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Analytical Methods

Affinity maturation of single-chain variable fragment specific for aflatoxin B_1 using yeast surface display

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ABSTRACT

As aflatoxin B_1 is one of the most toxic mycotoxins, it is important to detect and to quantify aflatoxin B_1 accurately by immunological methods. To enhance aflatoxin B_1-binding affinity of the single-chain variable fragment, yeast surface display technique combined with fluorescence-activated cell sorting was applied. A randomly mutated scFv library was subjected to 4 rounds of fluorescence-activated cell sorting, resulting in isolation of 5 scFv variants showing an affinity improvement compared to the parental wild type scFv. The best scFv with a 9-fold improvement in affinity for aflatoxin B_1 exhibited similar specificity to the monoclonal antibody. Most of the mutations in scFv-M37 were located outside of the canonical antigen-contact loops, suggesting that its affinity improvement might be driven by an allosteric effect inducing scFv-M37 to form a more favorable binding pocket for aflatoxin B_1 than the wild type scFv.

1. Introduction

Aflatoxins (AFs) are fungal toxic compounds with chemical similarity (polyketide-derived furanocoumarins). Aflatoxin B_1 (AFB_1), the most potent hepatotoxic carcinogen among AFs is a secondary metabolite produced by *Aspergillus parasiticus*, *Aspergillus flavus* and *Aspergillus nomius* which can colonize naturally on plenty of raw food commodities, such as nuts, seeds and legumes during pre- and post-harvest (Sudakin, 2003). AFB_1 is a highly thermostable small molecule (MW < 400 Da) and hence very difficult to be inactivated or removed after its contamination (Raters & Matissek, 2008). Its mutagenic or carcinogenic effect on hepatocarcinoma development has been well characterized to be classified as a group I carcinogens by the International Agency for Research on Cancer (Min, Kweon, Park, Park, & Seo, 2011). Long-term intake of AFB_1 with hepatitis B virus infection is the main risk factor for the progression and development of chronic liver disease or hepatocarcinoma (Kew, 2003). Therefore, highly sensitive and specific AFB_1-detection systems which can prevent distribution of AFB_1-contaminated foods and feeds worldwide are urgently required.

AFB_1 contamination has been monitored mainly via a chromatographic or immunological method. Various chromatographic techniques including high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography-tandem mass spectrometry (LC–MS/MS) have been developed for monitoring and quantifying AFB_1 (Cavaliere et al., 2007; Jaimez et al., 2000; Spanjer, Rensen, & Scholten, 2008). However, such chromatographic methods are inappropriate as an on-site detection system especially in developing countries due to time-consuming and cost-intensiveness. Therefore, the requirement for fast, portable and convenient detection tools of AFB_1 has led to the development of immunological methods such as enzyme-linked immunosorbent assay (ELISA), immuno-chromatographic strip or protein chip (Cho et al., 2005; Dorokhin, Haasnoot, Franssen, Zuilhof, & Nielen, 2011; Park, Kim, Kim, & Ko, 2014). So far monoclonal as well as polyclonal antibodies specific for AFB_1 have been used for various immunological detection modules and there are many commercialized kits (Groopman, Trudel, Donahue, Marshak-Rothstein, & Wogan, 1984; Haugen et al., 1981; Liu, Hsu, Lu, & Yu, 2013; Martin et al., 1984) which absolutely depend on their specificity and sensitivity.

However conventional monoclonal or polyclonal antibodies have some limitations in additional engineering approaches for *in vitro* affinity maturation, an increase of antibody stability, and prolonged half-life in the blood stream. It is also very difficult to produce the full-length structure of immunoglobulin G (IgG) type antibodies in various microbial systems. Besides, small sized recombinant antibodies such as single-chain variable fragment

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(scFv) or fragment antigen-binding (Fab) would be more effective as a detection probe for food toxins. In particular, scFv has been useful for AFB_1 detection, because of feasible antibody engineering tools for affinity or specificity, economical production using microorganisms such as *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae (S. cerevisiae)*, broad applicability for diverse detection modules. Antibody engineering technologies have been continuously advanced to improve the clinical or diagnostic functions of recombinant antibodies, such as an affinity enhancement via yeast surface display technology (Lee et al., 2010) or phage display (Hu et al., 2015), avidity increase by di- or polymerization (Zhu et al., 2010), thermal stability using nano-flow reversed-phase LC–MS (Hussack, Hirama, Ding, MacKenzie, & Tanha, 2011) or antibody solubility increase (Perchiacca, Ladiwala, Bhattacharya, & Tessier, 2012), prolonged half-life by PEGylation or increasing the affinity of fragment crystallizable region (Fc) for the Fc neonatal receptor (Patel et al., 2011). To obtain tailor-made antibodies, several technologies such as phage, ribosome, bacteria and yeast display have been developed and applied. Among them, yeast surface display technique coupled with fluorescence-activated cell sorter (FACS) enabled robust and high-throughput screening of huge library covering more than 10^9 cells and finally yielded reliable clones with high fidelity (Baek & Kim, 2014).

