A Thesis for the Degree of Master of Science

Production of functional single-chain variable fragment against Tumor Necrosis Factor alpha in engineered *Escherichia coli*.

재조합 대장균에서
종양괴사인자에 특이적인
재조합 단일사슬 항체의 생산

By

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School of Agricultural Biotechnology
Seoul National University
February 2016

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Advisor: Professor Jin-Ho Seo

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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ABSTRACT

Human TNF-alpha is a non-glycosylated protein of 17 kDa molecular weight and trimeric *in vivo*. Low levels of human TNFα aid in maintaining homeostasis and promoting the replacement of injured tissue, however, high levels of human TNFα cause autoimmune diseases such as reumatoid arthritis. To regulate the level of h-TNFα, many therapeutic antibodies for target h-TNFα have been produced. Production of small sized recombinant antibodies such as single-chain variable fragment (scFv) would be more effective than production of monoclonal antibodies. ScFv is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide of ten to about 25 amino acids. In previous research, genes for the scFv of a monoclonal antibody (mAb) against TNF-α were cloned and expressed in *Escherichia coli*. However, the scFv was expressed in insoluble form, so the purified scFv was refolded *in vitro* to acquire soluble and active scFv.

To produce soluble scFv against human TNF-α in recombinant *E. coli* C41(DE3), the expression vector for the scFv against TNFα fused with the maltose binding protein (MBP) was applied. The vector was constructed and transformed into *E. coli* C41(DE3). The resulting strain was able to express

the scFv successfully in soluble form. Second, the scFv and MBP fusion protein was purified by affinity chromatography using MBP as a ligand and the binding activity of the purified scFv against TNFα was verified by using Biacore T100. The association constant (k_a) of 2040 (1/M•s) and dissociation constant (k_d) of 0.001769 (1/s) were estimated. Finally, the K_D value defined as the equilibrium dissociation constant between the antibody and its antigen was determined to 8.671E-7 (M). The lower K_D value represents that the antibody has the higher affinity to an antigen. As typical antibodies have the K_D value of 10^{-7}~10^{-8} (M). The biological function of the scFv tw TNFα produced in *E. coli* could be confirmed.

Third, production of anti-TNFα scFv by fed-batch fermentation of engineered *E. coli* was attempted to produce 72.7 mg/L.

In conclusion, this thesis has demonstrated production of the functional scFv against human TNFα in recombinant *E. coli* for diagnosis and therapeutic applications.

Keywords : *Escherichia coli*, Tumor Necrosis Factor alpha, scFv, fusion protein, indirect enzyme linked immunosorbent assay, surface plasmon resonance

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I. INTRODUCTION

1. Tumor necrosis factor alpha (TNFα)

Human tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine. The naturally occurring form of human TNFα is glycosylated, but there are nonglycosylated recombinant human TNFα which have comparable biological activity. Human TNFα has two biological active forms such as a secreted, soluble form and as a membrane-anchored form. The human TNFα bound to the membrane appears as a 26 kDa polypeptide translation product with an uncleaved 76-residue signal sequence.

When the leader peptide removed, the 17 kDa secreted form of human TNFα transport to the outside of membrane, and mainly exists as a soluble trimeric 51 kDa complex molecule. Thus, human TNFα is produced from activated macrophages and monocytes, and induces immune reactions by activating other immune cells. When human TNFα had overexpressed, autoimmune diseases can occur such as rheumatoid arthritis, crohn's disease and multiple sclerosis.

2. Anti-TNFα

Anti-TNFα is an antibody which has affinity against TNFα. Anti-TNFα has been used as a pharmaceutical drug that suppresses response of TNFα by neutralizing TNFα (Figure 1). The anti-TNFα antibody takes an important part in the therapeutic antibody industry. Various types of anti-TNFα available in the market; infliximab (REMICADE of Janssen), eternacept (Enbrel of Pfizer) and adalimumab (HUMIRA of Abbott) (Figure 2). Most of therapeutic antibodies are monoclonal antibodies and produced in mammalian cell because of their large size and requirement of post-translational modification.

3. Single-chain variable region fragment antibody (scFv)

A single chain variable region fragment antibody (scFv) is a fusion protein of the variable region of the heavy and light chains (V_H and V_L) of immunoglobulin, connected with a short linker peptide of amino acids (Figure 3). This fragment is the smallest antibody which still retains affinity and specificity of its parent antibody and has many advantages in therapeutic

and diagnostic applications for its small size and easy mass production (L. Mohr et al., 2004). Compared to the much larger Fab′, which means one arm, antigen binding region of IgG including hinge region, and F(ab′)2, both arms of IgG, and IgG forms of monoclonal antibodies *in vivo*, scFv may clear more quickly from the blood and penetrate tissues with rapid distribution. Because scFv does not have the Fc domain. Fc domain induces an appropriate immune response by binding to immune molecules, so its immunogenicity is lower than that of the native antibody *in vivo*. In addition, recombinant scFv genes can be manipulated genetically to increase target sensitivity and specificity and to meet requirements of detection methods and therapy (Chowdhury et al., 1999). Up to date, scFv has been expressed in various systems such as mammalian cells, yeasts, bacteria, plants, and insect cells (Cupit et al., 1999, Sánchez et al., 1999, Galeffi et al., 2006, Choo et al., 2002). Usually, bacterial expression systems are used for the production of scFv compared to other various expression systems (Ahmad et al., 2012). ScFv can be expressed in a correctly folded and active form or aggregated form requiring *in vitro* refolding to make it active (Min et al., 2011).

4. Affinity tags

Soluble expression of proteins in *E. coli* is a major bottleneck in heterologous protein production. Most valuable proteins such as kinases, phosphatase, and many other enzymes are convoluted to produce as soluble form in *E. coli*. The rate of translation and protein folding in *E. coli* is faster than that in other microorganism systems. (Verma et al., 1998). Fast protein synthesis and slow protein folding in *E. coli* may make protein insoluble and aggregate (Widmann et al., 2000). Many approaches such as reduced temperatures or optimization of induction conditions have been tried to enhance soluble protein production (Kataeva et al., 2005). As another strategies, some affinity tags could improve the solubility of some of the companion proteins to which they were fused (Kapust et al., 1999 & Nygren et al., 1994) (Figure 4). There are numbers of commonly used solubility-enhancing fusion tags that can be applied to express proteins in *E. coli* (Table 1). The maltose binding protein (MBP) from *E. coli* is one of the most well-understood solubility factors and has significant evidence that MBP fusion is able to express soluble proteins as the unfused proteins are insoluble. Fusion tags such as histidine tag, polycationic amino acids tails are used for protein purification as well (Kweon et al., 2002). The solubility of Candida antarctica lipase B (CalB) which catalyzes a number of biochemical

reactions was improved remarkably by fusing 10-arginin tag at the C-Terminal (Jung et al., 2011). 6-lysine tagged ubiquitin fusion enhanced expression levels of a target protein (Kim et al., 2011).

5. *E. coli* strains for protein expression

In expression of heterologous proteins, *E. coli* is an eminent host organism due to the simplicity of its use, the high expression levels of heterologous proteins and the broad expression plasmids (Grisshammer et al., 1995). *E. coli* BL21(DE3) as a host strain is commonly used for the overexpression of both prokaryotic and eukaryotic proteins (Studier et al., 1990). A number of proteins have been produced with success to very high levels in BL21(DE3). But, BL21(DE3) have not always expressed high level of proper folded proteins. Some proteins was produced in inclusion body which is inactive form due to immoderate expression rate and toxicity to cell. To solve this drawback, C41(DE3) from BL21(DE3) is constructed and able to grow and continue to produce proteins at an elevated level (Miroux et al., 1996). The C41(DE3) strain has been applied to production of proteins that were expressed in low levels in BL21(DE3) (Sorenson et al., 2003) (Figure 5).

6. Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) measures differences in refractive index which presents the number of the complex of the ligand and the analyte according to affinity of two molecules. SPR determines whether a protein has affinity against a ligand and characterizes kinetic values such as association and dissociation rate constants.

SPR-based instruments use an optical method to measure the refractive index near (within ~ 300 nm) a sensor surface (Figure 6). In the BIAcore this surface is one side of a small flow cell (~ 20 - 60 nl) through which an aqueous solution (running buffer) passes at a continuous flow rate (1 - 100 µl/min). To detect interaction of molecules, one molecule (the ligand) is immobilized onto the sensor surface in advance. Then its binding partner (the analyte) is injected in appropriate buffer through the flow cell, also under continuous flow. When buffer which contains analyte passes the flow cell, the ligand and the analyte form complex which increases according to affinity of the two molecules. The accumulation of the complex on sensor surface increases refractive index (RI). This refractive index change is measured in real time and the result plotted as response units (RU) versus time (a sensorgram). This sensorgram may contain a background response, so the actual sensogram is calculated by subtracting a background response.

This background response will be generated if the refractive index of the running buffer is different from that of sample buffer. No ligand or an irrelevant ligand is immobilized to the sensor surface and the analyte is then injected through reference flow cell as a control. Thereby the background response is obtained. The association (k_a) and dissociation rate constants (k_d) and the corresponding affinity constants (K_D) are calculated by measure the association and dissociation of the binding interaction in real time.

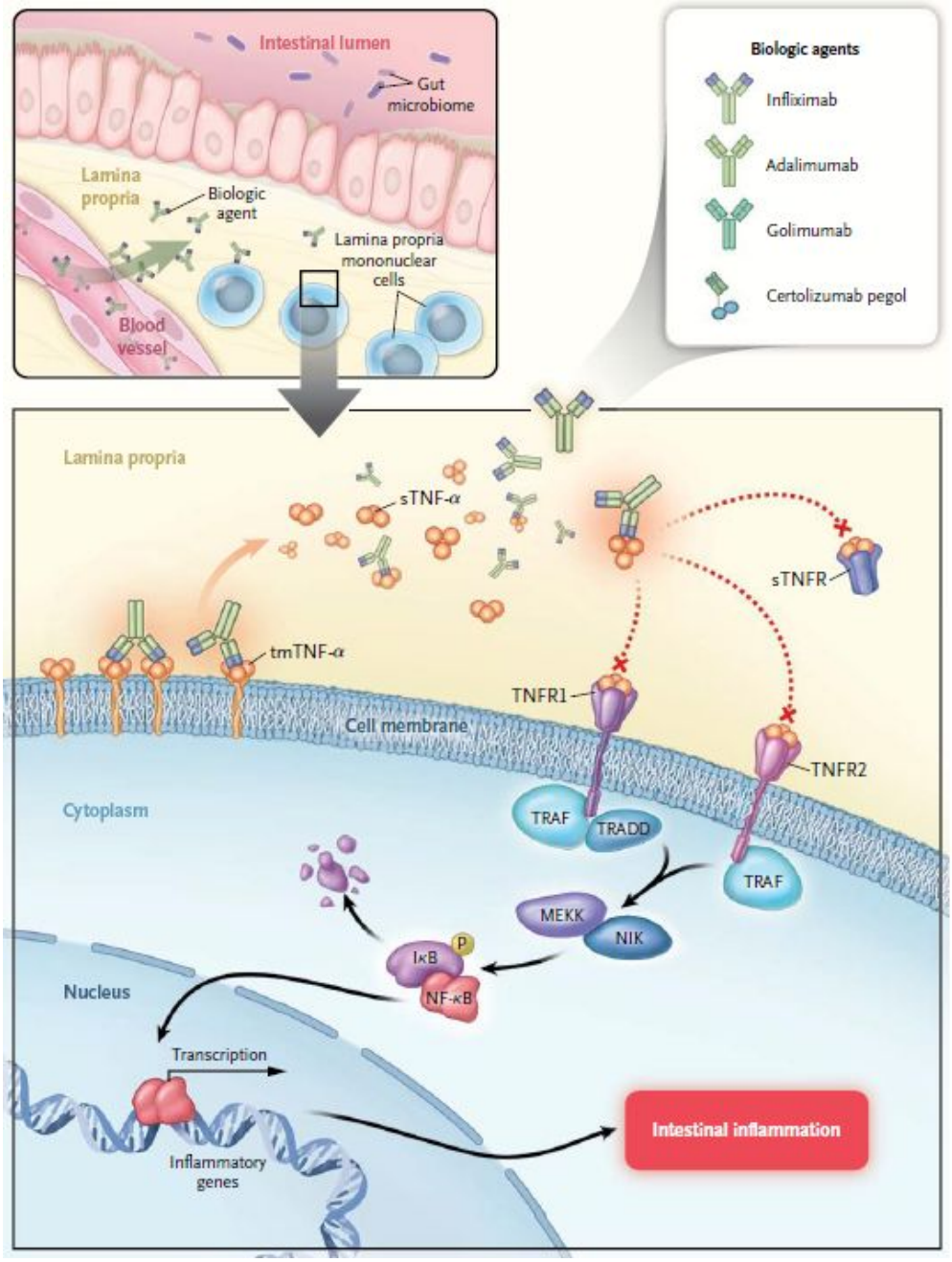

Figure 1. Mechanism of therapeutic effect of anti-TNFα antibodies in inflammatory bowel disease

(Nielsen et al., 2013)

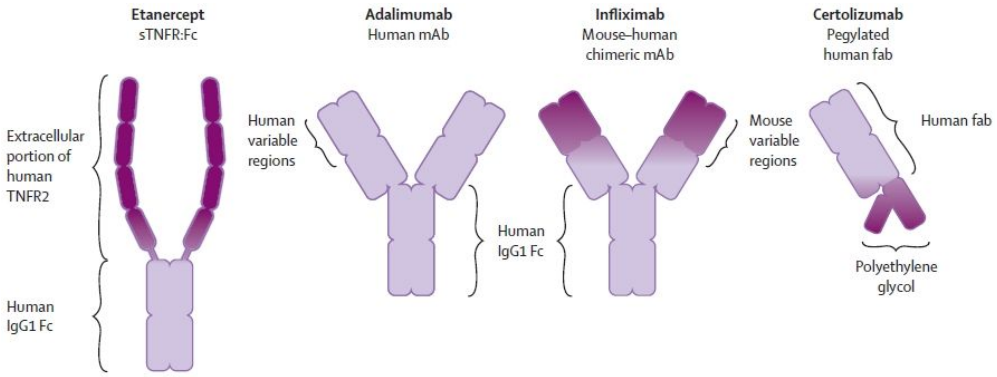

Figure 2. Structures of the TNF antagonists

(Wallis, 2008)

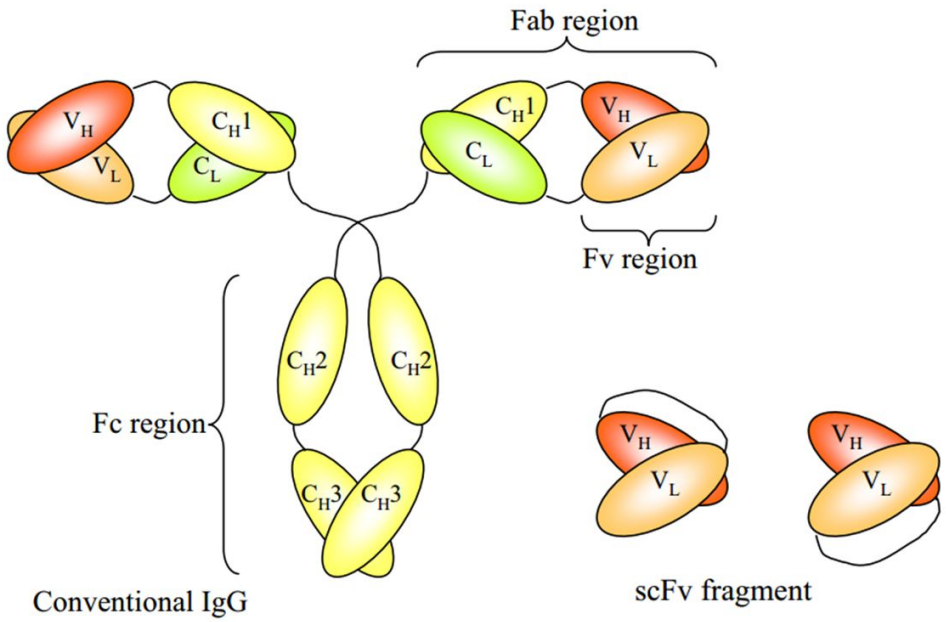

Figure 3. Subunit composition and domain distribution of immunoglobulin and single-chain variable fragment (scFv) antibody
(Joosten et al., 2003)

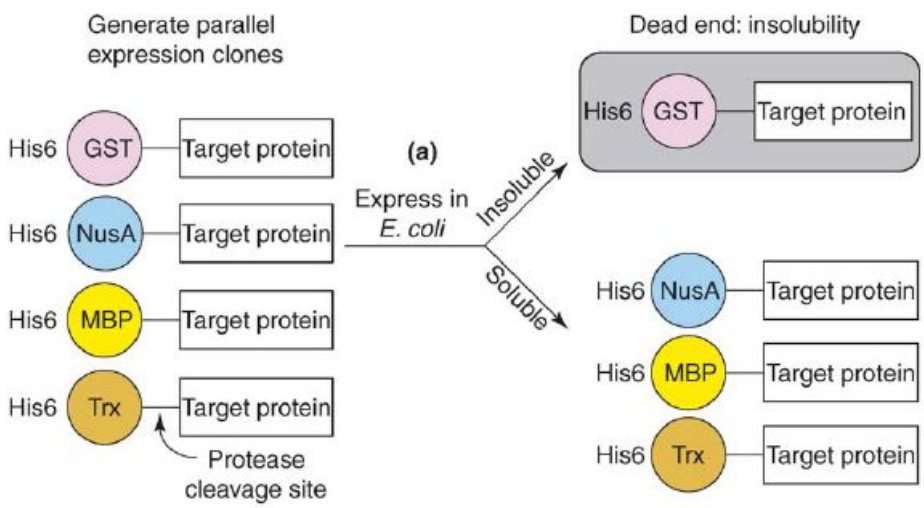

Figure 4. Protein expression using solubility tags

(Dominic et al., 2006)

Table 1. Generally used solubility-enhancing fusion partners

Tag	Source organism
MBP (Maltose-binding protein)	*Escherichia coli*
GST (Glutathione-S-trasferase)	*Schistosoma japonicum*
Trx (Thioredoxin)	*Escherichia coli*
NusA (N-Utilization substance)	*Escherichia coli*
SUMO (Small Ubiquitin-modifier)	*Homo sapiens*
SET (Solubility-enhancing tag)	Synthetic
DsbC (Disulfide bond C)	*Escherichia coli*
Skp (Seventeen kilodalton protein)	*Escherichia coli*
T7 PK (Phage T7 protein kinase)	Bacteriophage T7
GB1 (Protein G B1 domain)	*Streptococcus sp.*
ZZ (Protein A IgG ZZ repeat domain)	*Staphylococcus aureus*

