Expression of an Anti-Transferrin Receptor Antibody SCFV Fragment in *Escherichia coli* Using A L-Rhamnose-Based Tightly Regulated Promoter System

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Abstract

Transferrin receptor (TfR) levels are elevated in various types of cancer cells and correlate with the aggressive or proliferative ability of tumor cells. Therefore, TfR levels are considered useful as a prognostic tumor marker, and TfR is a potential target for drug delivery in therapy of malignant cells. In such kind of targeted delivery system, antibody fragments are frequently used as targeting moiety. Here, we report the generation of an anti-TfR single-chain antibody variable (scFv). The cDNA encoding the variable heavy and light chain domains of the scFv antibody fragment was derived from the anti-TfR monoclonal antibody LUCA31. The gene encoding the anti-TfR scFv fragment was codon optimized for expression in *Escherichia coli*, subsequently synthesized, and cloned into the pJExpress-804 Rhamex vector. The expression vector utilizes the *E. coli rha*B promoter and corresponding regulatory genes, and is tightly regulated by the presence of L-L-rhamnose. It is also tightly regulated in the absence of L-L-rhamnose by the addition of D-glucose. The His_6-tagged anti-TfR scFv fragment was expressed in *E. coli* NiCo21 and purified by means of immobilized metal chelate affinity chromatography on TALON™ matrix. In SDS-PAGE, a single band corresponding to a molecular mass of approximately 30 kDa was observed which corresponded to the predicted molecular mass based on the amino acid sequence.

Keywords: antibody fragment, L-L-rhamnose, Rhamex vector, scFv, transferrin receptor

Introduction

Transferrin receptor (TfR) levels are elevated in various types of cancer cells and correlate with the aggressive or proliferative ability of tumor cells (Elliot *et al.*, 1993). Therefore, TfR levels are considered useful as a prognostic tumor marker, and TfR is a potential target for drug delivery in therapy of malignant cells (Thorstensen & Romslo, 1993). In such kind of targeted delivery system, antibody fragments are frequently used as targeting moiety.

In this work, we report the generation of an anti-TfR single-chain antibody variable (scFv) and its purification by immobilized metal-ion affinity chromatography (IMAC). Immobilized metal ion affinity chromatography (IMAC) is known as the most frequently used and simplest method for isolating recombinant protein from a cell lysate in a single purification step. In this method, the target protein is tagged with a polyhistidine sequence (typically 6His), which mediates chelation to immobilized divalent metal ions such as nickel or cobalt (Robichon *et al.*, 2011).

Recombinant His-tagged proteins expressed in *Escherichia coli* and purified by immobilized metal affinity chromatography (IMAC) are commonly coeluted with native *E. coli* proteins, especially if the recombinant protein is expressed at a low level (Robichon *et al.*, 2011). To improve the final purity of expressed His-tagged protein, in this work we use NiCo21(DE3) competent *E. coli* (NEB), a derivative of *E. coli* BL21(DE3), that has been engineered to minimize contamination from common host metal binding proteins (http://www.neb.com).

Anti-TfR single-chain antibody variable fragment (scFv) was derived from the anti-TfR monoclonal antibody LUCA31. Monoclonal antibody LUCA31 can be produced from hybridoma cells from ATCC PTA-6055 (Mather *et al.*, 2009). The selection of antibody LUCA31 based on data showing that this antibody was specific against several cancer cells that produce transferrin receptor

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which was not covered by other antibodies such as Clone E6 (Mather *et al.*, 2009). The use of monoclonal antibody LUCA31 as the source of anti-TfR scFv leads this anti-TfR scFv to become toxic protein to *E. coli* as expression host. In consequence, tightly regulatable expression vector is critical for reproducible and functional expression of the protein.

