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# Development of high-affinity single chain Fv against foot-and-mouth disease virus

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ABSTRACT

Foot-and-mouth disease (FMD) is caused by the FMD virus (FMDV) and results in severe economic losses in livestock farming. For rapid FMD diagnostic and therapeutic purposes, an effective antibody against FMDV is needed. Here, we developed a high-affinity antibody against FMDV by FACS-based high throughput screening of a random library. With the FITC-conjugated VP1 epitope of FMDV and high-speed FACS sorting, we screened the synthetic antibody (scFv) library in which antibody variants are displayed in the periplasm of *Escherichia coli*. After three rounds of sorting, we isolated one antibody fragment (#138-scFv) against the VP1 epitope of FMDV. Next, to improve its affinity, a mutation library of #138-scFV was constructed by error-prone PCR and screened by FACS. After three rounds of sorting, we isolated one antibody (AM-32 scFv), which has a higher binding affinity ($K_D$ = 42.7 nM) than that of the original #138-scFv. We also confirmed that it specifically binds to whole inactivated FMDV.



## 1. Introduction

Foot-and-mouth disease (FMD) is one of the most devastating diseases in wild and domestic cloven-hoofed animals [1,2]. In 2011, a FMD outbreak in Korea consisting of 3.5 million infected livestock, especially swine and cattle, caused huge economical loss [3]. The FMD virus (FMDV), which causes FMD, is a picornavirus, the prototypic member of the genus *Aphthovirus*, and is classified with seven distinct serotypes [O, A, C, Asia 1, SAT1, SAT2, and SAT3] by antigenic diversity [4]. Serotype-O is the most prevalent and occurs worldwide, including in Korea [5]. To rapidly and effectively control FMD, we must identify FMDV infection at an early stage, which requires the development of rapid and reliable diagnostic systems. Several diagnostic methods, including ELISA and PCR, have been used, and the antigen-detecting ELISA with an antigen-specific antibody is currently preferred [6]. In these ELISA-based methods, the essential and key component is the antibody, which has a strong binding affinity and high specificity to FMDV serotypes.

Biotechnologies for antibody engineering allow the development of various forms of antibodies for diagnostic and therapeutic usage [7]. One of these techniques focuses on minimizing the size of the antibody, resulting in various antibody fragments that induce passive immunity and these can be used for biosensors, drug targeting, and drug delivery systems [8,9]. These antibodies, including monovalent antibody fragments (Fab), single-chain variable fragments (scFv), and single domains, are well established as an alternative to conventional monoclonal antibodies such as immunoglobulin G (IgG). Conventional monoclonal antibodies are fully structured and complex proteins, are mostly produced in mammalian hosts, and are relatively expensive. In contrast, recombinant small antibody fragments can be economically produced on a large scale from bacterial expression systems and the antibody properties (affinity, specificity) can be easily engineered [7,10]. Additionally, antibody fragments like scFv and Fab can be used to detect various antigens such as haptens, proteins, and whole pathogens [11].

FMDV has an icosahedral shell composed of 60 copies of each of four structural proteins (VP1–VP4), and among four proteins, VP1 is highly immunogenic and has been proposed to play a major role in serotype specificity and eliciting an immune response [1,4,12]. The G–H loop (amino acids 140–160 at the C terminus) of VP1 is a major antigenic site and is responsible for the induction of protective neutralizing antibodies [13]. Therefore, VP1 epitope could be a useful target for an antibody specific to FMDV. Here, we sought to isolate an scFv specific to the VP1 epitope of serotype-O FMDV by screening a synthetic antibody library constructed in *E. coli*. For the screening of library, we used a FITC-conjugated VP1 epitope

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of FMDV and a FACS-based high-throughput screening, which we previously established [14]. We improved the affinity of the isolated antibody by an affinity maturation process and determined its specific binding to inactivated FMDV.

## 2. Materials and methods

### 2.1. Bacterial strains

The *E. coli* strains and plasmids used are listed in Table 1. *E. coli* Jude-1 [15] was used for the library construction and screening. *E. coli* MG1655 was used for the production and purification of the isolated antibodies (scFv).