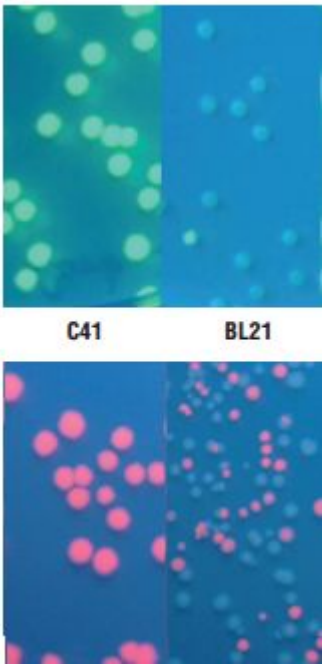

Figure 5. Toxic protein such as green fluorescent protein (top) or red fluorescence inducing protein (bottom) expressed in C41 and BL21 (Lucigen, United States)

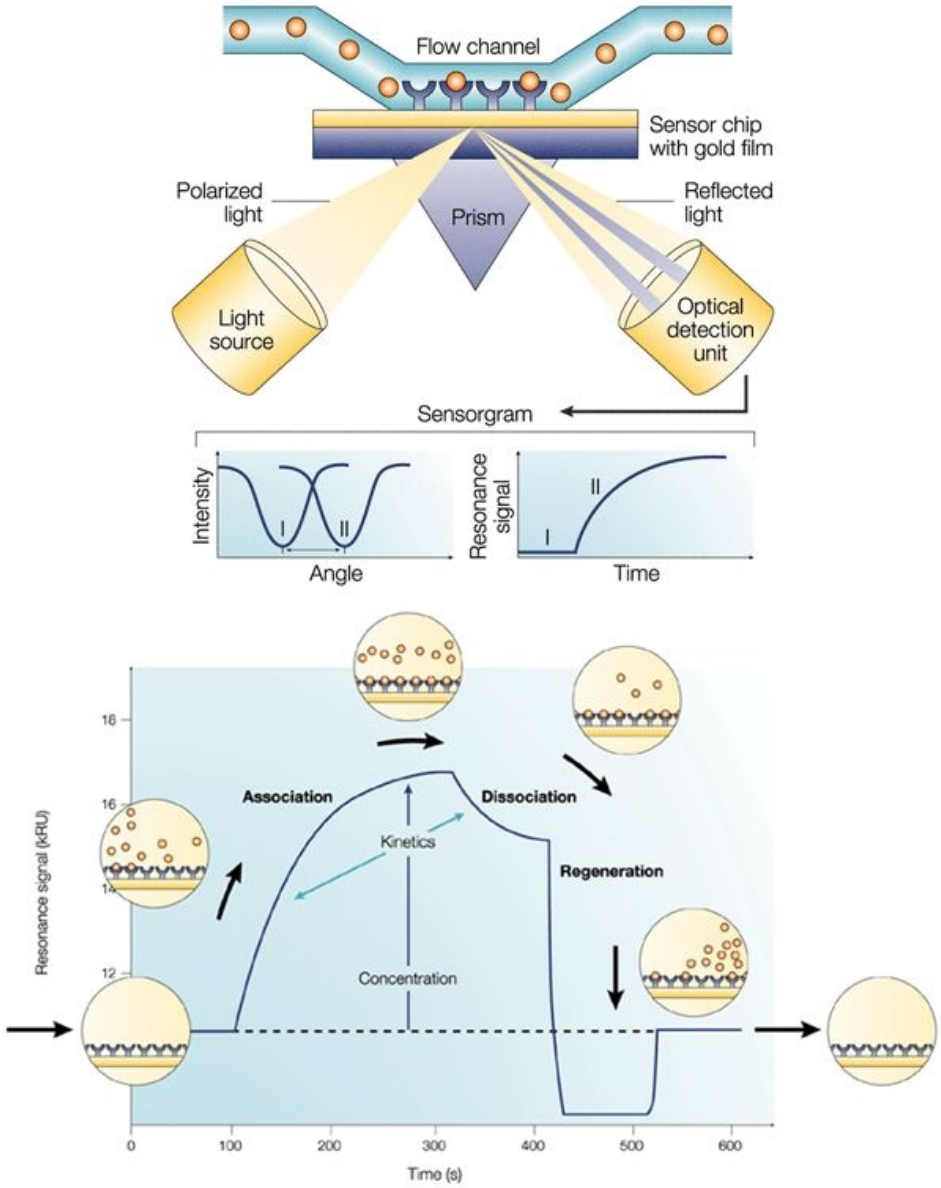

Figure 6. Typical set-up for an SPR biosensor and binding cycle (Cooper, 2002)

7. OBJECTIVES OF THE THESIS

This study is focused on production of single chain variable fragment (scFv) against TNFα in active form in *Escherichia coli*. This protein has affinity against human TNFα and characterization of the kinetic value of the scFv. The specific objectives of this research were as follows:

1) Construction of the soluble expression system of anti-TNFα scFv by fusing maltose binding protein and using *E. coli* C41(DE3) as host strain.

2) Analysis of the immunological properties of the purified anti-TNFα scFv against human TNFα and comparison of the characteristics among anti-TNFα fragments using ELISA and SPR.

3) Microbial production of the functional anti-TNFα recombinant antibody by fed-batch fermentation.

II. MATERIALS AND METHODS

1. Plasmids and strains

1.1 Enzymes and reagents

TNFα was bought from Sigma Aldrich Co. (St. Louis, USA). Restriction endonucleases, T4 DNA ligase, Taq polymerase, Klenow fragment, and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, USA). Pwo DNA polymerase and dNTPs were from Hoffmann-La Roche (Basel, Switzerland). Phosphate buffered saline (PBS: 0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl), phosphate buffered saline tween 20 (PBST: 0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl, 0.05 % Tween 20), carbonate-bicarbonate buffer capsules (0.05M carbonate-bicarbonate buffer, pH 9.6), bovine serum albumin (BSA), phosphate-citrate buffer tablets (0.05M phosphate-citrate buffer, pH 5.0, 1 tablet/100 ml), 5'-tetramethyl benzidine dihydrochloride (TMB), hydrogen peroxide were obtained from Sigma Chemical Co. (St. Louis, USA.). A molecular weight standard of DNA was obtained from New England Biolabs (Beverly, USA) and a protein standard for electrophoresis from Sigma Chemical Co. (St. Louis, USA). Agarose, ampicillin, ethidium

bromide, trizma base, imidazole were purchased from Sigma Chemical Co. (St. Louis, USA). Bacto-peptone, tryptone, yeast extract, and bacto-agar was purchased from Difco Laboratories (Detroit, USA). All chemicals were of reagent grade. Slide-A-Lyzer MINI Dialysis Device for buffer changing were purchased from Thermo Fisher Scientific, Inc. (Waltham, USA). HiTrapFF for purification of proteins fused with the His 6 residues, MBPTrap HP for purification of proteins fused with the maltose binding protein and Series S Sensor chip CM5, Amine Coupling Kit, type 2, HBS-EP+ 10X buffer, Acetate 5.5 for measuring affinity between hTNFα and anti-TNFα by using biacore T-100 were purchased from GE healthcare (Little Chalfont, UK). Anti-TNFα monoclonal antibody produced in mouse was purchased from Abcam (Cambridge, USA). Quick Start Bradford Protein Assay Kit 2 was purchased from Bio-rad (Hercules, USA).

1.2 Oligonucleotides

Oligonucleotide primers were synthesized by Bioneer Co. (Daejon, Korea). They were synthesized with different restriction enzyme sites on the ends according to the purpose of experiments, which were used in PCR amplification, vector construction and gene recombination.

1.3 Strains and plasmids

E. coli Top10 [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] (Invitrogen, Waltham, USA) was used for plasmid preparation for DNA manipulation. *E. coli* BL21(DE3) [F^- *omp*T *gal dcm lon* $hsdS_B$ (r_B^-, m_B^-) λ(DE3 [lacI lacUB6-T7 gene 1 lnd1 sam7 nin5])] (Novagen, Birmingham, Germany) and *E. coli* C41(DE3) [F^- *omp*T $hsdS_B$ (r_B^-, m_B^-) *gal dcm* (DE3)] (Lucigen, Middelton, USA) were used as host strains for the expression of target proteins.

Plasmids, pET19b were used as mother vectors which has the T7 promoter for expression of anti-TNFα scFv and maltose binding protein (MBP) fusion proteins. Anti-TNFα scFv gene was from pre-constructed plasmid (KT Bong, thesis, 2006) (Figure 6). MBP genes were amplified by polymerase chain reaction (PCR) from pMAL-p2X vector (New England Biolabs, Ipswich, USA).

1.4 Recombinant DNA techniques

All of cloning steps were carried out according to the procedure of Sambrook *et al* (Sambrook et al., 1989). Mini-scale plasmid preparation was

carried out with the High Pure Plasmid Isolation kit (Roche, Basel, Switzerland) and large-scale plasmid preparation was achieved using Plasmid Midi Kit (Qiagen, Venlo, USA). Digestion of DNAs with restriction endonuclease, and dephosphorylation were achieved under the conditions recommended by the supplier, and the results of digestion were analyzed by agarose gel electrophoresis. This system was provided by Bio-Rad (Hercules, USA). The isolation of DNA fragments from agarose gel, solubilization of the gels and recovery of the DNA fragments were conducted by "High Pure PCR Product Purification Kit" from Roche (Basel, Switzerland) or "QIAquick Gel Extraction Kit" from Qiagen (Venlo, Netherlands). Ligation of the DNA fragments was carried out by "DNA Ligation Kit" from Takara (Shiga, Japan).

1.4.1 Polymerase chain reaction (PCR)

All PCRs for amplification were performed with the GeneAmp 2400 (Applied Biosystems, Waltham, USA). AccuPower PCR PreMix (Bioneer Co., Daejon, Korea), a ready-to-use PCR reagent fully optimized for more accurate PCR amplification was used for the PCR reactions. PCRs for cloning of genes were performed containing 10 pM each of forward and reverse primers, and plasmid DNA as a template. Reaction time and temperature were optimized according to each reaction condition. The

amplified gene was confirmed by gel electrophoresis.

1.4.2 Construction of expression plasmids

To construct the expression vector of scFv against TNF-α, the scFv gene was amplified by the polymerase chain reaction (PCR) from the previously constructed vector (KT Bong, thesis, 2006) (Figure 7). with the corresponding PCR primers: F1 and R1 (Table 2). The amplified scFv gene was treated with DNA restriction enzymes of A and B, and inserted in the vector fragment which was obtained though treating plasmid from Hyo-Ran Lee (HR Lee, thesis, 2014) with same restriction enzymes and purifying the fragment eliminating scFv against aflatoxin B, plasmid pET19b s.s malE Xa scFv H6 was constructed. The ligation of the expression vectors was performed using the DNA ligation kit according to manufacturer's protocols.

1.5 DNA sequencing

DNA sequencing was performed by Mbiotech (Hanam, Korea). The results of DNA sequencing for selected clone candidates were compared each other, and then the selected DNAs were used in the subsequent experiments.

2. Expression of proteins

2.1 Transformation and expression of fusion proteins

Transformation of *E. coli* was carried out as described by Sambrook *et al.* (Sambrook et al., 1989). *E. coli* Top10 was incoculated in 5 mL LB medium, and precultured at 37°C overnight. 1% aliquot of the cultured cells was transferred into 50 mL of fresh LB medium and incubated with shaking until OD at 600 nm reached 0.5. The pellet which had been harvested by centrifugation at 6,000 rpm for 5 min at 4°C was resuspended cautiously in 5 mL of ice-cold 100 mM $CaCl_2$ solution and stored on ice for 30 min. The cell suspension was centrifuged (6,000 rpm for 5 min at 4°C) and the pellet was resuspended in 5 mL of 100 mM $CaCl_2$ solution. Resuspended cells were aliquoted to 100 μl, mixed with ligated DNA, and kept on ice for 30 min. They were subjected to heat-shock at 42°C for 45 seconds. One mL of LB medium was added to the cells and incubated at 37°C for 1 hour with slow agitation. An appropriate volume of the transformed cells was spread on an LB agar plate with a selection marker. BL21(DE3) strain was selected with kanamycin and C41(DE3) strain was selected with ampicillin.

Equal amounts of scFv and maltose binding protein fusion plasmids were used for transformation of *E. coli* BL21(DE3) and C41(DE3). The

transformed cells were spread on LB agar plates containing 50 μg/mL kanamycin, ampicillin (selection for plasmids) respectively.

E. coli BL21(DE3) and C41(DE3) colonies were picked from LB agar plates with appropriate antibiotics containing fresh transformed cells and cultured in 5 mL LB medium in the presence of with appropriate antibiotics for about 12 hr at 37°C. Flask cultures were carried out with LB medium in 500 mL baffled flasks with a 100 mL working volume. Each flask was inoculated with 1.0 mL of the transformed cell, pre-cultured in the log phase and was grown at 37°C in LB medium containing selective markers. Expression of scFv and MBP fusion proteins were induced by the addition of isopropyl-2-D-thio-galactopyranoside (IPTG) to final concentration of 0.2 mM.

2.2 SDS-PAGE

After induction, the induced cells were grown for about additional 4 h, centrifuged at 10,000 rpm, resuspended in 100 mM sodium phosphate buffer (pH 7.4) and then were treated by sonication for cellular protein assay. The crushed cells were centrifuged at 12,000 rpm at 4°C for 1 min and the supernatant, taken as the soluble fraction, was recovered carefully. The pellet, taken as the insoluble fraction, was resuspended in an equivalent volume of

the same phosphate buffer. Separation of proteins by molecular weight in SDS-PAGE, soluble and insoluble proteins were resuspended in sodium dodecyl sulfate (SDS) sample buffer and boiled at 100°C for 5 min. Heat-denatured fractions were analyzed by 10 ~ 15% SDS-polyacrylamide gel electrophoresis. Protein samples were electrophoresed on an SDS-containing discontinuous polyacrylamide gel electrophoresis unit using the Mini-Protein II system (Bio-Rad, Hercules, USA). 12.5% (w/v) separating gel was prepared usually from 33.5% (w/v) acrylamide/ 0.3% (w/v) N, N'-methylenebisacrylamide stock solution in 0.38 M Tris-HCl (pH 9.1) and 0.1% (w/v) SDS. The 4% (w/v) stacking gel was prepared from 30% (w/v) acrylamide/0.44% (w/v) N, N'-methylenebisacryl-amide stock solution in 0.125 M Tris-HCl (pH 6.8) and 0.1% (w/v) SDS. Both gels were polymerized with ammonium persulfate and TEMED. The running buffer was composed of 25 mM Tris base, 192 mM glycine, and 0.1% (w/v) SDS. Samples were mixed with equal volumes of 2X loading buffer [0.125 M Tris-Cl buffer (pH 6.8); 10% (v/v) β-mercaptoethanol; 4% (w/v) SDS; 20% (v/v) glycerol; a pinch of bromophenol blue] and boiled for 3 min before loading on the gel. Electrophoresis was carried out at 90 V for stacking and at 120 V for separating. When electrophoresis was finished, the gel was stained with commassie blue R-250 solution [0.2 % (w/v) Coomassie blue R-250; 50 % (w/v) methanol; 10 % (v/v) acetic acid] for 30 min with gentle

shaking and destained with destaining solution [20 % (v/v) methanol; 10 % (v/v) acetate].

2.3 Fed-batch fermentation

Fed-batch culture was performed in a 3 L jar fermentor (Fermentec, Cheongju, Korea) with 1 L working volume of a Riesenberg medium. E. coli was grown in a 500ml flask with 100 ml working volume at 37°C and 250 rpm for 12 hr. The pre-grown cells were inoculated to a fermentor for main culture. The 100 ml seed culture was prepared in a 500 ml flask and grown in a shaking incubator at 37°C and 250 rpm for 12 hr, and main culture was carried out. To maintain the dissolved oxygen (DO) level, agitation speed and aeration rate were set at 1200 rpm and 1 vvm, respectively. Acidity was automatically controlled at 6.8 by the pH-stat strategy. After depletion of 20 g/L sugar initially added, ammonia water and high concentration of carbon source feeding solutions (800 g/L glucose and magnesium sulfate heptahydrate 20 g/L) were used to keep the cell growth and basal level of sugar. Feeding solutions were conversed to organic acids by the metabolic processes of cells. When O.D. at 600 nm reached 150, IPTG was added to induce expression of scFv and MBP fusion proteins.

3. Purification and quantitative analysis of scFv

3.1 Purification

3.1.1 Affinity chromatography

The expressed protein was purified using the His tag and MBP tag (Figure 8). Purification with the His tag was conducted by gravity-flow and Ni-NTA agarose (Qiagen, Venlo, Netherlands) was used. Resin was washed with distilled water. After equilibrating the column with binding buffer [20 mM sodium phosphate (pH 8.0), 500 mM NaCl], the sample resuspended in binding buffer was loaded. The column loaded with the sample was washed with binding buffer, and the bound proteins were eluted with elution buffer [20 mM sodium phosphate (pH 8.0), 500 mM NaCl, 500 mM immidazole] under a constant level of immidazole concentration.

The purification with the MBP tag was carried out by the Äcta prime system (Amersham Bioscience, Uppsala, Sweden) using the MBPTrap HP column (GE healthcare, Little Chalfont, UK) used in MBP-tagged protein purification. Binding and elution buffer composition used in this experiment is as follows. Binding buffer [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA] (pH 7.4) and elution buffer [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM Maltose] (pH 7.4). Except for buffer composition, the procedure of purification using MBP tag was the same as purification using His tag.

Purified proteins were analyzed by SDS-PAGE.

3.1.2 Dialysis

To change the buffer in the purified protein solution, a Slide-A-Lyzer MINI Dialysis Device (Thermo Fisher Scientific, Inc., Waltham, USA) was used. The device was filled with desired buffer. After 4 hour, fresh buffer was loaded. About 2.5 mL of the protein sample was loaded on the membrane. Aggregated protein was removed and then soluble fraction was only used.