In a previous study, the hybridoma cell line 2C12 producing murine IgG highly sensitive and specific toward AFB_1 with nanomolar equilibrium dissociation constant value (K_D: 7×10^{-9} M) was established, even though AFB_1 is ~312.2 Da hapten that is not able to elicit an immune response without any adjuvants. The recombinant scFv against AFB_1 (scFv-WT) was cloned from cDNA of the hybridoma cell line 2C12 and the scFv-WT expressed as an inclusion body in *E. coli* cytoplasm was successfully refolded *in vitro* to recover affinity to AFB_1 (Min et al., 2011). However, its affinity was still not high enough to satisfy the most rigorous regulation detection limit of AFB_1 in foods and feeds established by European Food Safety Authority (<2 ppb). To improve the affinity of scFv-WT against AFB_1, a library containing diverse antibodies and an efficient antibody screening system was required.

In this study, a randomly mutated scFv library was constructed via error-prone PCR on the basis of scFv-WT gene and the scFv library was subjected to yeast surface display for high-throughput FACS screening. After sequential screening using flow cytometry with decrease in concentrations of the biotinylated AFB_1-BSA conjugate, 5 affinity-matured scFvs were isolated successfully and the best affinity-matured one exhibited ~9-fold-higher binding activity than scFv-WT and high specificity to other AFs in a similar level.

2. Materials and methods

2.1. Strains, plasmids and mycotoxins

The yeast strain for surface display of scFv was *S. cerevisiae* EBY100 (Invitrogen, Carlsbad, CA, USA), and *E. coli* DH5α and BL21(DE3) (Stratagene, La Jolla, CA, USA) were used for genetic manipulation and periplasmic expression of recombinant scFv, respectively. ScFv display plasmid pCTCON was donated from Prof. Yong Sung Kim at Ajou University (Chao et al., 2006; Lee et al., 2010). pET26b(+) (EMD Millipore, Darmstadt, Germany) was used for periplasmic expression of the hexahistidine tag-fused scFv. The bovine serum albumin (BSA) conjugates of AFB_1 (AFB_1-BSA conjugate), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), aflatoxin M_1 (AFM_1), aflatoxin M_2 (AFM_2), ochratoxin (OTA), deoxynivalenol (DON), fumonisin B_1 (FMB_1), fumonisin B_2 (FMB_2) and T-2 toxin were purchased from Fermentek Ltd. (Jerusalem, Israel). A milligram of lyophilized AFB_1-BSA conjugate was dissolved in 10 mL of 10% methanol solutions and stored at −20 °C. Biotin-XX Microscale Protein Labeling Kit (Life Technologies, Carlsbad, CA, USA) was applied to labeling the AFB_1-BSA conjugate for FACS screening.

2.2. Western blot using scFv-WT-displayed yeast cells

For construction of pCTCON plasmid displaying scFv-WT, the gene of scFv-WT was PCR-amplified using 5′-*Nhe*I and 3′-*BamH*I primers (Table 1) (Min et al., 2011). The PCR amplicon was digested with *Nhe*I and *BamH*I and then ligated into pCTCON linearized by the same restriction enzymes, resulting in the pCT-scFv-WT. The yeast cells harboring pCT-scFv-WT were induced by transferring to scFv display medium (SGCAA medium) and were cultivated at 17, 20, 23, 26 or 29 °C, respectively, to determine induction temperature for optimum display of scFv. The displayed scFv-WT proteins were released by the treatment of dithiothreitol (DTT) from outer membrane of induced cells and quantified using western blot. Displayed scFv at each temperature on the PVDF membrane (EMD Millipore, Darmstadt, Germany) was detected by the anti-c-Myc tag IgG [9E10] HRP conjugate (Abcam, Cambridge, MA, USA). Human recombinant purified His-tag c-Myc protein (~65 kDa) control (Alpha Diagnostic International, San Antonio, TX, USA) and yeast cells harboring pCTCON backbone plasmid were used as positive and negative controls, respectively. Development of transferred PVDF membrane was carried out using an Opti-4CN substrate kit (Bio-Rad, Hercules, CA, USA).

2.3. Random mutation of scFv-WT for construction of yeast library

A DNA pool with diverse scFv sequences was generated by error-prone PCR using nucleotide analogs, 8-oxo-2′-deoxyguanosine-5′-triphosphate (8-oxo-dGTP) and 2′-deoxy-p-nucleoside-5′-triphosphate (dPTP) (TriLink BioTechnologies, San Diego, CA, USA), and using HR forward and HR reverse primers (Table 1) (Chao et al., 2006). Mutation rate of error-prone PCR was modulated at 8–25 nucleotide changes in scFv-WT (~750 bp) by both a number of PCR amplification cycles (5, 10 & 20 cycles) and concentrations of 8-oxo-dGTP and dPTP (Colby et al., 2004). The error-prone PCR amplicons and pCTCON linearized by triple digestion with *Nhe*I, *BamH*I and *Sal*I were mixed at 9:1 M ratio and this mixture was transformed into *S. cerevisiae* EBY100 (10 μg DNA per 10^8 cells) based on Gietz's method (Gietz & Schiestl, 2007). Transformed cells were pooled into 100 mL of YPD medium (20 g dextrose, 20 g peptone and 10 g yeast extract in deionized-distilled H_2O (DDW) to a volume of 1 L) and the library was cultivated at 30 °C and 200 rpm for 1 h to rescue scarred cells upon transformation. Its serial diluents were plated onto selective SDCAA agar media (20 g dextrose, 6.7 g Difco yeast nitrogen base, 5 g Bacto™ casamino acids, 15 g Bacto™ agar, 5.4 g Na_2HPO_4 and 8.56 g $NaH_2PO_4 \cdot H_2O$ in DDW to a volume of 1 L) to estimate the library size. After additional cultivation for 2 h, an enriched library was transferred to 500 mL of fresh SDCAA medium, pre-incubated at 30 °C and cultivated at 30 °C and 200 rpm for 24 h. Aliquots of the over-grown library were stored under 15% glycerol at −80 °C and library stocks were prepared to contain at least 10-fold more cells than the library size which was calculated from colony counting.