### 2.2. Error-prone PCR

For the affinity maturation, random library was constructed by error-prone PCR protocol [16]. Briefly, 10 μL of 10 × PCR buffer (without $MgCl_2$, Takara Bio Inc., Shiga, Japan) was combined with dNTP mix (2.0 μL of 10 mM dGTP, 3.5 uL of 10 mM dATP, 4.0 μL of 10 mM dCTP, 1.4 μL of 100 mM dTTP), 10 μL of both 5 mM $MnCl_2$ and 25 mM $MgCl_2$, and 10 ng of DNA (#138-scFv). Polymerization was performed by low-fidelity rTaq DNA polymerase (Takara Co., Japan) with the primers Hv-Amp (5′-GATTGTTATTACTCGCGG) and Lgt-Amp (5′-GGCCGCGAATTCG). The PCR products were digested with *Eco*RI and *Nco*I and ligated into pMoPac16, which contains a pelB signal peptide for the periplasmic production of the scFv. The ligated DNA was transformed into *E. coli* Jude-1 by electroporation at 2.5 kV using a Gene Pulser (Bio-Rad Co., Hercules, CA, USA). After electroporation, cells were plated on SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) agar plates containing ampicillin (100 μg/mL) and incubated overnight at 37 °C.

### 2.3. Library screening by FACS

For the isolation of a primary antibody against FMDV, we used FACS to screen the previously constructed human synthetic scFv antibody library [14]. We inoculated 10 mL of Luria–Bertani (LB) media containing 2% (w/v) glucose and chloramphenicol (35 μg/mL) with the synthetic scFv antibody library. After growth overnight at 37 °C, 200 μL of the culture was transferred to 10 mL of fresh LB media. When cell density reached an $OD_{600}$ of 0.6–0.7, 1 mM isopropyl-$\beta$-d-thiogalactopyranoside (IPTG) was added. Four hours after induction, cells were harvested by centrifugation (6000 rpm, 4 °C, 10 min), and the cell pellets were washed two times with 10 mM Tris–HCl (pH 8.0) and resuspended with STE solution (0.5 M sucrose, 10 mM Tris–HCl, 10 mM EDTA, pH 8.0). After incubation at 37 °C for 30 min, cells were collected by centrifugation and resuspended in Solution A (0.5 M sucrose, 20 mM $MgCl_2$, 10 mM MOPS, pH 6.8) containing 1 mg/mL of hen egg lysozyme (Sigma–Aldrich, ST. Louis, MO, USA). After incubation at 125 rpm for 15 min at 37 °C, the spheroplasts were washed twice with 1 mL of phosphate buffered saline (PBS, 21.5 mM $Na_2HPO_4$, 685 mM NaCl, 13.5 mM KCl, 7 mM $KH_2PO_4$) pH 72. The resuspended cells were incubated with 5 μM FITC-conjugated VP1 epitope probe for 1 h at room termperature. The cells were then washed twice with 10 mM Tris–HCl (pH 8.0) and the fluorescent probe-labeled cells were sorted using a high-speed flow cytometer (MoFlo XDP, Beckman Coulter, Miami, FL, USA). After sorting in each round, the scFv genes were amplified from the sorted cells by PCR. After digestion with *Eco*RI and *Nco*I restriction enzymes, the scFv genes were cloned into pMoPac1 and transformed into *E. coli* Jude-1 for the next round screening.

### 2.4. Screening of affinity-maturated scFv candidates by FACS

After cultivation as described above, the cells were harvested by centrifugation (6000 rpm, 4 °C, 10 min). For labeling with fluorescent probes, cell pellets were resuspended in 5× PBS buffer. The resuspended cells were incubated with 5 μM of FITC-conjugated VP1 epitope peptide at 25 °C for 1 h. The cells were washed twice with 5× PBS, and the fluorescent probe-labeled cells were sorted using a high-speed flow cytometer. All *E. coli* cells sorted in each round of screening were immediately reused for the next round of FACS sorting without regeneration. The sorting was repeated until the highly fluorescent population was enriched. To obtain the required sample volume, 500 μL of sheath buffer was added to the sorted samples during each round. After the final sorting, the scFv genes were amplified from the sorted cells by PCR with primers, Assembly-F (5′-GATTGTTATTACTCGCGG) and Assembly-R (5′-GGCCGCGAATTCG). After digestion with *Eco*RI and *Nco*I, the amplified scFv genes were cloned into pMoPac16 and transformed into *E. coli* MG1655 for further analysis of the single clones.

