Original Article

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Purification and refolding of anti-T-antigen single chain antibodies (scFvs) expressed in *Escherichia coli* as inclusion bodies

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Summary **T-antigen (Galβ1-3GalNAcα-1-Ser/Thr) is an oncofetal antigen that is commonly expressed as a carbohydrate determinant in many adenocarcinomas. Since it is associated with tumor progression and metastasis, production of recombinant antibodies specific for T-antigen could lead to the development of cancer diagnostics and therapeutics. Previously, we isolated and characterized 11 anti-T-antigen phage clones from a phage library displaying human single-chain antibodies (scFvs) and purified one scFv protein, 1G11. More recently, we purified and characterized 1E8 scFv protein using a *Drosophila* S2 expression system. In the current study, four anti-T-antigen scFv genes belonging to Groups 1-4 were purified from inclusion bodies expressed in *Escherichia coli* cells. Inclusion bodies isolated from *E. coli* cells were denatured in 3.5 M Gdn-HCl. Solubilized His-tagged scFv proteins were purified using Ni^{2+}-Sepharose column chromatography in the presence of 3.5 M Gdn-HCl. Purified scFv proteins were refolded according to a previously published method of step-wise dialysis. Two anti-T-antigen scFv proteins, 1E6 and 1E8 that belong to Groups 1 and 2, respectively, were produced in sufficient amounts, thus allowing further characterization of their binding activity with T-antigen. Specificity and affinity constants determined using enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR), respectively, provided evidence that both 1E8 and 1E6 scFv proteins are T-antigen specific and suggested that 1E8 scFv protein has a higher affinity for T-antigen than 1E6 scFv protein.**

Keywords: T-antigen, ScFv, phage display, anti-carbohydrate antibodies, inclusion bodies

1. Introduction

Antibodies are a modular defense system that identifies and neutralizes foreign objects such as bacteria and viruses. Each antibody recognizes a specific, unique target antigen that binds at the antibody's antigen-binding site. This binding mechanism allows an antibody to tag a microbe and an infected cell to be attacked by other parts of the immune system as well as to directly neutralize its target. Antibodies contain variable domains characterized by structurally hypervariable regions, also known as complementarity determining regions (CDRs). The variable-region fragment (Fv fragment) is the smallest unit of an immunoglobulin molecule with functional antigen-binding activity. A single-chain variable fragment (scFv) consists of variable regions in the form of a heavy chain (VH) and a light chain (VL) that are joined by a flexible peptide linker and that are readily expressed in *E. coli* (*1*).

Alterations in cell surface carbohydrates are commonly found in cancerous tissues (*2*). T-antigen (Galβ1-3GalNAcα-1-Ser/Thr), also known as Thomsen-Friedenreich antigen (TF antigen), is an oncofetal antigen. It is commonly expressed as carbohydrate determinants of many adenocarcinomas and is also associated with tumor progression and metastasis (*3-5*). The availability of human antibodies against T-antigen would thus greatly facilitate the development of cancer diagnostics as well as cancer therapeutics.

In the authors' laboratory, phage display technology has been used to obtain scFv genes with specificities for carbohydrate moieties since many carbohydrates are self-antigens that seldom induce an immune response in animals (*6,7*). Genes encoding anti-Man3, anti-Lewis X,

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anti-Tn-antigen, and anti-T-antigen scFv proteins have been isolated from a phage library displaying human scFvs that had been characterized after purification of scFv proteins expressed in different expression systems (*8-12*).

Escherichia coli expression systems are the most prevalent systems for production of recombinant proteins (*13,14*). Efficient microbial production systems have allowed the production of heterologous proteins in sufficient amounts for diagnostic and therapeutic purposes as well as for structural studies (*15-18*). When heterologous proteins were highly expressed in *E. coli*, however, the proteins tended to form inclusion bodies in the cytoplasm. Purification of recombinant proteins from inclusion bodies requires refolding processes that are rather tedious, although the current authors recently reported purification and characterization of scFv proteins (*19,20*).

