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PREPARATION OF BIOLOGICALLY ACTIVE SINGLE-CHAIN VARIABLE ANTIBODY FRAGMENTS THAT TARGET THE HIV-1 GP120 V3 LOOP

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Abstract

KD-247 is a humanized monoclonal antibody that targets the third hypervariable (V3) loop of gp120. It can efficiently neutralize a broad panel of clade B, but not non-clade B, HIV-1 isolates. To overcome this limitation, we are seeking to prepare genetically-engineered single-chain variable fragments (scFvs) of KD-247 that will have broader neutralizing activity against both clade B and non-clade B HIV-1 isolates. Initial attempts of optimizing the expression of KD-247 scFv have resulted in the formation of insoluble protein. Therefore, we have established purification protocols to recover, purify, and refold the KD-247 scFv from inclusion bodies. The protocol involved step-wise refolding of denatured scFv by dilution, dialysis, and on-column nickel-affinity purification. Monomeric scFv was further purified by size-exclusion chromatography. Using far UV circular dichroism (CD) spectroscopy we confirmed the expected beta-sheet profile of the refolded KD-247 scFv. Importantly, the refolded KD-247 scFv showed neutralizing activity against replication-competent HIV-1 BaL and JR-FL Env pseudotyped HIV-1, at potency comparable to that of the native full-size KD-247 antibody. Ongoing studies focus on the application of this system in generating KD-247 scFv variants with the ability to neutralize clade B and non-clade B HIV-1 isolates.

Key words: KD-247, neutralizing antibody, HIV, gp120, V3 loop, scFv, purification, refolding.

Article information

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INTRODUCTION

The replication life-cycle of human immunodeficiency virus type 1 (HIV-1) begins with the cell-free virus attachment to the host cell, either a CD4-positive T lymphocyte or macrophage, through the interaction of HIV-1 glycoprotein 120 (gp120) with the host CD4 receptor (6,21). Conformational changes in the HIV-1 envelope glycoprotein (Env) trimer trigger the formation of the bridging sheet and the exposure of the third hypervariable (V3) loop, which are responsible for the binding to CCR5 or CXCR-4 co-receptor on the host cell (9,24,35,36,37,38,40,47). This is followed by additional conformational changes in the gp120/gp41 complex that eventually lead to fusion of the viral envelope with the cellular membrane to allow release of the viral contents into the host cell for viral replication (10,29,44,46). HIV-1 entry is a critical step to target in order to prevent infection and block viral replication.

For more than two decades since the discovery of HIV-1, exhaustive efforts have been put into the discovery of a vaccine against this devastating human pathogen. However, HIV-1 has evolved multiple mechanisms to evade the host antibody response, making the ideal mission of finding a cure against HIV-1 extremely challenging (23,32,43). Despite the failure of developing a successful HIV-1 vaccine, Highly Active Anti-Retroviral Therapy (HAART) has proven to be a highly effective treatment regimen against HIV-1 infection (33). HAART involves the administration of a combination of at least three antiretroviral drugs to overcome the ability of HIV-1 to become resistant against a particular drug. Among the 32 FDA-approved antiretroviral drugs against HIV-1, there are only two inhibitors of viral entry. Maraviroc targets the CCR5 co-receptor, while enfuvirtide binds to gp41 and blocks the fusion event (7,11,25). Therefore, the development of novel therapeutics against HIV-1 entry is still very crucial. Neutralizing antibodies discovered through the HIV-1 vaccine studies can be potential therapeutics for prophylaxis treatment (2,31,45). Although some broadly neutralizing antibodies such as 2G12, 2F5, 4E10, b12, PG9, PG16, HJ16, VRC01, VRC03 have been reported (16), there is a very large number of neutralizing antibodies with narrow neutralization profile that still can be improved through antibody engineering.

Development and advancement in the field of antibody engineering has enabled researchers to improve the properties of an antibody of interest using techniques such as phage display (3,4,28,41). To improve the effectiveness of clade-specific neutralizing antibodies, we have applied structural and molecular modeling approaches to help us engineer second-generation antibodies based on rational structure-based design. In this study, we are interested in investigating KD-247, a humanized monoclonal antibody (mAb) currently in clinical trials that binds the conserved V3 loop of gp120 and efficiently neutralizes many clade B HIV-1 isolates, which possess V3 loop with Glycine^{312}-Proline^{313}-Glycine^{314}-Arginine^{315} (GPGR) motif (12,27). Similar to other anti-V3 mAbs, KD-247 cannot neutralize

non-clade B HIV-1 isolates, which are the predominant HIV-1 infection cases arising today. Interestingly, Glycine^{312}-Proline^{313}-Glycine^{314}-Glutamine^{315} (GPGQ) motif is highly conserved among non-clade B HIV-1. To better understand the molecular interaction of KD-247 with its epitope, Isoleucine-Glycine-Proline-Glycine-Arginine (IGPGR) at positions 311 to 315, which is located at the tip region of HIV-1 V3 loop (12,27), we have constructed KD-247 in the form of a single-chain variable fragment (scFv).