### 2.5. Purification of the antibody fragment

After growth in a shake flask, cells were harvested by centrifugation (6000 rpm, 4 °C, 10 min), and cell pellets were washed twice with binding buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, pH 7.0). Crude extracts of the cells were prepared by sonication (20 min, 50% pulse, 20% amplitude) and the extracts were centrifuged (10,000 rpm, 4 °C, 10 min) to yield soluble lysates. The soluble lysates were purified with Talon metal-affinity resin (Clontech, Mountain View, CA, USA). The resin was washed twice with 10 mL of washing buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole, pH 7.0), and the attached scFvs were eluted using elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 150 mM imidazole, pH 7.0). The purified antibody fragments were stored at 4 °C for further analysis.

### 2.6. Enzyme-linked immunosorbent assay (ELISA)

The GST-fused VP1 epitope [17] was mixed with 0.05 M carbonate-bicarbonate coating buffer, pH 9.6 to a final concentration of 2 μM. Then 100 μL of antigen solution (GST-fused VP1 epitope) was loaded onto a 96-well ELISA plate and incubated for 2 h at 37 °C. Subsequently, each well was washed four times with PBS-T (PBS with 0.05% Tween-20 at pH 7.2) and blocked with 100 μL 5% skim milk in PBS-T. The plate was incubated for 1 h at 37 °C. After washing with PBS-T four times, each scFv analyte (soluble lysate or purified sample) was loaded onto the plate, and the plate was incubated for 2 h at room temperature. Each well was washed four times with PBS-T, after which monoclonal anti-6xHis tag antibody conjugated to horseradish peroxidase (HRP) (1:5000; Sigma–Aldrich) was added and the plates were incubated at room temperature for 1 h. Finally, the wells were washed with PBS-T, and tetramethylbenzidine (TMB) peroxidase substrate was added for the colorimetric detection of bound scFv clones. The reaction was arrested by adding 2 M $H_2SO_4$ stop solution. The absorbance was measured at 450 nm using a TECAN Infinite M200 Pro ELISA plate reader (Tecan Group Ltd., Männedorf, Switzerland).

The reactivity of scFv antibodies against inactivated serotype-O FMDV were analyzed by indirect ELISA. In this analysis, a serotype-O FMDV Manisa strain-coated plate (Prionics AG, Schlieren-Zurich, Switzerland) was used. The serially diluted scFvs were added to each well of the plate. After incubation at room temperature for 1 h, plates were washed four times with PBS-T and incubated with a HRP-conjugated anti-6xHis tag antibody (1:5,000) (Sigma–Aldrich) at room temperature for 1 h. Plates were washed four times with PBS-T and 100 μL TMB peroxidase substrate was added to initiate the peroxidase reaction. To stop the reaction, 50 μL of 2 M $H_2SO_4$

**Table 1**
Bacterial strains and plasmids used in this study.

| Strain or plasmid | Genotype | Source |
|---|---|---|
| *E. coli* strains | | |
| Jude-1 | *F-mcrAΔ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu) 7697 galU galK λ⁻ rpsL nupG tonA* | [15] |
| MG1655 | *F⁻ lambda⁻ ilvG⁻ rfb⁻⁵⁰ rph⁻¹ endA1 gyrA96(nal^R) thi⁻¹ recA1 relA1 lac glnV44* | Lab stock |
| Plasmids | | |
| pGEX-4T-1 | Ap^r, tac promoter, lacI^q | GE healthcare |
| pGST-VP1e | FMDV VP1 gene epitope in pGEX-4T-1 | [17] |
| pMoPac1 | Cm^r, lac promoter, NlpA leader peptide and 6 amino acids (CDQSSS) for APEx display | [14] |
| pMoPac16 | Ap^r, lac promoter, PelB signal peptide for secretory production | [14] |

