JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 5713−5722

pubs.acs.org/JAFC

# Enhancement and Analysis of Human Antiaflatoxin B1 (AFB1) scFv Antibody−Ligand Interaction Using Chain Shuffling

Kuntalee Rangnoi,[†] Kiattawee Choowongkomon,[‡] Richard O'Kennedy,[§] Florian Rüker,[∥] and Montarop Yamabhai*,[†]

[†]Molecular Biotechnology Laboratory, School of Biotechnology, Institute of Agriculture Technology, Suranaree University of Technology, Nakhon Ratchasima 3000, Thailand

[‡]Department of Biochemistry, Faculty of Science, Kasetsart University, 50 Ngam Wong Wan Road, Chatuchak, Bangkok 10900, Thailand

[§]School of Biotechnology and National Centre for Sensor Research, Dublin City University, Dublin 9, Ireland

[∥]Department of Biotechnology, University of National Resource and Life Sciences, Muthgasse 18, Vienna A-1190, Austria

Supporting Information

**ABSTRACT:** A human antiaflatoxin B1 (AFB1) scFv antibody (yAFB1-c3), selected from a naïve human phage-displayed scFv library, was used as a template for improving and analysis of antibody−ligand interactions using the chain-shuffling technique. The variable-heavy and variable-light (VH/VL)-shuffled library was constructed from the VH of 25 preselected clones recombined with the VL of yAFB1-c3 and vice versa. Affinity selection from these libraries demonstrated that the VH domain played an important role in the binding of scFv to free AFB1. Therefore, in the next step, VH-shuffled scFv library was constructed from variable-heavy (VH) chain repertoires, amplified from the naïve library, recombined with the variable-light (VL) chain of the clone yAFB1-c3. This library was then used to select a specific scFv antibody against soluble AFB1 by a standard biopanning method. Three clones that showed improved binding properties were isolated. Amino acid sequence analysis indicated that the improved clones have amino acid mutations in framework 1 (FR1) and the complementarity determining region (CDR1) of the VH chain. One clone, designated sAFH-3e3, showed 7.5-fold improvement in sensitivity over the original scFv clone and was selected for molecular binding studies with AFB1. Homology modeling and molecular docking were used to compare the binding of this and the original clones. The results confirmed that VH is more important than VL for AFB1 binding.

**KEYWORDS:** *aflatoxin, single-chain fragment variable (scFv), chain shuffling, enzyme-linked immunosorbent assay (ELISA)*

## INTRODUCTION

Mycotoxins are natural toxic secondary metabolites produced by several species of fungi. They contaminate a wide variety of agricultural products in the field and during storage.[1] Most mycotoxins are small-molecule haptens that can elicit an immune response only when attached to a large carrier protein.[2] Immunoanalysis or immunotherapy of mycotoxin ingestion requires antibodies that can bind to the free form of toxins that are found in nature; however, a large number of antibodies obtained from immunized animals can bind well only to conjugated toxins.[3]

Phage display technology is a powerful technique for the isolation of recombinant antibody fragments.[4] Small antibody-derived fragments such as scFv or Fab can be displayed on the surface of bacteriophage and subsequently selected for specific binding against various targets.[5] Phage-displayed antibody fragments selected from a naïve library tend to have low binding affinities ($K_D$) toward hapten antigens.[6] Nevertheless, this technique facilitates rapid evaluation of the binding activity of recombinant antibodies and is ideal for monitoring the efficacy of introduced modifications aimed at improving their properties, e.g., by various methods of affinity maturation such as site-directed mutation, saturation, or random mutagenesis, and chain shuffling.[7] Affinity maturation was previously successfully used to improve the binding affinity of different hapten-associated antibodies.[8,9] In this paper the application of chain shuffling[10] to enhance binding characteristics is reported.

Aflatoxins (AFBs) are carcinogenic toxins that could be produced as secondary metabolites from *Aspergillus flavus* and *Aspergillus parasiticus*.[11] These toxins are regularly found to contaminate food and feed supplies in tropical and subtropical regions. Thus, a highly effective method for the detection of aflatoxins is absolutely necessary. Polyclonal and monoclonal antibodies have been used as key reagents for the analysis of mycotoxin contamination by enzyme-linked immunosorbent assay (ELISA).[1] However, the incorporation of a highly engineered antibody that can be tailored to the performance requirements of any assay platform is greatly desirable, and the approach outlined here can address this need.

In the present study, phage display and chain-shuffling techniques were employed to unravel and improve the binding properties of recombinant human antiaflatoxin antibody. A human single-chain variable-fragment (scFv) antibody cloned,

Received: March 4, 2018
Revised: May 13, 2018
Accepted: May 21, 2018
Published: May 21, 2018







DOI: 10.1021/acs.jafc.8b01141
J. Agric. Food Chem. 2018, 66, 5713−5722

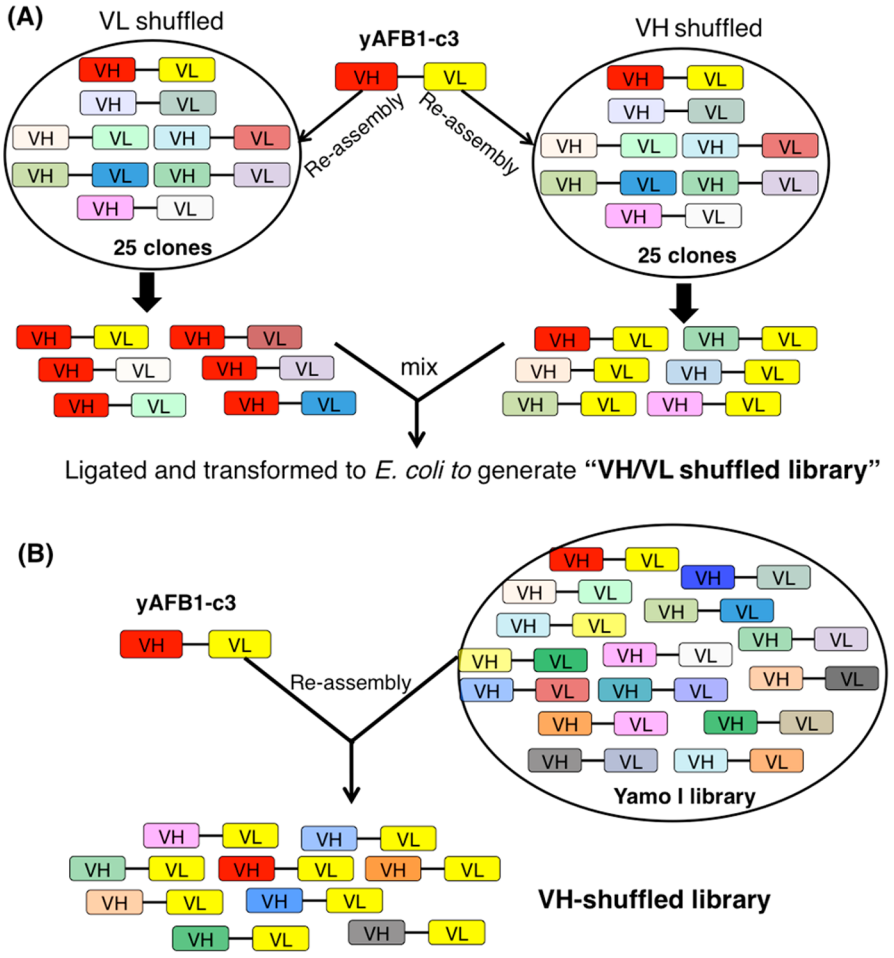


**Figure 1.** Schematic diagram for the construction of chain-shuffled libraries in this study. Two chain-shuffled libraries were constructed. (A) *Chain shuffled library I* (VH/VL shuffled library): VH (red) and VL (yellow) regions of clone yAFB1-c3 were shuffled with VL and VH of 25 affinity-selected clones from Yamo I and Tomlinson I library against AFB1-BSA. (B) *Chain shuffled library II* (VH-shuffled library): repertoire of VH genes from Yamo I library were recombined with the VL of yAFB1-c3 (yellow). This resulted in a VH-shuffled, VL-biased library. scFv shuffled products from chain-shuffled libraries I and II were ligated into phagemid vector (pMOD1.1) and transformed into *E. coli* DH5αF' and TG1, respectively.

