High Expression of Fusion Proteins Consisting of a Single-chain Variable Fragment Antibody Against a Tumor-associated Antigen and Interleukin-2 in *Escherichia coli*

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Abstract. *Background/Aim: The aim of this study was to establish a strategy for high-level production of single-chain variable fragment (scFv) antibodies fused with interleukin-2 (IL-2) in Escherichia coli. Materials and Methods: We constructed two fusion sequences consisting of a scFv gene derived from a mouse monoclonal antibody against a tumor-associated antigen (MK-1) and human Interleukin-2(IL-2) gene, ligated the fusions into pET15b and transformed into three different E. coli strains. The effects of temperature, isopropyl-β-D-thiogalactopyranoside (IPTG) concentration and duration of IPTG induction were investigated. Results: Employing E. coli strain Rosetta-gami B, which has an oxidizing cytoplasm that facilitates cytoplasmic disulfide bond formation, improved the level of soluble protein expression. Under optimal conditions, the highest levels of fusion protein expression and high percentages of the proteins were found in their soluble form. Specifically, 89.29% (0.28 g/l) of one fusion protein was soluble after a 10-h induction and 84.61% (0.26 g/l) of the other fusion protein was soluble after a separate 10-h induction. When analyzed by enzyme-linked immunosorbent assay, the partially-purified fusion proteins retained a specific binding activity to the cell lysate of Chinese hamster ovary (CHO) cells expressing MK-1. Conclusion: Taken together, the methods described herein permit the production of substantial amounts of the fusion proteins for conducting functional studies on the biological role of these fusion proteins.*

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MK-1, which possesses homotypic cell-cell adhesion activity, has been implicated in the differentiation and growth of epithelial cells under normal physiological conditions (1). This antigen is overexpressed in most carcinomas and has been therefore used as a target to classify cancers of the stomach and to detect the metastatic foci of gastrointestinal adenocarcinomas in the lymph nodes and other organs (2). MK-1 is a transmembrane glycoprotein with a molecular mass of 40 kDa and is encoded by the *GA733-2* gene (3). The GA733-2 antigen is also known as the 17-1 antigen, KS1/4 antigen, or Ep-CAM antigen (4). Previously, Watanabe *et al.* (1993) created FU-MK-1, a monoclonal antibody against MK-1, by immunizing mice with cancerous ascites derived from a poorly-differentiated adenocarcinoma of the stomach. FU-MK-1 is an IgG1 antibody that is reactive with a tumor-associated antigen expressed in most tumor tissues of epithelial origin, including gastric, colon, pancreatic, gallbladder, bile duct, breast and lung carcinomas (2).

Advances in genetic engineering and expression systems have enabled rapid progress in the development of antibodies fused to other proteins. Certain antibody fusion proteins, including antibodies specific for tumor-associated antigens, harbor novel properties. For example, antibodies targeting tumor antigens are fused to cytokines, such as interleukin-2 (IL-2) (5, 6). IL-2 is a cytokine produced by helper T-cells that stimulates T-cells to proliferate and transform into NK cells, which respond with increased cytotoxicity to tumor cells (7-10). Indeed, among the antibody/cytokine fusion proteins, antibody/IL-2 fusion proteins targeting cancer cells have been best characterized and the most broadly used fusion proteins in successful anti-tumor experiments using animal models (5, 6, 11).

A single-chain variable fragment (scFv) is a monoclonal antibody fragment in which the variable immunoglobulin domains of the heavy (V_H) and light κ ($V_κ$) chains are connected with a flexible linker to make a single polypeptide chain. Thus, some limitations of monoclonal antibodies (mAbs) can be overcome by scFv because it retains the original

antigen-binding site, allowing it to maintain its specific affinity for the antigen (12-14). Regarding the scFv/IL-2 fusion protein, Matsumoto *et al*. genetically fused recombinant human IL-2 (hIL-2) to a scFv(V_K-V_H) derived from FU-MK-1. The obtained FU-MK-1-scFv(V_K-V_H)/IL-2 was then expressed in the methylotrophic yeast *Pichia pastoris*, but its expression level was very low (0.002 g/l). The resulting FU-MK-1-scFv(V_K-V_H)/IL-2 fusion protein was applied to introduce specific cytotoxicity of lymphokine-activated killer cells to the tumor cells. It was reported that FU-MK-1-scFv(V_K-V_H)/IL-2 consequently suppressed the tumor growth in a severe-immunodeficiency genetic disorder (SCID) mouse xenograft model (15). Nevertheless, FU-MK-1-scFv(V_K-V_H)/IL-2 expression in *E. coli* has not yet been reported.

In the present study, the fusion gene encoding FU-MK-1-scFv(V_K-V_H)/IL-2 was amplified and rearranged the sequence to produce IL-2/FU-MK-1-scFv(V_H-V_K) and IL-2/FU-MK-1-scFv(V_K-V_H). These fusion genes were transformed into *E. coli* strains BL21(DE3), BL21(DE3)pLysS and Rosetta-gami B. Next, we compared the studied strains in terms of growth, productivity and product quality (solubility) in shake-flask cultivation. The effects of different temperatures, isopropyl-β-D-thiogalactopyranoside (IPTG) concentrations and induction periods on the expression levels of fusion proteins were investigated. The resulting fusion proteins were partially purified by Ni-affinity chromatography and quantified using the enzyme-linked immunosorbent assay (ELISA) method. Finally, the fusion proteins were tested against the specific binding antigen of a CHO cell line expressing MK-1 antigen.

Materials and Methods

Fusion gene. The fusion genes encoding anti-FU-MK-1-scFv(V_K-V_H)/IL-2 were obtained from Professor Kuroki Masahide, Department of Biochemistry, Fukuoka University School of Medicine, Jounan-ku, Fukuoka, Japan.

Escherichia coli expression strains. *E. coli* strains JM109 and DH5α (New England Biolabs Inc., Ipswich, MA, USA) were used for sub-cloning, sequencing, and plasmid amplification. The cells were grown in Luria Bertani (LB) medium (10 g/l bacto-tryptone, 10 g/l NaCl and 5 g/l yeast extract) containing 50 μg/ml ampicillin.

E. coli strains BL21(DE3) (Novagen®, Merck KGaA, Darmstadt, Germany), BL21(DE3)pLysS (Novagen®, Merck KGaA, Darmstadt, Germany) and Rosetta-gami B (EMD Biosciences, Madison, WI, USA) were used as expression hosts. All media used to cultivate cells or express proteins were supplemented with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol.