Previously, 11 anti-T-antigen phage clones were isolated from a phage library and categorized into four groups using a gycolipid, Galβ1-3GalNAcα-hexaethylene glycol-3,5-bis-8-dodecyloxy benzamide (T-antigen E6-BDB) (*11*). Of those, Group 4 scFv protein (1G11) was expressed in *E. coli* from its phagemid and purified to near homogeneity (*11*). However, the level of expression of the 1G11 scFv protein in *E. coli* was not sufficient for further characterization. More recently, a Group 1 1E8 scFv protein was successfully expressed and purified in a *Drosophila* S2 expression system (*21*). To compare scFv proteins belonging to Groups 1-4, the current study expressed and purified four different types of anti-T-antigen scFv genes using an *E. coli* expression system. Of four scFv proteins purified, levels of expression of 1E6 and 1E8 scFv proteins were high enough for characterization of their binding specificity and affinity for T-antigen. Enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR) analyses confirmed the binding activity of both scFv proteins to various T-antigen-presenting conjugates. Affinity constants determined using SPR suggested that 1E8 scFv protein bound to T-antigen with one-order higher affinity than 1E6 scFv protein. These results may provide a basis for the future development of cancer diagnostics and therapeutics.

2. Materials and Methods

2.1. *Materials*

E. coli strain BL21(DE3), Benzonase® Nuclease, and the pET22b(+) expression vector were obtained from Novagen (Germany). The vector was modified to encode the *pel*B leader and His-tag at the C-termini. *E. coli* strain JM109 was obtained from TaKaRa BIO (Shiga, Japan). Bovine serum albumin (BSA), human immunoglobulin G (hIgG), Trizma base, guanidine hydrochloride (Gdn-HCl), tween 20, oxidized L-glutathione (GSSG), reduced L-glutathione (GSH), lysozyme, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (St Louis, MO, USA). The synthesis of neoglycolipids including T-antigen E6-BDB was previously described (11). Human serum albumin (HSA)-conjugated T-antigen was purchased from Glyco Tech (Gaithersburg, MD, USA). Ni^{2+}-Sepharose 6 Fast Flow, and SA sensor chips, and an amine coupling kit were obtained from GE Healthcare (Uppsala, Sweden). ABTS reagent was obtained from Roche Diagnostics K.K. (Basel, Switzerland). B-PER® reagent and His Probe-HRP were obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Anti His-tag mouse monoclonal antibody was purchased from Abcam (Cambridge, MA, USA). Dithiothreitol (DTT), L(+) arginine, carbenicillin, isopropyl-β-D-thiogalactopyranoside (IPTG), and other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. *Anti T-antigen scFv-genes*

Anti-T-antigen scFv clones were obtained using a neoglycolipid, Galβ1-3GalNAcα-hexaethylene glycol-3,5-bis-8-dodecyloxy benzamide (T-antigen E6-BDB), as a probe from a phage library by panning and screening as previously described (*11*). Eleven phage clones were isolated and characterized using DNA sequencing and ELISA, which revealed four groups of clones with T-antigen binding activity. In this study, 1E8, 1E6, 1E10, and 1G11 scFv genes were selected as respective representatives of Groups 1, 2, 3, and 4 (*11*).

2.3. *Construction of scFv expression vectors for E. coli*

Four anti-T-antigen scFv genes, 1E8, 1E6, 1E10, and 1G11, were amplified by PCR from phagemids encoding respective scFv genes (*11*) as templates using corresponding sets of Fw and Rv primers as listed in Table 1. 1E8, 1E6, 1E10, and 1G11 scFv genes were ligated into *Bam*HI-*Not*I-digested pET22b to construct pET22b/1E8, pET22b/1E6, pET22b/1E10, and pET22b/1G11 expression vectors, respectively. *E. coli* JM109 and BL21(DE3) were used as host cells for plasmid preparation and expression of scFv proteins, respectively. The scFv sequences of pET22b/1E8, pET22b/1E6, pET22b/1E10, and pET22b/1G11 were confirmed by DNA sequencing.

2.4. *Expression, purification, and refolding of scFv proteins in E. coli BL21(DE3)*

E. coli BL21(DE3) cells transformed with pET22b/1E8, pET22b/1E6, pET22b/1E10, and pET22b/1G11 expression vectors were seeded into 60 mL of LB medium containing 50 μg/mL carbenicillin. After cells were grown to OD600 of ~0.5 by incubation at 37˚C

