid (clone no.41) had been verified by sequencing and was used for the construction of scFv::EGFP::HPR fusion protein. A short flexible linker $(G_4S)_n$ was incorporated between scFv and EGFP or between EGFP and HPR, where n was either 2 or 1. The linker was introduced by PCR primers during cloning of EGFP or HPR genes. Linker such as $(G_4S)_n$ was chosen in order to give flexibility in the structure of the fusion protein. Huang and Shusta (2006) used the same linker when fusing scFv or scTCR with GFP, except that they used 3 repeats of G_4S. Fusion protein of scFv::GFP using this type of linker was expressed better and formed a correct structure in yeast *S. cerevisiae* compared to the same fusion protein without linker, though the protein without linker could also be expressed as well (Huang & Shusta, 2006).

EGFP gene has been successfully cloned into pPICZα-scFv plasmid at the *ClaI* site, resulted in the recombinant plasmid pPICZα-scFv-EGFP. A number of 115 *E. coli* transformants were screened by colony PCR using AOX1F and AOX1R primers. Positive clones produced a PCR product with the size of 2 kb (Figure 1A). There were 23 colonies out of 115 which were found to have EGFP insert. However, only 14 clones exhibited the correct gene direction. They were confirmed by PCR using EGFP-F and AOX1-R primers. Clones having EGFP gene with correct direction showed DNA band of around 1000 bp (Figure 1B). Four clones were sequenced, and one clone showing the correct sequence was used for the next sub-cloning.

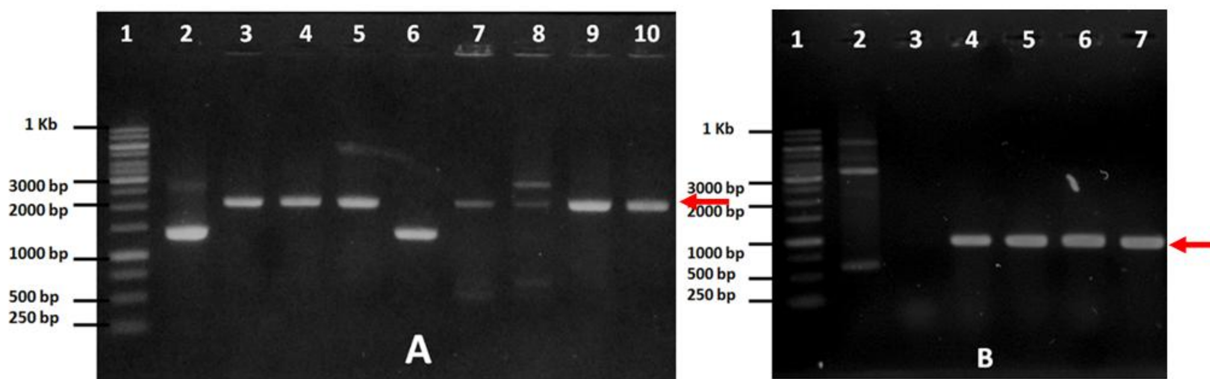

Figure 1. Subcloning of EGFP gene into pPICZα-scFv in *E. coli* TOP10F'. (A) Gene insertion was confirmed by PCR using a pair of primers (AOX1-F and AOX1-R). Positive clones having the right insert gave a DNA band of around 2kb. Lane 1: DNA ladder, lane 2: negative control, lane 3-10: different clones of *E. coli*. (B) Gene direction was confirmed by PCR using a pair of primers (EGFP-F and AOX1-R). Positive clones having the right gene direction showed a DNA band of around 907 bp. Lane 1: DNA ladder, lane 2: negative control, lane 3-7: different clones of *E. coli*.

Construction of a fusion protein scFv::EGFP::HPR.