**Table 1. Primers for Amplifying VH and VL Genes**[a]

| primer | sequence |
|---|---|
| VH amplification | |
| ShufFw5′ | 5′CCT TTC TAT GCG GCC CAG CCG GCC ATG GCC3′ |
| LinkBa3′ | 5′CCA CCA GAG CCG CCG CCG CCG CTA C3′ |
| VL amplification | |
| LinkFw5′ | 5′ GTA GCG GCG GCG GCG GCT CTG GTG G 3′ |
| ShufRv3′ | 5′CCC GTG ATG GTG ATG ATG ATG TGC GGC CGC ACC 3′ |

[a]Sequences corresponding to *Sfi*I and *Not*I site are underlined.

yAFB1-c3, previously selected from a compact, nonimmunized phage display scFv antibody library[12] was used as a template for improvement in this study. The VH/VL chain-shuffled libraries were constructed to isolate clones with improved binding activity to AFB1. Homology modeling and molecular docking were used to elucidate the anitibody−AFB1 interaction.

## MATERIALS AND METHODS

**Chemicals and Reagents.** All reagents were molecular grade or analytical grade. Antiaflatoxin scFv antibody clone yAFB1-c3 was generated in our laboratory.[12] Aflatoxin B1 standards and AFB1 conjugated with BSA were obtained from Aokin (Berlin, Germany). *Escherichia coli* TG1 (Zymo Research, CA, USA) and DH5αF' (New England Biolabs, MA, USA) was used for cloning and amplification of phage. *E. coli* HB2151 was obtained from the MRC (Cambridge, UK) and used for the production of soluble scFv fragments. All restriction enzymes (*Sfi* I, *Not* I, *Nco* I-HF), *Taq* DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (NEB, MA, USA). Mouse anti-M13-HRP was purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden), and Protein L peroxidase HRP was from Sigma (St. Louis, MO, USA).

**Construction of Chain-Shuffled Libraries.** Chain shuffling is an approach where either the heavy or the light chain of a clone is recombined with a repertoire of complementary chains to create diversification of a clone (yAFB1-c3). Then improved variants from the created libraries are subsequently affinity selected. In this study, two chain-shuffled libraries were constructed, as depicted in Figure 1. The first library (Figure 1A) was a small chain-shuffled library created by chain shuffling of clone yAFB1-c3 with VH and VL repertoires of


DOI: 10.1021/acs.jafc.8b01141
J. Agric. Food Chem. 2018, 66, 5713−5722

25 lead scFv genes previously affinity selected against AFB1-BSA from two human scFv libraries, i.e., Yamo I[13] and Tomlinson I (MRC, Cambridge, UK). The second library (Figure 1B) was a VH-shuffled library, created by assembling the repertoire of VH genes derived from Yamo I library with the VL gene of yAFB1-c3.

The variable-heavy and -light chains were amplified by PCR using a mixture of *Taq* and *Pfu* polymerases. Primers for amplification are listed in Table 1. PCR amplification was performed in a total volume of 50 μL, consisting of 1× *Pfu* DNA polymerase buffer, 0.2 mM dNTP (NEB, MA, USA), 2.5 U of *Taq* DNA polymerase, 1.5 U of *Pfu* DNA polymerase (Promega, WI, USA), 100 ng of DNA template, and 1 μM forward and reverse primers under the following conditions: 95 °C for 2 min, 30 cycles of 95 °C for 1 min, 66 °C for 30 s, 72 °C for 3 min, and a final extension of 72 °C for 5 min. The assembled reaction was performed in two steps. In the first step, assembly PCR was performed in the absence of primer in a total volume of 40 μL, consisting of 400 ng each of VH and VL, 1× *Pfu* DNA polymerase buffer, 0.2 mM dNTP, and 1.2 U of *Pfu* DNA polymerase. The mixture reaction was left at 95 °C for 2 min, then 25 cycles of 95 °C for 1 min, 66 °C for 30 s, 72 °C for 3 min, and a final extension of 72 °C for 5 min. In the second step, the assembled scFv fragments were amplified by PCR with pull-through primers (ShufFw5′ and ShufRv3′). The PCR reaction was performed in a total volume of 50 μL, comprised of 4 μL of assembled scFv, 1× of *Pfx*50 buffer, 0.2 mM dNTP, 1 μM each forward and reverse primer, and 5 U of *Pfx*50 DNA polymerase (Invitrogen, CA, USA). PCR was performed at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s, 66 °C for 30 s, 68 °C for 1 min, and a final extension of 68 °C for 5 min. The scFv DNA fragment products of approximately 800 bp were purified from gel using the Wizard SV clean up kit (Promega, WI, USA). The scFv DNA fragments and pMOD1.1 vector[13] were sequentially digested with *Not*I-HF and *Sfi*I at a ratio of 20 enzyme units per 1 μg of DNA. The scFv DNA, with equimolar quantities of VH shuffled and VL shuffled, were ligated to 1 μg of digested pMOD1.1 vector at a vector: insert molar ratio of 1:3 using 2400 T4 DNA ligase units. Finally, the ligated DNA was transformed into *E. coli* DH5αF′ and *E. coli* TG1 electrocompetent cells for chain-shuffled I and chain-shuffled II libraries, respectively. Cells were grown on TYE agar plates containing 100 μg/mL ampicillin and 1% (w/v) glucose. Phage-displayed scFv were amplified and rescued with M13 helper phage.[14] Then phage particles were purified from culture supernatant by precipitation with 20% (v/v) polyethylene glycol 8000 and 2.5 M NaCl.

**Affinity Selection against Immobilized AFB1-BSA.** To immobilize the target for biopanning, 50 μg of BSA-conjugated AFB1 in 100 μL of phosphate buffer saline (PBS) was added into wells of 96-well microtiter plates and incubated at 4 °C overnight. The target was reused for the next round of biopanning. The remaining sites on well were blocked with 2% (w/v) skim milk, dissolved in 0.01 M PBS buffer pH 7.4, for 1 h at room temperature. Before adding to AFB1-BSA-coated wells, the chain-shuffled libraries were preincubated in blocking buffer with 2% skim milk (w/v) and 1% (w/v) BSA dissolved in 0.01 M PBS buffer pH 7.4 at room temperature for 30 min. Then the libraries were incubated with AFB1 for 1 h at room temperature. Unbound phages were eliminated by washing the wells 10 times with PBS containing 0.05% (v/v) Tween 20 (PBST) followed by 10 times with PBS. For the second and third rounds of selection, the washing time number was increased by 15 times with PBST, followed by 15 times with PBS and 20 times with PBST, followed by 20 times with PBS, respectively. Biopanning was performed for two rounds with chain-shuffled library I and three rounds with chain-shuffled library II, respectively using three different elution methods. For elution method I, the bound phage was eluted with 100 μL of 1 μg/mL trypsin by incubating for 10 min, followed by incubation for another 10 min at room temperature with 50 μL of 100 mM glycine−HCl, pH 2.0. After glycine elution, the solution was neutralized with 50 μL of neutralization solution (0.2 M $NaH_2PO_4$, pH 7.5). Then both eluted solutions were mixed together before infecting *E. coli* TG1 or HB2151. For elution method II, bound phages were eluted by incubation with 50 μL of 50 μg/mL AFB1 for 15 min. For elution method III, soluble scFv antibody formats were directly obtained by allowing the phage to infect log phase *E. coli* HB2151 in 2xYT media, which was directly added to the well and incubated at 37 °C for 30 min. Infected *E. coli* was serially diluted and grown on 2xYT agar plates supplemented with 100 μg/mL ampicillin and 1% (w/v) glucose to obtain separated colonies. Then 86−190 colonies were randomly picked for enzyme-linked immunosorbent analysis (ELISA).

**Indirect ELISA.** After each round of biopanning, individual phage clones were rescued as previously described[12] before analysis by indirect ELISA. Immuno 96 wells plates (Nunc, Roskilde, Denmark) was coated with 1 μg of AFB1-BSA and 1% (w/v) BSA (negative control) in 100 μL of 0.01 M PBS buffer pH 7.4. Then the wells were blocked as previously described. After incubation for 1 h at room temperature, the wells were washed 3 times with PBS. After that 100 μL of phage supernatant or soluble scFv fragments and 50 μL of PBS were added into each well of the plate. Plates were left to stand on the bench for 1 h. After that unbound phage or soluble scFv fragments were washed away 3 times with PBST and 2 times with PBS. Subsequently, 100 μL of HRP-anti-M13 (1:5000 dilution in PBS) and HisProbe-HRP or Protein L-HRP were added to each well to detect bound phage and soluble scFv fragments, respectively. After incubation for 1 h at room temperature, the wells were washed again. Then, 100 μL of substrate solution, ABTS (Amresco, OH, USA) or TMB (Sigma, MO, USA), was added to each well and incubated at room temperature for 15 min to 2 h. Absorbance was measured at 405 nm. When using TMB as substrate, the reaction was stopped with 10% (v/v) HCl after incubation for 10−30 min. The yellow color was developed after adding HCl. Absorbance was measured at 450 nm.