Table 1. PCR primers used for scFv gene cloning and vector construction

Primers	Sequences[a]
pET-1E6 Fw	5'-GCATGC*GGATCC*GCAGGTGCAGCTGGTGGAG
pET-1E6Rv	5'-GCATGC*GCGGCCGC*TTTGATATCCACTTTGGTCCCAGG
pET-1E8 Fw	5'-GCATGC*GGATCC*GCAGGTGCAGCTGCAGCAG
pET-1E8 Rv	5'-GCATGC*GCGGCCGC*TAGGACGGTCAGCTTGGT
pET-1E10 Fw	5'-GCATGC*GAATTC*GCAGGTGCAGCTACAGCAG
pET-1E10 Rv	5'-GCATGC*GCGGCCGC*TTTGATTTCCACCTTGGTCCCTG
pET-1G11 Fw	5'-GCATGC*GAATTC*GCAGGTGCAGCTGCAGGAGT
pET-1G11 Rv	5'-GCATGC*GCGGCCGC*TTTAATCTCCAGTCGTGTCCCT

[a] Underlined italic sequences are restriction sites for enzymes.

with shaking, expression of scFv proteins was induced by the addition of 1 mM IPTG. Bacterial cells were incubated for 4 h and then harvested by centrifugation at 2,900× g for 30 min, after which they were stored at -30˚C for future experiments. Inclusion bodies were prepared from the bacterial cells by suspension in B-per Bacterial Protein Extraction Reagent according to the manufacturer's protocol. Briefly, 2.5 mL of the reagent containing 500 μg of lysozyme and 63 U of benzonase per 1 g of wet cells was added to bacterial cells. After the mixture was gently shaken for 10 min, pellets were collected by centrifugation at 14,500× g for 20 min and these were then resuspended in 15 mL of 10-fold diluted B-per reagent. After the mixture was gently shaken for 10 min, pellets were collected as inclusion bodies by centrifugation at 14,500 g for 20 min.

Inclusion bodies were solubilized in 20 mM sodium phosphate buffer, pH 7.4, containing 3.5 M Gdn-HCl and 0.2 M NaCl (3.5 M Gdn-HCl buffer), and centrifuged at 14,500× g for 30 min. Ni^{2+}-Sepharose slurry (0.5 mL) was added to test tubes containing the supernatant. After the tubes were inverted overnight at 25˚C, the Ni^{2}-Sepharose slurry was packed into columns. The columns were washed thoroughly with 3.5 M Gdn-HCl buffer containing 40 mM imidazole. ScFv proteins were then eluted with 3.5 M Gdn-HCl buffer containing 500 mM imidazole and 5 mM DTT. The eluted proteins were refolded as described below according to the method previously described (*16*) with some modifications. The scFv proteins purified in the presence of 3.5 M Gdn-HCl were refolded by stepwise dialyses as follows. The concentrations of the affinity-purified denatured scFv proteins were adjusted to 3.3 mM in 3.5 M Gdn-HCl buffer. The denatured scFv proteins in 20 mL were dialyzed against 300 mL of 50 mM Tris-HCl buffer, pH 8.0, containing 0.2 M NaCl and 1 mM EDTA (dialysis buffer) in the presence of 2 M Gdn-HCl, and then stepwise dialysis was done against (i) 80 mL of the dialysis buffer containing 1 M Gdn-HCl, 0.4 M L-arginine, 1 mM GSSG, and 2 mM GSH; (*ii*) 80 mL of the dialysis buffer containing 0.5 M Gdn-HCl, 0.4 M L-arginine, 1 mM GSSG, and 2 mM GSH; and (*iii*) 300 mL of PBS (10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl). Protein concentrations were determined with a Bio-Rad protein assay reagent using human IgG (Sigma-Aldrich) as standard protein.

2.5. *SDS-PAGE and Western blotting*

The purified scFv proteins were analyzed with SDS-PAGE (5-20 % gradient polyacrylamide gel) under reducing or non-reducing conditions. Separated proteins were stained with Coomassie Brilliant Blue (CBB). For immunoblotting, the proteins on the SDS-PAGE gel were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with PBS containing 3% BSA for 2 h at room temperature. The membrane was washed with PBS and then incubated with HRP-conjugated anti-His-Tag antibody at room temperature for 1 h. The membrane was washed five times with PBS containing 0.2% Tween 20. Bound primary antibodies were detected with HRP-conjugated secondary antibodies and color development using Ez West blue (ATTO Co., Tokyo, Japan). Alternatively, blotted scFv proteins were detected with His Probe-HRP.

2.6. *CD spectra of the purified scFv proteins*

CD measurements were performed with a J-820 CD spectrophotometer (JASCO Corporation, Japan). Far-UV CD spectra were recorded at 25˚C using 400 μL of each sample in a cuvette with a path length of 2 mm. Spectra were obtained after 10 scans and subtracted from the spectra of the buffer obtained under the same conditions. CD spectra were obtained for 1E6 and 1E8 scFv proteins in PBS buffer, pH 7.4.