We utilized an expression system based on *E. coli* L-rhamnose regulon *rha*BRS corresponding to the *rha*B promoter and the *rha*S and *rha*R genes (for simplicity, referred to here as pRHA). In the presence of L-L-rhamnose, transcription of *rha*R and *rha*S is activated by RhaR, resulting in an accumulation of RhaS. RhaS then acts as the L-L-rhamnose-dependent positive regulator of the *rha*B promoter (Via *et al.*, 1996). The expression and repression mechanism of the *rha*B promoter by L-L-rhamnose and D-glucose has been studied extensively (Tobin & Schleif, 1987, 1990; Wickstrum & Egan, 2004). The *rha*B promoter was capable of high-level recombinant protein expression in the presence of L-L-rhamnose (Via *et al.*, 1996; Giacalone *et al.*, 2006). This promoter is also subject to catabolite repression (Holcroft & Egan, 2000), so although this promoter is capable of high-level recombinant protein expression in the presence of L-L-rhamnose, it is also tightly regulated in the absence of L-L-rhamnose by the addition of D-glucose.

Materials and Methods

Establishment of Vector for Expression. The cDNA encoding the variable heavy and light chain domains of the scFv antibody fragment was derived from the anti-TfR monoclonal antibody LUCA31. Between the VL and VH domains, a flexible linker $(Gly_4Ser)_3$ was inserted to covalently unify both domains into a single-chain antibody variable fragment (scFv). At the C-terminal the sequence encoding six histidines and a cysteine (His_6Cys) was incorporated to facilitate protein purification, whereas at the N-terminal of the gene. A sequence encoding the signal peptide of *pel*B, derived from *Erwinia carotovora* pectate lyaseB, was added for localization of the protein into bacterial periplasm (Figure 1).
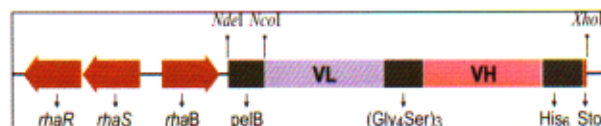

Figure 1. Establishment of vector for the expression of the scFv-fragment in *E. coli. pel*B signal sequence of pectate lyase B from *Erwinia carrotova* was used to direct the protein translocation into the bacterial periplasm. *rha*B: L-rhamnose promoter; *rha*R and *rha*S: gene regulator.

Transformation and Expression. *E. coli* NiCo21 (DE3) containing pRHA-pelB were grown on LB plates containing 100 µg/mL ampicillin. Single colonies were picked and individually grown overnight in 5 mL LB containing 100 µg/mL ampicillin at room temperature. This culture was then diluted 1:50 with LB-medium containing 100 µg/mL ampicillin and 2 g/L glucose, grown at room temperature in 250 mL shaking flask to an OD_{600} of 0.8–1.0, after which L-rhamnose was added to a final concentration of 1,000 µM and growth was continued overnight. The optimization of L-rhamnose concentration was previously done by varying L-rhamnose concentration from 0 µM to 1,000 µM. The cells were then harvested by centrifugation (6,000 rpm, 15 min, 4°C) and resuspended in 50 mM PBS containing 300 mM NaCl. After addition of lysozyme to a final concentration of 1 mg/mL and incubation at room temperature for 30 min, the His_6-tagged anti-TfR scFv fragment was isolated by centrifugation (20,000 rpm, 15 min, 4°C).

Purification. Purification of the His_6-tagged anti-TfR scFv fragment was performed by immobilized metal-ion affinity chromatography (IMAC) on TALONÔ chromatography matrix (Clontech) (Kusharyoto *et al.*, 2002). Two mL of the matrix were loaded into a PD-10 column (GE Bioscience) and equilibrated with 50 mM PBS pH 7.2 containing 300 mM NaCl, 10 mL periplasmic extract from 100 mL culture were applied to the column. After washing with 20 mL 20 mM imidazole in 50mM mM PBS pH 7.2 containing 300 mM NaCl, the His_6-tagged anti-TfR scFv fragment were eluted from the column with 200 mM imidazole in 50 mM PBS pH 7.2 containing 300 mM NaCl.

Bacterial Fractionation. Bacterial fractionation was done to ensure whether the His_6-tagged anti-TfR scFv fragment was expressed in the periplasmic space, cytoplasmic space or as inclusion body by using DTT and guanidinium chloride. Periplasmic

fraction, we mentioned as supernatant S1, was obtained by treating the expressed protein using 50 mM Tris HCl buffer pH 7.5 containing 2 M NaCl and 40 mM EDTA. Pellet obtained was then treated by using 50 mM Tris HCl buffer pH 7.5 containing 1.5 M guanidinium cloride to get cytoplasmic fraction (supernatant S2). Inclusion body fraction or supernatant S3 was obtained by treating pellet from cytoplasmic fraction using 50 mM Tris HCl buffer pH 7.5 containing 8 M guanidinium chloride and 10 mM DTT.