**Competitive ELISA.** Competitive or inhibition ELISA was performed as described for the normal ELISA method. For every assay, appropriate dilutions of scFv that showed a linear relationship by indirect ELISA were used. All antibody dilutions and standard AFB1 were dissolved in PBST buffer. The antibodies, at appropriate dilutions, were preincubated with varying concentrations of soluble aflatoxin-B1 (AFB1), ranging from 0.019 to 5 μg/mL. After incubation at 37 °C for 30 min, the mixture was then transferred to a previously coated and blocked ELISA plate and incubated for 1 h. The unbound antibodies were washed away 3 times with PBST and 2 times with PBS. Protein L-HRP or His Probe-HRP was added to the wells and incubated for 1 h at room temperature. The substrate solution was added as described in the indirect ELISA method. The positive clones were indicated by decreasing of OD values when the concentration of AFB1 was increased. A standard curve was plotted of absorbance ($A$) vs the logarithm of AFB1 concentration. A half-maximum inhibition ($IC_{50}$) was estimated at 50% $A/A_0$.

**Antibody Sequence Analysis.** Plasmid DNA was extracted and purified from overnight cultures using a commercial plasmid preparation kit (Mini Preps: Qiagen, Dusseldorf, Germany), and the DNA sequences were analyzed by automated DNA sequencing (Macrogen, Seoul, Korea) using primers: Yamo5′ Fw, 5′ CAG GAA ACA GCT ATG ACC 3′; 96 geneIII Rv, 5′ CCC TCA TAG TTA GCG TAA CG3′. Immunogenetic analysis of variable regions was performed using IMGT[15] and V-Base database.[16] The amino acid sequences were translated using Snapgene software (GSL Biotech, IL, USA). All sequences were aligned using Clustal Omega.

**Expression and Purification of scFv Fragments.** A single colony of *E. coli* HB2151 infected with phage-bearing positive clones was inoculated into 5 mL of 2xYT containing 100 μg/mL ampicillin and 2% (w/v) glucose and grown overnight at 37 °C with shaking. On the next day, 2 mL of overnight culture was inoculated into 200 mL of 2xYT broth containing 100 μg/mL ampicillin and 0.1% (w/v) glucose at 30 °C with shaking for approximately 3 h to reach an $OD_{600}$ of 0.9. After that the culture was induced with 1 mM IPTG and continued to grow with shaking for 6 h at 250 rpm. The scFv fragments were collected from the periplasmic extract as previously described.[17]

Soluble scFv were purified by immobilized metal-affinity chromatography (IMAC) using Ni-NTA and by an Akta purifier (GE Healthcare, Little Chalfont, UK). Before purification, cell debris was removed from the periplamic extract by filtering through a 0.2 μm membrane. A 1 mL His-Trap column (GE Healthcare, Little Chalfont, UK) was equilibrated with 10 mL of binding buffer (20 mM


DOI: 10.1021/acs.jafc.8b01141
J. Agric. Food Chem. 2018, 66, 5713−5722

**Table 2. Biopanning Results of the VH/VL Shuffled Library**

| biopanning scheme | round of selection | elution method | no. of clones that bind AFB1-BSA | no. of clones that bind free AFB1 | amino acid sequence analysis[a] | no. of original scFv (yAFB1-c3) | no. of improved clones |
|---|---|---|---|---|---|---|---|
| I | first | trypsin + glycine | 40/192 | 0 | 3/3 | 0 | 0 |
| | second | *E. coli* HB2151 | 46/86 | 0 | 3/4 | 0 | 0 |
| II | first | 50 $\mu$g/mL AFB1 | N/A | N/A | N/A | N/A | N/A |
| | second | *E. coli* HB2151 | 53/96 | 9 | 4/6 | 3 | 0 |

[a]The number of different scFv clones/the number of clones analyzed.

**VH amino acid sequence of yAFB1-c3 template, VH/VL- shuffled clones**

| | Family | Germline | FR1 | CDR1 | FR2 | CDR2 | FR3 | CDR3 | FR4 |
|---|---|---|---|---|---|---|---|---|---|
| yAFB1-c3(3) | VH1 | IGHV1-3*01 | QVQLVQSGAEVKKPGASVKVSCKAS | GY..TFTSYA | MHWVRQAPGQRLEWMGW | INAGNGNT | KYSQKFQGRVTITRDTS.ASTAYMELSSLRSEDTAVYYC | ARADDY.....GSGSYGFDY | WGQGTLVTVSS |
| slAF-IIh6 | VH1 | IGHV1-3*02 | -*-QL-*S-DEM-NR--SMM-SNN- | *Y..-S-TCD | RLR-V-HA--Q-Q*-I* | -ISA-SD- | T--PQLLHS-V-ISTA-SSTASNK-I-II**D---*--S | .-T-YD.....CCSW----- | ---------- |
| slAF-IIg3 | VH3 | IGHV3-23*04 | --*-----GGLVQ--G-LRL--A-- | -F..A-S--G | -N-------KG---VSA | -SGSG-S- | Y-ADSVK--F--S--N-.KN-L-LQMN---A-------- | VK-RS-.....YY--GSYYD | ---------- |
| slAF-IIb5 | VH3 | IGHV3-33*01 | --------GG-VQ--R-LRL--A-- | -F..--S--G | ---------KGP--VAV | -WYDGS-K | Y-ADSVK--F--S--N-.KN-L-LQMNG--A-------- | ---RTNRTVTTVPIYYYY--M-V | ----NPGHRLL |
| slAF-IIf9 | VH1 | IGHV1-18*01 | ----Q---------- | --..-----G | IS--------G------ | -S-Y---- | N-A--L-----M-T---.T--T---------D------- | --GSGW.....--.--R--- | ---------- |
| slAF-IIg2 | VH1 | IGHV1-45*02 | -----*-------T-S-M------- | --..-----D | IR--------A------ | -TPF---- | N-A----D---------.T----------D------- | --GSGW.....--.--R--- | ---------- |
| slAF-IIe1 | VH1 | IGHV1-45*02 | ---------- | --..-----Y | ----------G------ | -TPF---- | N-A----D-------R-.M----L-------D------- | --GSGW.....--.--R--- | ---------- |
| slAF-IIc6 | VH4 | IGHV4-39*01 | ----QE--PGLV--SETLSLT-TV- | DGSISGS--Y | WG-I--P--KG---I-T | TYY-S-T- | Y-NRSLKS----SG---.KNQFSLK-T-VTAA------- | --QL.....-R-YWA--I | -----M----- |
| slAF-I2d1 | VH3 | IGHV3-21*01 | --N-RE--GV-VQ--G-LRL--A-- | -F..--NN-N | -N-------KG---VSS | -SGDSRHI | Y-ADSVK--F--S--NA.KNSL-LQMN---A-------- | --VG.....-RHA--M-V | -----T----- |
| slAF-I1a3(2) | VH4 | IGHV4-45*02 | ----------T-S-M------- | --..---YRY | L---------A------ | -TPF---- | N-A----D-------R-.M----L----------M--- | -SG.....GPL-- | ---------- |