Construction of expression vectors. We amplified the fusion sequence coding for FU-MK-1-scFv(V_K-V_H)/IL-2 and re-arranged the sequence to produce IL-2/FU-MK-1-scFv(V_K-V_H) and IL-2/FU-MK-1-scFv(V_H-V_K). These fusion genes were successfully obtained through overlap PCR, verified by agarose gel electrophoresis and DNA sequencing (Macrogen Inc., Geumchen-gu, Seol, Korea). IL-2/FU-MK-1-scFv(Vκ-VH) and IL-2/FU-MK-1-scFv(V_H-V_K) were cloned into pET15b (Novagen®, Merck KGaA, Darmstadt, Germany) at the *Xho*I and *Bam*HI restriction sites (New England Biolabs Inc., Ipswich, MA, USA). The resulting plasmids, pET15b-IL-2/FU-MK-1-scFv(V_K-V_H) and pET15b-IL-2/FU-MK-1-scFv(V_H-V_K), contained the coding sequence for an N-terminal His-tag followed by a thrombin cleavage site. The recombinant plasmids were transformed into *E. coli* strains BL21(DE), BL21(DE3)pLysS, and Rosetta-gami B. All constructs were verified by DNA sequencing (Macrogen Inc., Geumchen-gu, Seoul, Korea).

Induction and expression of fusion proteins in E. coli under shake-flask cultivation. *E. coli* strains BL21(DE3), BL21(DE3)pLysS and Rosetta-gami B were separately transformed with pET15b-IL-2/FU-MK-1-scFv(V_K-V_H) or pET15b-IL-2/FU-MK-1-scFv(V_H-V_K). Single colonies of freshly transformed cells were grown in shake-flask cultures containing 10 ml of LB medium supplemented with 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol and were incubated at 37˚C and 200 rpm until reaching an OD_{600nm} of 0.6. The cells were harvested by centrifugation at 10160 × *g* and 4˚C for 15 min. Each cell pellet was inoculated into 50 ml of LB medium containing 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol. Protein expression was induced with the appropriate concentration of IPTG. The IPTG concentration and induction temperature varied according to the experimental requirements. The cells were grown for 12 h at 25˚C, 30˚C and 37˚C to determine the optimal induction conditions.

Detection of total protein and soluble fraction of the fusion proteins. Cell lysis was performed using the BugBuster® protein extraction kit (Novagen®, Merck KGaA, Darmstadt, Germany). Briefly, a cell pellet from 1 ml of culture induced at different conditions was resuspended in 300 μl of lysis buffer. A protease inhibitor cocktail used in purifying poly(His)-tagged proteins (Sigma-Aldrich Corp., St. Louis, MO, USA) was added to the cell suspension, incubated at room temperature for 10 min and centrifuged at 4˚C and 17418 ×*g* for 20 min. The pellets (insoluble fraction) containing inclusion bodies and the supernatant (soluble fraction) were separately mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, heated at 95˚C for 5 min and analyzed by SDS-PAGE.

SDS-PAGE and western blotting. SDS-PAGE analysis was performed according to the method of Laemmli (16). A mini-PROTEAN was used as instructed by the manufacturer (Bio-Rad Laboratories Inc., Hercules, CA, USA). The fusion protein concentration was estimated using the densitometry software ImageJ (http://rsb.info.nih.gov/ij/). Ovalbumin, with a molecular weight of 45 kDa (Wako Pure Chemical Industries, Ltd., Osaka, Japan), was used as a protein standard. Briefly, protein standards ranged from 0.01-1.0 g/l and the samples were run on a 12% SDS-PAGE gel, which was subsequently stained with a 0.1% (w/v) solution of Coomassie stain (Sigma-Aldrich Corp., St. Louis, MO, USA) and dried using a gel equilibrium drying set (Invitrogen Corp., Carlsbad, CA, USA). The dried gels were scanned on an HP ScanJet 4670 (Hewlett-Packard Company, Palo Alto, CA, USA) and loaded into the ImageJ software. The target protein bands were selected, plotted and compared with the protein standard. ImageJ generates a plot based on the intensities of the selected protein bands by measuring their peak areas. Protein molecular-weight markers (Fermentas Inc., Hanover, MD, USA) were used as protein standards to determine the molecular mass.

For western blots, proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories

Inc., Hercules, CA, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Inc., Hercules, CA, USA). The identities of the fusion proteins were confirmed by immunoblotting, which was performed using a WesternBreeze chromogenic detection kit (Invitrogen Corp., Carlsbad, CA, USA). Immunoblotting was conducted with a monoclonal rabbit anti-human IL-2 (hIL-2) antibody and an anti-rabbit antibody conjugated to alkaline phosphatase. The complex was monitored with an enzyme acting on the nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) as the substrates. The His-tag was detected by immunoblotting with a monoclonal anti-polyhistidine clone His-1 (Sigma-Aldrich Corp., St. Louis, MO, USA) that was conjugated to alkaline phosphatase and used along with the WesternBreeze chromogenic detection kit (Invitrogen Corp.).

Purification of fusion proteins by Ni-affinity chromatography. To purify the fusion proteins, the supernatant was clarified through a 0.22-µm membrane filter (Millex-GV, EMD Millipore Corp., Billerica, MA, USA) prior to subsequent purification steps. Ni-affinity chromatography was performed on a His·Bind®Kits 5-ml column (Novagen®, Merck KGaA, Darmstadt, Germany). In total, 2 ml of His·Bind resin was charged with 2 volumes of charging buffer (50 mM $NiSO_4$) and packed in the column under gravity flow. Next, the resin was equilibrated with 10 volumes of binding buffer (5 mM imidazole, 0.5 M NaCl and 20 mMTris-HCl; pH 7.9) and drained. Afterwards, 5 ml of the supernatant containing His-tagged protein was loaded into the column and incubated at 4˚C for 3 h. The resin bound with His-tagged protein was washed with 10 ml of binding buffer and subsequently washed with 6 ml of washing buffer (60 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl; pH 7.9) to remove non-specifically bound proteins. The protein was eluted with eluting buffer (5-1000 mM imidazole, 0.5 M NaCl and 20 mMTris-HCl; pH 7.9). Twenty fractions of 2 ml each were collected. Fractions containing the protein (analyzed by SDS-PAGE) were pooled and concentrated using Vivaspin 500 centrifugal concentrators (30,000 MWCO; Vivaproducts, Inc., Littleton, MA, USA). Finally, the protein was kept in a PBS solution until further analysis. The purity of the fusion proteins was determined by SDS-PAGE. The protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) with bovine serum albumin as a standard.