2.7. *ELISA of the purified and refolded scFv proteins from E. coli cells binding to neoglycolipids or human IgA (hIgA)*

Wells of a 96-well plate were coated with T-antigen-, Tn-antigen-, and Man3-E6-BDB pre-mixed with E6-

BDB at a ratio of 50 pmol:450 pmol per well. E6-BDB was used as a control. 1E6 and 1E8 scFv proteins were diluted with 20 mM Tris-HCl containing 1% BSA and 0.005% Tween 20 to a concentration of 1.67 μM and incubated at 37˚C for 1 h. Unbound scFvs were washed three times with 200 μL of 10 mM Tris-buffered saline, pH7.4 (TBS). Anti-His-tag mouse monoclonal antibody was used as a primary antibody for detection of scFv proteins. After HRP-conjugated second antibody treatment, 100 μL of ABTS solution was added for color development. The reaction was stopped by 100 μL of 1% oxalic acid. Absorbance at 415 nm was measured with a plate reader (Bio-Rad, Hercules, CA, USA).

Alternatively, human immunoglobulin A (hIgA) was used as a natural glycoprotein ligand since IgA contains six varieties of *O*-glycans, including T-antigen, in the hinge region (*22*). Wells of a 96-well plate were coated with human IgA class 1 (50 μg/mL) and then blocked with 3% BSA/TBS. ScFv proteins at concentrations of 85 nM and 420 nM were added to the hIgA-immobilized wells and incubated at 37˚C for 1h. HRP-conjugated RCA_{120} lectin (1 μg/mL), which recognizes galactose residues at the non-reducing termini, was used as a positive control. Detection of bound scFvs or RCA was carried out as described above.

2.8. *SPR analyses of purified scFv proteins against T-antigen*

Real-time measurements of the binding of 1E8 and 1E6 scFv proteins to T-antigen were performed with SPR at 25˚C on a Biacore 3000 biosensor (GE Healthcare, Uppsala, Sweden) using 10 mM HEPES, pH 7.4, containing 150 mM NaCl and 0.005% Tween 20 (running buffer) at a flow rate of at 20 μL/min. T-antigen pre-immobilized on the sensor chip (D301: Galβ1-3GalNAcα1-6Glc) was purchased from SuDx-Biotec Corp. (Kagoshima, Japan). Since this sensor chip does not contain a dextran matrix, non-specific binding is minimized in comparison to sensor chips such as the CM5 or CM3 that are normally used. 1E6 and 1E8 scFv proteins were injected to T-antigen for 2 min, and then dissociation phases were observed. Alternatively, 1E6 scFv protein was measured using T-antigen biotin-immobilized on the sensor chip SA with glucose-biotin as a negative control. To reduce non-specific binding to the sensor chip surface, NaCl was added to the analyte solutions to reach a final concentration of 0.3 M NaCl. Samples were injected to ligands for 4 min, and then dissociation phases were observed.

During SPR experiments, flow cells were regenerated by injection of the running buffer containing 3.5 M Gdn-HCl followed by thorough washing with the running buffer. Kinetics parameters were calculated using BIAevaluation software version 3.2. These sensorgrams were fitted to a 1:1 Langmuir binding model using a simultaneous non-linear program.

3. Results

3.1. *Expression, purification, and refolding of anti T-antigen 1E8, 1E6, 1E10, and 1G11 scFv proteins from E. coli cells*

Anti T-antigen 1E8, 1E6, 1E10, and 1G11 scFv proteins were purified from inclusion bodies, prepared from 40 mL of the culture medium, by solubilization with 3.5 M Gdn-HCl. These scFv proteins were subjected to Ni^{2+}-Sepharose chromatography in the presence of 3.5 M Gdn-HCl and they were then refolded by stepwise dialysis (*19,23*). Figures 1A and B show comparable results of SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining and Western blot analyses for the four scFv proteins purified and refolded from the respective inclusion bodies. Figure 1 shows the starting material for the purification of the scFv proteins in the inclusion bodies (lane 1) as well as the scFv proteins