scFvs are antibody fragments containing a single variable heavy (V_H) domain and a single variable light (V_L) domain connected by a flexible amino acid linker (4,5,13, 17,18,22,28). scFvs have been widely applied in the field of antibody engineering, especially in phage display for the discovery of antibodies specific for the antigen of interest and for the affinity maturation of the existing antibodies (28,41). Expression of scFv in the periplasmic space of *Escherichia coli* (*E. coli*) has been described (18,20). Alternatively, scFvs can be obtained by overexpression in the cytoplasm of *E. coli* using pET system with the caveat that they are usually expressed in inclusion bodies (14,15,26,39,42). Nonetheless, many studies have shown that the purification of scFvs from inclusion bodies is an obstacle that can be overcome through *in vitro* refolding (14,15,26,39,42). Here, we have established a system to obtain soluble, active KD-247 scFv, which we are now applying in our ongoing study to generate KD-247 variants to confirm the V3 loop binding site and to evaluate their neutralization profiles. This protocol can be useful for the successful purification of other scFvs that are expressed as inclusion bodies in bacterial systems.

MATERIALS AND METHODS

Construction of KD-247 scFv Expression Vector

The amino acid sequences of the variable domains of the heavy (V_H) and the light (V_L) chains of the KD-247 antigen binding fragment (F_{ab}) were obtained from the Protein Data Bank (PDB: 3NTC_H and 3NTC_L). The KD-247 scFv was designed in the order of the V_H sequence, a $(Glycine\text{-}Glycine\text{-}Glycine\text{-}Glycine\text{-}Serine)_4$ linker, and the V_L sequence. The gene of KD-247 scFv was optimized for protein expression in *E. coli* and synthesized by Epoch Life Science, Inc. Using *Bam HI* and *Hind III* restriction sites, the KD-247 scFv gene was subcloned into a pET28a3c plasmid, which was modified from pET28a(+) (Novagen, EMD4Biosciences) with insertion of the Rhinovirus 3C protease cleavage site downstream of a 6X-Histidine tag. The ligated product was transformed in *Escherichia coli* (*E. coli*) strain DH5α. The gene sequence of the pET28a3c-KD247-scFv construct was confirmed by sequencing using T7 promoter and T7 terminator primers (Novagen, EMD4Biosciences).

Optimization of KD-247 scFv Expression in E. coli

The KD-247 scFv expression vector (pET28a3c-KD247-scFv) was transformed into *E. coli* expression strain Origami 2 (DE3) pLysS (Novagen) by heat-shock. A single colony of transformed cell was inoculated in 10 ml Luria-Bertani broth (LB) containing 50 µg/ml kanamycin, 34 µg/ml chloramphenicol, and 10 µg/ml tetracycline at 37 °C with shaking at 225 rpm for overnight. 2 ml of the overnight culture was transferred into 200 ml LB containing the antibiotics and continue shaking at 37 °C until optical density at 600 nm (OD_{600nm}) reaches mid-log phase (0.6 - 0.8). 50 ml of culture was transferred into three other sterile flasks. The remaining culture was incubated at 37 °C with shaking for three hours without addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures in the three other flasks were induced with IPTG at final concentration of 0.25 mM, 0.5 mM, and 1 mM respectively and continue shaking at 37 °C for three hours. Cells were harvested by centrifugation at 4,200 x g for 15 min at 4 °C and pellets were stored at -20 °C. The same protocol was used for growing and inducing cultures at 30 °C. To optimize scFv expression in various *E. coli* expression strains, including BL21 (DE3), Rosetta 2 (DE3) (Novagen), BL21 Gold (DE3) pLysS (Stratagene), and BL21 Star (DE3) (Invitrogen), a similar protocol is used with modifications of the growing culture (in LB with the appropriate antibiotics) at 37 °C and inducing expression with 0.5 mM IPTG.

Each frozen cell pellet was thawed on ice and resuspended in 5 ml lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 250 µg/ml lysozyme. 10 µg/ml DNAse and 20 mM $MgSO_4$ were added to cell suspension and incubated on ice for 30 min before centrifugation at 13,000 x g for 20 min at 4 °C. Cell lysates in the supernatant were collected in new tubes. The pellets and lysates of both non-induced and induced samples were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of KD-247 scFv from Inclusion Bodies