Measurement of hIL-2 protein levels when expressing IL-2/FU-MK-1-scFv in E.coli. The levels of IL-2/FU-MK-1-scFv in the soluble protein fraction were quantified using a commercial ELISA kit (Human IL-2 Ready-SET-Go ELISA system; eBioscience Inc., San Diego, CA, USA) according to the manufacturer's instructions. In brief, ELISA 96-well plates (Corning Costar 9018 ELISA 96-well plates, Corning Incorporated, NY, USA) were coated with capture antibody in the coating buffer. Test samples and serial dilutions of the IL-2 standards were added and incubated for 1 h at room temperature. Biotin-conjugated rabbit anti-human IL-2, a detection antibody, was added after washing. Then, a 100 µl diluent containing avidin conjugated to horseradish peroxidase was added and incubated at room temperature for 1 h. After washing, 100 µl of tetramethylbenzidine (TMB; eBioscience Inc., San Diego, CA, USA) substrate solution was added and incubated for 15 min in the dark. After the addition of 50 µl of 2 N H_2SO_4, the plates were read at OD_{450nm} using a Bio-Rad microplate reader (Bio-Rad Laboratories Ltd., Hercules, CA, USA). Standard curves were generated with hIL-2 (eBioscience Inc., San Diego, CA, USA).

Characterization of the specific binding activity of the fusion protein. Briefly, 20 µg of the cell lysate from CHO cell lines expressing MK-1 antigen was mixed with100 µl of coating buffer (eBioscience, Inc., San Diego, CA, USA) and added to a 96-well plate in triplicate (Corning Costar 9018 ELISA 96-well plate; Corning Inc., Corning, NY, USA). After an overnight incubation at 4˚C, 4 µg of the fusion protein IL-2/FU-MK-1-scFv was diluted to 100 µl with dilution buffer (eBioscience Inc., San Diego, CA, USA) and added to each well. The solutions were then incubated at room temperature for 2 h. Then, 1:200 diluted biotin-conjugated anti-hIL-2 was added to the assay diluent, and the plate was incubated at room temperature for 1 h. Next, Avidin-HRP (eBioscience Inc., San Diego, CA, USA) was diluted 1:200 in assay diluent, added to the solution and subsequently incubated at room temperature for 30 min. At that time, 100 µl of TMB solution was added to each well and incubated at room temperature for 15 min. Finally, 50 µl of 2 N H_2SO_4 was added to quench the reaction and the plate was read at OD_{450nm} with a Bio-Rad microplate reader. The control experiment was performed using CHO cell lines that do not express MK-1 antigen and compared with the results obtained from CHO cell lines expressing MK-1 antigen.

Statistical analysis. The specific binding activity data are representative of three independent experiments and are the mean value±the standard deviation (SD) of these independent experiments. The results of the specific binding activity were analyzed using Student's *t*-test. A *p*-value of <0.05 was considered significant.

Results

Construction of plasmids. The fusion genes encoding IL-2/FU-MK-1-scFv(V_κ-V_H) and IL-2/FU-MK-1-scFv(V_H-V_κ) were successfully obtained using an overlap PCR technique, with the resulting sequences verified by agarose gel electrophoresis and DNA sequencing. The vectors pET15b-IL-2/FU-MK-1-scFv(V_κ-V_H) and pET15b-IL-2/FU-MK-1-scFv(V_H-V_κ) were constructed such that the target genes were fused after a His-tag and a thrombin cleavage site. Fusion gene expression was under the control of the T7 promoter. Our constructs differ from those of a previous report by Matsumoto *et al*. because the original fusion gene was in the FU-MK-1-scFv(V_H-V_κ)/IL-2 orientation and the plasmid lacked a His-tag. The molecular weight of the fusion protein FU-MK-1-scFv(V_H-V_κ)/IL-2 was previously reported to be 43 kDa (15). In the present study, a hexahistidine tag was added to the N-terminus, which increased the molecular mass by 1 kDa to the target mass of 44 kDa. The His-tag and the thrombin cleavage site were important in the subsequent detection, separation and purification steps. Moreover, the thrombin cleavage site allowed for removal of the His-tag.

Comparison of E. coli strains BL21(DE3), BL21(DE3)pLysS and Rosetta-gami B for fusion protein expression. The pET15b-IL-2/FU-MK-1-scFv(V_κ-V_H) and pET15b-IL-2/FU-MK-1-scFv(V_H-V_κ) vectors were separately transformed into *E. coli* strains BL21(DE3) (suitable for non-toxic protein expression and deficient in two key protease genes, *lon* and