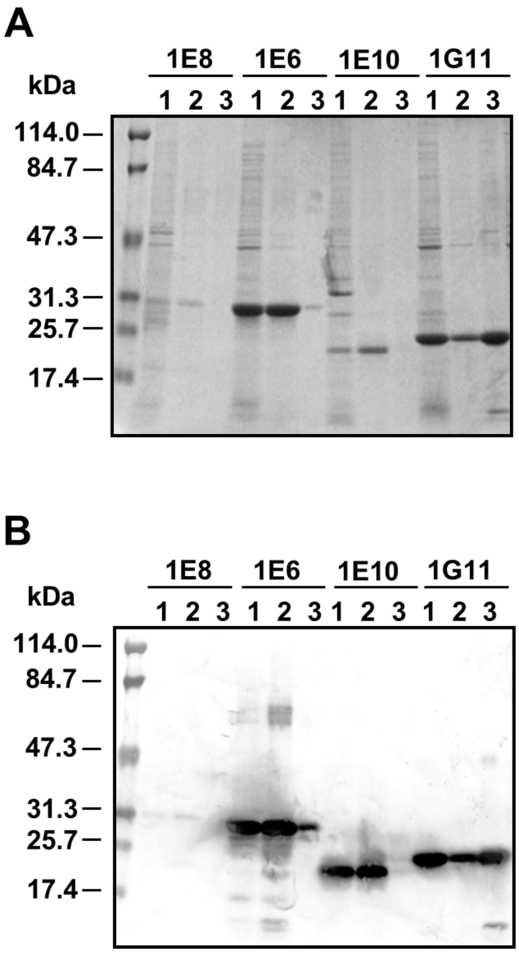

Figure 1. SDS-PAGE (A) and Western blot (B) analyses of 1E8, 1E6, 1E10, and 1G11 scFv proteins prepared from inclusion bodies by purification and refolding. Shown are results for inclusion bodies (lane 1) and soluble (lane 2) and insoluble (lane 3) fractions after purification and refolding. Proteins were visualized by CBB-staining **(A)**. Western blot analyses were carried out using an HRP-conjugated anti-His-tag mouse monoclonal antibody**(B)**.

Table 2. Summary of purification and refolding of scFv proteins expressed in *E. coli*

Items	scFv protein	1E8	1E6	1E10	1G11
Purification & refolding of scFvs from 60 mL culture	3.5 M Gdn-HCl soluble fractions (mg)	5.0	10.2	4.52	13.6
	Affinity-purified scFvs in 3.5 M Gdn-HCl (mg)	0.2	2.0	0.50	2.4
	Soluble fractions after refolding (mg) (% recovery from denatured scFvs)	0.2 (100)	1.9 (95)	0.53 (106)	0.31 (13)
Production yield of scFv protein (mg/L culture)		3.3	31.7	8.8	5.2

recovered from the soluble and insoluble fractions after refolding (lanes 2 and 3, respectively). A summary of purification and refolding of all four scFv proteins is presented in Table 2. 1E8, 1E6, and 1E10 scFv proteins were effectively recovered from the soluble fractions (Figure 1A, lane 2), indicating that most of the scFv proteins are folded. In contrast, 1G11 scFv protein was not readily recovered from the soluble fraction. While 1E8 expression was the lowest among the four scFv proteins, its efficient recovery from the soluble fraction allowed its further characterization. Each refolded scFv protein contained basically a single protein band (Figure 1), confirming it's near homogeneity. The molecular size of the 1E10 scFv protein was smaller than its theoretical molecular weight (Figure 1). 1E10 scFv protein moved faster than 1G11 scFv protein, which is an incomplete scFv lacking CDRs in its VL domain (*11*).

3.2. *Structural characterization of purified 1E6 and 1E8 scFv proteins*

CD spectroscopy was used to determine the secondary structures of the refolded scFv proteins (Figures 2A and 2B). Proteins with β-sheet structures are known to show a single negative peak with a minimum at 217 nm (*24*). The purified 1E6 and 1E8 scFv proteins showed a single negative peak with a minimum at approximately 217 nm. In the CD spectra, the molar ellipticity at 217 nm was −6,500 for the 1E6 scFv proteins and −2,500 for the 1E8 scFv proteins.