3.2 Quantitative analysis

3.2.1 Bradford assay

BSA (Bovine Serum Albumin) standard protein was prepared to determine a standard curve for quantitative analysis of scFv and MBP fusion proteins. BSA with concentrations of 0, 5, 10, 15 μg/ml for the standard assay was used. ScFv and MBP fusion protein samples were diluted with distilled water. 200 μl of Quick Start Bradford protein 1x dye reagent from Quick Start Bradford Assay Kit and 800 μl of protein were combined and vortexed. Combined samples were incubated at room temperature for 5 minutes, and measured absorbance at 595 nm.

4. Immunological analysis

4.1 Indirect ELISA

To examine the antigen-binding affinity of scFv and MBP fusion proteins indirect ELISA (Enzyme linked immunosorbent assay) was used (Fig. 12). The antigen-binding affinity was determined by detecting the signal of scFv and MBP fusion proteins according to concentrations of a TNFα.

An immune 96 microwell plate was coated with 0.1, 1, 10 μg/ml of human TNFα at room temperature overnight. The plate was washed with 200 μl of PBST buffer, and then was coated with 200 μl of 5% skim milk in 2 hours. The plate coated with skim milk was washed with PBST buffer and coated with 100 μl of the prepared soluble scFv and MBP fusion proteins and incubated at room temperature for 3 hours. Monoclonal antibody as positive control and 1% BSA as negative control were used. After washing, 100 μl of the anti-His antibody conjugated with HRP as secondary antibody was added to each well and incubated for 1 hour. After washing, 100 μl of 5'-tetramethyl benzidine dihydrochloride (TMB) substrate solution was added and incubated for 30 minutes. 2 M sulfuric acid solution was added to each well to stop color development. Absorbance of samples was measured by a microplate reader at 450 nm. To compare affinities between His-tag antibody

and MBP-tag antibody, additional experiments were conducted by using anti-MBP Ab and anti-His Ab as a secondary antibody (Figure 9).

4.2 Surface Plasmon Resonance

The antigen-binding affinity of scFv and MBP fusion proteins was characterized by SPR technique on a Biacore T100 (GE healthcare, Little Chalfont, UK) instrument.

To characterize kinetic values for affinity between anti-TNFα scFv and human TNFα, SPR experiment was performed. First, human TNFα (Sigma Aldrich, St. Louis, USA) in pH 5.5 acetate buffer was immobilized to the carboxymethyl dextran-coated CM5 sensor chips using covalent interaction of amine to obtain an immobilization level of ~350 RU. Second, the solution of the anti-TNFα scFv and MBP fusion protein at different concentrations was injected on the bound human TNFα at a flow rate of 30 µl/minute for 150 seconds for association. For dissociation of the anti-TNFα, HBS-EP+ buffer from GE Healthcare (Little Chalfont, UK) was injected at a flow rate of 30 µl/minute for 500 seconds. Biosensor matrices were regenerated using 0.5 mM NaOH. The Biacore T100 evaluation software was used to determine k_d (dissociation constant) and k_a (association constant) values. The value of K_D was determined by dividing k_d by k_a.

The affinity of anti-TNFα monoclonal antibody (Abcam, Cambridge, USA)

to human TNFα was also determined after immobilizing the receptor on CM5 sensor chips. The experiment was conducted by the same method as anti-TNFα scFv.

Table 2. Sequences of the primers used in this research

Primer name	Primer sequences (5′-3′)
TNF-NdeI-F	GCA ATT CCA TAT GGA TAT TGT GAT GAC GC
TNF-BamHI-R	CG GGA TCC TCA GTG GTG GTG GTG GTG G

CATATGGATATTGTGATGACGCAGTCTCCACTCTCCCTGCCTGTCA

GCTTTGGAGATCAAGTTTCTACCTCTTGCAGGTCTAGTCAGAGTCT

TGCAAACAGTTATGGGAACACCTATTTGTCTTGGTACCTGCACAA

GCCTGGCCAGTCTCCACAGCTCCTCATCTATGGGATTTCCAACAG

ATTTTCTGGGGTGCCAGACAGGTTCAGTGGCAGTGGTTCAGGGAC

AGATTTCACACTCAAGATCAGCACAATAAAGCCTGAGGACTTGGG

AATGTATTACTGTTTACAAGGTACACATCAGCCGTACACGTTCGG

AGGGGGGACCAAGCTGGAAATAAAAGGTGGCGGTGGCTCCGGCG

GTGGTGGCAGCGGTGGCGGCGGTTCTAGGGTCCAACTTCAGGAG

AATGGGGCTGAGCTGGTGAGGCCTCGGGCTTCAGTGAAGCTGTCC

TGCAAGGCTTCGGGCTACACATTTTCTGACTTTGAAATGCACTGG

GTGAAGCAGACACCTGTGCATGGCCTGGAATGGATTGGAGATATT

GATCCTGGAACTGGTGATACTGCCTACAATCTGAAGTTCAAGGGC

AAGGCCACACTGACTACAGACAAATCTTCCAGCACAGCCTACATG

GAGCTCCGCAGCCTGACATCTGAGGACTCTGCCGTCTATTACTGT

ACCCTCGGGGCCTTTGTTTACTGGGGCCAAGGGACTCTGGTCACT

GTCTCTGCACTCGAGCACCACCACCACCACCACTGA

Figure 7. Amino acid sequences of anti-TNFα scFv with His tag

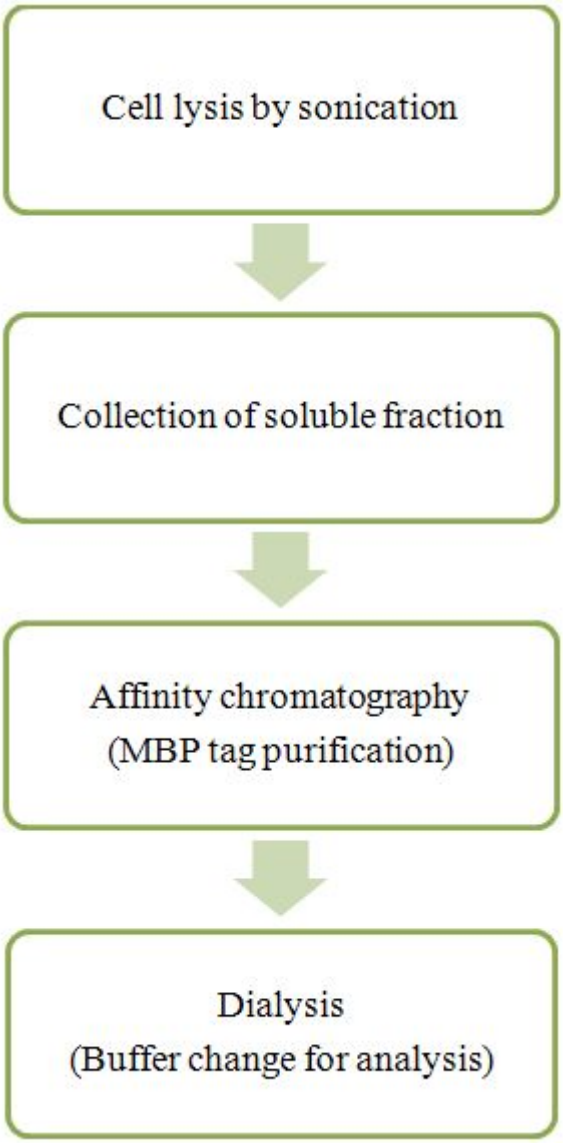

Figure 8. Purification process of MBP fusion scFv for analysis

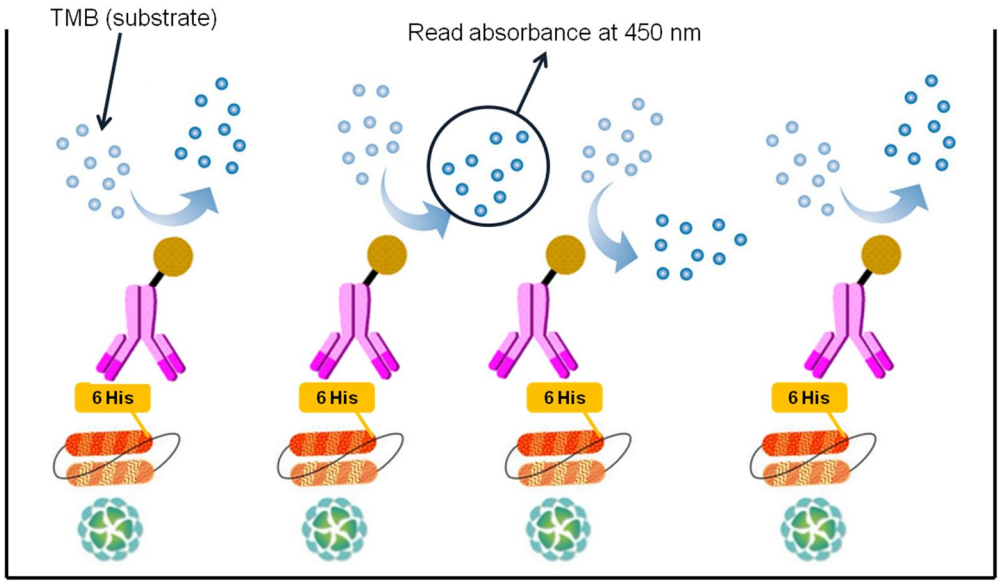

Figure 9. Diagram of indirect ELISA for identification of affinity between human TNFα and anti-TNFα

III. RESULTS AND DISCUSSIONS

1. Plasmids and strains

1.1 Construction of expression plasmids and strains

To obtain soluble and functional scFv *in vivo*, maltose binding protein which is expressed in high solubility in *E. coli* was fused with the anti-TNFα scFv. Plasmids for the expression of fusion protein were constructed that MBP was fused to the N-terminal of the scFv gene. At factor Xa protease site was inserted between the scFv and MBP genes. For purification, the 6 histidine tag was combined into expression plasmids. pET19b s.s. malE Xa scFv H6 plasmid was constructed for scFv expression (Figure 10).

2. Expression of proteins

2.1 ScFv and MBP fusion protein expression.

In order to express soluble and active scFv and to elevate the expression level of scFv, scFv was fused on C-terminal of the maltose binding protein. The MBP-fused scFv was inserted in expression vector containing

periplasmic targeting signal sequence. The vector of pET19 s.s malE Xa scFv H6 was transformed into *E. coli* C41(DE3). As a control strain, *E. coli* BL21(DE3) carrying the scFv gene without MBP was used. This scFv was expressed in BL21(DE3) in insoluble form and had 27kDa that is size of protein without MBP-tag (Figure 11).

To evaluate the effect of MBP and C41(DE3) on solubility of scFv, flask fermentations of the above two strains were performed. The soluble protein level of scFv with the MBP tag in C41(DE3) was higher than that of scFv without the MBP tag in BL21(DE3). These results suggested that an expression system of the fusion scFv with MBP in *E. coli* C41(DE3) was effective in expressing soluble scFv *in vivo* (Figure 11).

2.2 Fed-batch fermentation

Fed-batch fermentation of the recombinant strain which produced soluble scFv fused with MBP tag was performed using Riesenberg medium with 20 g/L glucose. After depletion of glucose initially added, the operation mode was controlled by the pH-stat fed-batch using feeding solution. When O.D. at 600 nm reached 100 ~ 150, IPTG (0.2 mM) was added for induction of scFv gene expression. After induction, scFv and MBP fusion protein was produced. Finally, the pH-stat fed-batch fermentation of C41(DE3) / pET19b

s.s malE Xa scFv H6 exhibited 86.14 mg/L dry cell weight, 72.7 mg/L scFv and MBP fusion protein concentration (Figure 12).

3. Purification and quantitative analysis of scFv

The scFv fused with MBP was purified by using the Ni-NTA resin which has specific affinity to the His-tag. SDS-PAGE was conducted with the purified scFv to verify the degree of purification. The band intensity of the MBP-fused scFv in SDS-PAGE was weak, suggesting that purification was not performed efficiently (Figure 13(a)).

For efficient purification of the scFv fused with MBP, the MBP Trap HP column (GE healthcare, Little Chalfont, UK) specific for MBP was used. FPLC was operated to purify the MBP fused scFv protein. SDS-PAGE analysis indicated that the target antibody was purified properly and detected at the size of molecular weight of 69 kDa, the size of the MBP fused scFv (Figure 13(b)). These results demonstrated that scFv and MBP fusion protein production system *in vivo* is constructed.

To quantify the scFv and MBP fusion protein, the Bradford assay was performed and standard curve was calculated using BSA. Using the standard curve, the concentration of the scFv and MBP fusion protein was measured

to be used for immunological analysis.

4. Immunological analysis of scFv

4.1 Indirect ELISA

Indirect ELISA analysis was performed for determining the binding activity of the anti-TNFα scFv and MBP fusion protein with human TNFα at 10 μg/mL of antigen concentration. First, the anti-His Ab conjugated with HRP as secondary antibody was used. But the absorbance of sample at 450 nm is similar with that of negative control (Figure 14). It was hypothesized that correct binding between anti-his Ab and his-tag would be difficult due to structure of protein where his-tag was located inside. Therefore, the anti-MBP Ab conjugated with HRP was used as a secondary antibody.

The scFv and MBP fusion protein with the anti-MBP antibody as a secondary antibody shows high affinity than using the anti-His antibody as a secondary antibody. Optical density at 450 nm of the scFv and MBP fusion protein with the anti-His antibody as a secondary antibody was similar to that of 1% BSA as negative control. On the other hand, optical density of the scFv and MBP fusion protein with anti-MBP antibody showed about 50% of

the anti-TNFα monoclonal antibody as positive control. These results demonstrated that the scFv and MBP fusion protein had affinity to human TNFα by using anti-MBP antibody as a secondary antibody (Figure 15).

4.2 Surface plasmon resonance

4.2.1 Anti-TNFα scFv fused with MBP tag

To characterize kinetic value like k_a, k_d and K_D between anti-TNFα scFv and TNFα, SPR technique on a Biacore T100 (GE Healthcare, Little Chalfont, UK) instrument was carried out.

In typical SPR experiment, binding reaction of antibody and antigen has association phase and dissociation phage. The association phase had gradually increase peak during flow of analyte and the dissociation phase was shown during flow of buffer without analyte (Figure 6). The SPR was performed with purified scFv to show gradually increase plot in 150 s of association phase and then decrease plot when the scFv supply ceased. The association value (k_a) and dissociation value (k_d) were calculated in 2040 (1/Ms) and $1.769*10^{-3}$ (1/s), respectively. Finally, K_D value was determined to $8.671*10^{-7}$ (M) (Figure 16).

K_D values of most antibodies are in the range from micromolar (10^{-6}) to

nanomolar (10^{-7} to 10^{-9}) range (Ramos-Vara et al., 2014). As the K_D value of scFv and MBP fusion protein was involved in this range, the scFv and MBP fusion protein was considered to have biological functions as antibody.

4.2.2 Anti-TNFα monoclonal antibody produced in mouse

To compare the efficiency of the MBP fusion scFv produced in this study with general antibody, SPR was performed with the monoclonal anti-TNFα antibody produced in a mouse. Before the SPR experiment with the MBP fusion scFv, the human TNFα was immobilized first in the same manner as the SPR with scFv performed above. In case of the monoclonal anti-TNFα, the association value (k_a), dissociation value (k_d) and K_D value were calculated in $2.984*10^4$ (1/Ms), $4.458*10^{-4}$ (1/s) and $1.494*10^{-8}$ (M), respectively (Figure 17). When comparing with MBP fusion scFv, K_D value of monoclonal anti-TNFα was 58-fold lower. This result represents that monoclonal anti-TNFα had higher affinity to human TNFα than MBP fusion scFv.

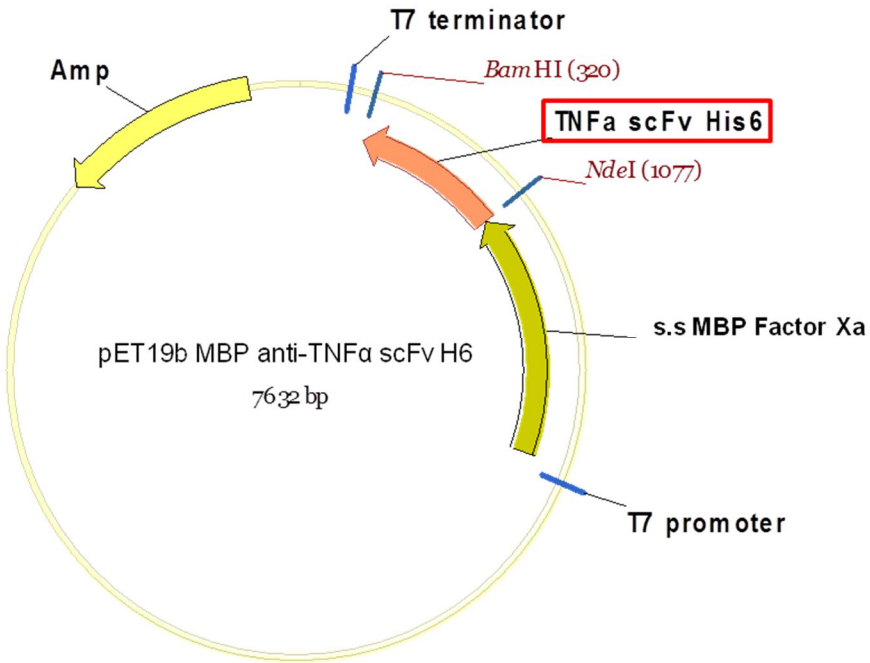

Figure 10. Genetic map of plasmid for expression of anti-TNFα scFv

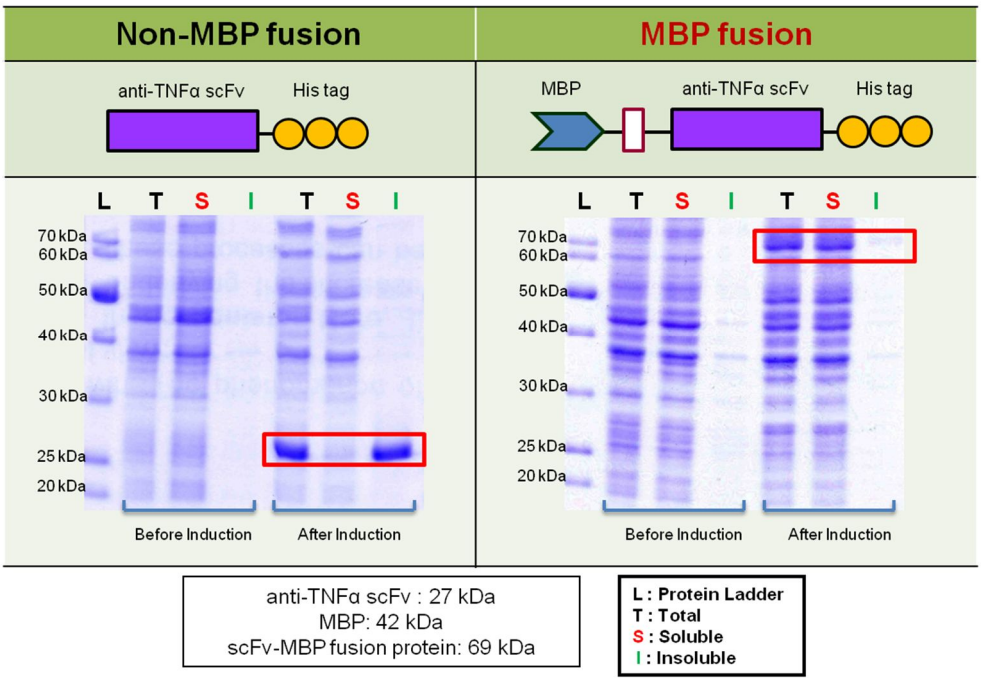

Figure 11. Expression of non-MBP fusion scFv in BL21(DE3) and MBP fusion scFv in C41(DE3) at 37°C with 0.2 mM IPTG