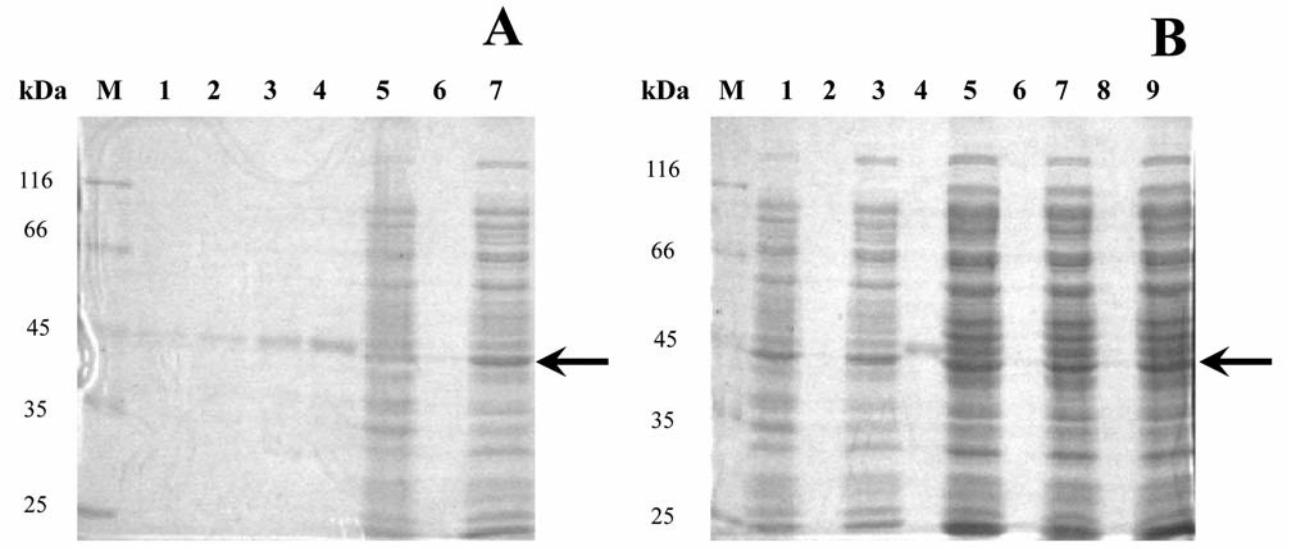

Figure 1. *Expression of soluble and total proteins in E. coli strain BL21(DE3)pLysS/pET15b-IL2/FU-MK-1-scFv(V_H-V_κ) at temperatures of 25˚C, 30˚C and 37˚C. (A) Lane M is a protein marker. Lanes 1, 2, 3 and 4 are the ovalbumin standard (45 kDa) at concentrations of 0.025, 0.050, 0.100 and 0.200 g/l, respectively. Lane 5 is the soluble protein from E. coli strain BL21(DE3)pLysS/pET15b (Control). Lane 7 is the soluble protein from E. coli strain BL21(DE3)pLysS/pET15b-IL2/FU-MK-1-scFv(V_H-V_κ) induced at 25˚C. (B) Lane M is a protein marker. Lanes 1 and 3 are the soluble protein from E. coli strain BL21(DE3)pLysS/pET15b-IL2/FU-MK-1-scFv (V_H-V_κ) induced at 30 and 37˚C, respectively. Lanes 5, 7 and 9 are the total protein from E. coli strain BL21(DE3)pLysS/pET15b-IL2/FU-MK-1-scFv(V_H-V_κ) induced at 25, 30 and 37˚C, respectively. Lane 4 is the ovalbumin standard (45 kDa) at a concentration of 0.2 g/l. The arrow indicates a molecular weight of 44 kDa.*

ompT), BL21(DE3)pLysS (suitable for toxic protein expression and produces T7 lysozyme) and Rosetta-gami B (suitable for rare codon expression and carries mutations for the reductase genes *trxB* and *gor* to facilitate cytoplasmic disulfide bond formation). The strains BL21(DE3) and BL21(DE3)pLysS are characterized by a reducing cytoplasm, and the strain Rosetta-gami B is characterized by an oxidizing cytoplasm. Thus, we determined the effect of an oxidizing cytoplasm on the organization of disulfide bonds in IL-2/FU-MK-1-scFv. The expression patterns from each strain were compared by SDS-PAGE and western blot. Initially, the expression analysis was performed with 0.5 mM IPTG at 37˚C for 12 h. The *E. coli* strain BL21(DE3) grew very slowly with low levels of expression and it was assumed that the fusion proteins may be toxic to this strain. Thus, in this study, the strain BL21(DE3) was not a suitable expression host. On the other hand, the fusion proteins were each expressed in both the reducing cytoplasm of *E. coli* strain BL21(DE3)pLysS and the oxidizing cytoplasm of the *trxB*/*gor* mutant *E. coli* strain Rosetta-gami B. The majority of the fusion proteins found in the cytoplasm of BL21(DE3)pLysS were in the insoluble fraction (>70%) (Figure 1B, Lanes 3 and 9 at 44 kDa). BL21(DE3)pLysS produced higher amounts of total fusion proteins (44 kDa) at all three temperatures (25, 30 and 37˚C) compared to Rosetta-gami B (Table I). However, BL21(DE3)pLysS had significantly lower amounts of soluble fusion proteins (44 kDa). We observed that BL21(DE3)pLysS and Rosetta-gami B had similar growth rates. In addition, for all three *E. coli* strains tested, the strains harboring pET15b-IL-2/FU-MK-1-scFv(V_H-V_κ) had significantly higher growth rates than the strains harboring pET15b-IL-2/FU-MK-1-scFv(V_κ-V_H). Despite the differences in growth rates, the differences in expression levels between IL-2/FU-MK-1-scFv(V_κ-V_H) and IL-2/FU-MK-1-scFv(V_H-V_κ) were small and there was no significant difference in the percentage of soluble fusion protein.

Effect of induction temperature. A common approach to reduce the *in vivo* aggregation of recombinant proteins is to lower the cultivation temperature (17). The production of fusion proteins IL-2/FU-MK-1-scFv(V_κ-V_H) and IL-2/FU-MK-1-scFv(V_H-V_κ) was investigated at three different induction temperatures (37˚C, 30˚C and 25˚C; Table I) and at a combination of temperatures (37˚C for 4 h followed by 25˚C for 12 h with IPTG induction; Table II). An induction temperature of 37˚C resulted in increased cell mass but lower levels of soluble fusion proteins. The induction at 30˚C yielded the highest total amount of fusion proteins for BL21(DE3)pLysS. Although Rosetta-gami B produced lower amounts of total fusion proteins, the soluble fraction was two-times higher in Rosetta-gami B than in BL21(DE3)pLysS. BL21(DE3)pLysS maintains a normal reducing environment in the cytoplasm and may not support disulfide bond formation. Rosetta-gami B carries mutations for the trxB and gor reductases to promote disulfide

Table I. *The production of the fusion proteins IL2/FU-MK-1-scFv(V_K-V_H) and IL2/FU-MK-1-scFv(V_H-V_K), expressed in E. coli strains BL21(DE3), BL21(DE3)pLysS and Rosetta-gami-B at 25˚C, 30˚C, or 37˚C. The protein expression was induced by 0.5 mM IPTG for 12 h.*

	Fusion protein	Total protein (g/l)	% of soluble fusion protein in total protein	Cell mass (g/l)
Temperature: 25˚C				
BL21(DE3)pLysS	IL2/FU-MK-1-scFv(V_K-V_H)	0.24	48	1.66
BL21(DE3)pLysS	IL2/FU-MK-1-scFv(V_H-V_K)	0.25	50	2.02
Rosetta-gami-B	IL2/FU-MK-1-scFv(V_K-V_H)	0.11	74	0.57
Rosetta-gami-B	IL2/FU-MK-1-scFv(V_H-V_K)	0.13	77	0.91
Temperature: 30˚C				
BL21(DE3)pLysS	IL2/FU-MK-1-scFv(V_K-V_H)	0.37	33	1.72
BL21(DE3)pLysS	IL2/FU-MK-1-scFv(V_H-V_K)	0.39	38	2.11
Rosetta-gami-B	IL2/FU-MK-1-scFv(V_K-V_H)	0.18	59	1.85
Rosetta-gami-B	IL2/FU-MK-1-scFv(V_H-V_K)	0.19	60	2.26
Temperature: 37˚C				
BL21(DE3)	IL2/FU-MK-1-scFv(V_K-V_H)	0.007	n/a	0.33
BL21(DE3)	IL2/FU-MK-1-scFv(V_H-V_K)	0.005	n/a	0.18
BL21(DE3)pLysS	IL2/FU-MK-1-scFv(V_K-V_H)	0.24	23	2.94
BL21(DE3)pLysS	IL2/FU-MK-1-scFv(V_H-V_K)	0.26	27	3.94
Rosetta-gami-B	IL2/FU-MK-1-scFv(V_K-V_H)	0.20	47	2.88
Rosetta-gami-B	IL2/FU-MK-1-scFv(V_H-V_K)	0.23	55	3.82

n/a=not detectable.