2.4. Screening of high affinity scFv clones using FACS

Frozen aliquot of scFv library was inoculated into 500 mL of SDCAA medium and grew overnight to an optical density of ~7 at 600 nm (OD_{600}). This passage culture was repeated to ensure complete elimination of dead cells during storage at −80 °C. The culture, freshly re-inoculated to ~0.1 at OD_{600} in SDCAA medium was incubate at 30 °C with shaking at 200 rpm overnight and was

Table 1
List of primers used in this study.

Name	Nucleotide sequence (5′–3′)
5′-*Nhe*I primer	ATACTTCGAAGGCTAGCATGGAGGTGAAGCTGCAGGAGTCTGGGGGAGGCTTA
3′-*BamH*I primer	CGAAGTATGGATCCTTCGGATCCACCTAGGACGAGTTTGGTTCCT
HR forward primer	CGACGATTGAAGGTAGATACCCATACGACGTTCCAGACTACGCTCTGCAG
HR reverse primer	CAGATCTCGAGCTATTACAAGTCTTCTTCAGAAATAAGCTTTTGTTC
5′-*Nco*I primer	TCCTCTTCGAAGCCATGGAGGTGAAGCTGCAGGAGTCTGGGGGAGGCTTA
3′-*Xho*I primer	ATTGCTTATCTCGAGTTCGGATCCACCTAGGACGAGTTTGGTTCCT

The underlined sequences correspond to restriction enzymes sites.

transferred to the same volume of SGCAA medium (galactose instead of dextrose in SDCAA), when the OD_{600} of culture reached nearly 2 for induction of scFv display of the yeast cells. Induced cells were incubated at 20 °C with shaking at 200 rpm overnight for full display of scFv of the library. Yeast libraries displaying scFv were labeled with the mouse monoclonal anti-c-Myc IgG [9E10] (1 μg/mL) (Santa Cruz Biotechnology, Santa Cruz, CA, US) at room temperature for 30 min followed by the fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG specific antibody (Sigma Aldrich, St. Louis, MO, USA) as well as the biotinylated AFB_1-BSA conjugate (50 μM) and the streptavidin γ-phycoerythrin conjugate (SAPE: Life Technologies, Carlsbad, CA, USA). Yeast clones exhibiting higher PE fluorescence signal relative to FITC fluorescence were screened using a FACSAria™ sorter or FACSCalibur™ (BD Biosciences, San Jose, CA, USA) and collected cells were pooled down to SDCAA medium for a next round of FACS screening. FACS screening progressed with decreasing concentrations of the biotinylated AFB_1-BSA conjugate (50 μM to 1 μM), and every screened library was stored at −80 °C. The final screened library was plated onto SDCAA plates, and individual colonies were picked for DNA sequencing and scFv characterization using surface plasmon resonance (SPR). To characterize the best affinity scFv using FACS, mean fluorescence intensities (MFI) of the best affinity scFv was calculated from events recorded by scFv-displayed populations only and its titration curve according to concentration of biotinylated AFB_1-BSA conjugate (10–50,000 nM) (Chao et al., 2006).

2.5. Periplasmic expression of functional scFvs

The scFv genes were PCR-amplified from pCTCON plasmids from the final selected clones with using 5′-*Nco*I and 3′-*Xho*I primers (Table 1) and sub-cloned into pET26b(+) in-frame for introducing the N-terminal pelB signal sequence for periplasmic expression and the C-terminal hexahistidine tag for purification. Periplasmic expression of scFv was induced at the logarithmic growth phase (~0.7 at OD_{600}) by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at final concentration of 0.2 mM and induced culture grew overnight at 20 °C. Induced medium was collected via centrifugation for 30 min at 15,000 ×*g* and 4 °C and concentrated using the Amicon® stirred cell (EMD Millipore, Darmstadt, Germany). A concentrated broth was loaded on a column packed with Ni–NTA Superflow (Qiagen, Valencia, CA, USA) in ÄKTA FPLC (GE Healthcare, Fairfield, CT, USA). The purity of the scFvs in the eluents was determined by densitometric analysis.

2.6. Determination of K_D using SPR analysis

The affinity of the scFvs for AFB_1 was measured using a BIAcore™ 2000 (GE Healthcare, Fairfield, CT, USA). Shortly described, the AFB_1-BSA conjugate and BSA were fed into the EDC/NHS-activated Fc-2 and Fc-1 of a CM5 sensor chip (GE Healthcare, Fairfield, CT, USA), respectively at a flow rate of 5 μL/min, after pH-scouting for optimum immobilization. Purified scFv proteins were diluted with a range of 10–1000 nM in a binding reaction buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20 at pH 7.4) and injected at 20 μL/min flow rate through the association and dissociation periods, each for 120 s. The SPR sensorgrams were analyzed using the BIAevaluation software 3.1 (GE Healthcare, Fairfield, CT, USA) and K_D was defined as the ratio of the dissociation rate constant (k_d) to the association rate constant (k_a).