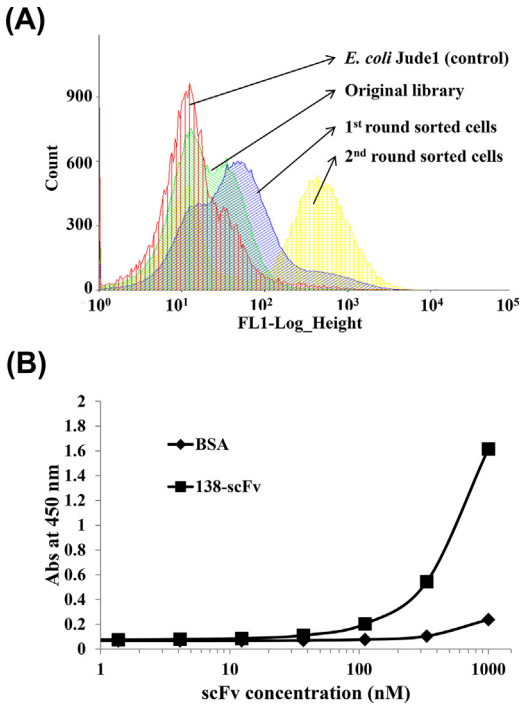


**Fig. 1.** FACS screening of synthetic antibody library and activity analysis of antibody fragments by ELISA. (A) Histogram of FACS screening. *E. coli* Jude-1 (red curve) was used as a negative control (host strain). The histograms of original library, 1st round sorted cell, 2nd round sorted cells are represented green, blue, yellow curves, respectively. (B) Activity analysis by ELISA. 2 μM of coating antigen were used as a FMDV VP1 epitope or BSA. The purifed #138-scFv (1 mM) was used as a analytes. The detection was used by anti-His-HRP monoclonal antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

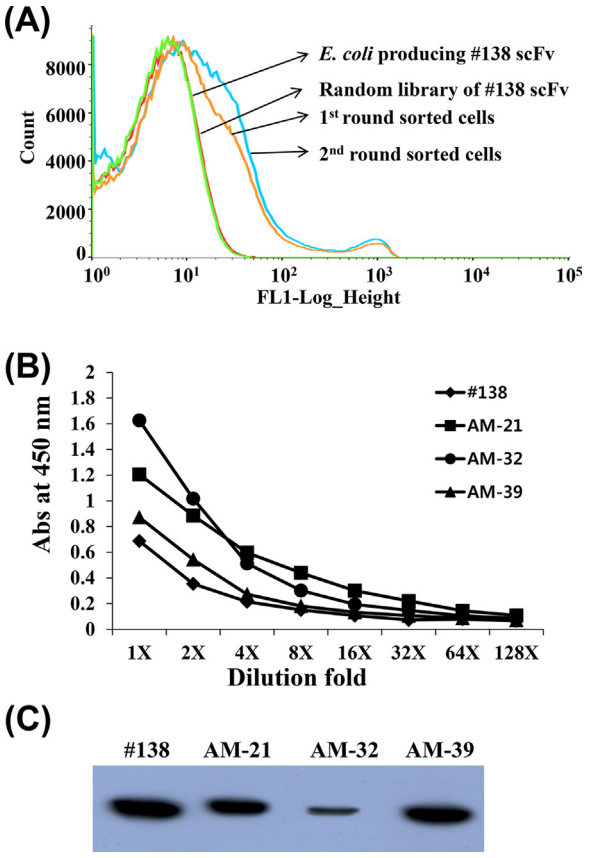


**Fig. 2.** FACS screening for affinity maturation and analysis of binding activity and expression level. (A) Histogram of FACS screening. *E. coli* producing #138-scFv was used as a control (red curve). The histograms of library, 1st round sorted cell, 2nd round sorted cells are represented green, orange, and blue curves, respectively. (B) With crude cell lysates (100 μL) as analytes, the binding activities were analyzed by ELISA. Symbols: diamond (♦), square (■), circle (●) and triangle (▲), indicate #138-scFv, AM-21 scFv, AM-32 scFv, and AM-39 scFv, respectively. (C) Analysis of antibody production by Western blotting. The band of protein interests were detected by anti-His-HRP monoclonal antibody. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