Table II. *Effects of temperature, induced concentration and duration on the production of the fusion proteins IL2/FU-MK-1-scFv(V_K-V_H) and IL2/FU-MK-1-scFv(V_H-V_K) in E. coli strain Rosetta-gami-B.*

	Post-induction time (h)						
	0	2	4	6	8	10	12
IL2/FU-MK-1-scFv(V_K-V_H)							
IPTG concentration (0.5 mM)							
Total soluble protein (g/l)	–	–	0.13	0.19	0.22	0.26	0.31
% of soluble fusion proteins	–	–	84.62	78.95	81.81	84.61	64.52
Soluble fusion proteins (g/l)	–	–	0.10	0.15	0.18	0.22	0.20
IPTG concentration (1.0 mM)							
Total soluble protein (g/l)	–	0.03	0.15	0.21	0.23	0.22	0.21
% of soluble fusion proteins	–	–	66.67	71.42	73.91	77.27	80.95
Soluble fusion proteins (g/l)	–	–	0.10	0.15	0.17	0.17	0.17
IL2/FU-MK-1-scFv(V_H-V_K)							
IPTG concentration (0.5 mM)							
Total soluble protein (g/l)	–	–	0.14	0.20	0.23	0.28	0.34
% of soluble fusion proteins	–	–	78.57	80.00	82.61	89.29	73.53
Soluble fusion proteins (g/l)	–	–	0.11	0.16	0.19	0.25	0.25
IPTG concentration (1.0 mM)							
Total soluble protein (g/l)	–	0.09	0.24	0.27	0.29	0.30	0.31
% of soluble fusion proteins	–	–	74.17	74.07	75.86	80.00	80.65
Soluble fusion proteins (g/l)	–	–	0.17	0.20	0.22	0.24	0.25

bond formation, which may result in enhanced solubility of the fusion proteins. The expression of fusion proteins was not significantly affected by lowering the temperature to 25˚C. However, a lower temperature increased the amount of soluble protein when compared to the strains with induction at 30˚C and 37˚C. We found that the *E. coli* strains Rosetta-gami-B/pET15b-IL-2/FU-MK-1-scFv(V_K-V_H) and Rosetta-gami-B/pET15b-IL-2/FU-MK-1-scFv(V_H-V_K) gave the highest soluble protein titers at 25˚C, with approximately 74% and 77%, respectively, of the total protein in soluble form. Because

our efforts to re-fold the fusion proteins from solubilized inclusion bodies by dialysis remained unsuccessful, we attempted to increase the yield of soluble protein produced in *E. coli* strain Rosetta-gami-B.

Effect of induction conditions. For the pET15b plasmid, the protein expression of genes cloned downstream of the *lac* promoter is induced by adding IPTG to the culture medium (18). IPTG binds to the *lac* repressor molecule, inhibiting the binding of this molecule to the *lac* operator and allowing transcription to occur. Because it has been reported that the expression of recombinant proteins is fully induced at concentrations above 0.4 mM IPTG, expression was induced with 0.5 mM compared to the conventionally used 1 mM IPTG, proposed by De Marco *et al*. (19). The final IPTG concentration should be optimized because IPTG greatly influences recombinant protein expression and can potentially inhibit cell growth. In this study, IPTG concentrations between 0.5 and 1.0 mM were examined. The cultivation temperature was a combination of temperatures: 37˚C for 4 h to allow for cell growth followed by 25˚C for 12 h with IPTG induction. After the addition of IPTG to the medium, the synthesis of a fusion protein began at 2 h for 1 mM IPTG and at 4 h for 0.5 mM IPTG. The optimal expression time was determined by analyzing samples at 2 h intervals for 12 h post-induction. The results of this experiment are shown in Table II. Upon induction with 1 mM IPTG, most of the fusion protein was induced with 66% of the total fusion protein found in the soluble fraction. The total amount of fusion protein increased over time in proportion to the percentage found in the soluble fraction and reached 0.21 g/l total fusion protein with 80.95% in the soluble fraction for IL-2/FU-MK-1-scFv(V_κ-V_H) and 0.31 g/l total fusion protein with 80.56% in the soluble fraction for IL-2/FU-MK-1-scFv(V_κ-V_H).

In this study, the production of fusion proteins in the oxidizing cytoplasm showed improved solubilization when the IPTG concentration was reduced from 1 mM to 0.5 mM. It was determined that the cells should be harvested 10 h after induction with 0.5 mM IPTG because this time point produced the maximum percentage of soluble His-tagged fusion protein IL-2/FU-MK-1-scFv(V_κ-V_H) and IL-2/FU-MK-1-scFv(V_H-V_κ) (Table II). The solubility of the expressed protein was investigated in detail. The pellets (insoluble fraction) containing inclusion bodies and the supernatant (soluble fraction) were obtained as described in the Materials and Methods section. Protein expression was characterized by running samples on 12% SDS-PAGE gels and staining with Coomassie brilliant blue. The majority of target protein (44 kDa) was detected in the cleared lysate (Figure 3A-D; lanes 3 and 4), indicating that the fusion proteins were expressed in a soluble form in Rosetta-gami B. The fusion protein concentration and the percentage of fusion protein found in the soluble fraction were highest (0.28 g/l and 89.29%, respectively) for IL-2/FU-MK-1-scFv(V_H-V_κ) and 0.26 g/l and 84.61%, respectively for IL-2/FU-MK-1-scFv(V_κ-V_H)) when induced with 0.5 mM IPTG for 10 h.

Production and purification of fusion proteins. Fusion protein expression in *E.coli* strain Rosetta-gami B was induced with a final concentration of 0.5 mM IPTG at 25˚C for 10 h. Cell lysis was performed as described in the Materials and Methods section. The pellets (insoluble fraction) were discarded and the supernatant (soluble fraction) was purified by Ni-chelating affinity chromatography utilizing the N-terminal His-tag present on the fusion proteins. Non-specifically bound proteins were removed by rinsing with wash buffers containing 5 mM imidazole. The proteins were eluted with Tris-HCl buffer containing 5-1000 mM imidazole. The elution fractions were collected and the fusion protein present in each fraction was quantified with a commercial ELISA kit (Figure 2). The IL-2/FU-MK-1-scFv fusion proteins were eluted from the His·Bind resin column using Tris-HCl containing 50-200mM imidazole (Figure 2C). The purified fusion proteins appeared as a single band of 44 kDa on immunoblots (Figure 2B, lane 7). Purification fraction from the 50 mM imidazole elution condition was analyzed by 12% SDS-PAGE and Western blot. The immunoblotting procedure used rabbit anti-hIL-2, which specifically binds IL-2 of the fusion protein, as a primary antibody and an anti-rabbit antibody conjugated to alkaline phosphatase as a secondary antibody. The expression signal was detected by a BCIP/NBT solution that reacts with alkaline phosphatase (Figures 3B and 3D; lanes 5 and 6). The purity of IL-2/FU-MK-1-scFv was assessed using SDS-PAGE and the concentration was evaluated by Bradford's method. It has been reported that affinity-tagged proteins can achieve up to 95% purity by Ni-chelating affinity chromatography (40, 41). With this purification step, SDS-PAGE analysis (Figures 3A and 3C; lanes 5 and 6) indicated high levels of purity (80%) and the fusion protein concentration was approximately 0.04 g/l with 20% protein recovery.