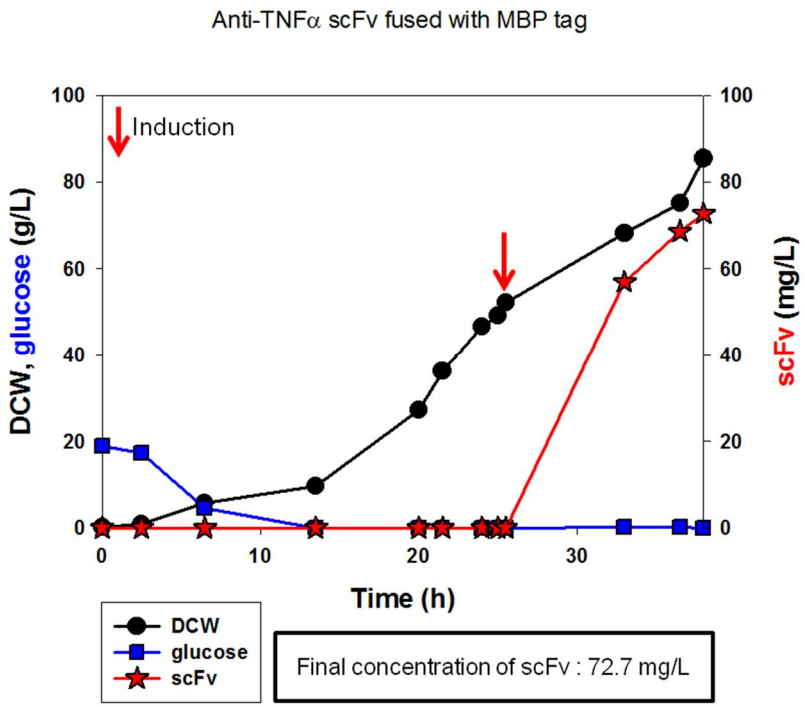

Figure 12. Profile of fed-batch fermentation

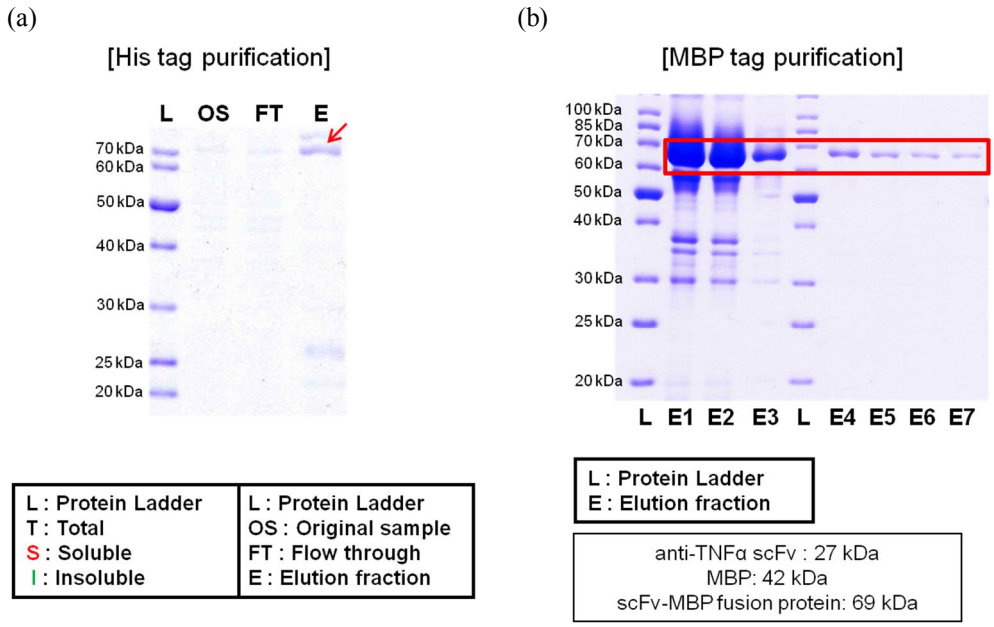

Figure 13. SDS-PAGE of MBP fusion scFv purified by HIS tag (a) and MBP tag (b) using affinity chromatography

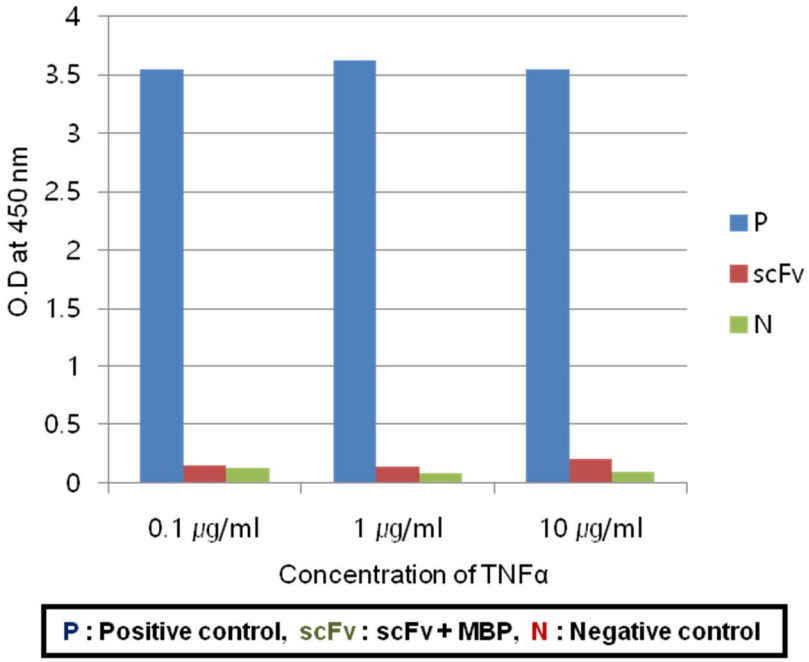

Figure 14. Comparison of antigen (human TNFα) binding activity of scFv and MBP fuison protein by indirect ELISA using anti-His Ab as secondary antibody

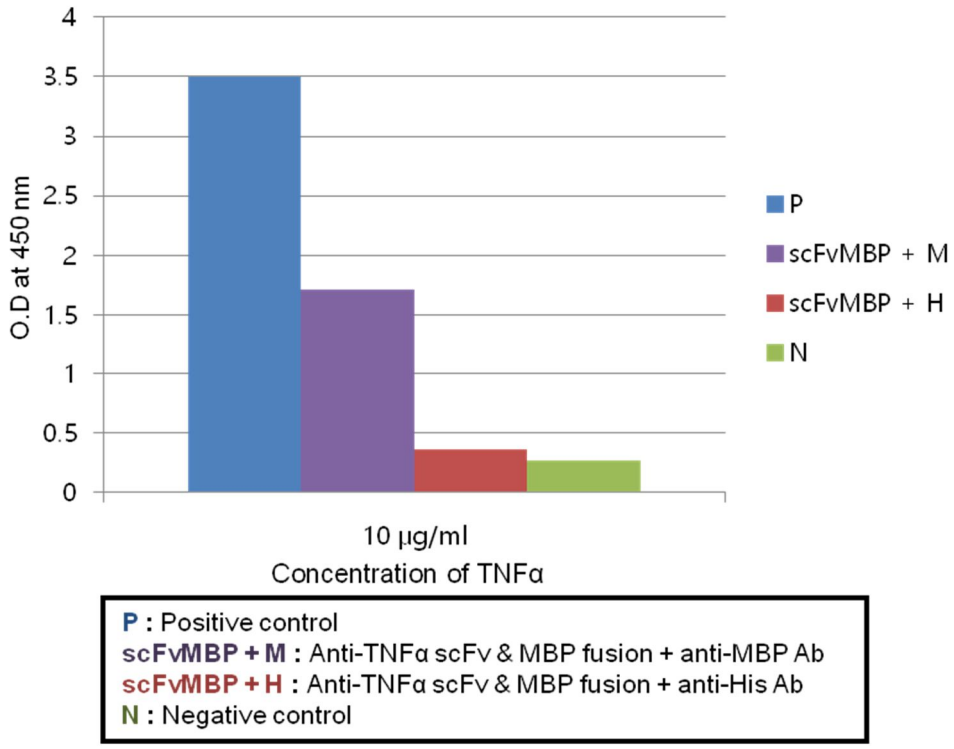

Figure 15. Comparison of antigen (human TNFα) binding activity of scFv and MBP fuison protein by indirect ELISA using anti-His Ab and anti-MBP Ab as secondary antibody

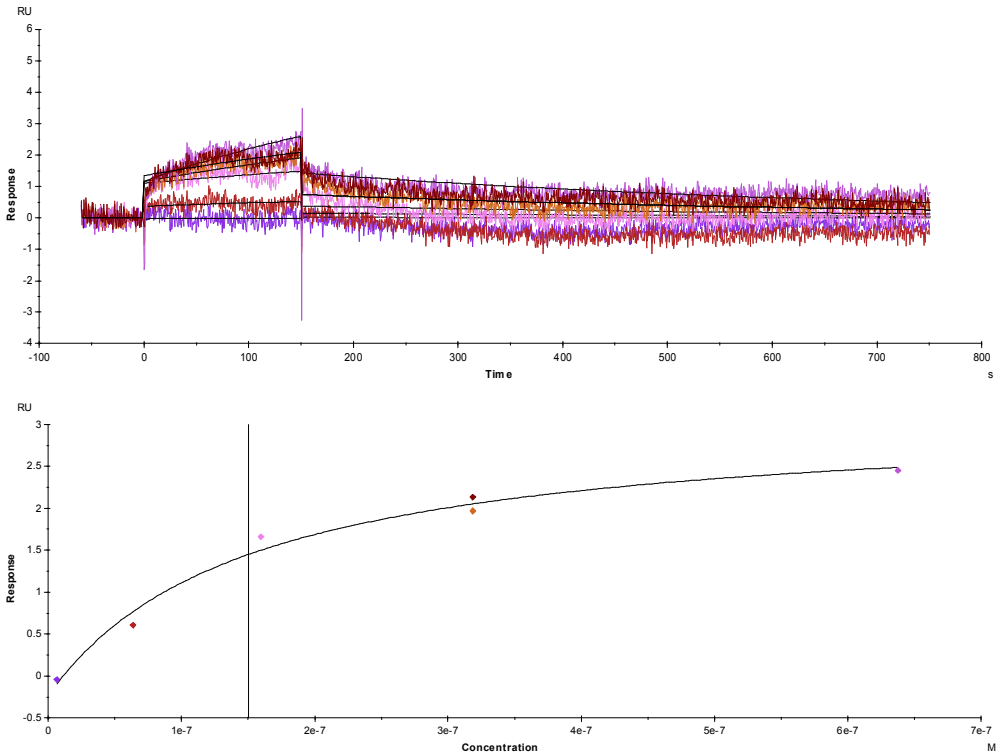

Figure 16. Antigen (human TNFα) binding activity of scFv and MBP fusion protein analyzed by SPR (Biacore T100)

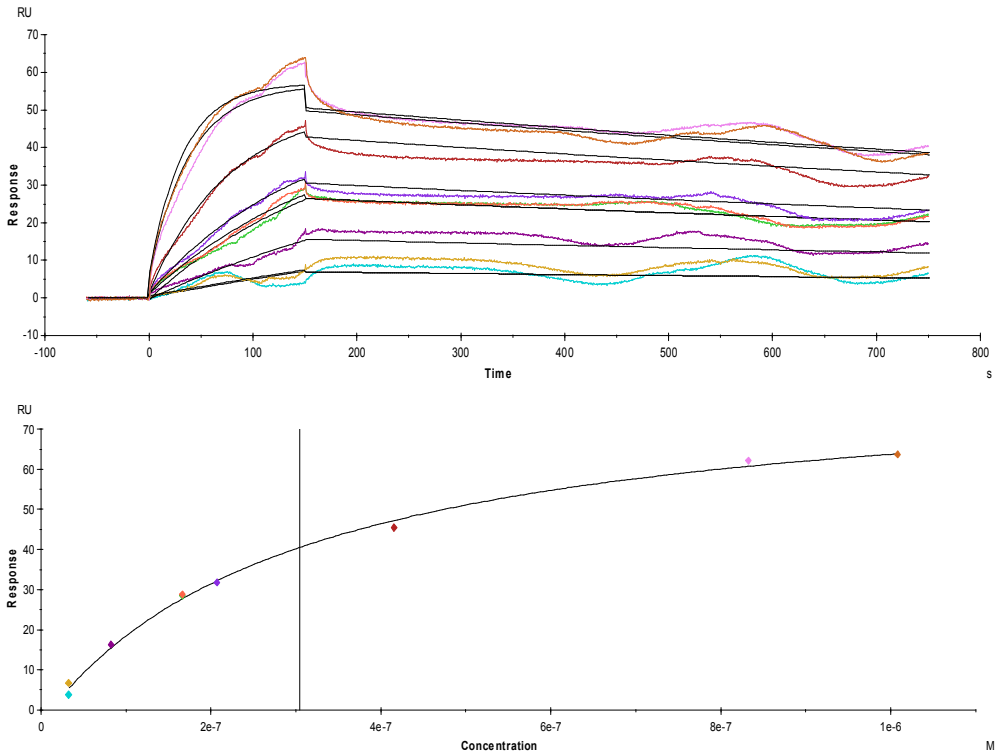

Figure 17. Antigen (human TNFα) binding activity of monoclonal antibody produced in mouse analyzed by SPR (Biacore T100)

IV. CONCLUSIONS

It was confirmed that the application of the MBP tag and *E. coli* C41(DE3) strain are effective to express the soluble and active anti-TNFα scFv. The results obtained in this research can be summarized as follows;

1) An expression system for anti-TNFα scFv by fusing the scFv gene with MBP in *E. coli* C41(DE3) was verified for production of soluble scFv.

2) The scFv and MBP fusion protein was purified by using the MBP tag as a ligand and immunological properties were measured with the purified protein. As a result, anti-TNFα scFv produced in *E. coli* had a binding affinity to human TNFα.

3) 72.7 mg/L scFv was produced in fed-batch fermentation of engineered *E. coli*.

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국 문 초 록

싸이토카인 종양괴사인자 알파(Tumor Necrosis Factor alpha) 는 17kDa 의 분자량을 갖는 단백질로 체내에서 감염에 대한 면역반응을 유도하는 proinflammatory cytokine이지만, 과발현 될 경우 류머티스 관절염과 같은 자가면역 질환이나 조직적인 부종, 고단백혈증, 호중구 감소증 등의 질병을 일으킬 수 있다. 이를 해결하기 위해 TNFα를 중화하는 여러 가지 anti-TNFα가 의약품으로 이용되고 있는데, 대부분의 anti-TNFα는 IgG의 형태로 존재한다. 그러나 IgG의 경우 크기가 크고 생산이 어렵다는 단점이 있어 light chain과 heavy chain의 가변영역만을 재조합 한 scFv (Single Chain Variable Fragment) 가 새로운 항체의 종류로 떠오르고 있다.

선행연구에 의해 첫째, 대장균에서 anti-TNFα scFv를 생산하였으나 insoluble하게 발현되어 그 자체로 제대로 된 기능을 하지 못하였다. 따라서 in vitro에서의 refolding이 필요 했는데, 이는 항체단백질을 생산하고자 할 때 시간적, 비용적인 손실을 일으켰다. 둘째로, 다른 항원(aflatoxin)에 대한 scFv생산 연구에서 MBP(Maltose binding protein) tag을 결합, C41균주에서 발현하자 soluble하게 발현되는 것을 확인하였다. 이를 종합하여 *in vivo*에서 기능적인 anti-TNFα scFv를 생산하고자 하였다.

먼저 추후 정제를 위한 His tag과 solubility를 향상시켜줄 MBP가 tagging된 anti-TNFα scFv를 발현할 수 있는 벡터를 구축하고 이를 C41균주에서 IPTG를 이용해 발현하여 soluble한 재조합 항체를 얻을 수 있었다. 이를 His tag과 MBP

tag을 각각 이용하여 정제 후 indirect ELISA를 통해 TNFα에 대한 affinity를 확인하고자 하였다. 2차 항체로 anti-His Ab를 사용했을 경우 His tag이 단백질 내부에 위치하여 2차 항체로 적절치 않다고 생각하여 anti-MBP Ab를 2차 항체로 사용하여 측정한 결과 affinity를 확인할 수 있었다. 정확한 affinity를 알아보고 다른 anti-TNFα와 비교하기 위하여 SPR(Surface Plasmon Resonance) 을 수행하였다. Monoclonal anti-TNFα의 경우 KD값이 1.494E-8 (M)로 측정되었고, anti-TNFα scFv w/ MBP fusion의 경우, KD값이 8.671E-7 (M)로 측정되었다. 그 후 유가식 배양을 통하여 최종농도 72.7 mg/L 의 scFv-MBP fusion protein을 생산하였다.

이 연구를 통해 치료용 단백질인 anti-TNFα의 재조합 scFv 항체를 미생물 발효공정을 통하여 생산 할 수 있음을 확인하였다.

주요어: *Escherichia coli*, scFv, fusion 단백질, tumor necrosis factor alpha, indirect ELISA, surface plasmon resonance

학번: 2014-20690

A Thesis for the Degree of Master of Science

Production of functional single-chain variable fragment against Tumor Necrosis Factor alpha in engineered *Escherichia coli*.