was added to each well. The absorbance was measured at 450 nm using an ELISA reader (Infinite M200 PRO). A bovine serum albumin (BSA) was used as a negative control.

### 2.7. Surface plasmon resonance (SPR) analysis

For the measurement of affinity ($K_D$) values of isolated scFv candidate, SPR analysis was performed. GST-fused VP1 epitope was modified with 2-(2-pyridinyldithio) ethaneamine (PDEA) of the thiol-coupling kit (GE Healthcare Biosciences AB, Uppsala, Sweden). The PDEA modified epitope protein was immobilized on the CM5 chip (GE Healthcare) modified with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/N-hydroxysuccinimide (NHS) solution. Binding signals of scFv candidates against VP1 epitope were detected in various concentrations. The $K_D$ values of the candidates were calculated using Biacore3000 software (GE Healthcare Biosciences).

## 3. Results and discussion

### 3.1. Primary screening of antibody fragments against VP1 epitope of FMDV

For the isolation of scFv antibodies against serotype-O FMDV, VP1 epitope (35 aa-length) of FMDV was used as an effective antigen [17]. And we used human synthetic antibody library instead of animal-derived antibody although the target is not a human but the cloven-hoofed animals. The ultimate goal of our work is the development of the high-sensitive and reliable diagnostic system with the engineered antibody, and for this purpose, we don't