Characterization of the specific binding activity of fusion proteins. Finally, IL-2/FU-MK-1-scFv(V_H-V_κ) and IL-2/FU-MK-1-scFv(V_κ-V_H) were tested for their ability to bind to CHO cells expressing the MK-1 antigen using cell lysates assayed by ELISA. As a control, the fusion proteins were also tested for their ability to bind to CHO cells that do not express MK-1. The OD_{450nm} values from the ELISA analyses of IL-2/FU-MK-1-scFv(V_H-V_κ) binding to cell lysates from CHO cells expressing MK-1 and to CHO cells that do not express MK-1 were 0.592±0.008 and 0.270±0.012, respectively. The results obtained for IL-2/FU-MK-1-scFv(V_κ-V_H) were similar to the OD_{450nm} values of 0.562±0.014 and 0.263±0.012for IL-2/FU-MK-1-scFv(V_κ-V_H) binding to cell lysates from CHO cells expressing MK-1 and to CHO cells that do not express

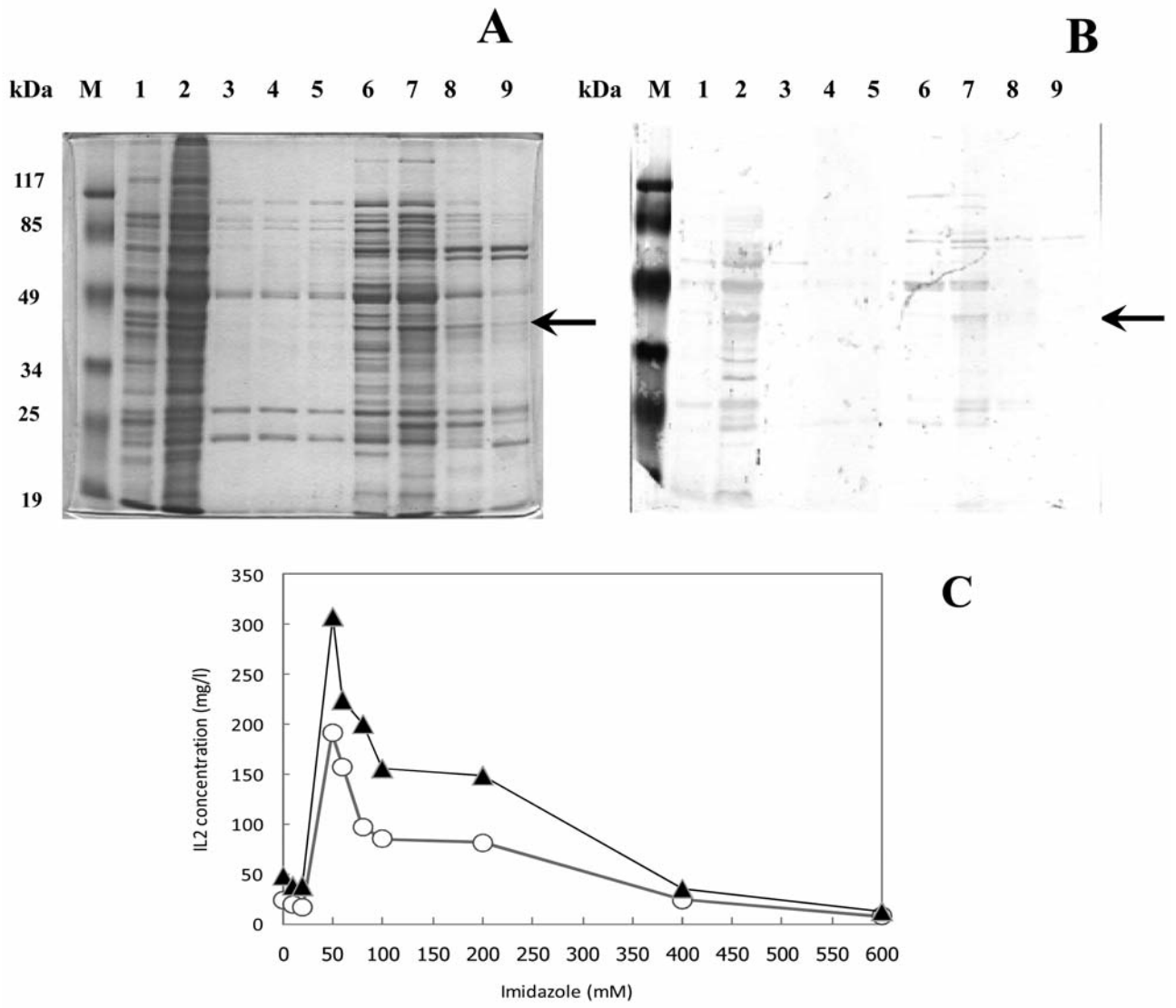

Figure 2. *Analysis of the purified fusion protein IL2/FU-MK-1-scFv(V_H-V_K) produced by inducing E. coli strain Rosetta-gami-B at 25°C and 0.5 mM IPTG for 10 h. (A) SDS-PAGE stained with Coomassie Brilliant Blue. (B) Western blot analysis. The purified fusion proteins separated by SDS-PAGE were electroblotted onto a PVDF membrane and immunoblotting was performed with a monoclonal rabbit anti-hIL-2 antibody (1:10,000) and an anti-rabbit antibody conjugated to alkaline phosphatase. Lane M, protein marker; Lane 1, the crude soluble fraction of the control (E. coli strain Rosetta-gami B/pET15b); Lane 2, crude soluble protein from E. coli strain Rosetta-gami B/pET15b-IL2/FU-MK-1-scFv(V_H-V_K); Lanes 3, 4, 5, 6, 7, 8 and 9, eluted fractions, with buffer containing 5, 10, 20, 40, 50, 100 and 600 mM imidazole, respectively. (C) The IL-2 concentration (mg/L) in each eluted fraction. The concentration of imidazole was increased stepwise from 0-600 mM. The arrow indicates a molecular weight of 44 kDa. Open circle, IL2/FU-MK-1-scFv(V_H-V_K); closed triangle, IL2/FU-MK-1-scFv(V_K-V_H).*

MK-1, respectively. The high OD_{450nm} values indicated the specificity of IL-2/FU-MK-1-scFv(V_H-V_K) and IL-2/FU-MK-1-scFv(V_K-V_H) for the MK-1 antigen, whereas the low OD_{450nm} values indicated reduced binding ability of IL-2/FU-MK-1-scFv(V_H-V_K) and IL-2/FU-MK-1-scFv(V_K-V_H) to CHO cells that do not express MK-1.