KD-247 scFv was expressed in *E. coli* BL21 (DE3) competent cells as described above with some modifications. 10 ml of overnight culture grown in the presence of kanamycin was transferred to 500 ml of LB containing kanamycin. Protein expression was induced at OD_{600nm} = 1.0 with addition of IPTG (0.5 mM) and incubation at 37 °C for three hours. Harvested cells in the pellet form were stored at -20 °C overnight. The cell pellet was resuspended in 25 ml Lysis Buffer (Table 1). The cell resuspension was then sonicated on ice with 30 seconds on-off cycle for 10 min. The cell lysate was centrifuged at 13,000 x g for 15 min at 4 °C. The pellet was first washed with 25 ml Wash Buffer A (Table 1) with gentle shaking at 4 °C overnight. After centrifugation at 13,000 x g for 15 min at 4 °C, the pellet was washed with 25 ml Wash Buffer B (Table 1) using the same procedure. The pellet containing inclusion bodies was solubilized in 10 ml Denaturing Buffer (Table 1) using the previous procedure. Finally, the denatured protein was collected from the supernatant after centrifugation and kept at 4 °C until use. Concentration of the denatured KD-247 scFv protein was estimated using a Nanodrop with the calculated extinction coefficient (62.3 x 10^3 $M^{-1}cm^{-1}$) and molecular weight (30.2 kDa).

Refolding of KD-247 scFv

The denatured KD-247 scFv protein was diluted in 100 ml Refolding Buffer A (Table 1). The diluted KD-247 scFv sample was dialyzed in Spectra/Por® Dialysis Membrane MWCO 3500 (Spectrum Laboratories, Inc.) against Refolding Buffer B (Table 1) at 4 °C overnight. These refolding steps were repeated four times using the same Refolding Buffer B. Partially refolded scFv was loaded onto a 5 ml HisTrap column (GE Healthcare) using an ÄKTAprime

plus FPLC system. Next, the column was washed with a gradient of Refolding Buffer B against Refolding Buffer C (Table 1). Column-bound refolded protein was eluted using a gradient of Refolding Buffer C against Elution Buffer (Table 1). Elution fractions corresponding to ~30 kDa protein were pooled and buffer exchanged against 1X phosphate-buffered saline (PBS) pH 7.6 using PD-10 desalting columns. Refolded KD-247 scFv was further separated by gel filtration on a HiPrep 26/60 Sephacryl S200 HR column (GE Healthcare) equilibrated with 1X PBS pH 7.6. Elution fractions corresponding to monomer (~30 kDa) were pooled and concentrated using Amicon Ultra Centrifugal Filters MWCO 10,000 (Millipore). The concentrated protein was filtered through a 0.22 µm filter before aliquoting for storage at -80 °C.

Polyacrylamide Gel Electrophoresis

Aliquots of protein samples collected at various purification steps were analyzed by 15% SDS-PAGE. Concentrated scFv was also analyzed by 6% Native-PAGE. Gels were stained using Coomassie-Blue Stain. After destaining with 40% methanol and 10% acetic acid, gel images were taken using the Fotodyne Imaging system (Fotodyne Inc.).

Circular Dichroism (CD) Spectroscopy

To confirm the secondary structure of refolded KD-247 scFv in comparison to KD-247 F_{ab}, we used far-UV CD, which measures the ellipticity (θ) of the protein sample from 200 to 240 nm wavelength. KD-247 F_{ab} and refolded scFv were diluted with 1X PBS to 200 µg/ml. 250 µl of each sample was analyzed at 25 °C or 37 °C using a 1 mm quartz cuvette in a J-815 CD Spectrometer (JASCO). For interaction with V3 peptide, 300 µl of 200 µg/ml sample was mixed with 6 µl of 1 mM V3 peptide (KRKRIHIGPGRAFYTT) derived from HIV-1 MN and incubated on ice overnight before analysis. Circular dichroism spectra were plotted using GraphPad Prism 5 (GraphPad Software Inc.).

Preparation of HIV-1 Virus and HIV-1 Env Pseudotyped Virus

pWT/BaL plasmid was obtained through the NIH AIDS Research and Reference Reagent Program (NIH ARRRP) from B.R. Cullen (Duke University). In a 75 cm^2 flask, 1.8 x 10^6 293T cells were transfected with 8 µg pWT/BaL using FuGENE 6 (Roche) in a 3:1 ratio. Supernatant containing virions was collected at 72 hours after transfection. After centrifugation at 1,100 rpm for 5 minutes at 4 °C, the supernatant was subsequently filtered through a 0.45 µm filter before storage at -80 °C. The same protocol was used for the production of HIV-1 Env pseudotyped virus. HIV-1 backbone plasmid with Env deletion, pSG3ΔEnv, was obtained through the NIH ARRRP from Drs. John C. Kappes and Xiaoyun Wu (University of Alabama at Birmingham). Plasmid for expression of JR-FL Env ($pEnv_{JR-FL}$) was provided by Dr. Shuzo Matsushita (Kumamoto University). Plasmid for ZM53M.PB12 Env ($pEnv_{ZM53M.PB12}$) was obtained through NIH ARRRP from Drs C.A. Derdeyn and E. Hunter (Emory University). 293T cells were co-transfected with 5.3 µg pSG3ΔEnv and 2.7 µg $pEnv_{JR-FL}$ (2:1 ratio). The amount of infectious virus in the supernatant was quantified using the $TCID_{50}$ assay as previously described (30).