3.3. *Binding of the 1E6 and 1E8 scFv proteins to T-antigen according to ELISA and SPR*

ELISA was performed using two types of T-antigen ligands: synthetic and natural. First, the binding of scFv proteins to neoglycolipids was analyzed (Figure 3A). Both 1E6 and 1E8 scFv proteins bound to T-antigen E6-BDB but not to GalNAcα-E6-BDB (Tn-antigen-E6-BDB) or mannotoriose-E6 BDB (Man3-E6-BDB). Second, human IgA (hIgA)-containing galactose at the non-reducing termini was used to determine the T-antigen activity of the 1E8 and 1E6 scFv proteins (Figure 3B). A galactose-specific lectin, RCA_{120}, was used as the positive control. Results clearly demonstrated that the 1E6 scFv protein had binding activity to hIgA whereas

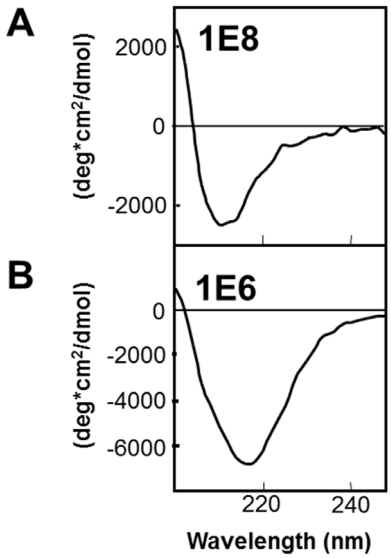

Figure 2. CD spectra of the purified and refolded scFv proteins. CD spectra of 1E8 and 1E6 scFv proteins were measured using 1E8 and 1E6 scFv proteins in PBS at 5.5 μM and 3.3 μM, respectively.

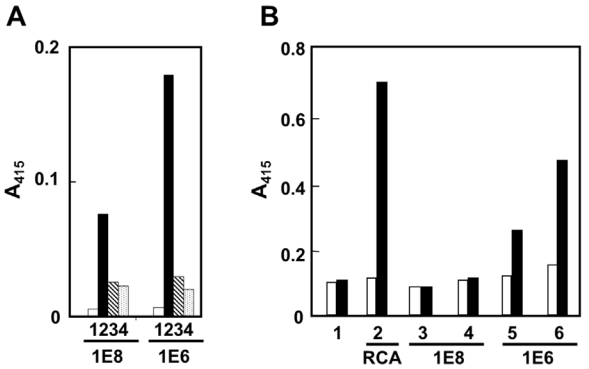

Figure 3. Binding of the purified and refolded scFv proteins to neoglycolipids (A) or human IgA (hIgA) (B) as analyzed with ELISA. In **(A)**, wells coated with E6-BDB (1; control; white bar), T-antigen-E6-BDB (2; black bar), Tn-antigen-E6-BDB (3; shaded bar), and Man3-E6-BDB (4; dotted bar) were incubated with 1E6 scFv or 1E8 scFv protein at a concentration of 1.67μM at 37˚C for 1 h. Anti-His-tag mouse monoclonal antibody was used as a primary antibody for detection of scFv proteins as described in the Methods. In **(B)**, hIgA containing six *O*-glycans in the hinge region was used as a glycoprotein anti-T-antigen substrate. 1E8 and 1E6 scFv proteins at 85 nM (3, 5) and 420 nM (4, 6) were analyzed. PBS and RCA_{120}-HRP (1μg/mL) were used as negative (1) and positive (2) control, respectively. White bars show binding to BSA (background control) whereas black bars show the binding of scFv proteins to hIgA.