SDS-PAGE and Western Blot. Whole cells or purified protein fractions were prepared for SDS-PAGE analysis by mixing 1 part sample with 1 part of Laemmli sample buffer (Biorad). Samples were analyzed by 15% SDS-PAGE. Proteins were visualized by Coomassie Brilliant Blue R-250 staining or transferred to nitrocellulose membrane (Biorad) for immunoblotting. The His_6-tagged protein was detected by His Detector™ Nickel-HRP (KPL) and colometric detection by TMB 1-component membrane substrate.

Results and Discussion

E. coli is currently the host of choice for producing antibody fragments. The pLac system is a popular expression vector for the production of nontoxic proteins. When toxic proteins are cloned into vectors of this class, low-level, untimely expression may stimulate a cascade of deleterious events ending in mutations that may affect target protein function, overproduction of target-directed proteases, or cell death (Giacalone *et al.*, 2006). Therefore, tight expression control prior to target protein induction is critical for reproducible and functional expression of host-toxic proteins. Two strategies have been described for expression of anti-TfR scFv-fragment derived from monoclonal antibody LUCA31 considered as low-level and host-toxic protein. Lower growth temperature before and during induction, also the utilization of L-rhamnose-based tightly regulated promoter vector was subject to reduce the probability of toxic event. The antibody fragment was not successfully expressed using vector with T5 promoter system (data not shown). Here, the vector pRhA-pelB (Figure 1) containing the gene encoding anti-TfR

scFv-fragment was transformed into and expression was performed in *E. coli* NiCo21(DE3).

To enable the expression of the antibody fragment in the periplasm, a sequence encoding the signal peptide of pelB, derived from *Erwinia carotovora* pectate lyaseB which has been used for effective translocation of various recombinant proteins in *E. coli* (Pines & Inouye, 1999), was added at the N-terminal of the protein. Secretory production of recombinant proteins provides several advantages compared to cytosolic, such as the N-terminal amino acid residue of the secreted product can be identical to the natural gene product after cleavage of the signal sequence by a specific signal peptidase (Choi & Lee, 2004). In addition, recombinant protein purification is simpler due to fewer contaminating proteins in the periplasm. Another advantage is enable the properly folded of protein with disulfide bond (scFv) and allows for the accumulation of proteins that are toxic in the cytoplasm (Cornelis, 2000). Localization of protein expression in bacterial periplasm was verified by bacterial fractionation (Figure 2). The expected band was shown in supernatant 1 (S1p) or periplasm fraction that was recovered from periplasm of the *E. coli* using Tris HCl buffer pH 7.5 and EDTA to disrupt the outer cell membrane. From the optimization of L-rhamnose concentration, we got 1,000 μM of L-rhamnose as the optimum condition (Figure 3); therefore this concentration was used for further protein expression.