TZM-bl Cell-Based Neutralization Assay

The ability of KD-247 scFv to neutralize HIV-1 virions was tested in a TZM-bl cell-based neutralization assay as previously described with some modifications (30). TZM-bl cells were obtained through the NIH ARRRP from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. Maraviroc (obtained through NIH ARRRP), KD-247 F_{ab} and scFv were diluted to various concentrations in PBS. HIV-1 BaL virus or JR-FL Env pseudotyped virus (final: 50 $TCID_{50}$) was diluted in infection medium (DMEM, 10% FBS, 1X Penicillin/Streptomycin, 20 µg/ml DEAE-Dextran). 50 µl of sample dilutions and virus or pseudotyped virus were pre-incubated at 37 °C for 1 hour prior to infection of TZM-bl cells (pre-seeded in 96-well plate with 100 µl of 1 x 10^4 cells per well). Luciferase activity of infected TZM-bl cells was determined at 48 hours post-infection using Bright-Glo Reagent (Promega) according to the manufacturer's instructions. Luminescence was measured using an EnSpire Plate Reader (Perkin Elmer). Neutralization assays were carried out at least two times with duplicates each time. The relative light unit (RLU) was adjusted based on luminescence reading of the cell control (non-infected cells). The mean RLU was used to calculate percent infection relative to the mean RLU of virus control (infected cells only). Percent infection was plotted as a function of sample concentration (in logarithmic scale) using GraphPad Prism 5 to determine 50% inhibitory or neutralizing concentration (IC_{50}).

RESULTS

Construction of KD-247 scFv

To better understand the molecular interaction of KD-247 with its epitope, Isoleucine^{311}-Glycine^{312}-Proline^{313}-Glycine^{314}-Arginine^{315} (IGPGR), which is located at the tip of HIV-1 V3 loop, we have constructed KD-247 in the form of a single-chain variable fragment (scFv). The V_H and V_L of KD-247 scFv are linked with a 20 amino acid-long linker, which consists of four repeats of the Glycine-Glycine-Glycine-Glycine-Serine sequence (Fig. 1A), to enable expression of monomeric scFv (18). The gene of KD-247 scFv optimized for *E. coli* expression was subcloned into the pET28a3c vector (Fig. 1B) for the overexpression of scFv with an N-terminal 6X Histidine (HIS_6) tag (Fig. 1A). In addition to the thrombin cleavage site, pET28a3c contains a 3C protease cleavage site for cleavage of the HIS_6 tag when necessary (Fig. 1B).

Optimization of KD-247 scFv Expression

To purify KD-247 scFv using an *E. coli* expression system, our first attempt was to express KD-247 scFv in Origami 2 (DE3) pLysS, which was engineered for the cytoplasmic expression of the recombinant protein with proper protein folding. When the scFv was expressed and induced at 37 °C using various IPTG concentrations, a distinct protein band of size approximately 30 kDa could be identified in the pellet fractions of cell lysates with the exception of the non-induced cells (Fig. 2A). This observation indicated that the overexpression of KD-247 scFvs in the cytoplasm had resulted in the aggregation of improperly folded protein in a form commonly known as inclusion bodies. Despite many efforts of optimizing the expression of KD-247 scFv, including inducing at 30 °C (Fig. 2B) and expressing in different *E. coli* strains (Fig. 2C), KD-247

scFv remained insoluble.