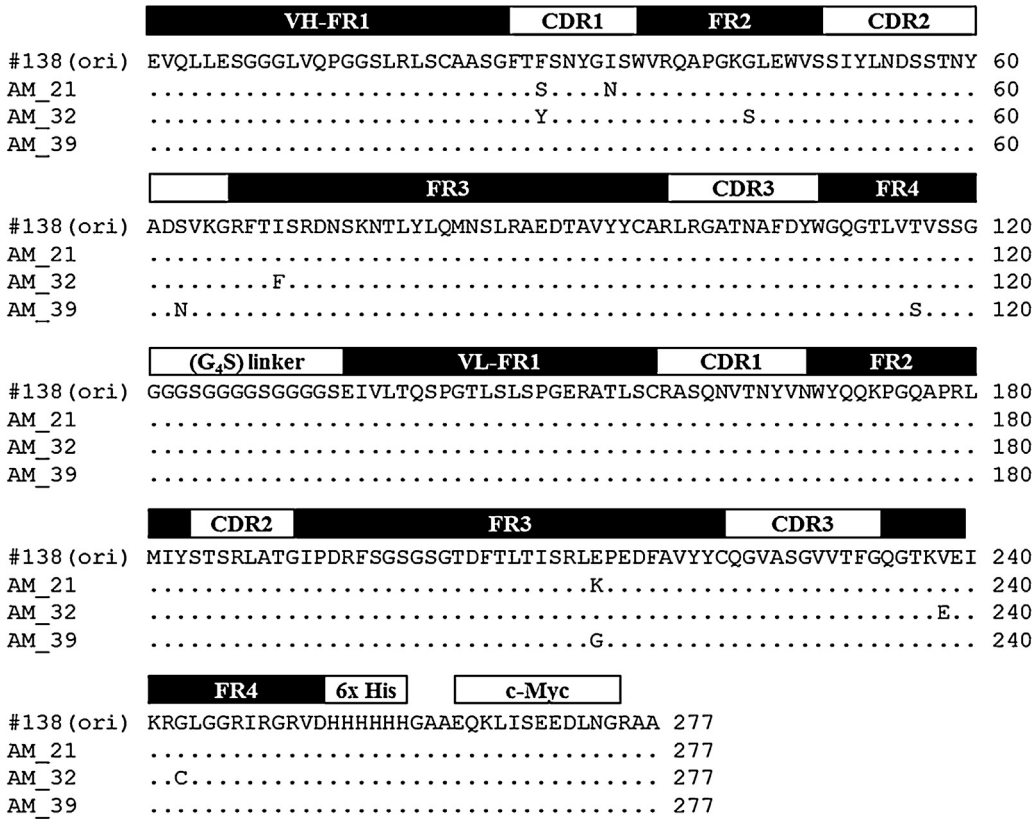


**Fig. 3.** Sequence analysis on mutational points in engineered scFvs (AM-21, AM-32 and AM-39). CDR means complementarity determining region, FR means framework region. Dots indicate same amino acids with original #138-scFv.

need to use animal-derived antibody. In the last two decades, there have been big progress in the human antibody engineering, and the development of many useful and potential tools have been focused on human antibodies than animal-derived antibodies [11,17]. We can do more efficient engineering of antibody using the human antibody platform, and more reliable diagnostic system can be developed. With the FITC-conjugated VP1 epitope, the synthetic antibody library ($3.3 \times 10^6$ cells) was screened by FACS. For the antibody display in the library, we employed an APEx (Anchored Periplasmic Expression) system [18], in which scFvs are fused to the first six amino acids (CDQSSS) of the *E. coli* NlpA lipoprotein and its signal peptide. Therefore, synthesized scFvs are anchored on the periplasmic side of the inner membrane after translocation into the periplasm. After cultivation and spheroplasting, cells were labeled with FITC-conjugated VP1 probe and sorted by ultrahigh-throughput FACS sorter selectively gating for high FITC fluorescence. In the first round of sorting, the most fluorescent cells (top ~2.6% of the total population, $3.8 \times 10^6$ cells) were collected, and in the second round of sorting, the top 8.2% of the populations were sorted and subjected to a final round of sorting (Fig. 1A). After the final round, the high-fluorescent cells were more enriched (Fig. 1A). The scFv genes from the sorted cells were amplified by PCR and ligated into pMoPac16 for expression of the antibody fragments in the periplasm. For individual clones, the activities of scFvs were analyzed by ELISA, and we selected one clone (#138), which exhibited selective binding activity against VP1 of FMDV (Fig. 1B). The selective antibody (#138-scFv) has specific affinity to VP1, but its binding affinity ($K_D$ > 200 nM) was not high enough for diagnostic and clinical use. Therefore, we decided to engineer the isolated #138-scFv toward affinity maturation.

### 3.2. Affinity maturation of the isolated antibody candidate

For the affinity maturation of anti-VP1 #138-scFv, its entire gene was mutagenized by error-prone PCR with a 0.5% error rate and transformed into *E. coli*, yielding a random library (approximately $1.42 \times 10^8$ cells). With the same FITC conjugated VP1 epitope probe, we again performed three rounds of FACS-based screening. From the random library, the most fluorescent cells (top ~1% of the total population) were sorted and, immediately after the first round of sorting, the sorted cells were applied to a second round of sorting by FACS. In the second round of sorting, the most fluorescent population of the sorted cells (top ~25% of total population) was sorted and after the third round of sorting, the highly fluorescent population was enriched (Fig. 2A). After third round screening, we collected 11,000 fluorescent cells and cloned the scFv genes into pMoPac16 for the periplasmic expression of scFvs. After transformation into *E. coli* MG1655, 40 colonies were randomly picked, and the binding activities of the isolated antibodies were analyzed by ELISA, in which samples were normalized to same cell concentration. Among 40 clones, three clones (AM-21, AM-32, and AM-39) (AM means 'affinity maturated') showing significantly higher affinities than those of other clones as well as the original #138-scFv were selected (Fig. 2B). For the selected clones, their expression levels were also analyzed by SDS-PAGE followed by Western blotting. In this analysis, cell densities were normalized and the equivalent volume of cell lysates were loaded on SDS-PAGE gel. Clones AM-21, AM-39, and original #138-scFv had similar expression levels while clone AM-32 exhibited a relatively lower gene expression level (Fig. 2C). The scFv of clone AM-32 exhibited much higher binding affinity than the original and other isolated clones despite lower