Comparison expression of pRHA-pelB-NiCo21(DE3) to controls was also done to ensure that the expected band was not native *E. coli* protein (Figure 4). Such host proteins are routinely copurified during IMAC procedures and are therefore referred to as contaminants. Several metal binding proteins that behave as IMAC contaminants have been identified in recent years. The most common *E. coli* proteins listed were CRP, Fur, ArgE, DnaK, SlyD, GlmS, GlgA, ODO1, ODO2, Can (YadF), ArnA (YfbG), AceE, GroES, and GroEL (Robichon *et al.*, 2011). In *E. coli* NiCo21(DE3), protein Can was commonly appear as contaminant.
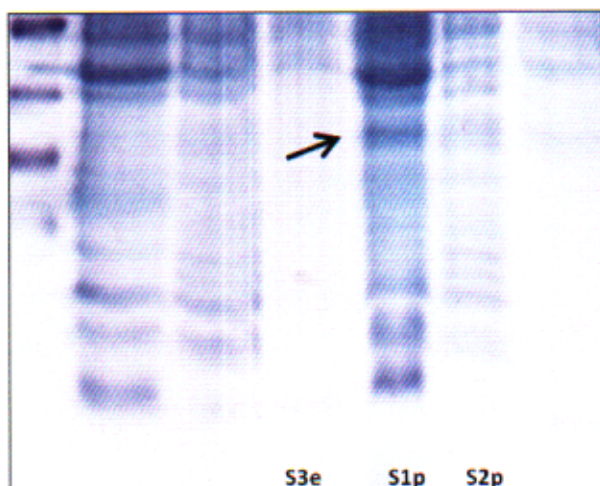
Figure 2. Bacterial fractionation. S: supernatan; e: supernatant *E. coli* without expression vector; p: supernatant *E.coli* containing expression vector. S1:cell was treated with lysozyme; S2: cell was sonicated and S3: treatment with DTT and 6M guanidinium chloride. S1, S2 and S3 showed protein expressed sequentially in periplasm, cytoplasm, or as inclusion body.
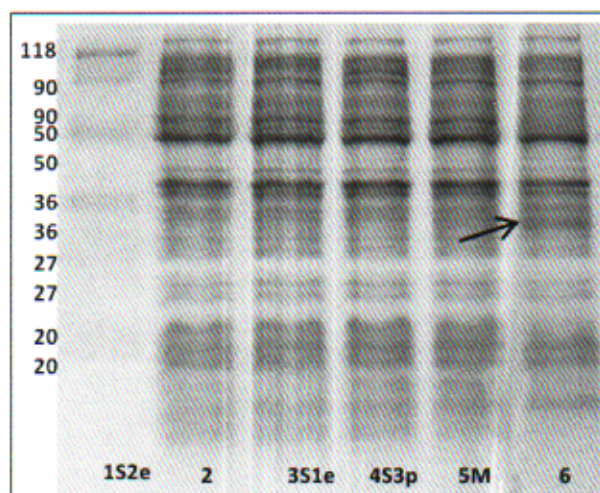

Figure 3. L-rhamnose concentration optimization in protein expression. 1:protein marker; 2: protein expression with 0 µM L-rhamnose; 3: protein expression with 1 µM L-rhamnose; 4: protein expression with 10 µM L-rhamnose; 5: protein expression with 100 µM L-rhamnose; 6: protein expression with 1,000 µM L-rhamnose.

From the result, it was shown that the *E. coli* NiCo21(DE3) without expression vector did not show the expected band although it was induced by L-rhamnose. The result also showed that both L-rhamnose and D-glucose play important role for the expression of pRHA-pelB-NiCo21(DE3), as the expected band could not be seen in the absence of each or both compounds. In SDS-PAGE (Figure 5), a single band corresponding to a molecular mass

of approximately 30 kDa was observed which corresponded to the predicted molecular mass based on the amino acid sequence. Western blot was performed to identify the His_6-tagged anti-TfR scFv fragment by using His DetectorÔ-Nickel-HRP (KPL). The band corresponding to a molecular mass of approximately 30 kDa was also identified (Figure 6).
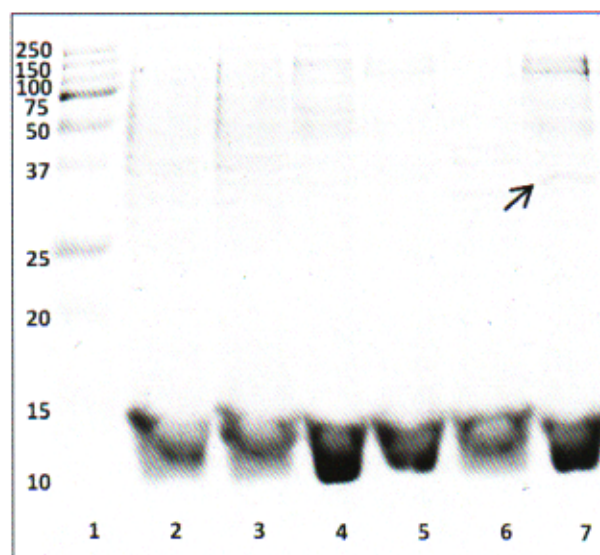