2.7. Measurement of specificity of scFv-M37

For competitive indirect ELISA, the 96-well ELISA plate (Nunc-Immuno™ MicroWell™ 96 well solid plates: Nalgene Nunc International, Penfield, NY, USA) was coated with 0.2 μg of the AFB_1-BSA conjugate overnight at 4 °C. The coated plates were treated with a blocker (5% skim milk in PBS buffer) to prevent non-specific binding. 100 μL of purified scFv-M37 (2 μg/mL) and same volume of AFs (AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1) or other mycotoxins (OTA, FMB_1, FMB_2, DON and T-2 toxin) in a dilution series (0–10 μg/mL) were added consecutively to each well. After 1 h incubation at 37 °C and 3 times washing with PBST buffer, HisProbe™-horse radish peroxidase (HRP) (Pierce Biotechnology, Rockford, IL, USA) was added and the plates were incubated again for 1 h at 37 °C. The ELISA plate was developed by adding 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) supplemented with H_2O_2 (Sigma Aldrich, St. Louis, MO, USA). After 2 N H_2SO_4 solution was added to stop HRP enzyme reaction, the absorbance was read at 450 nm on SpectraMax M Series (Molecular Devices, Sunnyvale, CA, USA). The specificity for AFs or other mycotoxins were calculated using half maximal inhibitory concentration (IC_{50}) according to the following equation: [Cross-reactivity = (IC_{50} of AFB_1/IC_{50} of AFs or IC_{50} of mycotoxins) × 100] (Min et al., 2011).

3. Results and discussion

3.1. Construction of scFv library for FACS screening

The pCT-scFv-WT construct was generated, transformed into *S. cerevisiae* EBY100 and the clone was induced to display scFv-WT on the cell surface. A distinct band of ~40 kDa, consistent with the molecular weight of the scFv-WT fusion protein (scFv fused with α-agglutinin II, hemagglutinin tag, linker, c-Myc and hexahistidine tag: ~40,394 Da) was observed in the western blot analysis (Supplementary Fig. 1A). The scFv-WT fusion protein was most strongly displayed in the densitometry analysis at 20 °C; subsequent induction was therefore performed at this temperature (Supplementary Fig. 1B). FACS bivariate dot-plots after a standard compensation procedure demonstrated that both the biotinylated AFB_1-BSA conjugate and anti-c-Myc tag IgG [9E10] were accessible to scFv displayed on the yeast outer membrane without any interference (Supplementary Fig. 2). The scFv-WT gene was randomly mutated and the size of the resulting library was ~2.08×10^7 cells. DNA sequencing of 30 clones arbitrarily selected from the library

revealed that 26 clones had unique gene sequences, and 4 to 25 amino acid substitutions were observed in the scFv-WT (data not shown). Therefore, the diversity of the scFv library covered $\sim 1.8 \times 10^7$ clones with unique genes.

3.2. Improved affinity clones from the scFvs library

4 rounds of screening were performed, and each round was monitored by FACS, and the results of monitoring are represented as bivariate dot-plots. As FACS screenings progressed, the polyclonal population of scFv clones exhibiting higher PE fluorescence signals (AFB_1-binding level) relative to FITC fluorescence (scFv display level) increased consequently (Fig. 1). We quantitatively screened for clones with high affinity and specificity for AFB_1 from those with broad cross-reactivities with other AFs under a 10-time higher concentration of the non-biotinylated AFs (0.5–10 μM) as a competitor (Boder & Wittrup, 2000). On the 10-μM biotinylated AFB_1-BSA conjugate, the 3rd screened library began to represent an abundant population containing scFv clones with a higher AFB_1-binding property than clones in the initial library, 1st, or 2nd round of library screening (Fig. 1). This result indicated that substantial rejection of scFv clones carrying any amino acid substitutions that could allow cross-reactivities with other AFs occurred during the 1st and 2nd screenings. The DNA sequencing of 20 clones of the final screened library revealed 6 unique scFv clones, one of which was a truncated form of scFv due to the presence of an amber stop codon occurring between V_H and the linker. These clones were named as scFv-M37, M39, M22, W3, and M72. Barring the truncated clone (Fig. 2), the full-length scFv variants had 4–12 substitutions throughout their amino acid sequences and only 6 of 42 substitutions were in the complementarity-determining regions (CDRs), indicating that most of the mutations were not clustered in the CDRs, but rather in the framework regions (FRs). Therefore, 5 scFv variants could bind with the biotinylated AFB_1-BSA conjugate under stringent selection pressure, i.e., in the presence of 10-fold higher concentrations of other non-biotinylated AFs and could still survive during the subsequent 4 rounds of FACS screening. Therefore, we inferred that these 5 variants might be highly specific for AFB_1.

3.3. Production of soluble scFv antibodies via E. coli periplasmic secretion

Many scFvs have been reported to have a strong propensity for insoluble accumulation in the *E. coli* cytoplasm and even in the periplasm (Bothmann & Plückthun, 2000; de Marco, 2009). Indeed, a considerable portion of the scFv variant, expressed at 20 °C (over 90% of total scFv protein) was aggregated as the inclusion body in the cytoplasm and periplasm (Min et al., 2010).