**VL amino acid sequence of yAFB1-c3 template, VH/VL- shuffled clones**

| | Family | Germline | FR1 | CDR1 | FR2 | CDR2 | FR3 | CDR3 | FR4 |
|---|---|---|---|---|---|---|---|---|---|
| yAFB1-c3(3) | VK1 | IGKV1-39*01 | DTVMTQSPSSLSASVGDRVTITCRAS | QS.....ISSY | LNWYQQKPGKAPRLLIY | AAS | ....SLQSGVPSRFSGNGS..GTDFTLTISSLQPEDFATYYC | QQSY.STPYA | FGQGTKVEIKRA |
| slAF-IIh6 | VK1 | IGKV1-39*01 | ---------- | --.....---- | ---------- | --- | ....---------..---------- | ----.----- | ----------.. |
| slAF-IIg3 | VK1 | IGKV1-39*01 | ---------- | --.....---- | ---------- | --- | ....---------..---------- | ----.----- | ---------- |
| slAF-IIb5 | VK4 | IGKV4-1*01 | -IQ------D--AV-LGE-A--N-KS- | --VLYSSNNK-- | -A-------QP-K--V- | W-- | ....TRE----D----S--..----------A--V-V--- | ----.----T | ----PSWKSN.. |
| slAF-IIf9 | VK1 | IGKV1-39*01 | ---------- | --.....---- | ---------- | --- | ....---------..---------- | ----.----- | ---------- |
| slAF-IIg2 | VK1 | IGKV1-39*01 | ---------- | --.....---- | ---------- | --- | ....---------..---------- | ----.----- | ---------- |
| slAF-IIe1 | VK1 | IGKV1-39*01 | ---------- | --.....---- | ---------- | --- | ....---------..---------- | ----.----- | ---------- |
| slAF-IIc6 | VL5 | IGLV5-45*03 | QAVL--.-------P-ASARL--TLR | SGLN...VGAYR | MY---K---SP-QF-LM | YK- | ESSNHRA--LS-----SKDSSANEGI-S--G--SA-A-D--- | MIWH.NSVVE | I-G----TVLG- |
| slAF-I2d1 | VL5 | IGKV5-45*03 | QAVL--.-------P-ASASL--TLR | SGIN...VGAYR | IY-------SP--Y-LM | YK- | DSDKHQA---------SKDSSANAGI-L--G--S--E-D--- | VIWH.-SAVV | --G----TVL-- |
| slAF-I1a3(2) | VL2 | IGLV2-8*01 | QSAL--.-A-A-G-P-QS---S-TGT | G-DV...GGYN- | VS----H-----K-M-- | EV- | ....KRH----D----SK-..-NTAS--V-G--A--E-D--- | SSYAG-NNLV | --G----TVL-- |

**Figure 2.** Amino acid sequences of the VH and VL of VH/VL-shuffled clones. Deduced amino acid alignment of the template yAFB1-c3 and newly selected scFv from the VH/VL-shuffled library. Amino acid residues, which are different from those found in yAFB1-c3, are shown in each scFv. Dashed lines represent sequence identity with yAFB1-c3. Dots represents unoccupied amino acid positions; number in brackets, number of replicate sequence; FR, framework region; CDR, complementarity determining region; *, stop codon.

$NaH_2PO_4$, 500 mM NaCl, 20 mM imidazole, pH 7.5). Then the filtered periplasmic extract was loaded into the column. To remove loosely bound protein, the column was washed with the same buffer. The scFv were eluted from the column by increasing volumes of a high concentration of imidazole (20 mM $NaH_2PO_4$, 500 mM NaCl, 500 mM imidazole, pH 7.5) until the UV peak was clearly visible. The antibody fraction was dialyzed with PBS buffer using 10 kDa snakeskin dialysis tubing (Thermo Scientific, IL, USA). Protein concentration was quantified using a Nanodrop ND2000 spectrophotomer (Thermo Scientific, IL, USA).

**Binding Kinetics of scFv Antibodies by Surface Plasmon Resonance (SPR).** The binding kinetics of purified scFv antibodies were evaluated by Biacore 3000 (GE Healthcare, Little Chalfont, UK). The AFB1-BSA was immobilized on the CM5 chip (GE Healthcare, Little Chalfont, UK) using amide-coupling chemistry according to the manufacturer's instructions. BSA was immobilized as a control in another flow cell on the same sensor chip. Unbound ligand was washed away with HEPES containing 0.01% (v/v) Tween 20 and the inactivated sites blocked by 30 $\mu$L of 1 M ethanolamine, pH 8.5. Purified scFv at different concentrations were each injected over the captured AFB1-BSA at a constant flow of 30 $\mu$L/min with a 3 min contact time and a 9 min dissociation time. The captured surface was regenerated between each binding reaction with 15 mM NaOH at a flow rate of 30 $\mu$L/min for 1 min. BIAvaluation software was used to analyze the kinetic binding data which was then fitted globally to a 1:1 binding model.

**Antibody Modeling and Molecular Docking.** Homology modeling of the three-dimensional (3D) structures of scFv yAFB1-c3, sAFH-3e3, sAFH-3e11, and sAFH-3f11 were generated from the amino acid sequences using the SWISS-MODEL Web site.[18] The templates (PDB code 3uzq) were chosen by sequence identity analysis. Models were visualized with the program PyMoL.[19] The MolProbity Web site was used to analyze Ramachandran values of the scFv model (http://molprobity.biochem.duke.edu). To understand the molecular interaction between AFB1 and the scFv antibody, GOLD software was used to analyze antigen−antibody docking.[20] An AFB1 molecule was obtained from the PubChem compound database. ChemPLP scoring function was tested in separate runs. The active site for docking was defined as the position CB for Ala33 of yAFB1-c3, CB for His33 of sAFH-3e3, and CG for Tyr33 of sAFH-3e11 and sAFH-3f11 within the 6 Å radius. The best-scored solution was considered and viewed in Discovery Studio Visualizer.[21]

## RESULTS AND DISCUSSION

**Construction and Biopanning of Chain-Shuffled Library I (VH/VL-Shuffled Library).** The preselected 25 scFv clones that were used for the construction of chain-shuffling library I included 2 clones (yAFB1-c3 and TomI-f6) previously shown to bind soluble AFB1[12] and 23 clones from Yamo I library that bind only to AFB1-BSA but not the free toxin. The VH and VL of these 25 clones were shuffled with the VH and VL of yAFB1-c3 as described in the Materials and Methods section. The library diversity was $7.15 \times 10^4$ cfu/mL with 0.38% background. The biopanning results are shown in Table 2. The biopanning scheme I using a standard method of elution yielded no binder for free AFB1 as determined by competitive ELISA. This result was not unexpected because out of 25 clones, which were the templates for library construction, there were only 2 clones that can bind to soluble AFB1. When soluble AFB1 was used for elution in the first round of biopanning scheme II we could retrieve clones that could bind to free toxin. Unfortunately, no clone showed an improvement


DOI: 10.1021/acs.jafc.8b01141
J. Agric. Food Chem. 2018, 66, 5713−5722

**Table 3. Biopanning Results of VH-Shuffled Library**

| round of selection | elution method | phage/scFv | no. of clones that bind AFB1-BSA | no. of clones that bind free AFB1 | amino acid sequence analysis[a] | no. of original scFv (yAFB1-c3) | no. of improved clone |
|---|---|---|---|---|---|---|---|
| first | trypsin + glycine | phage | 93/95 | 11/11 | | | |
| | | scFv | 95/95 | 28/29 | 6/11 | 5/11 | 0 |
| third | trypsin + glycine | phage | 90/95 | 5/5 | 1/2 | 1/2 | 1 |
| | | scFv | 89/95 | 5/5 | 3/5 | 2/5 | 2 |

[a]The number of different scFv clones/the number of clones sequenced.

| Heavy chain | FR1 | CDR1 | FR2 | CDR2 | FR3 | CDR3 | FR4 |
|---|---|---|---|---|---|---|---|
| yAFB1-c3(8) | QVQLVQSGAEVKKPGASVKVSCKAS | GYTFTSYA | MHWVRQAPGQRLEWMGW | INAGNGNT | KYSQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYC | ARADDYGSGSYGFDY | WGQGTLVTVSS |
| sAFH-3e3(2) | G----E---------- | --S--N-H | ---------- | -------- | ---------- | --------- | ---------- |
| sAFH-3e11 | --------S-L---------- | -------Y | V--------- | -------- | ---------- | --------- | ---------- |
| sAFH-3f11 | -------A---------- | -----G-Y | ---------- | -------- | ---------- | --------- | ---------- |
| sAFH-g5 | E----E---------S--------- | -------- | ---------- | -------- | ---------- | --------- | ---------- |
| sAFH-g1 | ---------- | -------- | ---------- | -------- | ------K---------- | --------- | ---------- |
| sAFH-f8 | --------S-L---------- | -------- | ---------- | -------- | ---------- | --------- | ---------- |
| sAFH-b5(2) | E---L--A---------- | -------- | ---------- | -------- | ---------- | --------- | ---------- |
| sAFH-d1 | ---------- | -------G | IS--------G-----G | -IPIF-TA | N-A---------ADE-T---------- | --------- | ---------- |

**Figure 3.** Amino acid sequences of the VH domain of selected VH-shuffled clones. Alignment was performed using the Clustal Omega database. Amino acids sequence, which were different from the original clone (yAFB1-c3), are indicated. Dashes indicates sequence identity with yAFB1-c3. Number in brackets, number of replicate sequence; FR, framework region; CDR, complementarity determining region.

in binding sensitivity. Nevertheless, these results confirmed previous observations that the elution method can affect the binding properties of the antibody selected.[22] To study antibody-AFB1 interactions, 13 clones from both schemes I and II of biopanning were selected for amino acid sequence analysis.