Figure 4. Comparison expression of pRHA-pelB-NiCo21(DE3) to controls. 1:protein marker; 2: NiCo21(DE3) without expression vector; 3: NiCo21(DE3) without expression vector, yet L-rhamnose was added as inducer; 4: pRHA-pelB-NiCo21(DE3) without glucose; 5: pRHA-pelB-NiCo21(DE3) without L-rhamnose; 6: pRHA-pelB-NiCo21(DE3) without D-glucose and L-rhamnose; 7: pRHA-pelB-NiCo21(DE3)with D-glucose and L-rhamnose.
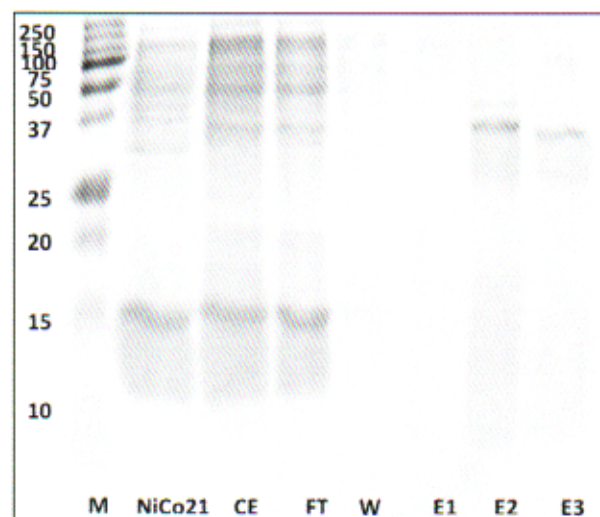

Figure 5. SDS-PAGE of fractions obtained during protein purification by IMAC on Talon™ matrix. NiCo21: *E. coli* cell extract; CE: cell extract; FT: flow-through; W: washing step; E: elution steps and M: protein marker.
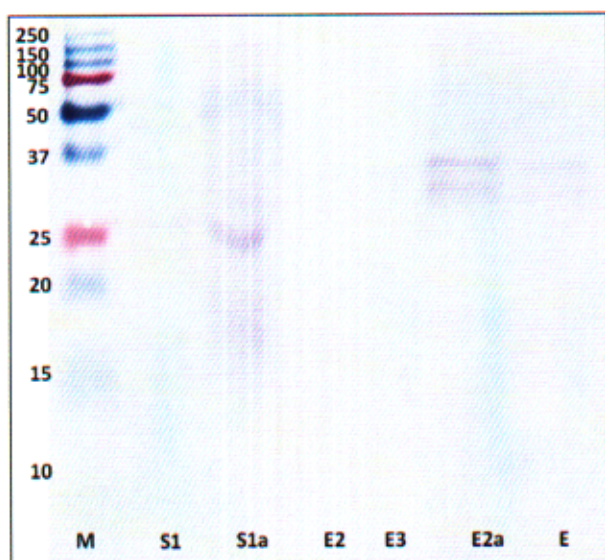
Figure 6. Western blot of fractions obtained during protein purification by IMAC on Talon™ matrix and protein fractination. S1: supernatant from bacterial fractination; E: elution steps and M: protein marker; a: sample precipitated by using acetone.

However, contamination by the *E. coli* NiCo21(DE3) proteins could also be seen. The band at approximately 28 kDa might correspond to the protein Can-CBD, a carbonicanhydrase which was tagged with the chitin binding domain (CBD) sequence in *E. coli* NiCo21(DE3) (Robichon *et al.*, 2011). This protein exhibits four zinc-binding sites that confer a significant affinity for metal chelating resins. Moreover, Can-CBD expression increases during slow growth, or during stress and starvation, in other words, conditions typically encountered during over expression of recombinant proteins (Merlin *et al.*, 2003). To overcome this, in future work the presence of Can-CBD can be eliminated by purification using chitin beads. The CBD has a high affinity for the chitin beads which allows a better recovery of the protein.

Conclusions

The strategy of using pRHA expression system represents an alternative technology for cloning and expression of protein that was found to be toxic. The His$_6$-tagged anti-TfR scFv fragment was successfully expressed in *E. coli*NiCo21(DE3). Contamination by *E. coli* NiCO21(DE3) protein, especially by Can-CBD, still persisted after purification by IMAC on TALON™. Contamination by other CBD-tagged proteins of *E. coli* NiCO21(DE3) was less significant.

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