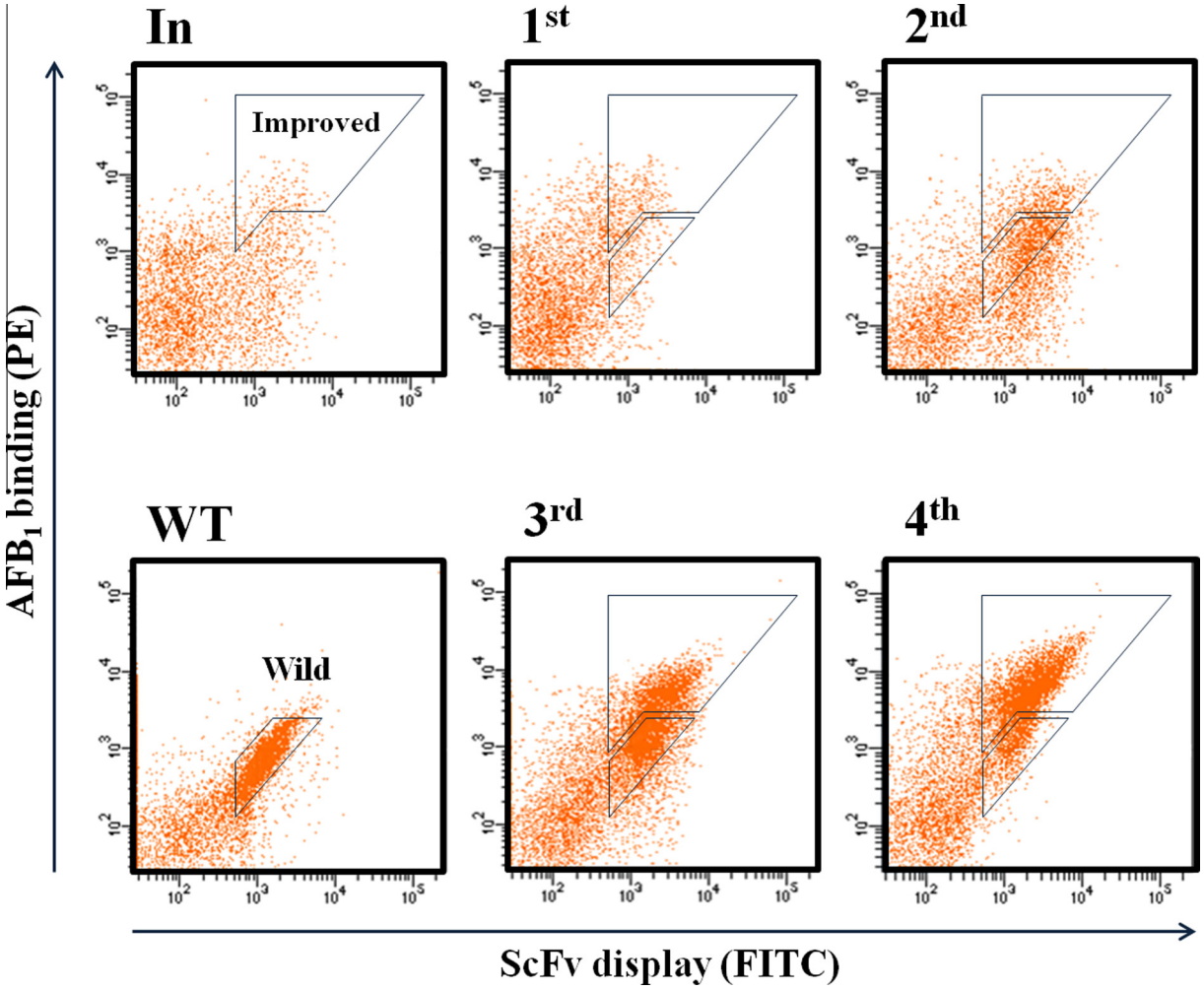

Fig. 1. Bivariate dot-plots of FACS screened libraries. Total 10,000 cells (apricot dots) were recorded by FACSAria™ sorter and the trapezoid (wild) and the diagonal (improved) windows represent the populations of cells displaying scFv-WT and cells showing improved affinity for AFB_1, respectively. In, initial scFv library before screening; WT, scFv-WT; 1st–4th, each round of FACS screened library.