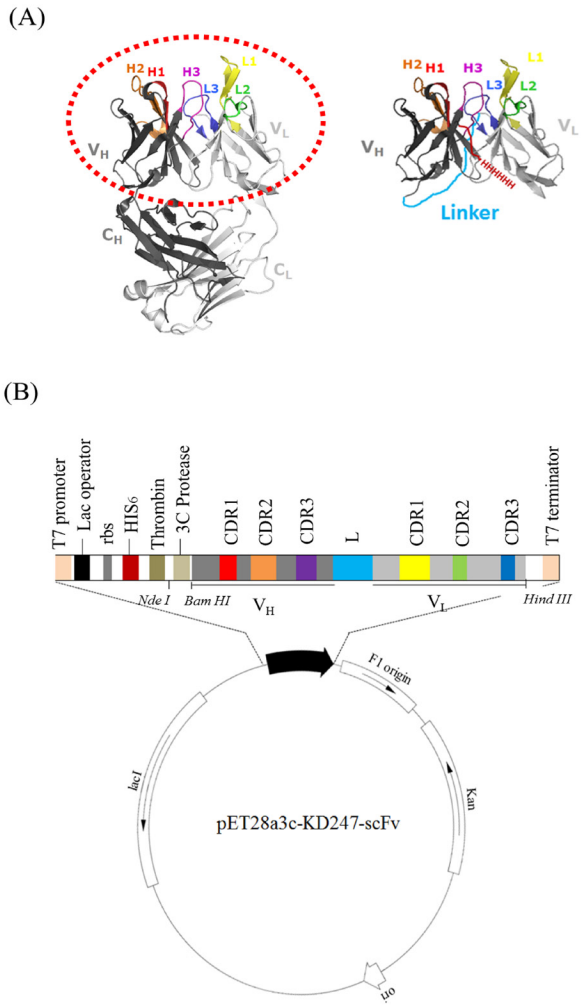

Figure 1. Schematic diagram of pET28a3c-KD247-scFv. (A) The crystal structure of KD-247 F_{ab} (1.5 Å resolution, PDB: 3NTC) is shown with the complementarity determining regions (CDRs) highlighted in different colors. [Red: CDR 1 heavy chain (H1); Orange: CDR 2 heavy chain (H2); Purple: CDR 3 heavy chain (H3); Yellow: CDR 1 light chain (L1); Green: CDR 2 light chain (L2); Dark blue: CDR3 light chain (L3)]. The expected structure of KD-247 scFv was illustrated as a model. Figures were generated using PyMOL (8). (B) The variable domains of the heavy chain (V_H) and light chain (V_L) of KD-247 are connected with a peptide linker (L) to form the scFv. The twenty amino acid long peptide linker consists of four repeats of Glycine-Glycine-Glycine-Glycine-Serine, $(GGGGS)_4$. The scFv construct was subcloned at the N-terminal 6X Histidine tag (HIS_6) of the pET28a3c vector.

Purification of KD-247 scFv from Inclusion Bodies

Although the overexpression of KD-247 scFv failed to yield soluble protein, we were able to establish a system to recover and purify KD-247 scFv from the inclusion bodies (Fig. 3). Briefly, inclusion bodies isolated from cell lysate after sonication were washed extensively to eliminate the majority of cellular debris before denaturation using 6 M guanidine-hydrochloride (Gu-HCl) in the presence of β-mercaptoethanol. Denatured scFv was dissolved using buffer containing 6 M urea. Next, dialysis of denatured scFv against buffer containing 0.8 M urea enabled gradual refolding of denatured scFv to partially folded intermediates with the assistance of a cysteine-cystine redox reagent to help with disulfide bond formation. Finally, partially folded intermediates were immobilized on a nickel column for gradient wash to remove excess denaturing reagent. SDS-PAGE shows that scFv of ~ 30 kDa can be detected in the pellet of cell lysate, supernatant of denaturation (Fig. 4A), and the elution from nickel column (Fig. 4B). The refolded scFvs consist of multiple folded species and can be separated using size-exclusion chromatography (Fig.4C). The monomeric scFv was collected and concentrated for downstream assays. On native-PAGE, the concentrated KD-247 scFv appeared as a distinct band and migrated faster than the KD-247 F_{ab}, confirming that its native form is a monomer.

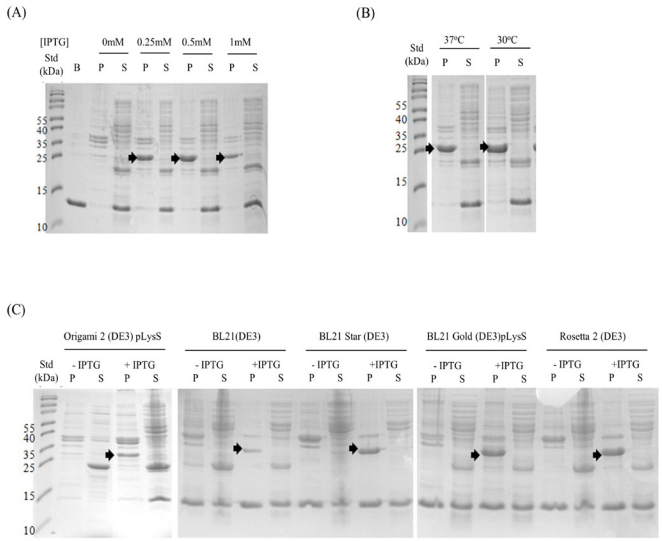

Figure 2. SDS-PAGE showing the optimization of KD-247 scFv overexpression in *E. coli*. (A) Overexpression of KD-247 scFv in Origami 2 (DE3) pLysS at various IPTG concentrations (0.25 mM, 0.5 mM, and 1 mM) were examined. (B) KD-247 scFv expression was induced at 37 °C or 30 °C. (C) Overexpression of KD-247 scFv in various *E. coli* strains was compared. As shown by the arrows, KD-247 scFv was expressed as inclusion bodies in *E. coli*. [Std: Protein standards; P: Pellet; S: Supernatant; B: Lysis Buffer].