Insertion of HPR gene into the vector pPICZα-scFv-EGFP resulted in around 18 colonies of transformants harboring the recombinant plasmid pPICZ-scFv-EGFP-HPR. A short linker (G_4S) was also introduced between EGFP and HPR genes. Screening of the transformant colonies was performed by colony PCR using AOX1F and AOX1R primers, yielding a DNA band of 2400 bp (not shown). Due to a single restriction site that we used (*XbaI*), the recombinant clones should be confirmed further by PCR method for correct HPR gene direction. We have 16 out of 18 clones that showed a DNA band of 650 bp using HPR-F and AOX1R primers (Figure 2A), which confirmed the correct direction of HPR within the whole construct. For further analysis, the recombinant plasmid pPICZα-scFv-EGFP-HPR was digested with *XbaI* and should give 400bp DNA fragment after digestion (Figure 2B). The so verified plasmid clones were further verified by DNA sequencing. Two clones were sequence-verified (clones no.2a and 11b) and showed correct whole sequence of fusion gene construct. The map of the final plasmid construct pPICZ-scFv-EGFP-HPR is depicted in Figure 3. HPR gene was intently placed at the C-terminal domain of the construct to avoid any steric hindrance that might result from the structure of the fusion construct. Short linker peptides that were introduced between those three domains of the protein were expected to ensure flexibility of protein folding of each domain. So far, we have not found any report yet, on the fusion of three domains of different proteins including antibody fragment, GFP and another protein within a single construct for expression in *P. pastoris*. A fusion construct of A33scFv-GFP was successfully expressed in *P. pastoris*, placing the GFP at the N-terminal and the scFv at the C-terminal orientation (Petrausch *et al*., 2007). Relative position of GFP and scFv, either at N- or C-terminal, did not affect secretion level of the fusion protein in yeast, nor the length of the peptide linker used (Huang and Shusta, 2006). Zewe *et al*. (2007) also constructed a fusion of human pancreatic RNase with a scFv against an internalizing antigen, the transferrin receptor (CD71) and expressed it in *E. coli*. A fusion protein which has a very low immunogenic potential in human with no inherent toxicity may be a potent cytotoxin for tumor cells subsequent to antibody. The human pancreatic RNase, however, is per se a non-toxic enzyme that only becomes harmful to cells after being internalized by antibody-mediated endocytosis. This fusion protein is therefore a promising reagent, either alone or in combination, for the therapy of tumors that overexpress transferrin receptor (Zewe *et al.*, 2007).

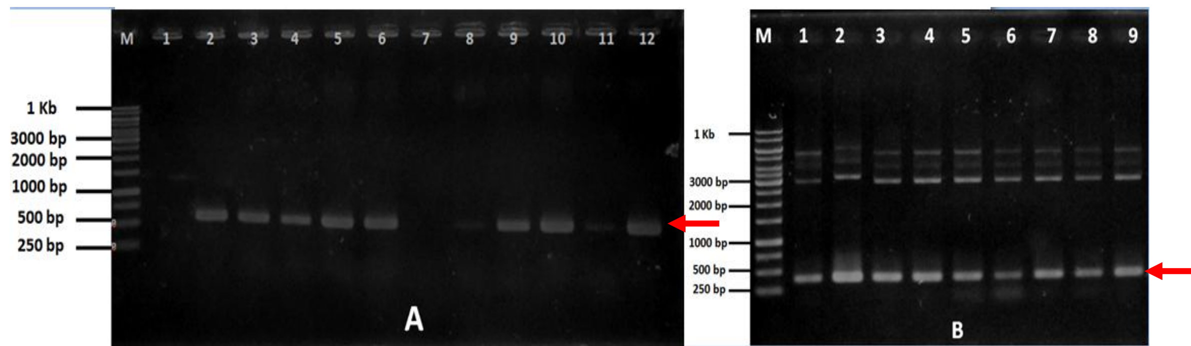

Figure 2. Subcloning of HPR gene into pPICZα-scFv-EGFP in *E. coli* TOP10F'. (A) Confirmation of gene insertion and orientation in the recombinant plasmid using PCR method and primers HPR-F and AOX1-R; M: DNA ladder; lane 1: negative control; lane 2~12: different clones of *E. coli*. Positive clones having the right direction of insert give a DNA band of around 584 bps. (B) Several clones of recombinant plasmids were cut off with *Xba*I. Positive clones showed a DNA band of 400 bps (marked by an arrow).