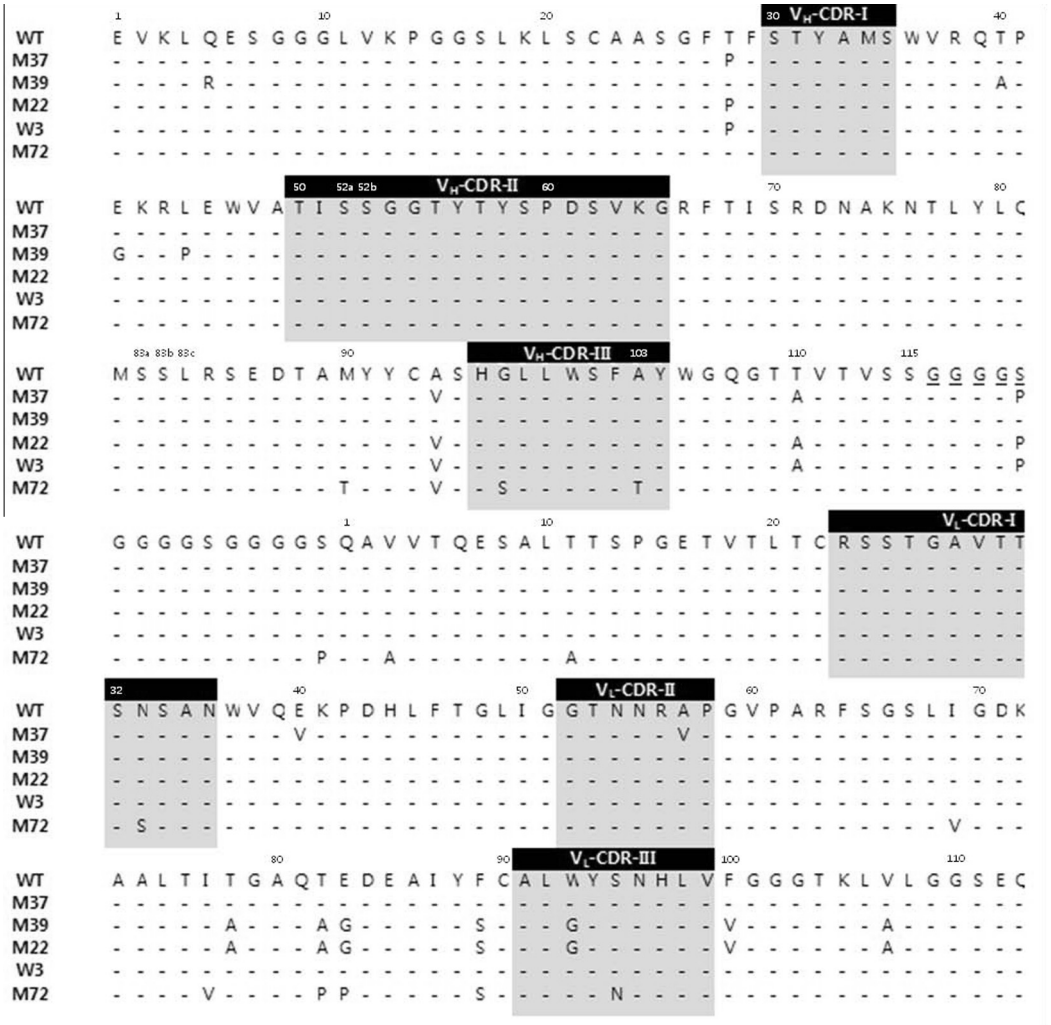

Fig. 2. Amino acid sequences alignment of affinity-matured scFv variants. Antibody numbering, and all CDRs and FRs of the V_H and V_L domains were determined according to Chochia antibody sequence database's rule. The different residues were presented as one letter code of amino acid and the same amino acids were presented as hyphen (−). The underlined sequences correspond to glycine and serine residues in synthetic linker between V_H and V_L.

Nevertheless, a small portion of soluble scFv could be observed in the culture medium via periplasmic leakage and each scFv variant in the culture medium was successfully purified by Ni–NTA affinity chromatography. The purity of the eluents reached up to ~90% in densitometry and was then applied to the BIAcore™ 2000 system for further analysis.

3.4. K_D comparison of affinity-matured scFv variants

We determined the binding kinetics of the purified scFv-WT protein using the BIAcore™ 2000 system. ScFv-WT showed k_a of $0.7 \times 10^{-4}\,M^{-1}s^{-1}$ and k_d of $6 \times 10^{-3}\,s^{-1}$ to yield a K_D value of 82.7×10^{-8} M. The overall K_D values of the 5 scFv isolates were ~3–9 folds lower than that of scFv-WT, indicating an approximately ~3–9 folds improvement in binding affinity relative to the scFv-WT. The best affinity-matured scFv was scFv-M37 which showed a 9-fold decrease in the K_D value compared to the wild type (Table 2). An increase in k_a and a decrease in k_d of scFv-M37 synergistically contributed to an improvement in its binding affinity for AFB_1 (Fig. 3A). Likewise, the MFI titration graph obtained from FACS bivariate dot-plots also clearly showed that scFv-M37 possessed a higher binding profile toward AFB_1 than scFv-WT (Fig. 3C and D).

3.5. Specificity of scFv-M37

As shown in Fig. 3B, scFv-M37 had low cross-reactivity with AFG_1 (2%) and no cross-reactivity with AFB_2, AFG_2, AFM_1 or AFM_2 (<0.01%) at a concentration range of $1–10^5$ ng/mL; this specificity pattern was similar to that of IgG 2C12 reported previously (Min et al., 2011). As expected, no other mycotoxins such as OTA or DON competed with AFB_1. Eventually, scFv-M37 containing 6 mutations could maintain its primary specificity to AFB_1, as observed for IgG 2C12, indicating that sequential FACS screenings were successfully conducted under highly stringent conditions to wash out scFv clones showing any conspicuous cross-reactivities with AFs.

3.6. Sequence analysis of affinity-improved scFv variants

A mutation (L_K:Ser5Pro) occurred in the $(Gly_4Ser)_3$ synthetic linker between V_H and V_L which allowed them to be folded separately, resulting in formation of a favorable interface between V_H and V_L for the binding of various antigens. To evaluate this mutation for an affinity increase of scFv-M37, the reverse mutant of scFv-M37 in which the proline at linker:5 of scFv-M37 was restored to the original linker residue (serine), was analyzed using BIAcore™ 2000. This back mutation of proline to serine resulted in a slight change in k_d but ultimately showed a similar K_D value to

Table 2
Summary of binding kinetics of affinity-matured scFv variants using SPR. Chi^2 values show information about the goodness of fitting in generating theoretical sensorgram data.