Table 1. Buffers for KD-247 scFv Purification.

Lysis Buffer	50 mM Tris-HCl pH 8.2, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM PMSF, 100 µg/ml lysozyme
Wash Buffer A	50 mM Tris-HCl pH 8.2, 150 mM NaCl, 3% Triton X-100, 1M Gu-HCl
Wash Buffer B	50 mM Tris-HCl pH 8.2, 150 mM NaCl
Denaturing Buffer	50 mM Tris-HCl pH 8.2, 150 mM NaCl, 6 M Gu-HCl, 10 mM β-mercaptoethanol
Refolding Buffer A	6 M Urea, 50 mM Tris-HCl pH 8.2, 150 mM NaCl
Refolding Buffer B	0.8 M Urea, 50 mM Tris-HCl pH 8.2, 150 mM NaCl, 2 mM Cysteine, 0.4 mM Cystine
Refolding Buffer C	50 mM Tris-HCl pH 8.2, 150 mM NaCl
Elution Buffer	50 mM Tris-HCl pH 8.2, 150 mM NaCl, 500 mM Imidazole

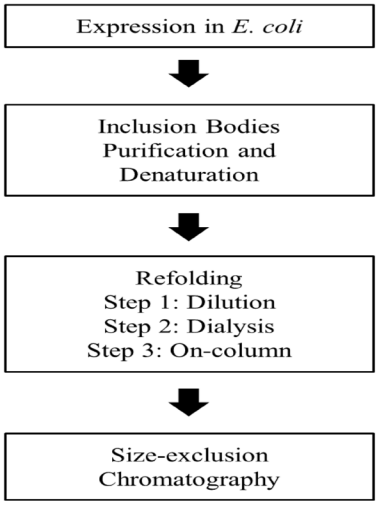

Figure 3. Schematic representation of the purification and refolding of KD-247 scFv. KD-247 scFv was overexpressed in the BL21 (DE3) strain using the pET system. The pellet recovered after cell lysis was washed twice before denaturation with 6 M Gu-HCl containing β-mercaptoethanol. Denatured protein was refolded by first diluting in 6 M Urea and then dialyzing against 0.8 M Urea containing redox reagents cysteine and cystine. Partially refolded scFv was immobilized on a nickel column for further purification to remove residual urea. KD-247 scFv contains HIS_6 tag at the N-terminus, which enables nickel-affinity purification. scFvs eluted from the column were subjected to size-exclusion chromatography to obtain monomeric scFv.

Far-UV Circular Dichroism Spectroscopy

To confirm the proper folding of scFv into the immunoglobulin structure which consists primarily of β-sheets (1, 18), we performed far-UV circular dichroism (CD) spectroscopy (19). CD spectra of refolded KD-247 scFv with and without V3 peptide are very similar to that of KD-247 F_{ab} (Figs. 5A &B). This indicates that the bacterially expressed KD-247 scFv can be refolded into a form that is comparable to KD-247 F_{ab}, which is obtained from a eukaryotic expression system. Furthermore, the β-sheet profiles of KD-247 scFv do not show significant differences when incubated at 37 °C for 1 hour and overnight (Fig. 5C).

Neutralization Assay

To further evaluate the biological activity of KD-247 scFv, we performed an HIV-1 neutralization assay using TZM-bl cells. As shown in Table 2, the refolded KD-247 scFv is able to neutralize clade B HIV-1 or pseudotyped HIV-1 (with GPGR V3 loop), but not clade C pseudotyped HIV-1 (with GPGQ V3 loop). KD-247 scFv neutralizes HIV-1 BaL and JR-FL Env pseudotyped HIV-1 with 50% neutralizing concentration (IC_{50}) comparable to the KD-247 F_{ab}. As expected, both KD-247 scFv and F_{ab} cannot neutralize ZM53M.PB12 (Clade C) Env pseudotyped HIV-1. Maraviroc, which targets the CCR5 coreceptor, is able to inhibit the entry of the HIV-1 and pseudotyped HIV-1 viruses examined in this assay.