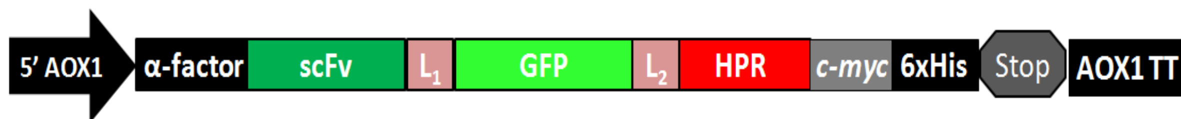

Figure 3. Schematic description of the gene fusion construct scFv::EGFP::HPR in pPICZα plasmid. 5'AOX1= promoter for alcohol oxidase gene; α-factor= *S. cerevisiae*-derived secretion signal sequence; scFv= anti-EGFRvIII antibody fragment; GFP= enhanced-GFP (EGFP); HPR= *human pancreatic ribonuclease* gene; c-myc= an epitope marker; 6xHis= polyhistidine tag; Stop= stop codon; AOX1 TT= translation terminator sequence. There are short linkers between scFv and GFP (L_1) (GGGSIDGGGGS), and between GFP and HPR (L_2)(IDSRGGGGS).

Transformation, selection and analysis of *P. pastoris* transformants

After confirmation by sequencing followed by linearization with *Sac*I enzyme, the recombinant plasmid pPICZα-scFv-GFP-HPR was transformed into yeast cells by electroporation, so that the recombinant plasmid could be stably integrated into the yeast genome and express the protein. The recombinant protein expressed is a fusion protein containing α-factor secretion signal at the N-terminal and myc epitope and 6xHis-tag at the C-terminal. The transformation process yielded 51 individual transformed colonies of *P. pastoris* with the transformation efficiency of 1.02×10^2 cfu/µg of plasmid DNA. Cell-growth state, cell density, incubation time, medium used, and the amount of DNA used influence the transformation efficiency. The transformation efficiency obtained here was slightly lower than the average value of 10^3 cfu/µg of plasmid DNA using the same electroporation protocol. This plasmid (pPICZα) does not contain any yeast replicon fragment, therefore integration of the plasmid into the genome was absolutely required for the transformants to be stable (Gietz and Woods, 2001).

Some expression vectors for *Pichia* can increase the number of gene copies in *P. pastoris*, so that the amount of expressed proteins will be higher. The pPICZα vector also carries zeocin resistance gene, so that the selection of transformants carrying multiple copies of the integrated vector can be conducted. Multiple insertions can be identified through increased resistance to zeocin. Genetic stability analysis were selected from single colonies growing on YPD agar medium containing 100, 200, 500, and 1000 µg/ml zeocin, respectively (Figure 4). YPD agar medium without zeocin was also used as control. Figure 4 showed that most of transformed colonies look stable in medium with zeocin concentration up to 500 µg/ml. Growth of several colonies was stunted in zeocin concentration of 1000 µg/ml, thus approximately only 53% of colonies were considered stable. When determining the gene copy number within the genome of

transformed yeast, Norden *et al.* (2011) affirmed that there were variations in the dose of antibiotic tolerance due to differences in the recombinant genes among different clones. Assuming that the *Sh ble* gene is incorporated in the same ratio as the AOX1 TT sequence, an estimated 1 copy (minimum) of the gene *Sh ble* zeocin resistance is required for growth at 100 µg/ml zeocin, 4 copies at 500 µg/ml, 9 copies at 1000 µg/ml, and clones with as many as 17 copies of gene are found from a petridish with the highest antibiotic concentration of 2000 µg/ml (Norden *et al*., 2011).