Clone ID	K_a [$(M^{-1}\ s^{-1}) \times 10^{-4}$]	K_d [$(s^{-1}) \times 10^{-3}$]	K_D [$(M) \times 10^{-8}$]	Fold improvement in affinity	Chi^2 value
WT	0.7	6	82.7	1	3.01
M37	2.1	1.9	9.2	9	2.98
M39	1.7	5.1	30	3	4.7
M22	1.5	2.2	15	6	3.5
W3	2	2.3	11.3	7	1.77
M72	2.5	6.3	25.4	3	1.06

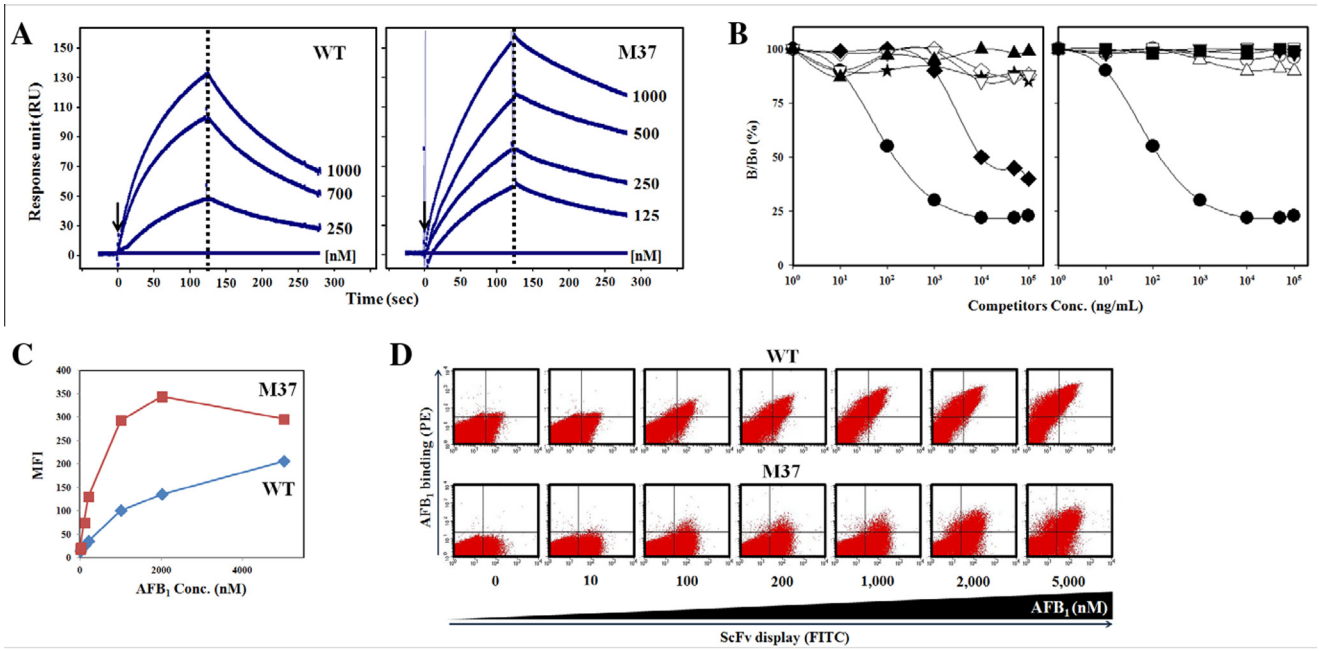

Fig. 3. Characterization of scFv-M37 using SPR (A), ciELISA (B) and FACS (C and D). (A) Sensorgrams for binding of scFv-WT (left panel) and scFv-M37 (right panel) to AFB_1. Various concentrations of scFv-WT (WT) and scFv-M37 (M37) as analytes were injected into the AFB_1-BSA conjugate immobilized on CM5 chip in BIAcore™ 2000. Each data was monitored by BIAevaluation software 3.1. Black arrows (↓) indicate start points of analyte injection. (B) Specificity assay of scFv-M37 using ciELISA. For AFs (left panel), a range of 1–10^5 ng/mL of AFB_1 (●), AFB_2 (◇), AFG_1 (♦), AFG_2 (★), AFM_1 (▽) and AFM_2 (▲) were used as competitors. For other mycotoxins (right panel), 1–10^5 ng/mL of OTA (□), FMB_1 (○), FMB_2 (△), DON (▼) and T-2 toxin (■) were used. B_0 is an absorbance at 450 nm without competitors and B is an absorbance at 450 nm with various concentrations of each competitor. All data showed the standard deviation less than 10%. (C) MFI titration curves and (D) Bivariate dot-plots of scFv-WT (WT;♦) and scFv-M37 (M37;■) under various concentrations of AFB_1. The scFv-WT or scFv-M37 was displayed on the surface of yeast, and binding to various concentrations of biotinylated AFB_1-BSA conjugate (0–5 μM) together with mouse monoclonal anti-c-Myc IgG [9E10] treatment, followed by labeling with SAPE and FITC-anti-mouse IgG antibody simultaneously.