재조합 대장균에서
종양괴사인자에 특이적인
재조합 단일사슬 항체의 생산

By

Ji-Na Kim

School of Agricultural Biotechnology
Seoul National University
February 2016

A Thesis for the Degree of Master of Science

Production of functional single-chain variable fragment against Tumor Necrosis Factor alpha in engineered *Escherichia coli.*

Advisor: Professor Jin-Ho Seo

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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ABSTRACT

Human TNF-alpha is a non-glycosylated protein of 17 kDa molecular weight and trimeric *in vivo*. Low levels of human TNFα aid in maintaining homeostasis and promoting the replacement of injured tissue, however, high levels of human TNFα cause autoimmune diseases such as reumatoid arthritis. To regulate the level of h-TNFα, many therapeutic antibodies for target h-TNFα have been produced. Production of small sized recombinant antibodies such as single-chain variable fragment (scFv) would be more effective than production of monoclonal antibodies. ScFv is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide of ten to about 25 amino acids. In previous research, genes for the scFv of a monoclonal antibody (mAb) against TNF-α were cloned and expressed in *Escherichia coli*. However, the scFv was expressed in insoluble form, so the purified scFv was refolded *in vitro* to acquire soluble and active scFv.

To produce soluble scFv against human TNF-α in recombinant *E. coli* C41(DE3), the expression vector for the scFv against TNFα fused with the maltose binding protein (MBP) was applied. The vector was constructed and transformed into *E. coli* C41(DE3). The resulting strain was able to express

the scFv successfully in soluble form. Second, the scFv and MBP fusion protein was purified by affinity chromatography using MBP as a ligand and the binding activity of the purified scFv against TNFα was verified by using Biacore T100. The association constant (k_a) of 2040 (1/M•s) and dissociation constant (k_d) of 0.001769 (1/s) were estimated. Finally, the K_D value defined as the equilibrium dissociation constant between the antibody and its antigen was determined to 8.671E-7 (M). The lower K_D value represents that the antibody has the higher affinity to an antigen. As typical antibodies have the K_D value of 10^{-7}~10^{-8} (M). The biological function of the scFv tw TNFα produced in *E. coli* could be confirmed.

Third, production of anti-TNFα scFv by fed-batch fermentation of engineered *E. coli* was attempted to produce 72.7 mg/L.

In conclusion, this thesis has demonstrated production of the functional scFv against human TNFα in recombinant *E. coli* for diagnosis and therapeutic applications.

Keywords : *Escherichia coli*, Tumor Necrosis Factor alpha, scFv, fusion protein, indirect enzyme linked immunosorbent assay, surface plasmon resonance

Student Number : 2014-20690

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I. INTRODUCTION

1. Tumor necrosis factor alpha (TNFα)

Human tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine. The naturally occurring form of human TNFα is glycosylated, but there are nonglycosylated recombinant human TNFα which have comparable biological activity. Human TNFα has two biological active forms such as a secreted, soluble form and as a membrane-anchored form. The human TNFα bound to the membrane appears as a 26 kDa polypeptide translation product with an uncleaved 76-residue signal sequence.

When the leader peptide removed, the 17 kDa secreted form of human TNFα transport to the outside of membrane, and mainly exists as a soluble trimeric 51 kDa complex molecule. Thus, human TNFα is produced from activated macrophages and monocytes, and induces immune reactions by activating other immune cells. When human TNFα had overexpressed, autoimmune diseases can occur such as rheumatoid arthritis, crohn's disease and multiple sclerosis.

2. Anti-TNFα

Anti-TNFα is an antibody which has affinity against TNFα. Anti-TNFα has been used as a pharmaceutical drug that suppresses response of TNFα by neutralizing TNFα (Figure 1). The anti-TNFα antibody takes an important part in the therapeutic antibody industry. Various types of anti-TNFα available in the market; infliximab (REMICADE of Janssen), eternacept (Enbrel of Pfizer) and adalimumab (HUMIRA of Abbott) (Figure 2). Most of therapeutic antibodies are monoclonal antibodies and produced in mammalian cell because of their large size and requirement of post-translational modification.

3. Single-chain variable region fragment antibody (scFv)

A single chain variable region fragment antibody (scFv) is a fusion protein of the variable region of the heavy and light chains (V_H and V_L) of immunoglobulin, connected with a short linker peptide of amino acids (Figure 3). This fragment is the smallest antibody which still retains affinity and specificity of its parent antibody and has many advantages in therapeutic

and diagnostic applications for its small size and easy mass production (L. Mohr et al., 2004). Compared to the much larger Fab′, which means one arm, antigen binding region of IgG including hinge region, and F(ab′)2, both arms of IgG, and IgG forms of monoclonal antibodies *in vivo*, scFv may clear more quickly from the blood and penetrate tissues with rapid distribution. Because scFv does not have the Fc domain. Fc domain induces an appropriate immune response by binding to immune molecules, so its immunogenicity is lower than that of the native antibody *in vivo*. In addition, recombinant scFv genes can be manipulated genetically to increase target sensitivity and specificity and to meet requirements of detection methods and therapy (Chowdhury et al., 1999). Up to date, scFv has been expressed in various systems such as mammalian cells, yeasts, bacteria, plants, and insect cells (Cupit et al., 1999, Sánchez et al., 1999, Galeffi et al., 2006, Choo et al., 2002). Usually, bacterial expression systems are used for the production of scFv compared to other various expression systems (Ahmad et al., 2012). ScFv can be expressed in a correctly folded and active form or aggregated form requiring *in vitro* refolding to make it active (Min et al., 2011).

4. Affinity tags

Soluble expression of proteins in *E. coli* is a major bottleneck in heterologous protein production. Most valuable proteins such as kinases, phosphatase, and many other enzymes are convoluted to produce as soluble form in *E. coli*. The rate of translation and protein folding in *E. coli* is faster than that in other microorganism systems. (Verma et al., 1998). Fast protein synthesis and slow protein folding in *E. coli* may make protein insoluble and aggregate (Widmann et al., 2000). Many approaches such as reduced temperatures or optimization of induction conditions have been tried to enhance soluble protein production (Kataeva et al., 2005). As another strategies, some affinity tags could improve the solubility of some of the companion proteins to which they were fused (Kapust et al., 1999 & Nygren et al., 1994) (Figure 4). There are numbers of commonly used solubility-enhancing fusion tags that can be applied to express proteins in *E. coli* (Table 1). The maltose binding protein (MBP) from *E. coli* is one of the most well-understood solubility factors and has significant evidence that MBP fusion is able to express soluble proteins as the unfused proteins are insoluble. Fusion tags such as histidine tag, polycationic amino acids tails are used for protein purification as well (Kweon et al., 2002). The solubility of Candida antarctica lipase B (CalB) which catalyzes a number of biochemical

reactions was improved remarkably by fusing 10-arginin tag at the C-Terminal (Jung et al., 2011). 6-lysine tagged ubiquitin fusion enhanced expression levels of a target protein (Kim et al., 2011).

5. *E. coli* strains for protein expression

In expression of heterologous proteins, *E. coli* is an eminent host organism due to the simplicity of its use, the high expression levels of heterologous proteins and the broad expression plasmids (Grisshammer et al., 1995). *E. coli* BL21(DE3) as a host strain is commonly used for the overexpression of both prokaryotic and eukaryotic proteins (Studier et al., 1990). A number of proteins have been produced with success to very high levels in BL21(DE3). But, BL21(DE3) have not always expressed high level of proper folded proteins. Some proteins was produced in inclusion body which is inactive form due to immoderate expression rate and toxicity to cell. To solve this drawback, C41(DE3) from BL21(DE3) is constructed and able to grow and continue to produce proteins at an elevated level (Miroux et al., 1996). The C41(DE3) strain has been applied to production of proteins that were expressed in low levels in BL21(DE3) (Sorenson et al., 2003) (Figure 5).

6. Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) measures differences in refractive index which presents the number of the complex of the ligand and the analyte according to affinity of two molecules. SPR determines whether a protein has affinity against a ligand and characterizes kinetic values such as association and dissociation rate constants.

SPR-based instruments use an optical method to measure the refractive index near (within ~ 300 nm) a sensor surface (Figure 6). In the BIAcore this surface is one side of a small flow cell (~ 20 - 60 nl) through which an aqueous solution (running buffer) passes at a continuous flow rate (1 - 100 µl/min). To detect interaction of molecules, one molecule (the ligand) is immobilized onto the sensor surface in advance. Then its binding partner (the analyte) is injected in appropriate buffer through the flow cell, also under continuous flow. When buffer which contains analyte passes the flow cell, the ligand and the analyte form complex which increases according to affinity of the two molecules. The accumulation of the complex on sensor surface increases refractive index (RI). This refractive index change is measured in real time and the result plotted as response units (RU) versus time (a sensorgram). This sensorgram may contain a background response, so the actual sensogram is calculated by subtracting a background response.

This background response will be generated if the refractive index of the running buffer is different from that of sample buffer. No ligand or an irrelevant ligand is immobilized to the sensor surface and the analyte is then injected through reference flow cell as a control. Thereby the background response is obtained. The association (k_a) and dissociation rate constants (k_d) and the corresponding affinity constants (K_D) are calculated by measure the association and dissociation of the binding interaction in real time.

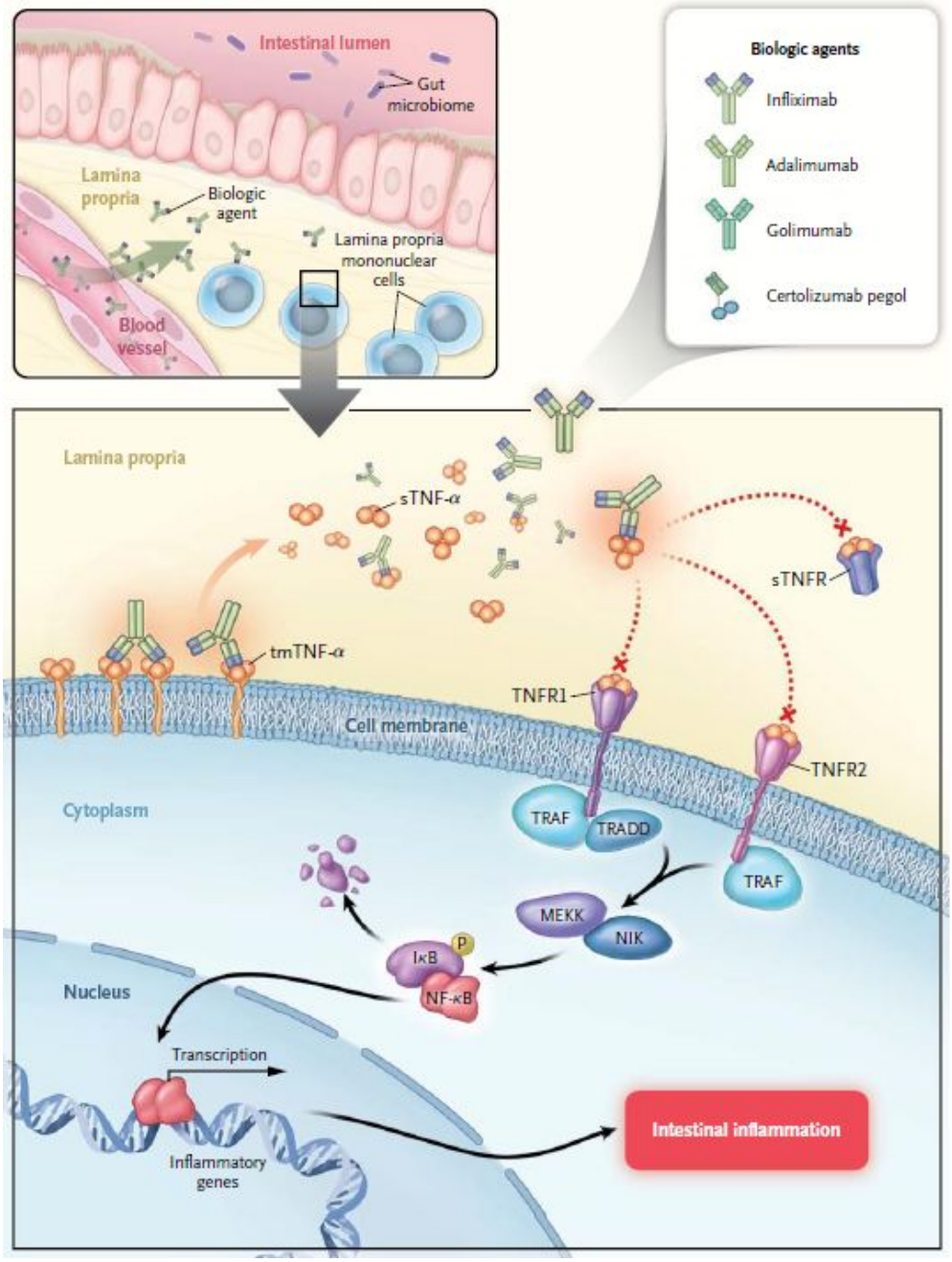

Figure 1. Mechanism of therapeutic effect of anti-TNFα antibodies in inflammatory bowel disease

(Nielsen et al., 2013)

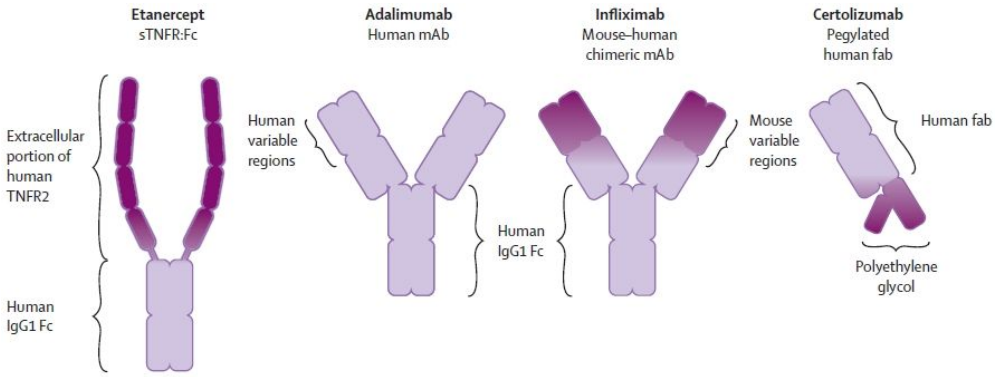

Figure 2. Structures of the TNF antagonists

(Wallis, 2008)

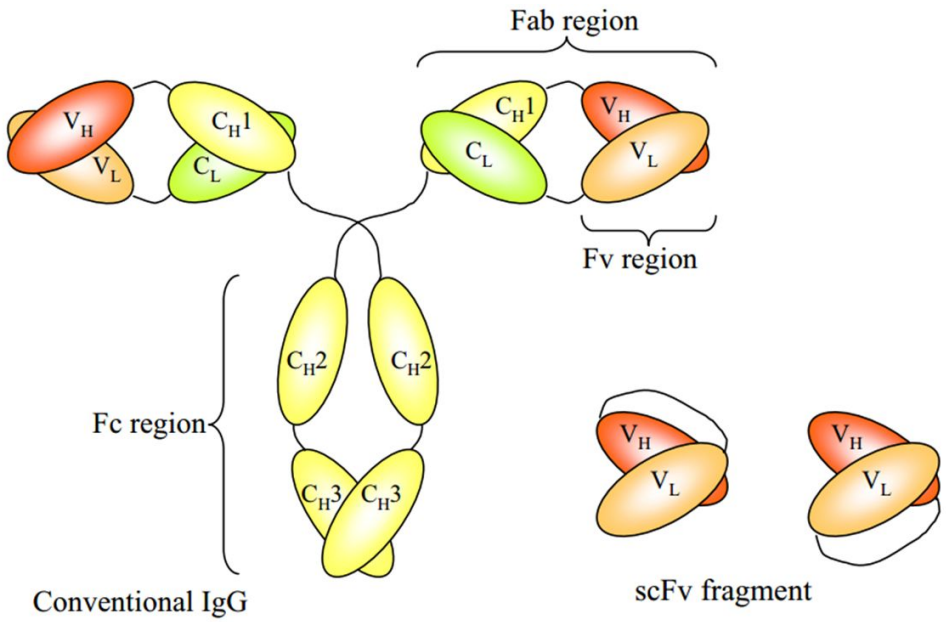

Figure 3. Subunit composition and domain distribution of immunoglobulin and single-chain variable fragment (scFv) antibody
(Joosten et al., 2003)

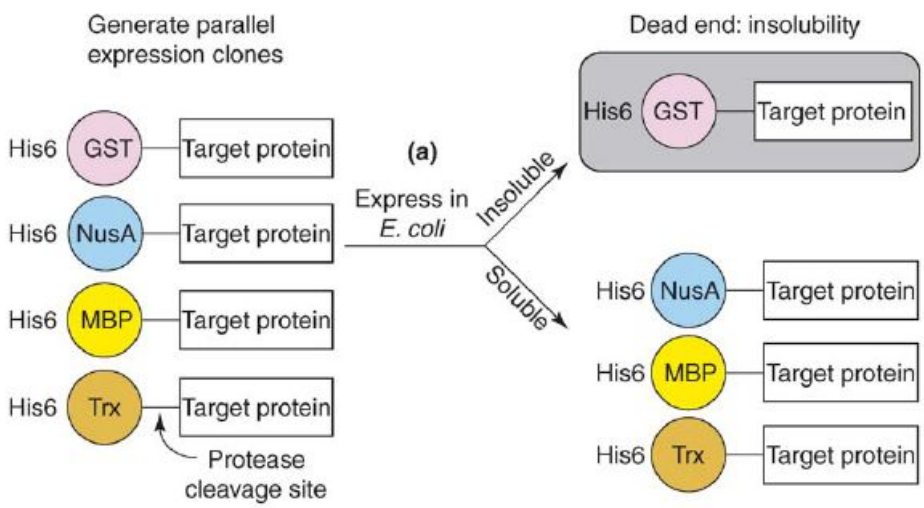

Figure 4. Protein expression using solubility tags

(Dominic et al., 2006)

Table 1. Generally used solubility-enhancing fusion partners

Tag	Source organism
MBP (Maltose-binding protein)	*Escherichia coli*
GST (Glutathione-S-trasferase)	*Schistosoma japonicum*
Trx (Thioredoxin)	*Escherichia coli*
NusA (N-Utilization substance)	*Escherichia coli*
SUMO (Small Ubiquitin-modifier)	*Homo sapiens*
SET (Solubility-enhancing tag)	Synthetic
DsbC (Disulfide bond C)	*Escherichia coli*
Skp (Seventeen kilodalton protein)	*Escherichia coli*
T7 PK (Phage T7 protein kinase)	Bacteriophage T7
GB1 (Protein G B1 domain)	*Streptococcus sp.*
ZZ (Protein A IgG ZZ repeat domain)	*Staphylococcus aureus*