**Amino Acid Sequence Analysis of Binders from Library I.** DNA sequences of binders were translated to amino acid sequences using Snapgene software and aligned using Clustal Omaga software. The origin of the V gene and complementarity-determining region (CDR) were determined using V-BASE immunoglobulin the V gene database and the IMGT database. The closest germline sequences for antiaflatoxin scFv antibody were identified from the database. From 13 clones, 3 clones were identical; these clones were the original yAFB1-c3 clone. The VH and VL of the original clone belonged to VH3 (IGHV1-3*01) and VK1 (IGKV1-39*01), respectively. The alignment of the deduced amino acid sequence of nine clones, which showed different amino acid sequences, is shown in Figure 2. Nine clones could be classified into two groups, based on the ability to bind free AFB1. Group I is composed of 3 clones that can bind to soluble AFB1, i.e., s1AF-IIh6, s1AF-IIg3, and s1AF-IIb5. The VH of this group belonged to the VH1 and VH3 families (IGHV1-3*02, IGHV3-23*04, and IGHV3-33*01). The VL of clone s1AF-IIh6 and s1AF-IIg3 were identical to the VL of the original clone, while that of s1AF-IIb5 belonged to VK4 (IGKV4-1*01). Group II is composed of another six clones, which were not inhibited by soluble AFB1 by competitive ELISA. The VH of group II belonged to VH1, VH3, and VH4 (IGHV1-18*01, IGHV1-45*02, IGHV3-21*01, IGHV4-39*01, and IGHV4-45*02). Clones s1AF-IIf9, s1AF-IIg2, and s1AF-I1e1 have the same VL as the original clone, whereas VL of clone s1AF-IIc6, s1AF-I 2d1, and s1AF-IIa3 belonged to VL5 and VL2 (IGLV5-45*03 and IGLV2-8*01). Since the VL gene of clone yAFB1-c3 was found in both groups these results indicated that the VH is predominantly responsible for the binding of the scFv to free toxin. This finding confirmed a previous observation that the heavy chain plays a bigger role in antigen interaction than the light chain.[6]

**Construction and Affinity Selection of Improved Binders from the Chain-Shuffled Library II (VH-shuffled library).** On the basis of the previous results, which indicated that the VH was more important for the binding of the scFv antibody to AFB1 than the light chain, a second chain-shuffled library was constructed by combining a repertoire of the VH genes from the Yamo I library with the VL gene from clone yAFB1-c3 (Figure 1B). The diversity of the chain-shuffled library II was $3.48 \times 10^5$ cfu/mL with 0.43% background. The biopanning result is shown in Table 3. After the first round of biopanning, almost all phage-displayed scFv and souble scFv clones tested could bind to free AFB1. An example of competitive ELISA of selected soluble scFv clones from the first round of biopanning is shown in Supplementary Figure S-1A. Unfortunately, none of the clones showed improved binding when compared with the original clone (yAFB1-c3). Competitive ELISA of phage-displayed scFv clones from the first round of biopanning also showed no clone with improved binding sensitivity (data not shown).

After 3 rounds of biopanning, competitive ELISA results of phage-displayed scFv and soluble scFv clones showed that all of the phage and scFv clones could bind to free AFB1 (Supplementary Figure S-1B,C). Four clones, namely, sAFH-3e11, sAFH-3e3, sAFH-3f11, and sAFH-3d4, showed improved binding sensitivity to AFB1 compared to the original clone. Eighteen clones from a total of 50 clones were selected for amino acid sequence analysis in the next step

**Amino Acid Sequence Analysis of Affinity-Improved scFv Clones.** The gene of VH domain of all 18 clones belonged to family VH1 and was derived from germline IGHV1-3*01 except for clone sAFH-d1, which was derived from germline IGHV1-69*01. Eight shuffled clones are identical to the original clone (yAFB1-c3). For the four improved clones (sAFH-3e11, sAFH-3e3, sAFH-3f11, and sAFH-3d4) clones sAFH-3e3 and sAFH-3d4 were identical. The deduced amino acid sequences of VH domain of improved clones (sAFH-3e11, sAFH-3e3, and sAFH-3f11) and non-


DOI: 10.1021/acs.jafc.8b01141
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improved clones are illustrated in Figure 3. For the improved clones, there were 3−5 amino acid differences from the original clone at positions in framework 1 (FR 1) and CDR1 regions. For nonimproved clones, there were some minor amino acid changes in FR1 and FR2 but not in the CDR1 region. Clone sAFH-d1, which showed the worst binding affinity, contained amino acid changes in CDR1, FR2, CDR2, and FR3 areas. It is interesting to note that there were no mutations in the CDR3 region, which has been shown from crystal structures of an antibody−antigen complex to be the most important domain for molecular interactions between antibody and hapten.[23,24] Therefore, in order to further improve the binding sensitivity, other methods of mutagenesis focusing on the CDR3 region can be attempted to enhance the affinity of the antibody.

To confirm the binding properties of the three improved clones, competitive ELISAs were performed and the $IC_{50}$ was determined in comparison to the original yAFB1-c3 clone (Figure 4). The $IC_{50}$ of the improved heavy chain shuffled

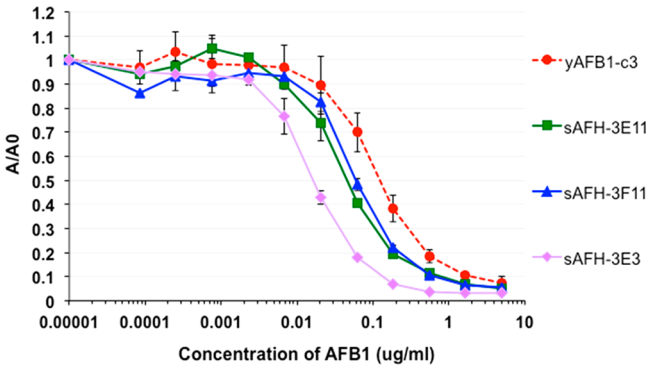


**Figure 4.** Competitive ELISA of improved scFv clones. Optimal dilution of soluble scFv antibodies was incubated with decreasing concentrations of AFB1 from 5000 to 0.028 ng/mL. Bound scFv antibody was detected with a His Probe-HRP. Results are shown as $A/A_0$, where the absorbance values of the evaluated samples ($A$) are normalized by expressing them as a function of the blank standard ($A_0$). VH chain-shuffled clones (sAFH-3e11, sAFH-3f11, and sAFH-3e3) and yAFB1-c3 (original) AFB1-specific clones showing $IC_{50}$ values of 0.02, 0.05, 0.06, and 0.15 $\mu$g/mL, respectively.

cloned varied between 0.02 and 0.06 $\mu$g/mL, which was 2.5−7.5-fold higher than that of the original clone. These results were similar to those obtained from the chain shuffling of a human scFv antibody against hepatitis B virus, which demonstrated an increase in the affinity by factors of 2.8−6.5 compared to the parental clone from the VH-shuffled library observed.[25] This result is comparable to another method of affinity maturation using a combination of random mutation and yeast display method, of which the best mouse scFv (obtained from a hybridoma) against AFB1 could be improved 9-fold compared to the parental clone.[26] This improvement was much lower when compared to previous reports on the affinity maturation of chicken scFv antibody against halofuginone, an antiprotozoal drug, where a 185-fold improvement over the original scFv could be obtained by affinity selection from a heavy chain-biased, light-chain-shuffling library.[8] The difference could be due to the nature of the antigen. In order to further improve the binding property of the antibody, another method of affinity maturation such as CDR walking mutagenesis, which was shown to improve in affinity of a human anti-HIV-1 antibody, could be applied.[27]

**Purification and Kinetic Analysis of Improved scFv Clones.** To further study the binding characteristic of the three improved scFv clones in comparison to the original yAFB1-c3, the antibody fragments were overexpressed in *E. coli*, purified from periplasmic extracts using HIS Trap columns, and subsequently analyzed by SDS-PAGE. The SDS-PAGE results showed that a scFv-6xHis fusion protein of about 32 kDa could be expressed, but some contaminated bands were visible (Supplementary Figure S-2). Each clone showed a different level of expression. The yields of clones yAFB1-c3, sAFH-3e11, sAFH-3f11, and sAF-3e3 were 4.96, 3.08, 6.36, and 3.85 mg/L of culture volume, respectively.