scFv-M37 (Data not shown). This indirectly suggested that the L_K:Ser5Pro mutation might only have a minor contribution to affinity improvement of scFv-WT. Ironically, the substituted residues in the 6 mutations are considerably rare among natural IgG repertoires (Fig. 4). Among these 6 residues, the residue that occurred most frequently in nature (<4%) is Val at V_H:94 of scFv-M37 (Fig. 4). It is also likely that simultaneous generation of these 6 mutations would be rare in a natural immune system, specifically via somatic hypermutation or clonal selection of B lymphocytes. While only a single mutation (V_L:Ala57Val) was located in the position of the canonical CDR loop (V_L-CDR-II), the others were located outside of the CDR loops, in a region known to be the critical domain to determine an antibody's affinity and specificity for an antigen. In particular, 2 of the 6 mutations (V_H:Thr28Pro and V_H:Ala94Val) in scFv-M37 occurred at the so-called 'Vernier zone' of V_H. Even in the case of CDR grafting or humanization of a murine antibody, these residues in the 'Vernier zone' should be conserved to maintain inherent binding affinity and specificity. This is because some critical residues at the 'Vernier zone' do not directly interact with an antigen but are rather located in the β-sheet framework underlying the CDRs to play a role in adjustment of the loop structures to facilitate contact with antigens (Lee, Tullman-Ercek, & Georgiou, 2006). The K_D values of scFv-M37, -M22 and -W3 showed more than a ~6-fold decrease, and interestingly, these 3 scFv variants shared the same mutations (V_H:Thr28Pro, V_H:Ala94Val, V_H:Thr100Ala and L_K:Ser5Pro). Most likely, these 4 common substitutions arose at an early stage during error-prone PCR and might have been preserved among a large fraction of the scFv clones during FACS screening, due to their selective effect on affinity and not specificity. The comparison of amino acid sequences, revealed that scFv-W3 (7-fold decrease in K_D) had only 4 common mutations only and scFv-M37 (9-fold decrease in K_D) and scFv-M22 (6-fold decrease in K_D) had an additional 2 (V_L:Glu40Val and V_L:Ala57Val) and 8 mutations (V_L:Thr78Ala, V_L:Thr82Ala, V_L:Glu83Gly, V_L:Phe89Ser, V_L:Trp93Gly, V_L:Phe100Val, V_L:Pro100Ser and V_L:Val107Ala) located in the V_L domain, respectively; the additional mutations did not match among the clones. Moreover, it is difficult to define the effectiveness of the individual mutations in these affinity-matured scFv variants. With respect to their K_D values, 2 mutations in V_L of the scFv-M37 clone appeared to play a positive role in affinity improvement (i.e., a decrease in K_D) with 4 conserved mutations in V_H, but the 8 mutations of scFv-M22 did not show an improved affinity effect.

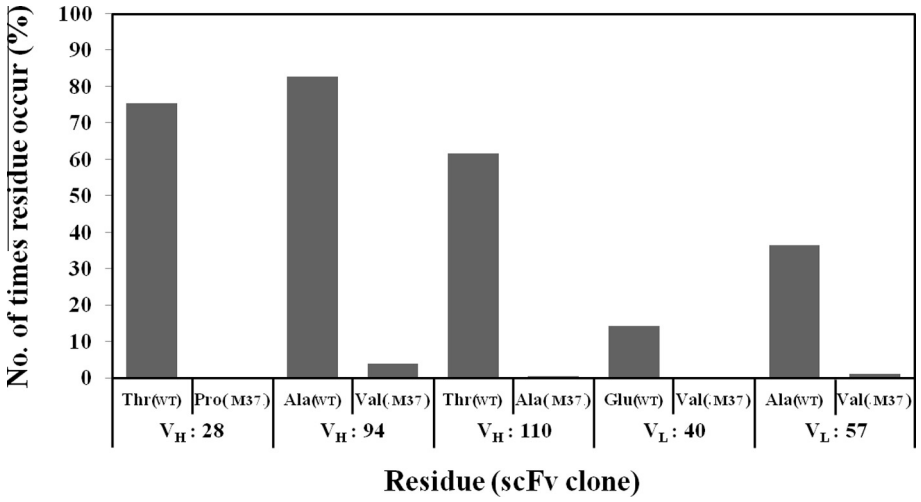

Fig. 4. Probability of residues naturally occurring at mutated position of scFv-M37. Amino acid residues were presented as 3 letter code.

4. Conclusion

Recombinant scFv is a versatile alternative to the conventional IgG for the detection of food-borne mycotoxins, because of the cost-effective production using microbial systems and its capability to be improved for its affinity, stability or specificity via use of antibody library technology incorporating high-throughput screening systems. In this study, AFB_1 was chosen as a model mycotoxin for affinity improvement with high specificity. After 4 rounds of a competitive FACS screening in the presence of 10-times higher concentrations of AFs than of AFB_1, 5 affinity-matured scFv variants against AFB_1 were successfully isolated from a yeast library that can display scFv mutants generated by error-prone PCR on the surface of a yeast cell. Among these variants, scFv-M37 showed the most improved binding towards AFB_1, with a 9-fold improvement compared to scFv-WT, which was confirmed by its lower K_D value in an SPR assay and its higher MFI profile in FACS analysis in comparison with those of scFv-WT. The results of ciELISA showed that scFv-M37 also maintained a desirable level of specificity inherited from the parental IgG 2C12. Thus, further studies are warranted to achieve additional improvement of the affinity of scFv-M37 for AFB_1 via chain shuffling using DNA pools of 5 affinity-matured scFv variants or via an *in silico* design to build a more favorable binding pocket for AFB_1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.04.117.

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