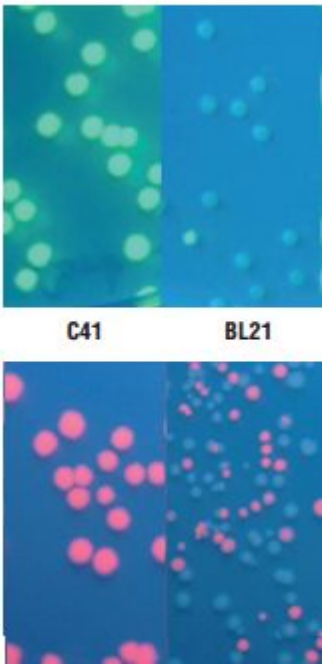

Figure 5. Toxic protein such as green fluorescent protein (top) or red fluorescence inducing protein (bottom) expressed in C41 and BL21 (Lucigen, United States)

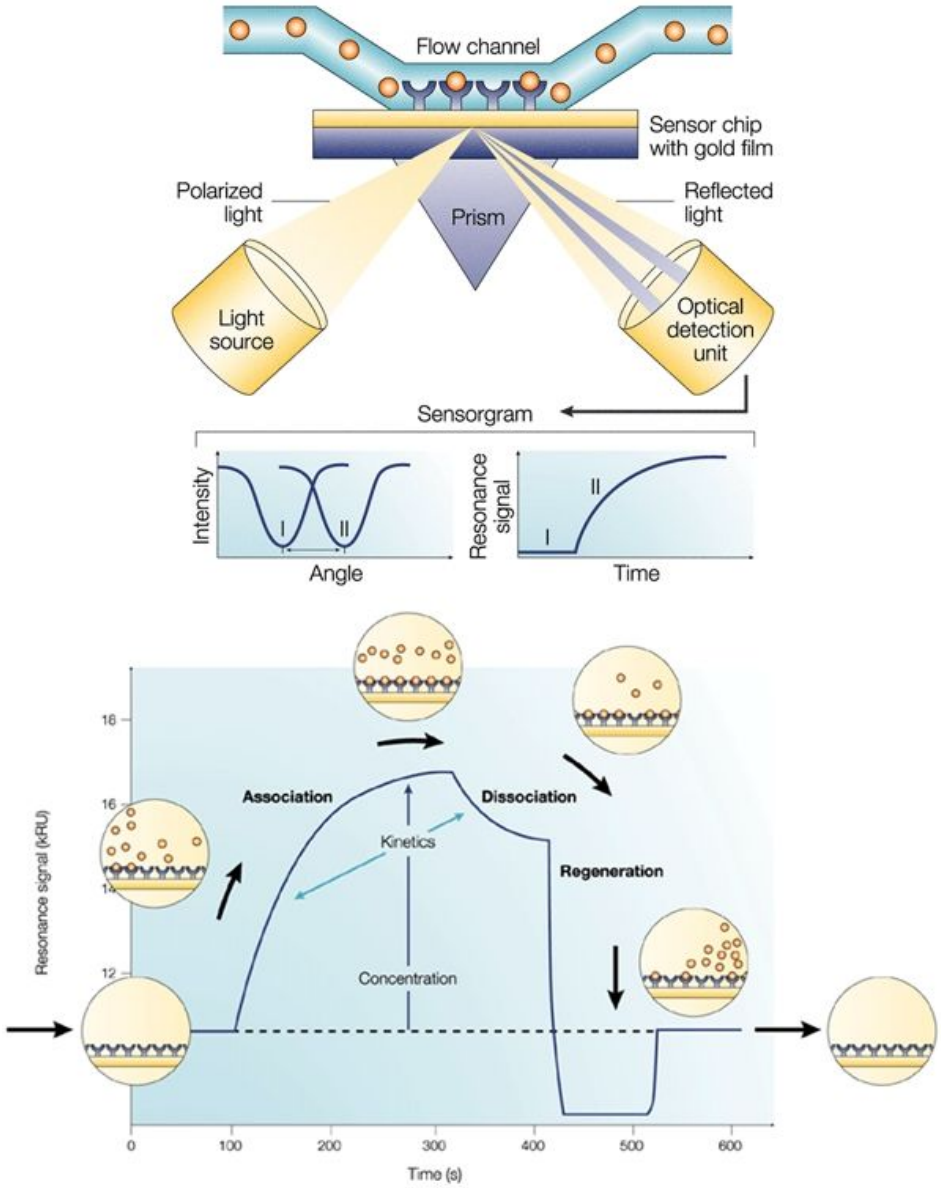

Figure 6. Typical set-up for an SPR biosensor and binding cycle (Cooper, 2002)

7. OBJECTIVES OF THE THESIS

This study is focused on production of single chain variable fragment (scFv) against TNFα in active form in *Escherichia coli*. This protein has affinity against human TNFα and characterization of the kinetic value of the scFv. The specific objectives of this research were as follows:

1) Construction of the soluble expression system of anti-TNFα scFv by fusing maltose binding protein and using *E. coli* C41(DE3) as host strain.

2) Analysis of the immunological properties of the purified anti-TNFα scFv against human TNFα and comparison of the characteristics among anti-TNFα fragments using ELISA and SPR.

3) Microbial production of the functional anti-TNFα recombinant antibody by fed-batch fermentation.

II. MATERIALS AND METHODS

1. Plasmids and strains

1.1 Enzymes and reagents

TNFα was bought from Sigma Aldrich Co. (St. Louis, USA). Restriction endonucleases, T4 DNA ligase, Taq polymerase, Klenow fragment, and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, USA). Pwo DNA polymerase and dNTPs were from Hoffmann-La Roche (Basel, Switzerland). Phosphate buffered saline (PBS: 0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl), phosphate buffered saline tween 20 (PBST: 0.01 M phosphate buffer with 0.138 M NaCl, 0.0027 M KCl, 0.05 % Tween 20), carbonate-bicarbonate buffer capsules (0.05M carbonate-bicarbonate buffer, pH 9.6), bovine serum albumin (BSA), phosphate-citrate buffer tablets (0.05M phosphate-citrate buffer, pH 5.0, 1 tablet/100 ml), 5'-tetramethyl benzidine dihydrochloride (TMB), hydrogen peroxide were obtained from Sigma Chemical Co. (St. Louis, USA.). A molecular weight standard of DNA was obtained from New England Biolabs (Beverly, USA) and a protein standard for electrophoresis from Sigma Chemical Co. (St. Louis, USA). Agarose, ampicillin, ethidium

bromide, trizma base, imidazole were purchased from Sigma Chemical Co. (St. Louis, USA). Bacto-peptone, tryptone, yeast extract, and bacto-agar was purchased from Difco Laboratories (Detroit, USA). All chemicals were of reagent grade. Slide-A-Lyzer MINI Dialysis Device for buffer changing were purchased from Thermo Fisher Scientific, Inc. (Waltham, USA). HiTrapFF for purification of proteins fused with the His 6 residues, MBPTrap HP for purification of proteins fused with the maltose binding protein and Series S Sensor chip CM5, Amine Coupling Kit, type 2, HBS-EP+ 10X buffer, Acetate 5.5 for measuring affinity between hTNFα and anti-TNFα by using biacore T-100 were purchased from GE healthcare (Little Chalfont, UK). Anti-TNFα monoclonal antibody produced in mouse was purchased from Abcam (Cambridge, USA). Quick Start Bradford Protein Assay Kit 2 was purchased from Bio-rad (Hercules, USA).

1.2 Oligonucleotides

Oligonucleotide primers were synthesized by Bioneer Co. (Daejon, Korea). They were synthesized with different restriction enzyme sites on the ends according to the purpose of experiments, which were used in PCR amplification, vector construction and gene recombination.

1.3 Strains and plasmids

E. coli Top10 [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] (Invitrogen, Waltham, USA) was used for plasmid preparation for DNA manipulation. *E. coli* BL21(DE3) [F^- *omp*T *gal dcm lon* $hsdS_B$ (r_B^-, m_B^-) λ(DE3 [lacI lacUB6-T7 gene 1 lnd1 sam7 nin5])] (Novagen, Birmingham, Germany) and *E. coli* C41(DE3) [F^- *omp*T $hsdS_B$ (r_B^-, m_B^-) *gal dcm* (DE3)] (Lucigen, Middelton, USA) were used as host strains for the expression of target proteins.

Plasmids, pET19b were used as mother vectors which has the T7 promoter for expression of anti-TNFα scFv and maltose binding protein (MBP) fusion proteins. Anti-TNFα scFv gene was from pre-constructed plasmid (KT Bong, thesis, 2006) (Figure 6). MBP genes were amplified by polymerase chain reaction (PCR) from pMAL-p2X vector (New England Biolabs, Ipswich, USA).

1.4 Recombinant DNA techniques

All of cloning steps were carried out according to the procedure of Sambrook *et al* (Sambrook et al., 1989). Mini-scale plasmid preparation was

carried out with the High Pure Plasmid Isolation kit (Roche, Basel, Switzerland) and large-scale plasmid preparation was achieved using Plasmid Midi Kit (Qiagen, Venlo, USA). Digestion of DNAs with restriction endonuclease, and dephosphorylation were achieved under the conditions recommended by the supplier, and the results of digestion were analyzed by agarose gel electrophoresis. This system was provided by Bio-Rad (Hercules, USA). The isolation of DNA fragments from agarose gel, solubilization of the gels and recovery of the DNA fragments were conducted by "High Pure PCR Product Purification Kit" from Roche (Basel, Switzerland) or "QIAquick Gel Extraction Kit" from Qiagen (Venlo, Netherlands). Ligation of the DNA fragments was carried out by "DNA Ligation Kit" from Takara (Shiga, Japan).

1.4.1 Polymerase chain reaction (PCR)

All PCRs for amplification were performed with the GeneAmp 2400 (Applied Biosystems, Waltham, USA). AccuPower PCR PreMix (Bioneer Co., Daejon, Korea), a ready-to-use PCR reagent fully optimized for more accurate PCR amplification was used for the PCR reactions. PCRs for cloning of genes were performed containing 10 pM each of forward and reverse primers, and plasmid DNA as a template. Reaction time and temperature were optimized according to each reaction condition. The

amplified gene was confirmed by gel electrophoresis.

1.4.2 Construction of expression plasmids

To construct the expression vector of scFv against TNF-α, the scFv gene was amplified by the polymerase chain reaction (PCR) from the previously constructed vector (KT Bong, thesis, 2006) (Figure 7). with the corresponding PCR primers: F1 and R1 (Table 2). The amplified scFv gene was treated with DNA restriction enzymes of A and B, and inserted in the vector fragment which was obtained though treating plasmid from Hyo-Ran Lee (HR Lee, thesis, 2014) with same restriction enzymes and purifying the fragment eliminating scFv against aflatoxin B, plasmid pET19b s.s malE Xa scFv H6 was constructed. The ligation of the expression vectors was performed using the DNA ligation kit according to manufacturer's protocols.

1.5 DNA sequencing

DNA sequencing was performed by Mbiotech (Hanam, Korea). The results of DNA sequencing for selected clone candidates were compared each other, and then the selected DNAs were used in the subsequent experiments.

2. Expression of proteins

2.1 Transformation and expression of fusion proteins

Transformation of *E. coli* was carried out as described by Sambrook *et al.* (Sambrook et al., 1989). *E. coli* Top10 was incoculated in 5 mL LB medium, and precultured at 37°C overnight. 1% aliquot of the cultured cells was transferred into 50 mL of fresh LB medium and incubated with shaking until OD at 600 nm reached 0.5. The pellet which had been harvested by centrifugation at 6,000 rpm for 5 min at 4°C was resuspended cautiously in 5 mL of ice-cold 100 mM $CaCl_2$ solution and stored on ice for 30 min. The cell suspension was centrifuged (6,000 rpm for 5 min at 4°C) and the pellet was resuspended in 5 mL of 100 mM $CaCl_2$ solution. Resuspended cells were aliquoted to 100 μl, mixed with ligated DNA, and kept on ice for 30 min. They were subjected to heat-shock at 42°C for 45 seconds. One mL of LB medium was added to the cells and incubated at 37°C for 1 hour with slow agitation. An appropriate volume of the transformed cells was spread on an LB agar plate with a selection marker. BL21(DE3) strain was selected with kanamycin and C41(DE3) strain was selected with ampicillin.

Equal amounts of scFv and maltose binding protein fusion plasmids were used for transformation of *E. coli* BL21(DE3) and C41(DE3). The

transformed cells were spread on LB agar plates containing 50 μg/mL kanamycin, ampicillin (selection for plasmids) respectively.

E. coli BL21(DE3) and C41(DE3) colonies were picked from LB agar plates with appropriate antibiotics containing fresh transformed cells and cultured in 5 mL LB medium in the presence of with appropriate antibiotics for about 12 hr at 37°C. Flask cultures were carried out with LB medium in 500 mL baffled flasks with a 100 mL working volume. Each flask was inoculated with 1.0 mL of the transformed cell, pre-cultured in the log phase and was grown at 37°C in LB medium containing selective markers. Expression of scFv and MBP fusion proteins were induced by the addition of isopropyl-2-D-thio-galactopyranoside (IPTG) to final concentration of 0.2 mM.

2.2 SDS-PAGE

After induction, the induced cells were grown for about additional 4 h, centrifuged at 10,000 rpm, resuspended in 100 mM sodium phosphate buffer (pH 7.4) and then were treated by sonication for cellular protein assay. The crushed cells were centrifuged at 12,000 rpm at 4°C for 1 min and the supernatant, taken as the soluble fraction, was recovered carefully. The pellet, taken as the insoluble fraction, was resuspended in an equivalent volume of

the same phosphate buffer. Separation of proteins by molecular weight in SDS-PAGE, soluble and insoluble proteins were resuspended in sodium dodecyl sulfate (SDS) sample buffer and boiled at 100°C for 5 min. Heat-denatured fractions were analyzed by 10 ~ 15% SDS-polyacrylamide gel electrophoresis. Protein samples were electrophoresed on an SDS-containing discontinuous polyacrylamide gel electrophoresis unit using the Mini-Protein II system (Bio-Rad, Hercules, USA). 12.5% (w/v) separating gel was prepared usually from 33.5% (w/v) acrylamide/ 0.3% (w/v) N, N'-methylenebisacrylamide stock solution in 0.38 M Tris-HCl (pH 9.1) and 0.1% (w/v) SDS. The 4% (w/v) stacking gel was prepared from 30% (w/v) acrylamide/0.44% (w/v) N, N'-methylenebisacryl-amide stock solution in 0.125 M Tris-HCl (pH 6.8) and 0.1% (w/v) SDS. Both gels were polymerized with ammonium persulfate and TEMED. The running buffer was composed of 25 mM Tris base, 192 mM glycine, and 0.1% (w/v) SDS. Samples were mixed with equal volumes of 2X loading buffer [0.125 M Tris-Cl buffer (pH 6.8); 10% (v/v) β-mercaptoethanol; 4% (w/v) SDS; 20% (v/v) glycerol; a pinch of bromophenol blue] and boiled for 3 min before loading on the gel. Electrophoresis was carried out at 90 V for stacking and at 120 V for separating. When electrophoresis was finished, the gel was stained with commassie blue R-250 solution [0.2 % (w/v) Coomassie blue R-250; 50 % (w/v) methanol; 10 % (v/v) acetic acid] for 30 min with gentle

shaking and destained with destaining solution [20 % (v/v) methanol; 10 % (v/v) acetate].

2.3 Fed-batch fermentation

Fed-batch culture was performed in a 3 L jar fermentor (Fermentec, Cheongju, Korea) with 1 L working volume of a Riesenberg medium. E. coli was grown in a 500ml flask with 100 ml working volume at 37°C and 250 rpm for 12 hr. The pre-grown cells were inoculated to a fermentor for main culture. The 100 ml seed culture was prepared in a 500 ml flask and grown in a shaking incubator at 37°C and 250 rpm for 12 hr, and main culture was carried out. To maintain the dissolved oxygen (DO) level, agitation speed and aeration rate were set at 1200 rpm and 1 vvm, respectively. Acidity was automatically controlled at 6.8 by the pH-stat strategy. After depletion of 20 g/L sugar initially added, ammonia water and high concentration of carbon source feeding solutions (800 g/L glucose and magnesium sulfate heptahydrate 20 g/L) were used to keep the cell growth and basal level of sugar. Feeding solutions were conversed to organic acids by the metabolic processes of cells. When O.D. at 600 nm reached 150, IPTG was added to induce expression of scFv and MBP fusion proteins.

3. Purification and quantitative analysis of scFv

3.1 Purification

3.1.1 Affinity chromatography

The expressed protein was purified using the His tag and MBP tag (Figure 8). Purification with the His tag was conducted by gravity-flow and Ni-NTA agarose (Qiagen, Venlo, Netherlands) was used. Resin was washed with distilled water. After equilibrating the column with binding buffer [20 mM sodium phosphate (pH 8.0), 500 mM NaCl], the sample resuspended in binding buffer was loaded. The column loaded with the sample was washed with binding buffer, and the bound proteins were eluted with elution buffer [20 mM sodium phosphate (pH 8.0), 500 mM NaCl, 500 mM immidazole] under a constant level of immidazole concentration.

The purification with the MBP tag was carried out by the Äcta prime system (Amersham Bioscience, Uppsala, Sweden) using the MBPTrap HP column (GE healthcare, Little Chalfont, UK) used in MBP-tagged protein purification. Binding and elution buffer composition used in this experiment is as follows. Binding buffer [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA] (pH 7.4) and elution buffer [20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM Maltose] (pH 7.4). Except for buffer composition, the procedure of purification using MBP tag was the same as purification using His tag.

Purified proteins were analyzed by SDS-PAGE.

3.1.2 Dialysis

To change the buffer in the purified protein solution, a Slide-A-Lyzer MINI Dialysis Device (Thermo Fisher Scientific, Inc., Waltham, USA) was used. The device was filled with desired buffer. After 4 hour, fresh buffer was loaded. About 2.5 mL of the protein sample was loaded on the membrane. Aggregated protein was removed and then soluble fraction was only used.