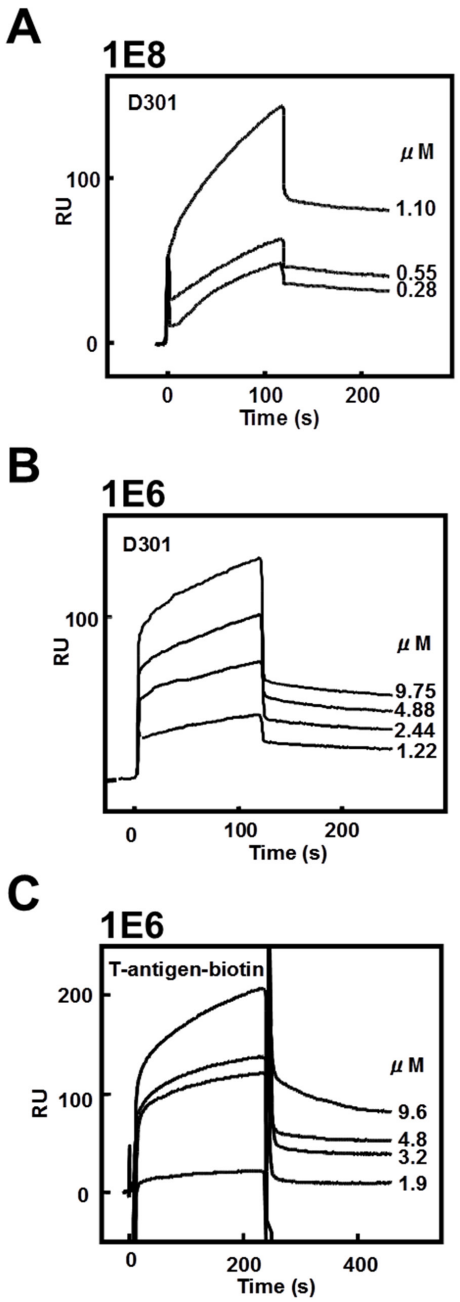

Figure 4. SPR analyses of purified and refolded 1E8 (A) and 1E6 scFv (B & C). Two different types of sensor chips were used: T-antigen immobilized sensor chip D301 **(A & B)** purchased from SuDx Biotec and T-antigen-biotin immobilized sensor chip SA. Binding of the purified and refolded 1E8 scFv **(A)** and 1E6 scFv **(B)** to D301 was measured at scFv protein concentrations of 0.28 μM, 0.55 μM, and 1.1 μM and 1.22 μM, 2.44 μM, 4.88 μM, and 9.75 μM, respectively. In **(C)**, the binding kinetics of 1E6 scFv protein were further analyzed using biotin-immobilized T-antigen on the SA sensor chip at concentrations of 1.9 μM, 3.2 μM, 4.8 μM, and 9.6 μM.

the 1E8 scFv protein did not.

SPR analyses were performed to further confirm the T-antigen binding activity of both the 1E6 and 1E8 scFv proteins (Figure 4). Kinetic parameters of 1E8 scFv and 1E6 scFv binding to T-antigen are summarized in Table 3. Both the 1E8 and 1E6 scFv proteins had good affinity for D301 (T-antigen-immobilized sensor chip) (Figures 4A and 4B, respectively), as indicated by respective *KD* values of 8.3×10^{-8} M and 1.3×10^{-6} M (Table 3). 1E6 scFv protein was analyzed using the biotinylated T-antigen sensor chip (Figure 4C) and was found to have $KD = 8.0 \times 10^{-7}$ M (Table 3).

4. Discussion

The current authors and their colleagues previously isolated genes encoding human scFv proteins with specificity against various carbohydrate moieties from a phage library displaying human scFv proteins (*8-12*). Eleven anti-T-antigen phage clones were isolated from a phage library and categorized into four groups (*11*). An anti-T-antigen 1G11 scFv protein from Group 4 was purified by DEAE-cellulose and Ni^{2+}-Sepharose affinity chromatography from the solubilized fraction of *E. coli* cells transfected with a phagemid bearing the 1G11 scFv gene (*11*). More recently, a Group 1 1E8 scFv protein was successfully expressed and purified in a *Drosophila* S2 expression system (*21*).

The current study used an *E.coli* expression system to purify and characterize four anti-T-antigen scFv proteins from Groups 1-4 (*11*). Two anti-T-antigen scFv proteins, 1E8 and 1E6 respectively belonging to Groups 1 and 2, were successfully purified for further characterization. *E. coli* strains, such as BL21(DE3), produce native bacterial proteins that exhibit a high affinity for divalent cations like nickel. Such native bacterial proteins may be co-purified with His-tagged recombinant proteins *via* Ni^{2+}-Sepharose chromatography (*25*). The use of inclusion bodies consisting of mostly recombinant proteins as the starting material could help avoid this problem of contamination. Inclusion bodies were denatured under mild conditions in 3.5 M Gdn-HCl and His-tagged scFv proteins were purified using Ni^{2+}-Sepharose column chromatography in the presence of 3.5 M Gdn-HCl. The purified scFv proteins were refolded by step-wise dialysis in the presence of arginine according to previously reported procedures (*23*). As shown in Table 2, 1E6 scFv protein was purified with the highest yield

Table 3. Kinetics parameters of 1E8 scFv and 1E6 scFv binding to T-antigen

scFv protein/Ligand	*ka* (1/Ms)	*kd* (1/s)	*KD* (M)	Sensorgrams
1E8/D301	8.8×10^{3}	7.3×10^{-4}	8.3×10^{-8}	Figure 4A
1E6/ D301	5.5×10^{2}	7.2×10^{-4}	1.3×10^{-6}	Figure 4B
1E6/ T-antigen biotin	1.1×10^{3}	9.2×10^{-4}	8.0×10^{-7}	Figure 4C

among the four scFv proteins. The level of production, 30 mg from a 1 L culture, was within the range expected from this procedure. Interestingly, when an alternative *Drosophila* S2 cells expression system was used to produce anti-T-antigen scFv proteins, we found that in contrast to this study, properly-folded 1E8 scFv protein was more efficiently expressed than 1E6 scFv protein (*21*).