The binding kinetics against AFB1-BSA was measured using a BIAcore 3000 system (Table 3). While the original scFv (yAFB1-c3) showed a $K_D$ value of $5.22 \times 10^{-8}$ M, the binding affinity of the three improved VH-shuffled clones were 3−20-fold better relative to the parental clone. The best affinity-matured scFv was clone sAF-3e3, showing the lowest $K_D$ of $2.29 \times 10^{-9}$ M. The kinetic values suggested that the affinity enhancement was a result of a combination of an increase in the $K_{on}$ and a decrease in the $K_{off}$, Table 4.

**Table 4. Binding Kinetics of scFv Antibodies by SPR[a]**

| scFv | $K_{on}$ ($M^{-1}$ $s^{-1}$) | $K_{off}$ ($s^{-1}$) | $K_D$ (M) |
|---|---|---|---|
| yAFB1-c3 | $2.97 \times 10^4$ | $1.55 \times 10^{-3}$ | $5.22 \times 10^{-8}$ |
| sAFH-3e11 | $3.56 \times 10^4$ | $6.69 \times 10^{-4}$ | $1.88 \times 10^{-8}$ |
| sAFH-3f11 | $3.33 \times 10^4$ | $3.86 \times 10^{-4}$ | $1.16 \times 10^{-8}$ |
| sAFH-3e3 | $1.29 \times 10^5$ | $2.95 \times 10^{-4}$ | $2.29 \times 10^{-9}$ |

[a] $K_{on}$ association constant, $K_{off}$ dissociation constant, and $K_D$ equilibrium dissociation constant ($K_D = K_{off}/K_{on}$).

**Molecular Docking.** The 3D homology models of scFv were generated based on a previously published scFv template (PDB code 3uzq) with a resolution of 1.6 Å. The percent similarities with template scFv were 64.41 for yAFB1-c3, sAFH-3e11, and sAFH-3f11 and 62.40 for sAFH-3e11. The quality of models was checked for the Ramachandran plot analysis via the MolProbity Web site. The plot showed that all four-scFv antibodies had 93.33% in favored regions, 6.2% in allowed regions, and 0.8% in the outlier regions. The three-dimensional models of scFv yAFB1-c3 and sAFH-3e3 are shown in Figure 5. The amino acids of sAFH-3e3 that are different from the corresponding positions in the original scFv yAFB1-c3 in the FR1 and CDR1 of VH region are labeled.

Molecular docking analysis indicated that the $V_H$ domain of both antibodies provided the main contact to AFB1. As shown in Figure 6, the AFB1 binding pocket was surrounded with residues from the heavy chain. This is in agreement with the result obtained from competitive ELISA. This finding was consistent with a previous study, which demonstrated that the VH is generally more important for the interaction with the antigen than the VL.[28] The capability of the variable domain of the heavy chain to bind to AFB1 has been exemplified by an alpaca heavy chain antibody (VHH) or nanobody (Nb) obtained from phage display library of VHH of an immunized alpaca.[29] The diversity in the CDR3 region of VH has been reported to be sufficient for most antibody specificities;[24] however, in this study none of the residues of the improved scFv clones are located in CDR3. Therefore, the binding affinity of the antibody might be improved by mutagenesis of the CDR3 region.[30,31]


DOI: 10.1021/acs.jafc.8b01141
J. Agric. Food Chem. 2018, 66, 5713−5722

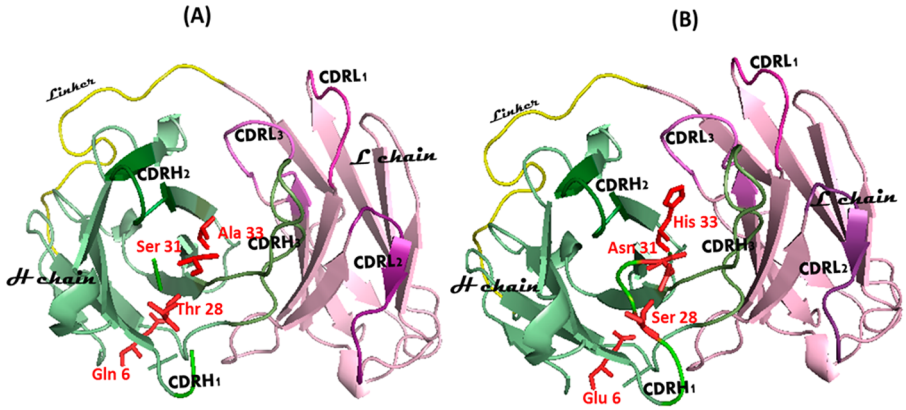


**Figure 5.** Three-dimensional structure prediction of scFv. VH and VL of scFv yAFB1-c3 (A) and scFv sAFH-3e3 (B) are shown in green and pink, respectively. Complementarity-determining region (CDR) of VH and VL are labeled. Mutated amino acid residues and their number in sAFH-3e3 and the corresponding positions in original scFv yAFB1-c3 are indicated in red.

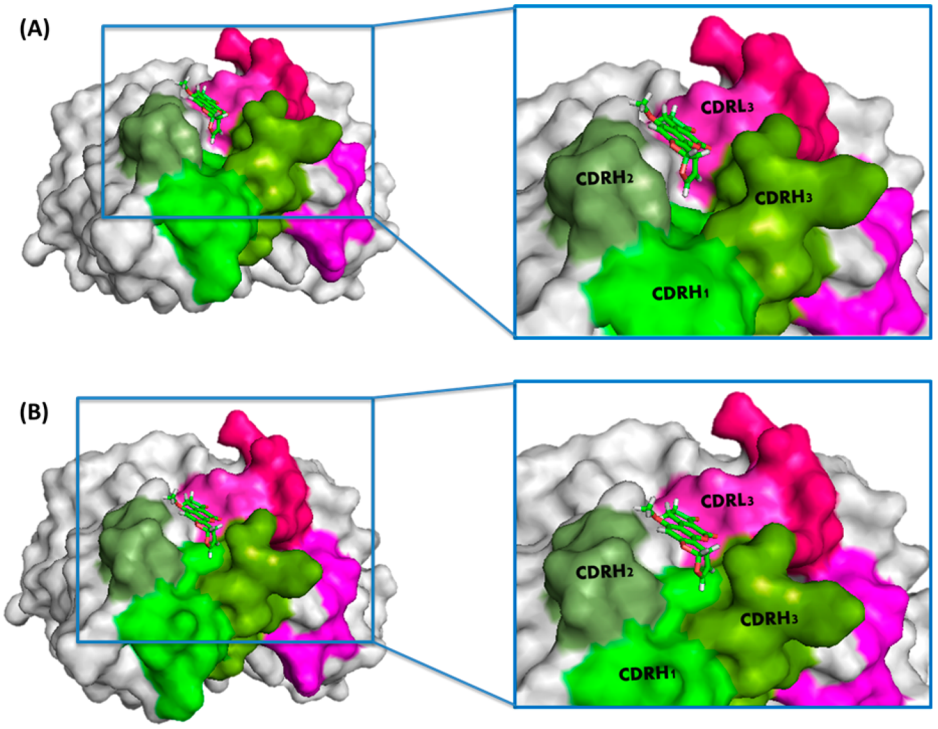


**Figure 6.** Docking model of the scFv binding pocket. Complementarity-determining region (CDR) of VH and VL of scFv yAFB1-c3 (A) and sAFH-3e3 (B) are labeled. Close-up views of AFB1 interaction with the antigen-binding site indicated that the AFB1 was surrounded by amino acids from the VH domain. This figure was generated using PyMoL.