3.2 Quantitative analysis

3.2.1 Bradford assay

BSA (Bovine Serum Albumin) standard protein was prepared to determine a standard curve for quantitative analysis of scFv and MBP fusion proteins. BSA with concentrations of 0, 5, 10, 15 μg/ml for the standard assay was used. ScFv and MBP fusion protein samples were diluted with distilled water. 200 μl of Quick Start Bradford protein 1x dye reagent from Quick Start Bradford Assay Kit and 800 μl of protein were combined and vortexed. Combined samples were incubated at room temperature for 5 minutes, and measured absorbance at 595 nm.

4. Immunological analysis

4.1 Indirect ELISA

To examine the antigen-binding affinity of scFv and MBP fusion proteins indirect ELISA (Enzyme linked immunosorbent assay) was used (Fig. 12). The antigen-binding affinity was determined by detecting the signal of scFv and MBP fusion proteins according to concentrations of a TNFα.

An immune 96 microwell plate was coated with 0.1, 1, 10 μg/ml of human TNFα at room temperature overnight. The plate was washed with 200 μl of PBST buffer, and then was coated with 200 μl of 5% skim milk in 2 hours. The plate coated with skim milk was washed with PBST buffer and coated with 100 μl of the prepared soluble scFv and MBP fusion proteins and incubated at room temperature for 3 hours. Monoclonal antibody as positive control and 1% BSA as negative control were used. After washing, 100 μl of the anti-His antibody conjugated with HRP as secondary antibody was added to each well and incubated for 1 hour. After washing, 100 μl of 5'-tetramethyl benzidine dihydrochloride (TMB) substrate solution was added and incubated for 30 minutes. 2 M sulfuric acid solution was added to each well to stop color development. Absorbance of samples was measured by a microplate reader at 450 nm. To compare affinities between His-tag antibody

and MBP-tag antibody, additional experiments were conducted by using anti-MBP Ab and anti-His Ab as a secondary antibody (Figure 9).

4.2 Surface Plasmon Resonance

The antigen-binding affinity of scFv and MBP fusion proteins was characterized by SPR technique on a Biacore T100 (GE healthcare, Little Chalfont, UK) instrument.

To characterize kinetic values for affinity between anti-TNFα scFv and human TNFα, SPR experiment was performed. First, human TNFα (Sigma Aldrich, St. Louis, USA) in pH 5.5 acetate buffer was immobilized to the carboxymethyl dextran-coated CM5 sensor chips using covalent interaction of amine to obtain an immobilization level of ~350 RU. Second, the solution of the anti-TNFα scFv and MBP fusion protein at different concentrations was injected on the bound human TNFα at a flow rate of 30 µl/minute for 150 seconds for association. For dissociation of the anti-TNFα, HBS-EP+ buffer from GE Healthcare (Little Chalfont, UK) was injected at a flow rate of 30 µl/minute for 500 seconds. Biosensor matrices were regenerated using 0.5 mM NaOH. The Biacore T100 evaluation software was used to determine k_d (dissociation constant) and k_a (association constant) values. The value of K_D was determined by dividing k_d by k_a.

The affinity of anti-TNFα monoclonal antibody (Abcam, Cambridge, USA)

to human TNFα was also determined after immobilizing the receptor on CM5 sensor chips. The experiment was conducted by the same method as anti-TNFα scFv.

Table 2. Sequences of the primers used in this research

Primer name	Primer sequences (5′-3′)
TNF-NdeI-F	GCA ATT CCA TAT GGA TAT TGT GAT GAC GC
TNF-BamHI-R	CG GGA TCC TCA GTG GTG GTG GTG GTG G

CATATGGATATTGTGATGACGCAGTCTCCACTCTCCCTGCCTGTCA

GCTTTGGAGATCAAGTTTCTACCTCTTGCAGGTCTAGTCAGAGTCT

TGCAAACAGTTATGGGAACACCTATTTGTCTTGGTACCTGCACAA

GCCTGGCCAGTCTCCACAGCTCCTCATCTATGGGATTTCCAACAG

ATTTTCTGGGGTGCCAGACAGGTTCAGTGGCAGTGGTTCAGGGAC

AGATTTCACACTCAAGATCAGCACAATAAAGCCTGAGGACTTGGG

AATGTATTACTGTTTACAAGGTACACATCAGCCGTACACGTTCGG

AGGGGGGACCAAGCTGGAAATAAAAGGTGGCGGTGGCTCCGGCG

GTGGTGGCAGCGGTGGCGGCGGTTCTAGGGTCCAACTTCAGGAG

AATGGGGCTGAGCTGGTGAGGCCTCGGGCTTCAGTGAAGCTGTCC

TGCAAGGCTTCGGGCTACACATTTTCTGACTTTGAAATGCACTGG

GTGAAGCAGACACCTGTGCATGGCCTGGAATGGATTGGAGATATT

GATCCTGGAACTGGTGATACTGCCTACAATCTGAAGTTCAAGGGC

AAGGCCACACTGACTACAGACAAATCTTCCAGCACAGCCTACATG

GAGCTCCGCAGCCTGACATCTGAGGACTCTGCCGTCTATTACTGT

ACCCTCGGGGCCTTTGTTTACTGGGGCCAAGGGACTCTGGTCACT

GTCTCTGCACTCGAGCACCACCACCACCACCACTGA

Figure 7. Amino acid sequences of anti-TNFα scFv with His tag

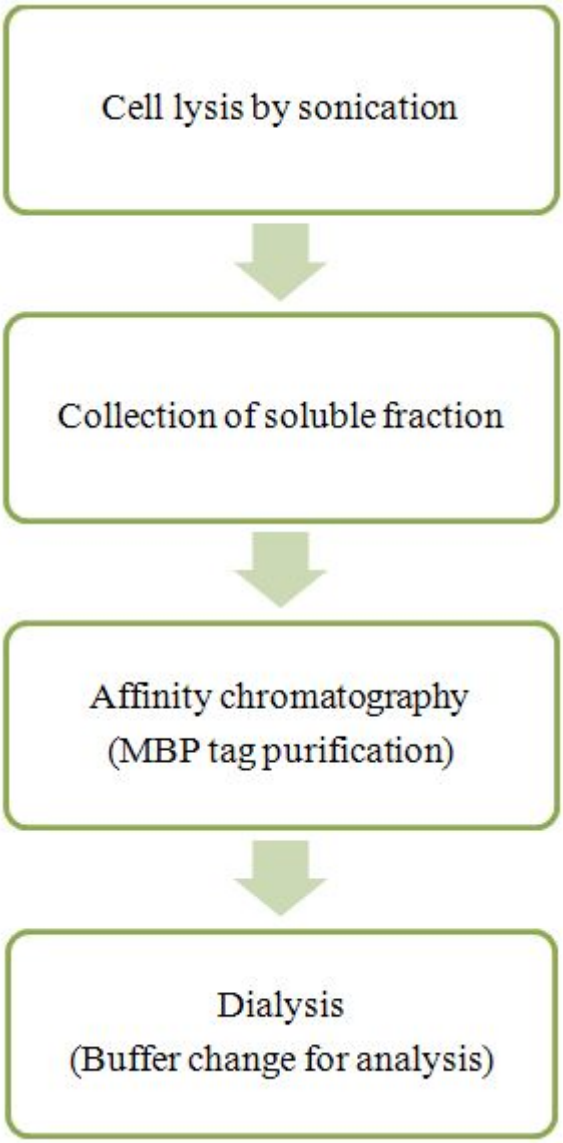

Figure 8. Purification process of MBP fusion scFv for analysis

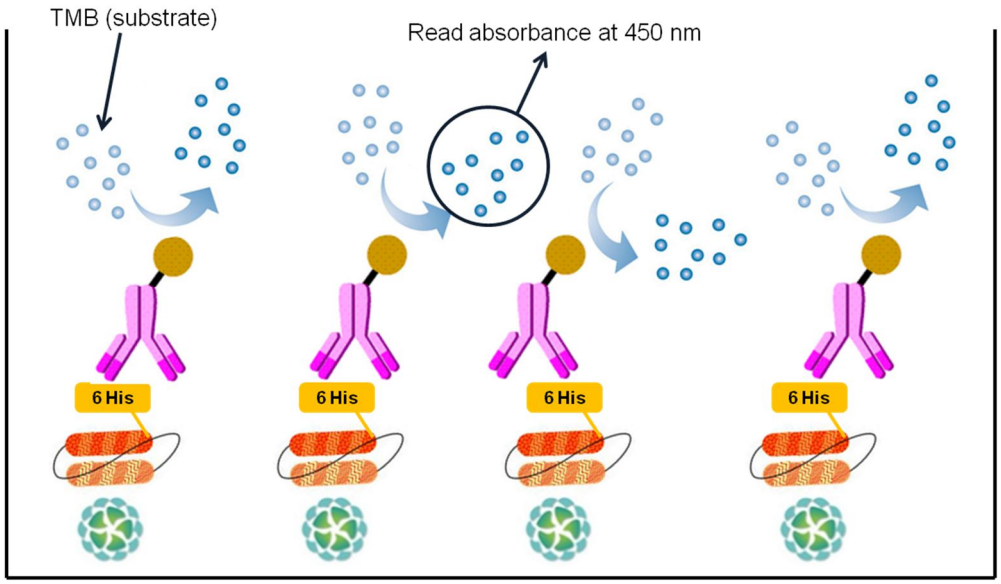

Figure 9. Diagram of indirect ELISA for identification of affinity between human TNFα and anti-TNFα

III. RESULTS AND DISCUSSIONS

1. Plasmids and strains

1.1 Construction of expression plasmids and strains

To obtain soluble and functional scFv *in vivo*, maltose binding protein which is expressed in high solubility in *E. coli* was fused with the anti-TNFα scFv. Plasmids for the expression of fusion protein were constructed that MBP was fused to the N-terminal of the scFv gene. At factor Xa protease site was inserted between the scFv and MBP genes. For purification, the 6 histidine tag was combined into expression plasmids. pET19b s.s. malE Xa scFv H6 plasmid was constructed for scFv expression (Figure 10).

2. Expression of proteins

2.1 ScFv and MBP fusion protein expression.

In order to express soluble and active scFv and to elevate the expression level of scFv, scFv was fused on C-terminal of the maltose binding protein. The MBP-fused scFv was inserted in expression vector containing

periplasmic targeting signal sequence. The vector of pET19 s.s malE Xa scFv H6 was transformed into *E. coli* C41(DE3). As a control strain, *E. coli* BL21(DE3) carrying the scFv gene without MBP was used. This scFv was expressed in BL21(DE3) in insoluble form and had 27kDa that is size of protein without MBP-tag (Figure 11).

To evaluate the effect of MBP and C41(DE3) on solubility of scFv, flask fermentations of the above two strains were performed. The soluble protein level of scFv with the MBP tag in C41(DE3) was higher than that of scFv without the MBP tag in BL21(DE3). These results suggested that an expression system of the fusion scFv with MBP in *E. coli* C41(DE3) was effective in expressing soluble scFv *in vivo* (Figure 11).

2.2 Fed-batch fermentation

Fed-batch fermentation of the recombinant strain which produced soluble scFv fused with MBP tag was performed using Riesenberg medium with 20 g/L glucose. After depletion of glucose initially added, the operation mode was controlled by the pH-stat fed-batch using feeding solution. When O.D. at 600 nm reached 100 ~ 150, IPTG (0.2 mM) was added for induction of scFv gene expression. After induction, scFv and MBP fusion protein was produced. Finally, the pH-stat fed-batch fermentation of C41(DE3) / pET19b

s.s malE Xa scFv H6 exhibited 86.14 mg/L dry cell weight, 72.7 mg/L scFv and MBP fusion protein concentration (Figure 12).

3. Purification and quantitative analysis of scFv

The scFv fused with MBP was purified by using the Ni-NTA resin which has specific affinity to the His-tag. SDS-PAGE was conducted with the purified scFv to verify the degree of purification. The band intensity of the MBP-fused scFv in SDS-PAGE was weak, suggesting that purification was not performed efficiently (Figure 13(a)).

For efficient purification of the scFv fused with MBP, the MBP Trap HP column (GE healthcare, Little Chalfont, UK) specific for MBP was used. FPLC was operated to purify the MBP fused scFv protein. SDS-PAGE analysis indicated that the target antibody was purified properly and detected at the size of molecular weight of 69 kDa, the size of the MBP fused scFv (Figure 13(b)). These results demonstrated that scFv and MBP fusion protein production system *in vivo* is constructed.

To quantify the scFv and MBP fusion protein, the Bradford assay was performed and standard curve was calculated using BSA. Using the standard curve, the concentration of the scFv and MBP fusion protein was measured

to be used for immunological analysis.

4. Immunological analysis of scFv

4.1 Indirect ELISA

Indirect ELISA analysis was performed for determining the binding activity of the anti-TNFα scFv and MBP fusion protein with human TNFα at 10 μg/mL of antigen concentration. First, the anti-His Ab conjugated with HRP as secondary antibody was used. But the absorbance of sample at 450 nm is similar with that of negative control (Figure 14). It was hypothesized that correct binding between anti-his Ab and his-tag would be difficult due to structure of protein where his-tag was located inside. Therefore, the anti-MBP Ab conjugated with HRP was used as a secondary antibody.

The scFv and MBP fusion protein with the anti-MBP antibody as a secondary antibody shows high affinity than using the anti-His antibody as a secondary antibody. Optical density at 450 nm of the scFv and MBP fusion protein with the anti-His antibody as a secondary antibody was similar to that of 1% BSA as negative control. On the other hand, optical density of the scFv and MBP fusion protein with anti-MBP antibody showed about 50% of

the anti-TNFα monoclonal antibody as positive control. These results demonstrated that the scFv and MBP fusion protein had affinity to human TNFα by using anti-MBP antibody as a secondary antibody (Figure 15).

4.2 Surface plasmon resonance

4.2.1 Anti-TNFα scFv fused with MBP tag

To characterize kinetic value like k_a, k_d and K_D between anti-TNFα scFv and TNFα, SPR technique on a Biacore T100 (GE Healthcare, Little Chalfont, UK) instrument was carried out.

In typical SPR experiment, binding reaction of antibody and antigen has association phase and dissociation phage. The association phase had gradually increase peak during flow of analyte and the dissociation phase was shown during flow of buffer without analyte (Figure 6). The SPR was performed with purified scFv to show gradually increase plot in 150 s of association phase and then decrease plot when the scFv supply ceased. The association value (k_a) and dissociation value (k_d) were calculated in 2040 (1/Ms) and $1.769*10^{-3}$ (1/s), respectively. Finally, K_D value was determined to $8.671*10^{-7}$ (M) (Figure 16).

K_D values of most antibodies are in the range from micromolar (10^{-6}) to

nanomolar (10^{-7} to 10^{-9}) range (Ramos-Vara et al., 2014). As the K_D value of scFv and MBP fusion protein was involved in this range, the scFv and MBP fusion protein was considered to have biological functions as antibody.

4.2.2 Anti-TNFα monoclonal antibody produced in mouse

To compare the efficiency of the MBP fusion scFv produced in this study with general antibody, SPR was performed with the monoclonal anti-TNFα antibody produced in a mouse. Before the SPR experiment with the MBP fusion scFv, the human TNFα was immobilized first in the same manner as the SPR with scFv performed above. In case of the monoclonal anti-TNFα, the association value (k_a), dissociation value (k_d) and K_D value were calculated in $2.984*10^4$ (1/Ms), $4.458*10^{-4}$ (1/s) and $1.494*10^{-8}$ (M), respectively (Figure 17). When comparing with MBP fusion scFv, K_D value of monoclonal anti-TNFα was 58-fold lower. This result represents that monoclonal anti-TNFα had higher affinity to human TNFα than MBP fusion scFv.

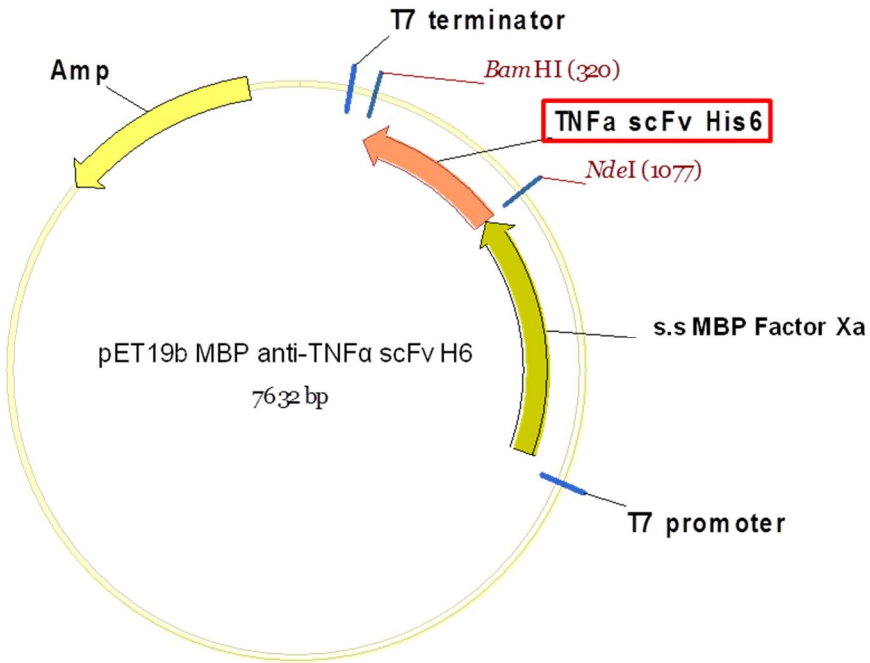

Figure 10. Genetic map of plasmid for expression of anti-TNFα scFv

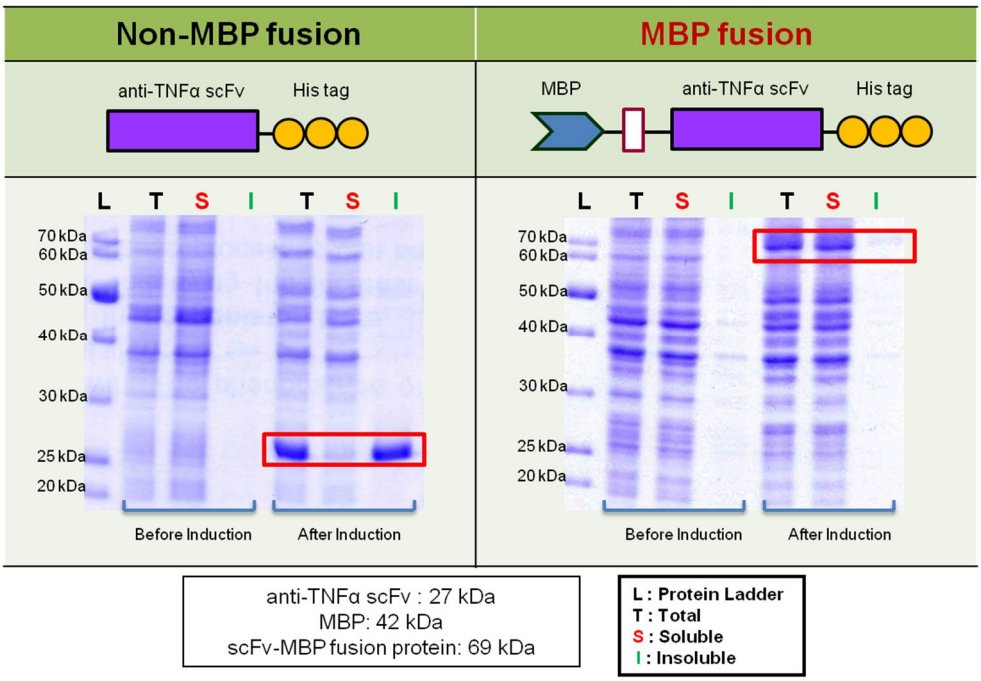

Figure 11. Expression of non-MBP fusion scFv in BL21(DE3) and MBP fusion scFv in C41(DE3) at 37°C with 0.2 mM IPTG