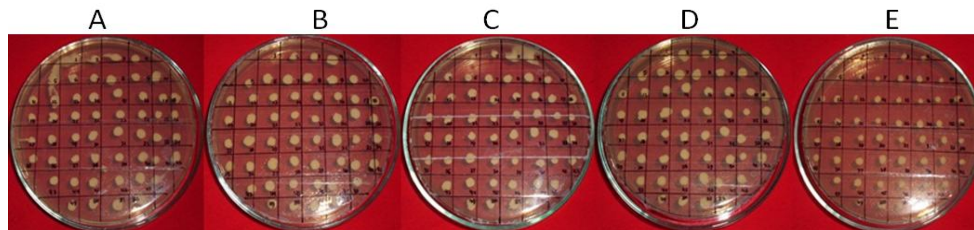

Figure 4. Screening of genetically stable transformed yeast cells on zeocin plates containing various concentration of zeocin : (A) 0 µg/µl (as control), (B) 100 µg/µl, (C) 200 µg/µl, (D) 500 µg/µl, and (E) 1000 µg/µl.

Observation under fluorescence microscopy revealed that transformed *Pichia* cells exhibited green fluorescence (Figure 5). It proved that GFP has been functionally expressed from the transformed cell. Since this GFP is fused at both end with scFv and HPR, this fact may deduce the functionality structure of those both domains. GFP has been widely used as a probe for intracellular dynamics and localization (Huang and Shusta, 2006). Given that the secretion efficiencies of both unfused scFv and scFv fusions varied dramatically from one scFv class to another, they have investigated the possibility of using GFP as probe for the secretory pathway of heterologous proteins. The fusion protein was expected to be secreted as a full-length product and also possessed active GFP fusion moieties. Nevertheless, the difference in relative position of fusion proteins (GFP and scFv, either at N- or C-terminal) was not resulting different amounts of fluorescent protein but resulting different intracellular localization patterns (Huang and Shusta, 2006). The formation of these fluorescent particles occurs because GFP protein folded and trapped in the cytoplasm This is likely to occur as a consequence of the high level solubility of GFP within the cytoplasm because of the very high expression, and also the possibility of a transfer of GFP into some organelles (Zupan *et al*., 2004).

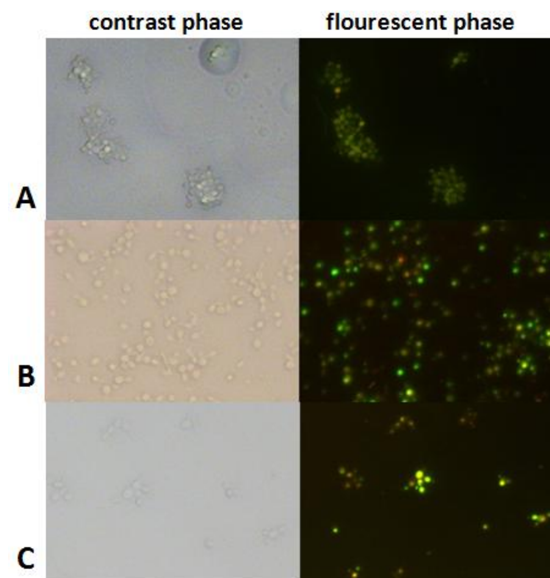

Figure 5. Confocal microscopy of transformed and non-transformed *P. pastoris* cells on contrast (left) and fluorescent (right) phases. Non-transformed cells (no EGFP) as control (A). Transformed cells show fluorescence (marked by arrow) from clone no.48 (B) and clone no.50 (C).

The fusion protein was constructed with a His-tag at the very end of the C-terminal. His-tag is mostly used in the production of proteins to facilitate purification and detection of the desired protein. The result of immunoblotting assay (slot blot) of five selected samples showed that the recombinant protein was detected by anti-His antibody (Figure 6A). The

recombinant protein was also displayed on SDS-PAGE (Figure 6B). Slot blot analysis is a semi-qualitative technique to detect and characterize total recombinant protein expressed from the host cells, whereas western blot may confirm the yield of protein with high sensitivity and a clear molecular weight of analyzed protein (Zhu *et al.*, 2005). Western blot analysis was also performed to determine the molecular weight of the recombinant scFv fusion protein as shown in Figure 7B.