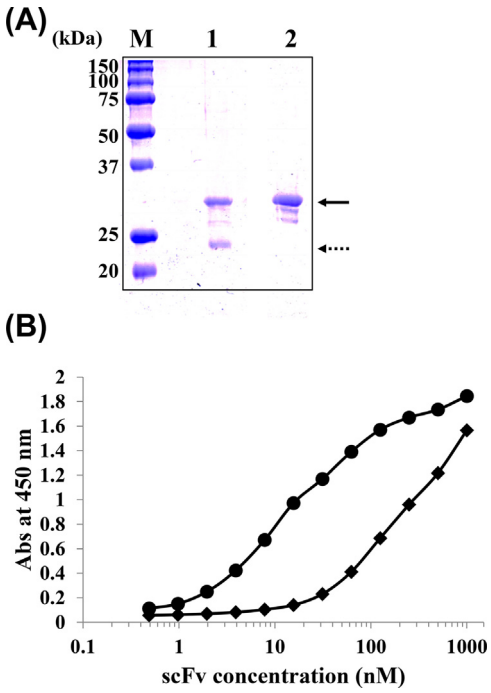


**Fig. 4.** Analysis of binding activities of purified scFvs by indirect ELISA. (A) SDS-PAGE analysis of the purified scFvs. Lane M, molecular weight markers (kDa); lane 1, AM-32 scFv; lane 2, #138-scFv. The solid and dashed arrows indicate scFv and SlyD proteins. (B) ELISA for binding affinity against VP1 epitope. Symbols: diamond (♦) indicates original #138-scFv, and circle (●) indicates AM-32 scFv.

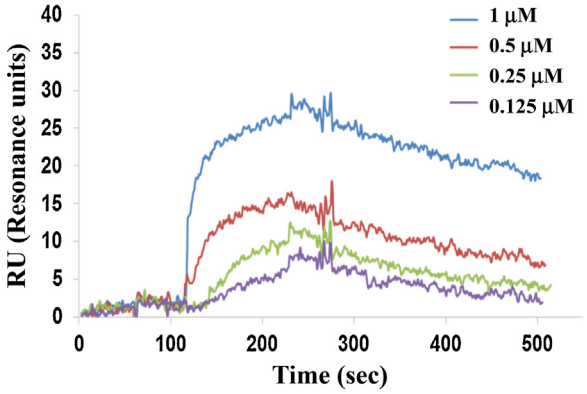


**Fig. 5.** SPR analysis of AM-32 scFv against GST-fused VP1 epitope.

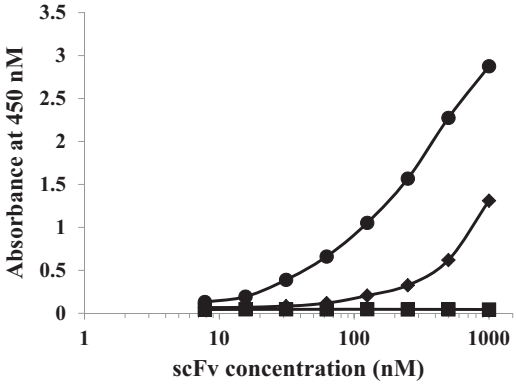


**Fig. 6.** The activities of original or engineered scFv against inactivated FMDV. With the purified antibodies #138 and AM-32 scFvs, the binding activity against whole (inactivated) FMDV were analyzed by ELISA. Symbols: diamond (♦), circle (●) and square (■) indicate original #138-scFv, AM-32, and no scFv (negative control).

expression level, and this result indicates that the scFv of clone AM-32 has a much higher specific affinity than others.