His-H33 of sAFH-3e3 and Tyr-H33 of sAFH-3e11 and sAFH-3f11, which corresponds to the Ala-H33 of yAFB1c3, were used as the center for docking analysis. The molecular docking disposition of sAFH3e3 was similar to yAFB1-c3, whereas those for the other two mutated scFv antibodies (sAFH-3e11 and sAFH-3f11) were different. Detailed analysis of the docking model of sAFH-3e3 and yAFB1-c3 showed that the common AFB1-interacting residues included four H bonds (one in Thr-L231 of CDR L3, two in Tyr-L233 of CDR L3, and one in Asn-H57 of CDR H2) and T-shaped $\pi$ stacking of the aromatic side chain of Trp-H50 of FR H2 with the benzofuranyl ring of AFB1. More interactions with AFB1 within the binding pocket of sAFH-3e3 than those of yAFB1-c3 could be an explanation for the higher binding affinity ($K_D$). For sAFH-3e3, the Gly-H103 in CDR H3 formed a carbon−hydrogen bond and the His-H33 in CDR H1 formed a $\pi$ alkyl interaction with AFB1. The corresponding position Ala-H33 of yAFB1-c3 showed no interaction with AFB1; instead, the Asn-52 in FR2 formed hydrogen bonding with AFB1 (Figure 7). For sAFH-3e11 and sAFH-3f11, the common AFB1 interacting residues included two H bonds (one in Asn-H52 of CDR H1 and one in Tyr-H33 of CDR H1). Gly-H31 in CDR H1 of sAFH-3f11 formed a carbon−hydrogen bond with AFB1 at the same position with Ser-H31 in CDR H1 of sAFH-3e11. These differences may influence the sensitivity of antibody against AFB1. In addition to H bonding, because of the relatively large hydrophobic areas of the aflatoxin, the hydrophobic interaction might contribute to the interaction between scFv and AFB1 as well. The His-H33 in sAFH-3e3 and Gly-H31 in sAFH-3f11, which corresponded to a small alanine and serine in the original


DOI: 10.1021/acs.jafc.8b01141
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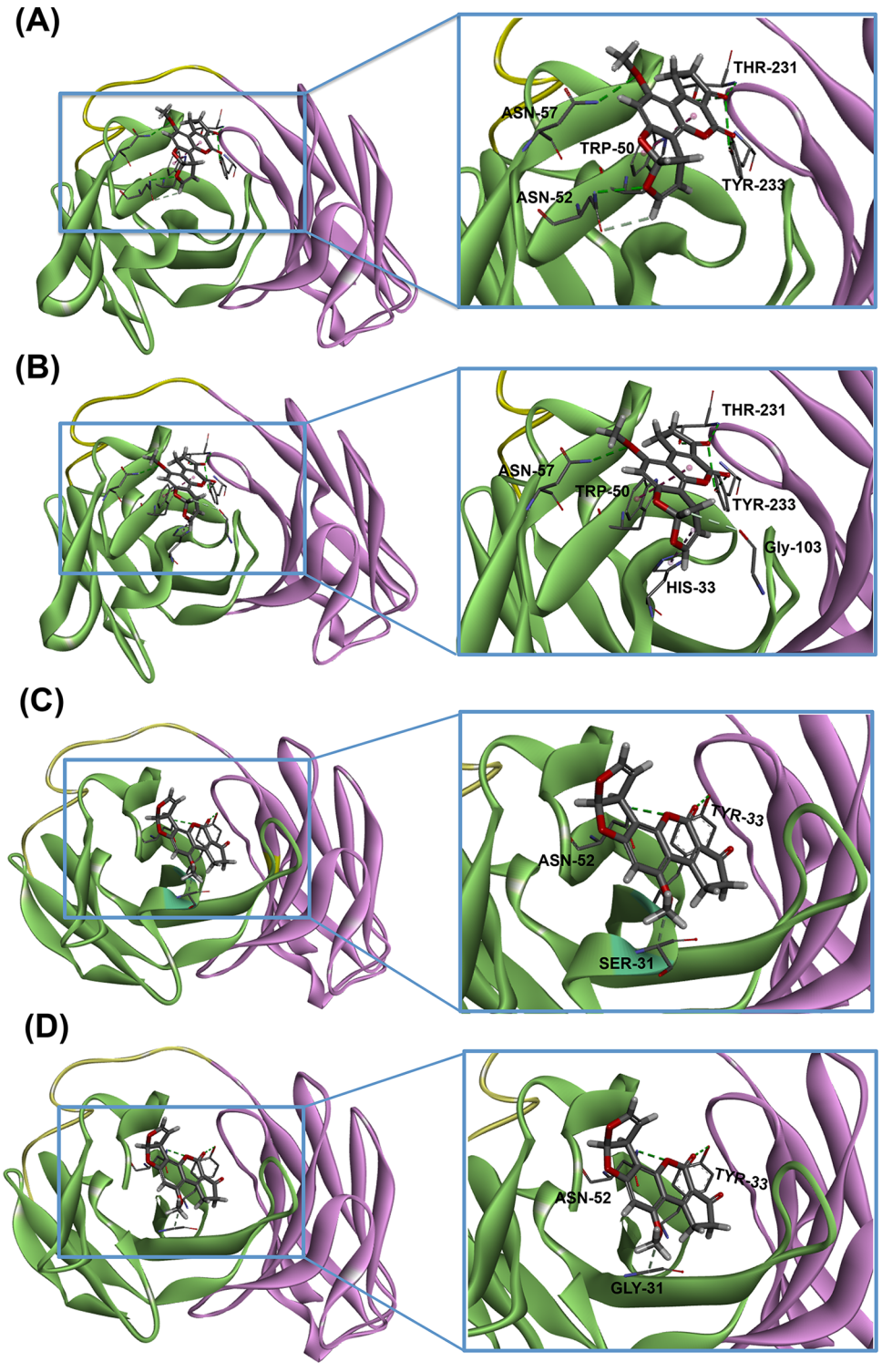


**Figure 7.** Molecular docking of the scFv–AFB1 interaction. Molecular docking of yAFB1-c3 (A), sAFH- 3e3 (B), sAFH-3e11 (C), and sAFH-3f11 (D) scFv with aflatoxin B1 generated using Gold software. Heavy chain, light chain, and linker are shown in green, pink, and yellow, respectively. Amino acid residues involved in the interactions with the AFB1 are labeled. Hydrogen bonds are shown as green dashed lines. Binding dispositions of sAFH-3e3 to AFB1 and yAFB1-c3 are similar. His-H33 and Gly-H103 of sAFH-3e3 form a $\pi$ interaction and carbon hydrogen bond, whereas Asn-H52 of yAFB1-c3 forms a hydrogen bond with AFB1 around the same area. For sAFH3e11 and sAFH-3f11, the amino acid residues involved in the interaction with AFB1 are similar but different from sAFH-3e3 and yAFB1-c3. Ser-H31 of sAFH3e11 form a carbon–hydrogen bond with AFB1, instead of Gly-H31 of sAFH 3f11.

clone, was located in the binding pocket and appeared to be important for hydrophobic interactions with AFB1. The contribution of both hydrogen-bonding and hydrophobic interactions for binding of AFB1 is in agreement with a previous report.[32] The mutations that were located outside the canonical antigen-contact loops suggested favorable allosteric effects that could not be predicted by 3D modeling as previously suggested.[26] Therefore, our result also confirmed



DOI: 10.1021/acs.jafc.8b01141
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that case-to-case analysis is necessary to understand the contribution of antibody chains to their binding properties.[33]

In conclusion, application of the chain-shuffling technique for improvement of the binding characteristic of human antiaflatoxin (yAFB1-c3) scFv antibody was demonstrated. The best-improved clone, which showed a 7.5-fold increase in the binding sensitivity, was obtained from the VL-biased, VH-shuffled library. Three-dimensional structure analysis indicated that the hydrogen-bonding and hydrophobic interactions of the variable region of the heavy chain play a major in the binding of the antibody to AFB1.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b01141.

Competitive ELISA of binders selected from the VH-shuffled library; SDS-PAGE of improved scFv fragments (PDF)

## ■ AUTHOR INFORMATION


### Corresponding Author

*Tel: +66 44 224388. Fax: +66 44 224154. E-mail: montarop.g@sut.ac.th.

### ORCID

Montarop Yamabhai: 0000-0003-2674-2419



### Funding

This work was supported by Grants from Suranaree University of Technology (SUT) and Agricultural Research Development (Public Organization) [ARDA], grant no. CRP550701085. K.R. was supported by a SUT and Technology grant (TSA Sandwich) from the Austrian Agency for International Cooperation in Education and Research (OeAD-GmbH) grant, no. ZI.:ICM-2013-05756.


### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS


We thank Romer Laboratories Co., especially Barbara Cvak and Julia Mayer, in Tulln, Austria, for the facility and expertise that involve aflatoxin analysis. We also thank Dr. Jenny Fitzgerald, Dr. Paul Leonard, and Dr Hui Ma from Dublin City University for kind technical support.


## ■ REFERENCES


(1) Wild, C.; Miller, J.; Groopman, J. *Mycotoxin control in low- and middle-income countries*; IARC Working Group Report No. 9; International Agency for Research on Cancer: Lyon, France, 2015; available from https://www.ncbi.nlm.nih.gov/books/NBK350558/.

(2) Singh, M. K.; Srivastava, S.; Raghava, G. P.; Varshney, G. C. HaptenDB: a comprehensive database of haptens, carrier proteins and anti-hapten antibodies. *Bioinformatics* **2006**, *22*, 253−255.

(3) Janeway, A. C.; Travers, P.; Walport, M.; Shlomshik, J. M. *Immunobiology*; Garland Publisher: New York, 2008.

(4) Finlay, W. J.; Bloom, L.; Cunningham, O. Phage display: a powerful technology for the generation of high specificity affinity reagents from alternative immune sources. *Methods Mol. Biol.* **2011**, *681*, 87−101.

(5) Sidhu, S. S. *Phage Display in Biotechnology and Drug Discovery*; Taylor and Francis: Boca Raton, London, New York, Singapore, 2005; Vol. *748*.

(6) Irving, R. A.; Kortt, A. A.; Hudson, P. J. Affinity maturation of recombinant antibodies using *E. coli* mutator cells. *Immunotechnology* **1996**, *2*, 127−143.

(7) Sheedy, C.; MacKenzie, C. R.; Hall, J. C. Isolation and affinity maturation of hapten-specific antibodies. *Biotechnol. Adv.* **2007**, *25*, 333−352.

(8) Fitzgerald, J.; Leonard, P.; Darcy, E.; Danaher, M.; O'Kennedy, R. Light-chain shuffling from an antigen-biased phage pool allows 185-fold improvement of an anti-halofuginone single-chain variable fragment. *Anal. Biochem.* **2011**, *410*, 27−33.

(9) Moghaddam, A.; Løbersli, I.; Gebhardt, K.; Braunagel, M.; Marvik, O. J. Selection and characterisation of recombinant single-chain antibodies to the hapten Aflatoxin-B1 from naïve recombinant antibody libraries. *J. Immunol. Methods* **2001**, *254*, 169−181.

(10) Marks, J. D.; Griffiths, A. D.; Malmqvist, M.; Clackson, T. P.; Bye, J. M.; Winter, G. By-passing immunization: building high affinity human antibodies by chain shuffling. *Nat. Biotechnol.* **1992**, *10*, 779−783.

(11) Kensler, T. W.; Roebuck, B. D.; Wogan, G. N.; Groopman, J. D. Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. *Toxicol. Sci.* **2011**, *120*, S28−S48.

(12) Rangnoi, K.; Jaruseranee, N.; O'Kennedy, R.; Pansri, P.; Yamabhai, M. One-step detection of aflatoxin-B(1) using scFv-alkaline phosphatase-fusion selected from human phage display antibody library. *Mol. Biotechnol.* **2011**, *49*, 240−249.

(13) Pansri, P.; Jaruseranee, N.; Rangnoi, K.; Kristensen, P.; Yamabhai, M. A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnol.* **2009**, *9*, 6.

(14) Kristensen, P.; Winter, G. Proteolytic selection for protein folding using filamentous bacteriophages. *Folding Des.* **1998**, *3*, 321−328.

(15) Lefranc, M.-P. IMGT-ONTOLOGY and IMGT databases, tools and Web resources for immunogenetics and immunoinformatics. *Mol. Immunol.* **2004**, *40*, 647−659.

(16) Retter, I.; Althaus, H. H.; Münch, R.; Müller, W. VBASE2, an integrative V gene database. *Nucleic Acids Res.* **2004**, *33*, D671−D674.

(17) Kay, B. K.; Winter, J.; McCafferty, J. *Phage display of peptides and proteins: a laboratory manual*. Academic Press: 1996.

(18) Biasini, M.; Bienert, S.; Waterhouse, A.; Arnold, K.; Studer, G.; Schmidt, T.; Kiefer, F.; Cassarino, T. G.; Bertoni, M.; Bordoli, L.; Schwede, T. SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res.* **2014**, *42*, W252−W258.

(19) Schrödinger, L. The PyMOL Molecular Graphics System. *The PyMOL Molecular Graphics System*, Version 1.8;, Schrödinger, LLC, 2016.

(20) Jones, G.; Willett, P.; Glen, R. C.; Leach, A. R.; Taylor, R. Development and validation of a genetic algorithm for flexible docking. *J. Mol. Biol.* **1997**, *267*, 727−748.

(21) Biovia, D. S. Discovery studio modeling environment. *Discovery studio modeling environment*; Dassault Systemes, San Diego, CA, 2015.

(22) Schier, R.; Marks, J. D. Efficient in vitro affinity maturation of phage antibodies using BIAcore guided selections. *Hum. Antibodies* **1996**, *7*, 97−105.

(23) Morea, V.; Tramontano, A.; Rustici, M.; Chothia, C.; Lesk, A. M. Conformations of the third hypervariable region in the VH domain of immunoglobulins. *J. Mol. Biol.* **1998**, *275*, 269−294.

(24) Xu, J. L.; Davis, M. M. Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* **2000**, *13*, 37−45.

(25) Park, S. G.; Lee, J. S.; Je, E. Y.; Kim, I. J.; Chung, J. H.; Choi, I. H. Affinity maturation of natural antibody using a chain shuffling technique and the expression of recombinant antibodies in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **2000**, *275*, 553−557.

(26) Min, W. K.; Kim, S. G.; Seo, J. H. Affinity maturation of single-chain variable fragment specific for aflatoxin B(1) using yeast surface display. *Food Chem.* **2015**, *188*, 604−611.

(27) Yang, W.-P.; Green, K.; Pinz-Sweeney, S.; Briones, A. T.; Burton, D. R.; Barbas, C. F., III CDR walking mutagenesis for the

affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. *J. Mol. Biol.* **1995**, *254*, 392−403.
(28) Lopez-Requena, A.; Rodriguez, M.; de Acosta, C. M.; Moreno, E.; Puchades, Y.; Gonzalez, M.; Talavera, A.; Valle, A.; Hernandez, T.; Vazquez, A. M.; Perez, R. Gangliosides, Ab1 and Ab2 antibodies II. Light versus heavy chain: An idiotype-anti-idiotype case study. *Mol. Immunol.* **2007**, *44*, 1015−1028.
(29) He, T.; Wang, Y.; Li, P.; Zhang, Q.; Lei, J.; Zhang, Z.; Ding, X.; Zhou, H.; Zhang, W. Nanobody-based enzyme immunoassay for aflatoxin in agro-products with high tolerance to cosolvent methanol. *Anal. Chem.* **2014**, *86*, 8873−8880.
(30) Rajpal, A.; Beyaz, N.; Haber, L.; Cappuccilli, G.; Yee, H.; Bhatt, R. R.; Takeuchi, T.; Lerner, R. A.; Crea, R. A general method for greatly improving the affinity of antibodies by using combinatorial libraries. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 8466−8471.
(31) Krykbaev, R. A.; Liu, W. R.; Jeffrey, P. D.; Margolies, M. N. Phage display-selected sequences of the heavy-chain CDR3 loop of the anti-digoxin antibody 26−10 define a high affinity binding site for position 16-substituted analogs of digoxin. *J. Biol. Chem.* **2001**, *276*, 8149−8158.
(32) Li, X.; Li, P.; Zhang, Q.; Li, Y.; Zhang, W.; Ding, X. Molecular characterization of monoclonal antibodies against aflatoxins: a possible explanation for the highest sensitivity. *Anal. Chem.* **2012**, *84*, 5229−5235.
(33) Al Qaraghuli, M. M.; Palliyil, S.; Broadbent, G.; Cullen, D. C.; Charlton, K. A.; Porter, A. J. Defining the complementarities between antibodies and haptens to refine our understanding and aid the prediction of a successful binding interaction. *BMC Biotechnol.* **2015**, *15*, 99.


DOI: 10.1021/acs.jafc.8b01141
*J. Agric. Food Chem.* 2018, 66, 5713−5722