The secondary structure formed by the refolded 1E8 and 1E6 scFv proteins was assessed using CD spectrometry. The far-UV CD spectra of the refolded scFv proteins revealed that both scFvs possess a β-sheet structure that is characteristic of such immunoglobulin fragments (*24,26*). The CD spectrum of 1E6 scFv indicates that it has a β-sheet structure with most of its proteins folded correctly. The CD spectrum of 1E8 scFv indicates the presence of a β-strand structure that is not as deep. This CD spectrum indicates that both correctly folded and unfolded proteins may be present in the refolded 1E8 scFv sample, suggesting that improvements on refolding procedures are required for purification of 1E8 scFv protein from inclusion bodies. The 1E8 scFv protein expressed in *Drosophila* S2 cells, however, was found to be qualitatively much better than *E. coli*-expressed and properly refolded as judged by CD and NMR (*21*).

1E10 scFv protein should have a molecular mass similar to those of 1E6 and 1E8, but the 1E10 scFv protein moved faster than both 1E6 and 1E8 scFv proteins according to SDS-PAGE (Figure 1). This indicates that the expressed 1E10 scFv protein may not be intact. There is no apparent explanation for the production of 1E10 scFv protein with a smaller molecular mass, so further study is needed.

Both 1E8 and 1E6 scFv proteins specifically bound to T-antigen-E6-BDB, which was the T-antigen probe used to screen phages displaying scFv proteins (Figure 3A). When phage antibodies representing 1E8, 1E6, 1E10, or 1G11 scFv proteins were originally analyzed for specificity, 1E8, 1E6, and 1E10 bound to T- and Tn-antigen equally whereas 1G11 bound specifically to T-antigen (*11*). The results of the current study confirm that both 1E8 and 1E6 scFv proteins exhibit T-antigen specificity. 1E6 scFv bound to hIgA, but binding of 1E8 scFv to hIgA was not detected (Figure 3B). The reason for the poor binding of 1E8 scFv protein to hIgA is not apparent. The binding activity of 1E8 scFv protein with T-antigen has been confirmed since it bound to T-antigen moieties conjugated to lipid (Figure 3A) and gold surfaces (Figure 4A).

Table 3 summarizes the kinetic parameters calculated from the sensorgrams shown in Figure 4. The results indicate that the 1E6 scFv protein bound similarly to T-antigen that was immobilized covalently on the gold surface (D301), or *via* biotin to SA, with a *KD* of approximately 1×10^{-6} M. 1E8 scFv bound to D301 with an affinity of approximately 1×10^{-7} M. These results demonstrate that both 1E6 and 1E8 scFv proteins have a definite affinity for T-antigen, with the former having a lower affinity (one order) than the latter. Since protein-carbohydrate interactions are generally weak, these human scFv proteins with rather high affinities for oncofetal T-antigen have high potential for future development of cancer therapeutics. For example, an anti-T-antigen monoclonal antibody JAA-F11 has been shown to block the adhesion of human and mouse tumor cells in metastatic models in *in vitro* and *in vivo* studies (*27*). Further characterization of 1E8 and 1E6 scFv proteins, their affinity improvement, and their effects on tumor growth and metastasis will be needed for future clinical application of these scFv proteins.

In conclusion, this study successfully purified and compared four anti-T-antigen scFv proteins from inclusion bodies expressed in *E. coli* cells. Although four anti-T-antigen scFv genes belonging to Groups 1-4 were expressed and produced in an *E. coli* expression system, 1E6 and 1E8 scFv proteins were produced at sufficient levels for further characterization of their T-antigen binding. Specificity and affinity constants determined using ELISA and SPR, respectively, provided evidence that Group 1 and 2 scFv proteins are T-antigen-specific and suggested that 1E8 scFv protein has a higher affinity for T-antigen than 1E6 scFv protein.

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