### 3.3. Sequence analysis of the isolated scFvs

The sequences of the antibodies in three isolated clones (AM-21, AM-32, and AM-39) were determined by a sequencing experiment. All clones have a different set of three to five mutations (Fig. 3) although all clones had mutations within some of the same regions. All clones had at least one mutation in the complementary determining region (CDR) of the variable heavy chain (VH): F29S and I34N in VH-CDR1 of AM-21, F29Y in VH-CDR1 of AM-32, and S63N in VH-CDR2 of AM-39. Similarly, all clones had mutations in the variable light chain (VL), specifically in the framework region (FR) including E213 position of VL-FR3 (Fig. 3). Although six CDRs in variable regions are mainly responsible for the antibody-antigen interaction, mutations in the FR can also have a significant effect on the binding characteristics of the antibody [19]. With the current information regarding the mutations in our clones, it is difficult to identify the critical residue for affinity maturation. In our future work, we will identify the critical residue by using substitutions at each mutation and analyzing their effect on the binding activities of the antibody.

### 3.4. Binding affinity of the isolated scFv against VP1-epitope of FMDV

For AM-32 scFv, which had relatively higher binding activity than the other mutants, we purified it to analyze its specific activity. After flask cultivation, the 6xHis-tag fused scFv was successfully purified by affinity column chromatography (Fig. 4A). In this purification, we saw an additional protein band (dashed arrow), which might be SlyD (21 kDa), a common contaminating protein in the eluate from His-tag affinity columns [20,21]. We analyzed the specific activities of the purified AM-32 and original #138-scFv against the GST-fused VP1 epitope of FMDV by ELISA. As shown in Fig. 4B, the binding activity of AM-32 scFv was much higher than that of #138-scFv. The signal of AM-32 scFv was saturated at 100 nM analytes, while the parental #138-scFv was saturated at 1 μM analytes, indicating that AM-32 has at least a 10 times or higher affinity than #138-scFv.

We determined the binding activities ($K_D$) of the isolated antibody against the antigen by SPR analysis. An antigen (GST-fused VP1 epitope) was immobilized on a CM5 chip, the purified antibody was loaded, and the binding affinity was analyzed. In the SPR analysis, the clone AM-32 exhibited a $K_D$ of 42.7 nM, with a $k_{off}$ of $1.28 \times 10^{-3}$ $M^{-1}$ $s^{-1}$, corresponding to an AM-32-scFv-VP1 half-life of 0.15 h (Fig. 5).

### 3.5. Binding affinity of the isolated scFvs against whole FMDV

Finally, we examined the binding activity of the AM-32-scFv against whole inactivated serotype-O FMDV by ELISA. Compared to the negative control (no treatment of scFv), #138 and AM-32 scFvs showed higher affinity against inactivated FMDV (Fig. 6). The AM-32-scFv antibody had two times higher affinity than that of #138-scFv, suggesting that the affinity maturated antibody against serotype-O FMDV was successful and can be tested for therapeutic or diagnostic applications.

## 4. Conclusions

In this work, we successfully isolated a scFv antibody against serotype-O FMDV with high affinity ($K_D$ = 42.7 nM), which is low enough for the development of FMDV diagnostic systems includ-

ing ELISA or various types of biosensors. Production of the isolated antibody fragments in *E. coli* and bacterial hosts are more efficient than conventional monoclonal antibody production in mammalian hosts [22–24]. Using the antibody fragment (scFv), the manufacturing cost for diagnostic system can be lowered and the diagnostic systems is made easier due to the easy handling and small size. Therefore we believe the isolated AM-32 scFv can be a useful tool for the development of efficient and cost-effective FMDV diagnostic system. In this work, we also successfully demonstrated the usefulness of FACS-based high throughput screening strategy for the isolation and affinity maturation of antibody against serotype-O FMDV. The affinity of the AM-32 scFv was clearly confirmed by SPR analysis, and we also found that it has higher affinity than those of the initially-isolated antibody as well as the antibody reported previously [14]. This screening strategy can be also easily applied for the isolation of antibodies for other FMDV serotype antigens (A, C, Asia 1, SAT1, SAT2, and SAT3), and through the isolation of those antibodies, more reliable diagnostic system can be developed.

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