Statistical analysis of the data presented in Figure 4 clearly indicates that the OD_{450nm} values for the specific binding ability of IL-2/FU-MK-1-scFv(V_H-V_K) and IL-2/FU-MK-1-scFv(V_K-V_H) to CHO cells expressing MK-1 are significantly elevated ($p<0.05$, Student's *t*-test) compared with their ability to bind to CHO cells that do not express MK-1. Therefore, both fusion proteins expressed in *E. coli* strain Rosetta-gami B were able to bind to CHO cells expressing MK-1 antigen, but the proteins did not bind to CHO cells that do not express the MK-1 antigen.

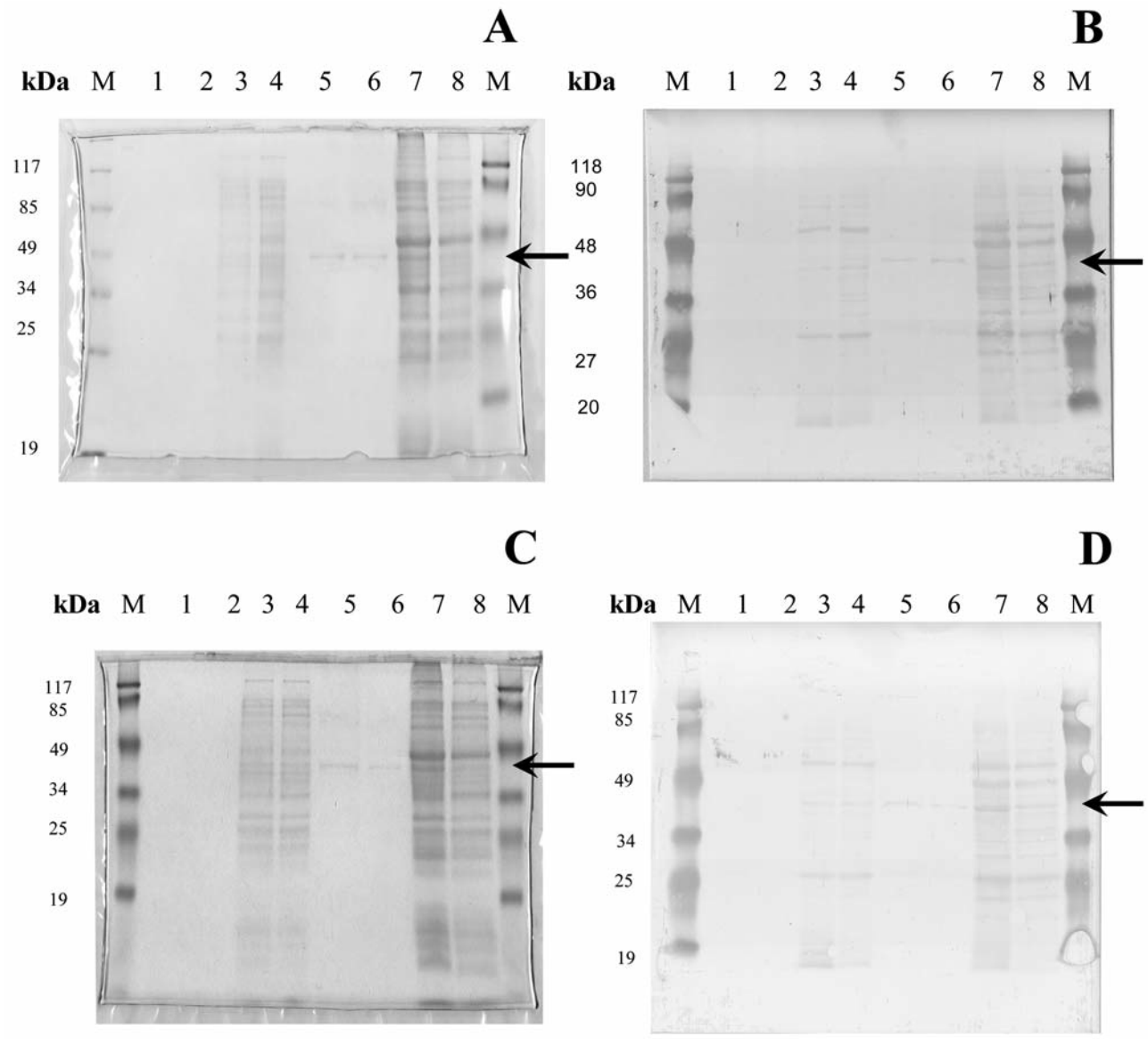

Figure 3. *SDS-PAGE analysis and western blot analysis (1:10,000 anti-hIL-2 antibody) of the purified fusion proteins IL2/FU-MK-1-scFv(V_H-V_K) and IL2/FU-MK-1-scFv(V_K-V_H) produced by inducing E. coli strain Rosetta-gami-B at 25˚C and 0.5 mM IPTG for 10 h. (A) SDS-PAGE and (B) western blot analysis. Lane M, protein marker. Lanes 1 and 2 are the crude soluble protein of the control (E. coli strain Rosetta-gami-B/pET15b). Lanes 3 and 4 are the crude soluble protein of E. coli strain Rosetta-gami-B/pET15b-IL2/FU-MK-1-scFv(V_H-V_K). Lanes 5 and 6 are purified IL2/FU-MK-1-scFv(V_H-V_K). Lanes 7 and 8 are the crude total protein of E. coli strain Rosetta-gami-B/pET15b-IL2/FU-MK-1-scFv(V_H-V_K). (C) SDS-PAGE and (D) western blot analysis. Lane M, protein marker. Lanes 1 and 2 are the crude soluble protein of the control (E. coli strain Rosetta-gami-B/pET15b). Lanes 3 and 4 are the crude soluble protein of E. coli strain Rosetta-gami-B/pET15b-IL2/FU-MK-1-scFv(V_K-V_H). Lanes 5 and 6 are purified IL2/FU-MK-1-scFv(V_K-V_H). Lanes 7 and 8 are the crude total protein of E. coli strain Rosetta-gami-B/pET15b-IL2/FU-MK-1-scFv(V_K-V_H). The arrow indicates a molecular weight of 44 kDa.*