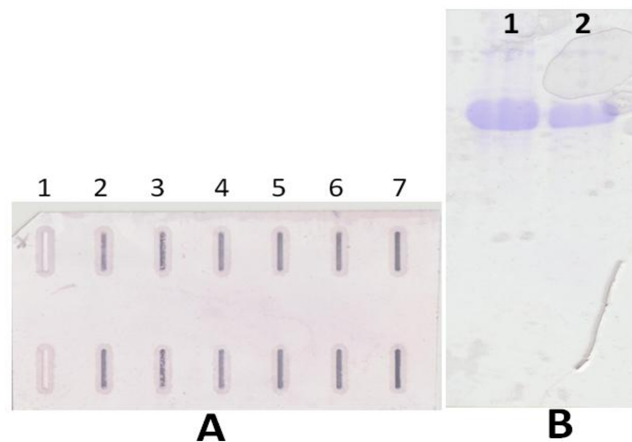

Figure 6. Expression of *P. pastoris*-derived scFv::EGFP::HPR fusion protein from several yeast transformant clones (6 clones), analyzed using slot-blot (A) and gel electrophoresis methods (B). (A) In slot-blot analysis each column represents protein samples in duplicate; column 1: negative control; column 2-7: extracellular protein from culture medium of yeast transformants. (B) Concentrated recombinant chimera protein detected using gel electrophoresis; lane 1: clone no.48; lane 2: clone no.50.

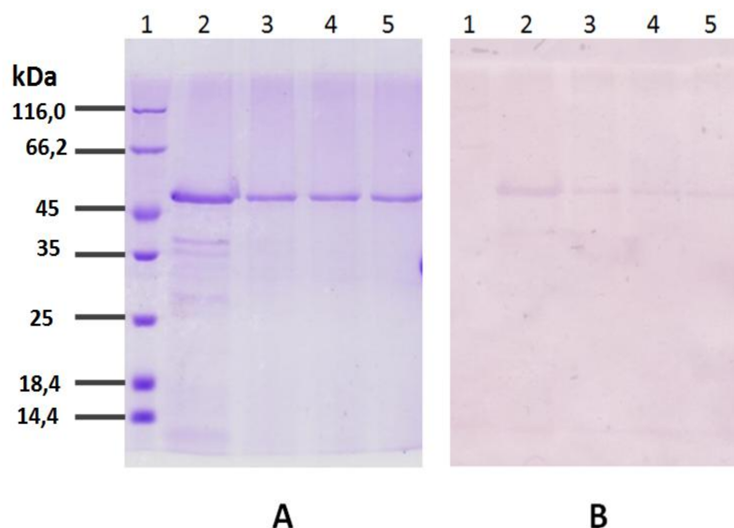

Figure 7. Isolation and purification of *P. pastoris*-derived recombinant scFv::EGFP::HPR fusion protein. Protein was analyzed with SDS-PAGE (A) and (B) Western blot. Lane 1: protein MW marker; lane 2: concentrated crude protein; lane 3-5: elution fractions of purified protein.

The recombinant fusion protein expressed in *P. pastoris* was purified using column affinity chromatography on Ni-NTA agarose (Qiagen). Protein contaminants were efficiently washed with washing buffer containing 10 mM imidazole. The recombinant protein containing Histag was efficiently maintained in the column matrix. Elution was performed with 100 mM imidazole. Purified product was then verified with SDS-PAGE (Figure 7A) and western blot (Figure 7B). Determination of protein concentration was performed for the first three elution fractions where most of the target

protein was eluted. Purified protein concentrations were 720, 484 and 376 µg/ml for the first three elution fractions respectively. The estimated amount of the total recombinant protein produced per liter of yeast culture was 15,85 mg. According to Maeng *et al.* (2012) the yield of the purified anti-BNP scFv expression in Pichia was 150 µg/ml and the purity was >95% based on SDS-PAGE analysis. A yield of 5 to 20 mg of purified protein per L culture medium has been achieved for production of scFv against ED-B domain of fibronectin in *P. pastoris* (Marty *et al*., 2001).

Conclusion

The fusion gene scFv-GFP-HPR has been successfully incorporated into the vector pPICZα and was confirmed by sequencing. The fusion gene was successfully transformed in *P. pastoris* with a transformation efficiency of 102 cfu/ug of DNA. The fusion protein was produced in *P. pastoris* as 55 kDa in size. The total amount of the recombinant protein produced per liter of yeast culture was estimated to be 15.85 mg.

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