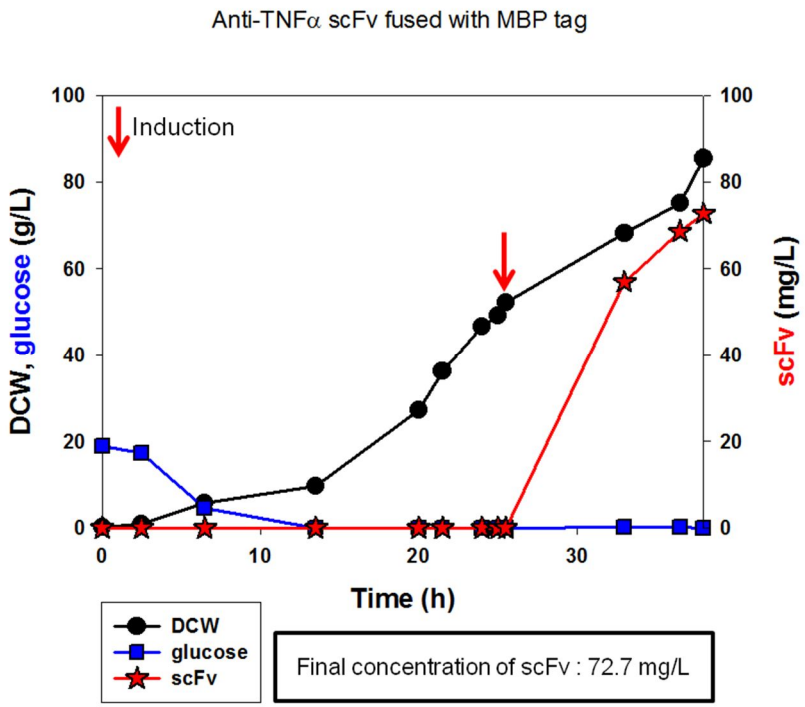

Figure 12. Profile of fed-batch fermentation

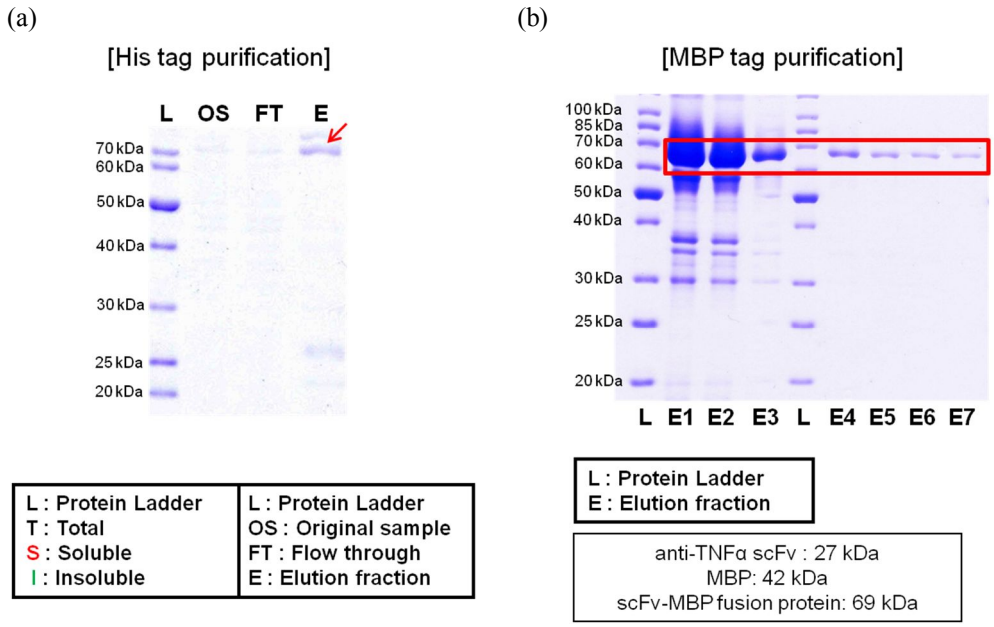

Figure 13. SDS-PAGE of MBP fusion scFv purified by HIS tag (a) and MBP tag (b) using affinity chromatography

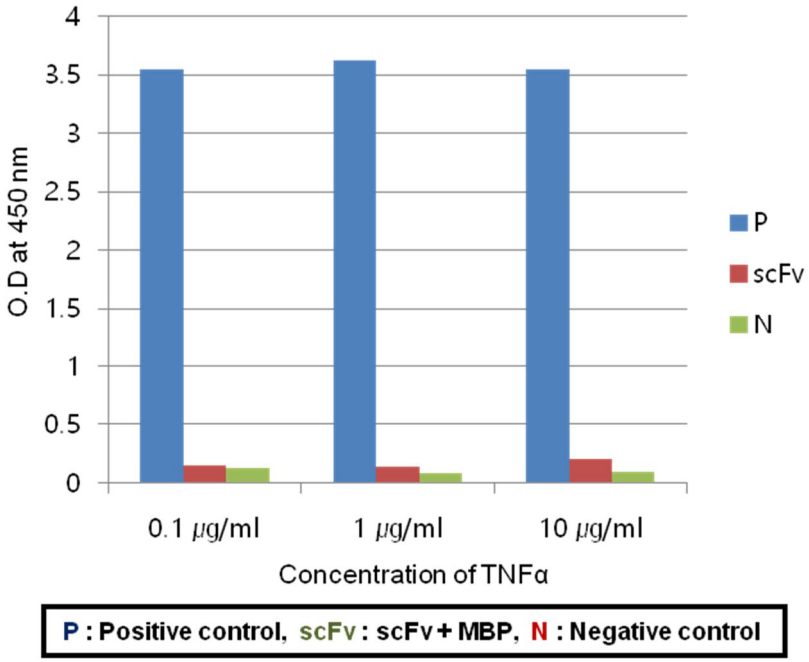

Figure 14. Comparison of antigen (human TNFα) binding activity of scFv and MBP fuison protein by indirect ELISA using anti-His Ab as secondary antibody

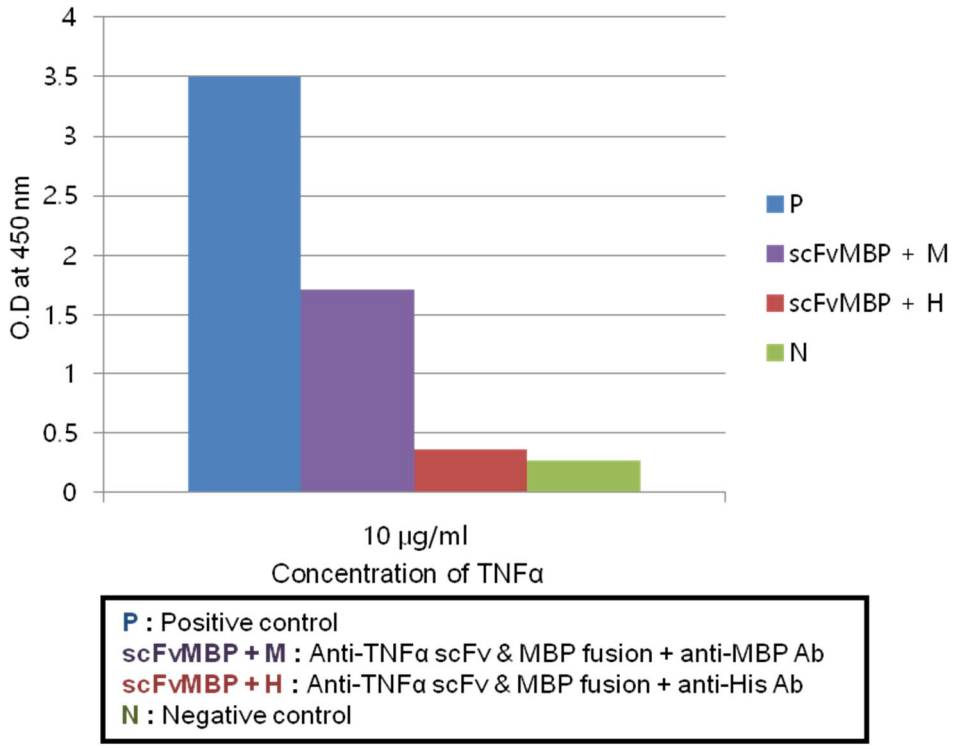

Figure 15. Comparison of antigen (human TNFα) binding activity of scFv and MBP fuison protein by indirect ELISA using anti-His Ab and anti-MBP Ab as secondary antibody

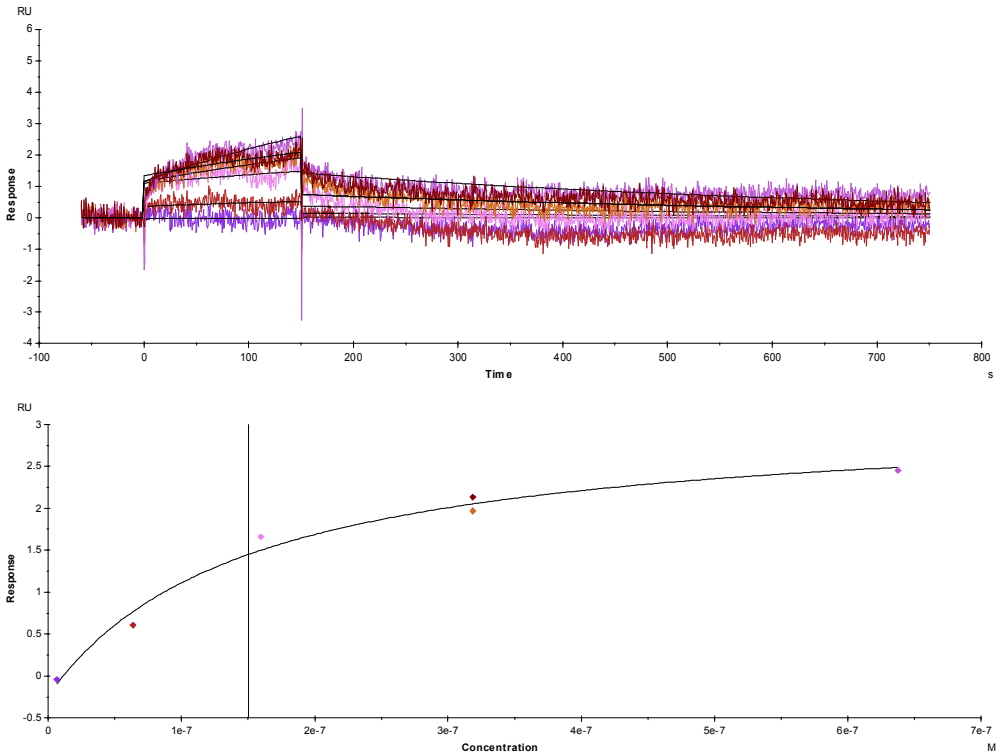

Figure 16. Antigen (human TNFα) binding activity of scFv and MBP fusion protein analyzed by SPR (Biacore T100)

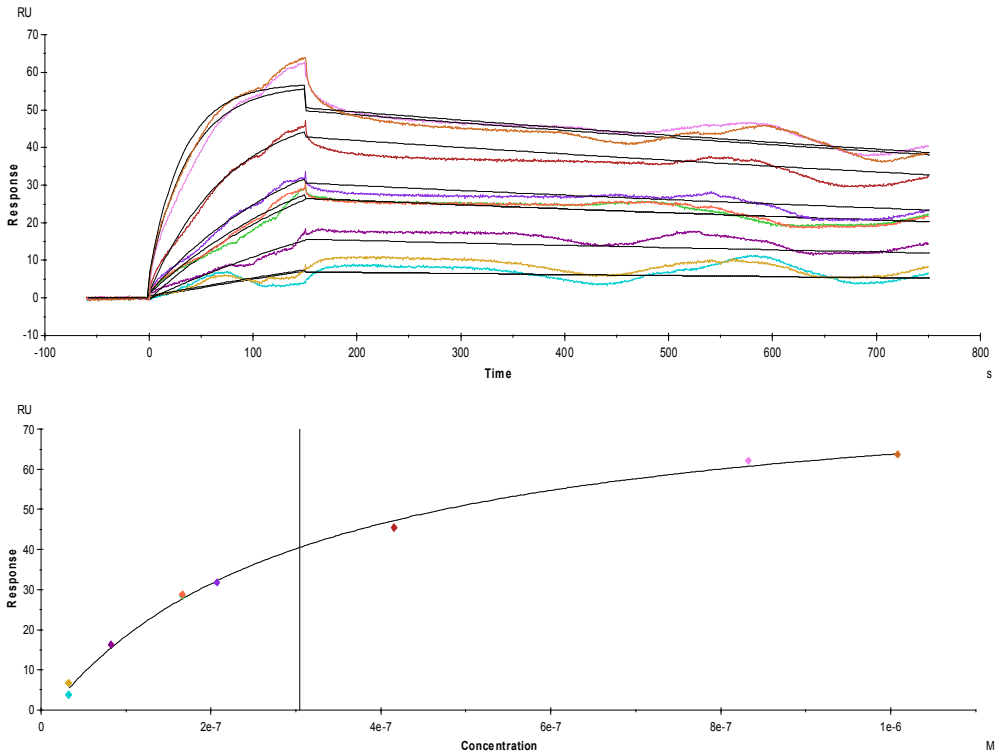

Figure 17. Antigen (human TNFα) binding activity of monoclonal antibody produced in mouse analyzed by SPR (Biacore T100)

IV. CONCLUSIONS

It was confirmed that the application of the MBP tag and *E. coli* C41(DE3) strain are effective to express the soluble and active anti-TNFα scFv. The results obtained in this research can be summarized as follows;

1) An expression system for anti-TNFα scFv by fusing the scFv gene with MBP in *E. coli* C41(DE3) was verified for production of soluble scFv.

2) The scFv and MBP fusion protein was purified by using the MBP tag as a ligand and immunological properties were measured with the purified protein. As a result, anti-TNFα scFv produced in *E. coli* had a binding affinity to human TNFα.

3) 72.7 mg/L scFv was produced in fed-batch fermentation of engineered *E. coli*.

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국 문 초 록

싸이토카인 종양괴사인자 알파(Tumor Necrosis Factor alpha) 는 17kDa 의 분자량을 갖는 단백질로 체내에서 감염에 대한 면역반응을 유도하는 proinflammatory cytokine이지만, 과발현 될 경우 류머티스 관절염과 같은 자가면역 질환이나 조직적인 부종, 고단백혈증, 호중구 감소증 등의 질병을 일으킬 수 있다. 이를 해결하기 위해 TNFα를 중화하는 여러 가지 anti-TNFα가 의약품으로 이용되고 있는데, 대부분의 anti-TNFα는 IgG의 형태로 존재한다. 그러나 IgG의 경우 크기가 크고 생산이 어렵다는 단점이 있어 light chain과 heavy chain의 가변영역만을 재조합 한 scFv (Single Chain Variable Fragment) 가 새로운 항체의 종류로 떠오르고 있다.

선행연구에 의해 첫째, 대장균에서 anti-TNFα scFv를 생산하였으나 insoluble하게 발현되어 그 자체로 제대로 된 기능을 하지 못하였다. 따라서 in vitro에서의 refolding이 필요 했는데, 이는 항체단백질을 생산하고자 할 때 시간적, 비용적인 손실을 일으켰다. 둘째로, 다른 항원(aflatoxin)에 대한 scFv생산 연구에서 MBP(Maltose binding protein) tag을 결합, C41균주에서 발현하자 soluble하게 발현되는 것을 확인하였다. 이를 종합하여 *in vivo*에서 기능적인 anti-TNFα scFv를 생산하고자 하였다.

먼저 추후 정제를 위한 His tag과 solubility를 향상시켜줄 MBP가 tagging된 anti-TNFα scFv를 발현할 수 있는 벡터를 구축하고 이를 C41균주에서 IPTG를 이용해 발현하여 soluble한 재조합 항체를 얻을 수 있었다. 이를 His tag과 MBP

tag을 각각 이용하여 정제 후 indirect ELISA를 통해 TNFα에 대한 affinity를 확인하고자 하였다. 2차 항체로 anti-His Ab를 사용했을 경우 His tag이 단백질 내부에 위치하여 2차 항체로 적절치 않다고 생각하여 anti-MBP Ab를 2차 항체로 사용하여 측정한 결과 affinity를 확인할 수 있었다. 정확한 affinity를 알아보고 다른 anti-TNFα와 비교하기 위하여 SPR(Surface Plasmon Resonance) 을 수행하였다. Monoclonal anti-TNFα의 경우 KD값이 1.494E-8 (M)로 측정되었고, anti-TNFα scFv w/ MBP fusion의 경우, KD값이 8.671E-7 (M)로 측정되었다. 그 후 유가식 배양을 통하여 최종농도 72.7 mg/L 의 scFv-MBP fusion protein을 생산하였다.

이 연구를 통해 치료용 단백질인 anti-TNFα의 재조합 scFv 항체를 미생물 발효공정을 통하여 생산 할 수 있음을 확인하였다.

주요어: *Escherichia coli*, scFv, fusion 단백질, tumor necrosis factor alpha, indirect ELISA, surface plasmon resonance

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