Discussion

In this study, we reported our efforts to use *E. coli* to express IL-2/FU-MK-1-scFv fusion proteins. We aimed to establish a highly-producing strategy available for any other fusion proteins consisting of scFv and IL-2. We compared three different *E. coli* host strains under various conditions intending to increase their efficiency to produce soluble recombinant fusion proteins. Initially, three strains, BL21(DE), BL21(DE3)pLysS and Rosetta-gami B, were tested for the expression of fusion proteins using 0.5 mM IPTG at 37˚C for 12 h. Although, BL21(DE3) was identified as the most widely

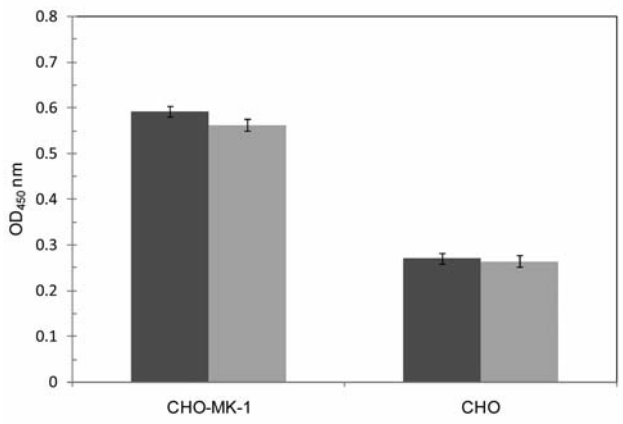

Figure 4. *The specific binding activity of IL2/FU-MK-1-scFv(V_K-V_H) and IL2/FU-MK-1-scFv(V_H-V_K) to MK-1-expressing and non-expressing CHO cells by a cell lysate-based ELISA method ($p<0.05$). Dark grey bars, IL2/FU-MK-1-scFv(V_H-V_K); light grey bars, IL2/FU-MK-1-scFv(V_K-V_H).*

used host for high-yield protein expression, our results demonstrate that BL21(DE3) growth is negatively affected by the expression of these fusion proteins. Our experiments led to a clear preference for the *E. coli* strain Rosetta-gami B, which lacks thioredoxin (*trxB*) and glutathione (*gor*) reductases, for the expression of IL-2/FU-MK-1-scFv(V_K-V_H) and IL-2/FU-MK-1-scFv(V_H-V_K) compared to BL21(DE3)pLysS, which has a reducing environment in the cytoplasm (20, 21). In the present study, the levels of fusion protein expression in *E. coli* strain BL21(DE3)pLysS were twice as high as titers from *E. coli* strain Rosetta-gami B. However, Rosetta-gami B provided a higher percentage of soluble fusion protein. Thus, improvement insolubility was observed when fusion proteins were expressed in an oxidizing environment.

We have shown that lowering the cultivation temperature and reducing IPTG concentration potentiated fusion protein solubility. A temperature of 25˚C stimulated higher production of soluble protein compared to a temperature of 37˚C. Cultivation at higher temperature (37˚C) favored bacterial growth and production of insoluble protein in both strains. Our results are in agreement with the reported beneficial effects of lower expression temperatures on the solubility of recombinant proteins (19, 22, 23). It has been reported that reducing the cultivation temperature decelerates overall metabolism and therefore reduces the quantity of newly synthesized protein. Additionally, it has been shown that hydrophobic interactions and therefore aggregation are impeded at lower temperatures (24, 25). Zhang *et al*. also reported that high temperature (37˚C) with a short induction time (4 h) results in a high yield of scFv with activity against fibroblast growth factor receptor 3 containing 9-arginine (scFv-9R); however, most of recombinant proteins do not exist in the soluble fraction but are instead found in inclusion bodies. In contrast, a low temperature (20˚C) and long induction time (20 h) can significantly promote scFv-9R expression in the soluble fraction, which contained more than 90% of the recombinant protein (26). In our case, the *E. coli* strain Rosetta-gami-B expressing the fusion proteins at 25˚C provided reasonable yields of soluble protein (77%). Our results clearly indicate that different orientations of the heavy-chain and κ light-chain variable genes of FU-MK-1-scFv influence cell growth. The fusion protein IL-2/FU-MK-1-scFv(V_K-V_H) may be toxic to *E. coli* because the expression levels of IL-2/FU-MK-1-scFv(V_K-V_H) and IL-2/FU-MK-1-scFv(V_H-V_K) were nearly identical, but the growth rates of both *E. coli* strains harboring IL-2/FU-MK-1-scFv(V_K-V_H) were twice as low as the growth rates of strains harboring IL-2/FU-MK-1-scFv(V_H-V_K). Similarly, it was reported that, in the case of antibody fragments, the solubility and level of scFv expression may be significantly affected by the order in which the V_H and V_L domains are expressed (27).

Another strategy to influence the expression of the target protein is to vary the concentration of IPTG, which may result in increased titers of soluble protein (21). In the current study, *E. coli* Rosetta-gami-B were chosen for fusion protein expression. The fusion proteins were expressed with reasonable yields and high percentages were found in the soluble form (0.25 g/l IL-2/FU-MK-1-scFv(V_H-V_K) with 89.29% soluble and 0.26 g/l IL-2/FU-MK-1-scFv(V_K-V_H) with 84.61% soluble). The varying IPTG concentrations showed no noticeable effect on the total amount of fusion protein obtained in Rosetta-gami-B. However, we concluded that the solubility of fusion proteins correlate with the concentration of IPTG. In the current study, it appears that the solubility and proper folding of fusion proteins benefited greatly from the lower transcription rates that occurred when less than the standard inducer concentration of 1 mM IPTG was used.

The fusion proteins were successfully purified by eluting with a buffer containing 50 mM imidazole. This study shows that by choosing appropriate cultivation conditions, we could either produce soluble fusion protein or avoid re-folding steps during purification. In summary, fusion proteins IL-2/FU-MK-1-scFv(V_H-V_K) and IL-2/FU-MK-1-scFv(V_K-V_H) have been successfully expressed in *E. coli* and partially purified. Our results also demonstrate that the fusion proteins IL-2/FU-MK-1-scFv(V_H-V_K) and IL-2/FU-MK-1-scFv(V_K-V_H) produced in this study retained reactivity to CHO cells expressing the MK-1 antigen but did not react significantly to CHO cells that do not express MK-1 antigen, thus providing preliminary evidence for the potential usefulness of scFvs in immunotherapy applications for cancer. Taken together the results mentioned, the methods described in this study permit the production of substantial amounts of the fusion proteins for conducting functional studies on the biological role of these fusion proteins.

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