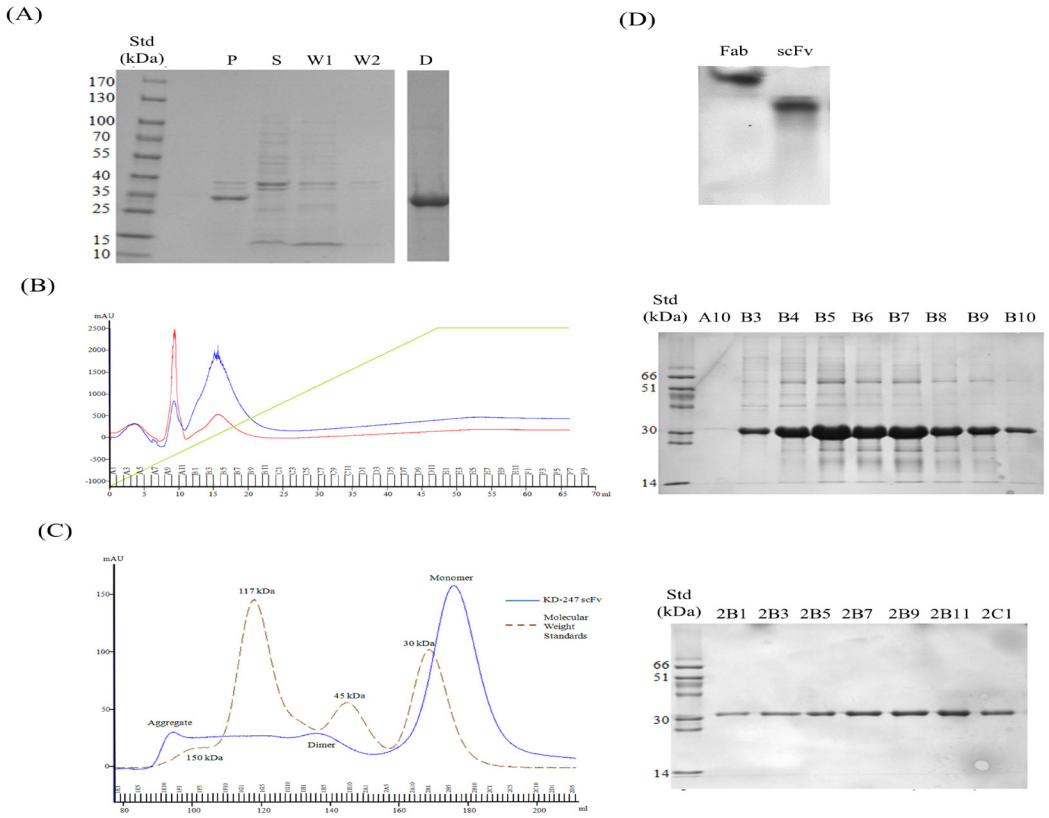

Figure 4. Purification of KD-247 scFv from inclusion bodies. (A) SDS-PAGE showed proteins collected at various steps of purification. [P: pellet of lysed cells; S: supernatant of lysed cells; W1: supernatant after washing pellet with Wash Buffer A; W2: supernatant after washing pellet with Wash Buffer B; D: denatured scFv (diluted in 50 mM Tris-HCl pH 8.2, 150 mM NaCl)]. (B) Chromatogram showing the elution of scFvs from the nickel column. Blue line represents absorbance at 280 nm, red line represents absorbance at 254 nm. Elution fractions collected were analyzed by SDS-PAGE. (C) Size-exclusion chromatogram of KD-247 scFv in various refolded forms (blue solid line) compared to molecular weight standards (brown dashed line). Eluted scFvs in the monomeric fractions were analyzed by SDS-PAGE. (D) Native-PAGE of KD-247 F_{ab} compared to the monomeric KD-247 scFv recovered after concentration.

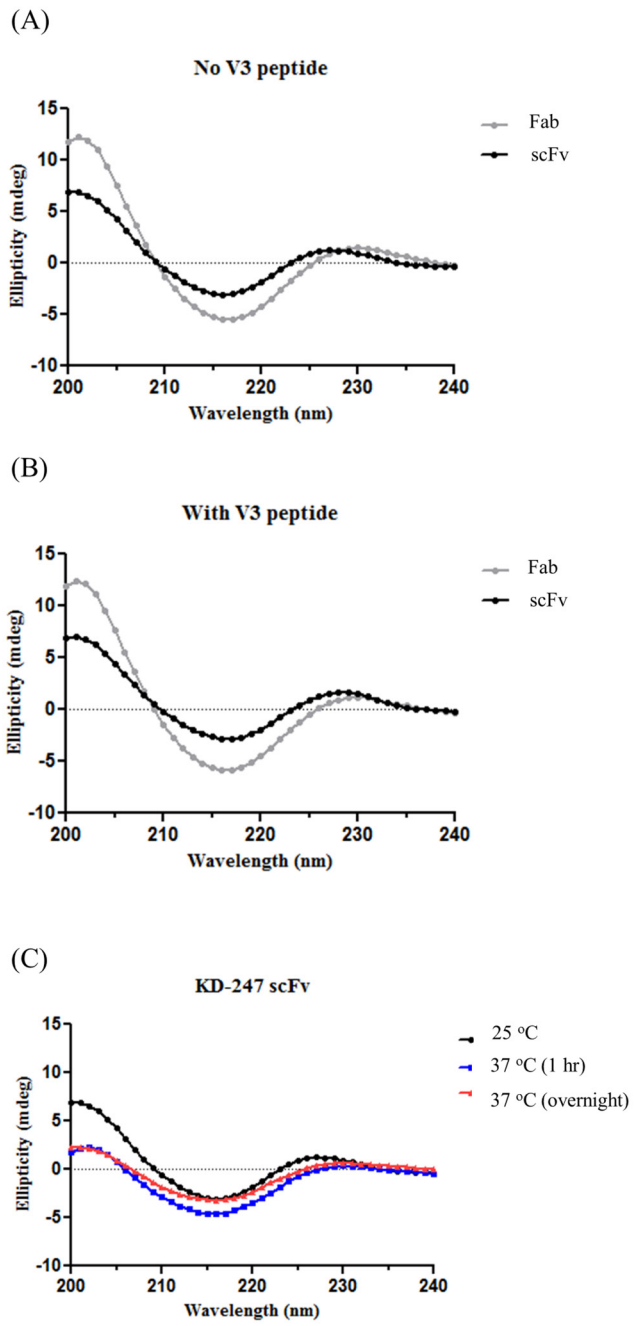

Figure 5. Far-UV circular dichroism (CD) spectra to evaluate secondary structures. The refolded KD-247 scFv (black) was compared to KD-247 F_{ab} (grey) in the absence of V3 peptide (A) and in the presence of V3 peptide (B). (C) CD of the refolded scFv at 25 °C (black), after 1 hour incubation at 37 °C (blue) and after overnight incubation at 37 °C (red).

DISCUSSION

In human adaptive immune system, B lymphocytes play the role of producing antibodies that are specific to a foreign antigen, such as virus, microbes and parasites (1). Human neutralizing antibody produced in nature are consist of a pair of identical heavy and light chains with three constant domains on the heavy chain and one constant domain on the light chain. Each variable domain on the heavy and light chains contains the complementarity determining regions (CDRs) that are responsible for the binding to the antigen (1,4,28). Innovative recombinant DNA technologies have made possible the modification of an antibody into smaller binding fragments such as scFv, in which the antigen binding sites can be retained (1,17,18,22,28,34). scFv format has been widely used in the phage display system because of its relatively small size to allow genetic engineering in the bacterial system (1,4,18,22,28,41). In this study, we have shown that KD-247 scFv, which was expressed in a bacterial system, can be successfully recovered from inclusion bodies through refolding. The refolded KD-247 scFv showed secondary structure and neutralizing activity comparable to KD-247 F_{ab}, which was obtained from the eukaryotic expression system.

To date, several refolding systems from insoluble scFv have been reported (15,26,39,42). Tsumoto K. and colleagues have described the use of glutathione and the addition of L-arginine in the refolding process of scFv (42). The on-column refolding approach based on the nickel-affinity chromatography has been described by other groups (15,26). Our system reported here is based on a combination of these protocols performed in a step-wise fashion with the addition of the redox reagents cysteine and cystine. The presence of disulfide bonds in the scFv can be a challenge in the proper expression of recombinant protein in the reducing environment of the *E. coli* cytoplasm (14). To address the issue of obtaining soluble scFv, we are still investigating the use of other protein expression vectors containing fusion tags that may help with the expression of soluble recombinant protein.

Using the purification protocol described here, we have purified KD-247 scFv from inclusion bodies and refolded the protein *in vitro* under the artificial control of cysteine and cystine to enable formation of intradomain disulfide bonds. The yield of purified, refolded scFv was comparably lower than the yield of soluble protein obtained from overexpression. This is possibly due to the loss of protein at each step of purification and during concentration. Al-

Table 2. 50% neutralizing activity (IC_{50}) of KD-247 scFv by TZM-bl cell assay.

	Clade B HIV-1 Env		Clade C HIV-1 Env
	BaL	**JR-FL**	**ZM53M.PB12**
Maraviroc	0.001 µM	0.003 µM	0.003 µM
KD-247 Fab	0.1 µM	0.5 µM	> 5 µM
KD-247 scFv	0.2 µM	0.6 µM	> 5 µM

though the yield is sufficient for subsequent assays such as far-UV CD and HIV-1 neutralization assay, optimization of this system will be needed to obtain sufficiently large quantity of protein for X-ray crystallography studies.

The refolded KD-247 scFv showed comparable neutralization profile compared to KD-247 F_{ab}. Their abilities to neutralize only clade B HIV-1 (GPGR V3 loop) but not clade C HIV-1 (GPGQ V3 loop), also further confirm the neutralization studies as previously described using KD-247 mAb (12,27). Using this system, we are currently engineering KD-247 scFv variants, which are designed based on our molecular modeling studies to alter the binding site of KD-247 and which we expect to exhibit improved neutralizing profiles in future studies.

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Other articles in this theme issue include references (48-75).

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