# One-Step Detection of Aflatoxin-B1 Using scFv-Alkaline Phosphatase-Fusion Selected from Human Phage Display Antibody Library

**Article** *in* Molecular Biotechnology · April 2011

DOI: 10.1007/s12033-011-9398-2 · Source: PubMed

CITATIONS
14

READS
483

**5 authors**, including:

Kuntalee Rangnoi
Suranaree University of Technology
**7** PUBLICATIONS **123** CITATIONS
SEE PROFILE

Nanthanit Jaruseranee
Mae Fah Luang University
**7** PUBLICATIONS **215** CITATIONS
SEE PROFILE

Richard O'Kennedy
**52** PUBLICATIONS **1,568** CITATIONS
SEE PROFILE

Potjamas Pansri
**4** PUBLICATIONS **128** CITATIONS
SEE PROFILE

Mol Biotechnol (2011) 49:240–249
DOI 10.1007/s12033-011-9398-2

RESEARCH

# One-Step Detection of Aflatoxin-$B_1$ Using scFv-Alkaline Phosphatase-Fusion Selected from Human Phage Display Antibody Library

**Kuntalee Rangnoi · Nanthnit Jaruseranee · Richard O'Kennedy · Potjamas Pansri · Montarop Yamabhai**

Published online: 5 April 2011


**Abstract** A unique human phage display library was used to successfully generate a scFv to the highly carcinogenic toxin aflatoxin B1. Such an antibody has major potential applications in therapy and diagnostics. To further exploit its analytical capacity, the scFv was genetically fused to alkaline phosphatase, thereby generating a novel and highly sensitive self-indicating reagent. The performance of this reagent was further characterized, demonstrating its efficacy. The sensitivity of scFv-AP fusion was three-fold better than that of the scFv form. The ability of this human library to generate antibodies to a small hapten was clearly demonstrated and this is linked to its intrinsic diversity, which exceeds many existing conventional human libraries. Our results indicate that demography may influence the diversity of the repertoire of the library in terms of its capacity to generate antibodies to specific targets. Equally, the approach demonstrated should also be applicable for other haptens and larger antigens.

**Keywords** Phage display · scFv · Antibody engineering · Alkaline phosphatase · ELISA · Aflatoxin · Human · Hapten · Competitive · Recombinant antibody

**Abbreviations**

| | |
|---|---|
| AFB | Aflatoxin B |
| AFG | Aflatoxin G |
| AFM | Aflatoxin M |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| scFv | Single chain fragment variable |
| AP | Alkaline phosphatase |
| BAP | Bacterial alkaline phosphatase |
| $IC_{50}$ | Inhibitory concentration at 50% |
| ELISA | Enzyme-linked immunosorbent assay |

## Introduction

Haptens are low molecular weight molecules that cannot elicit an immune response unless they are coupled to appropriate carriers [1]. Examples of common haptens include pesticides, herbicides, insecticides, drugs, vitamins, steroids, hormones, explosives, dyes, and toxins. Other haptens are reagents commonly used in biological research such as fluorescein, biotin, digoxigenin, and dinitrophenol [2]. Thus, obtaining antibodies that can interact specifically with these haptens will be very important and beneficial for various applications, such as immunoassays, immunobiosensors, immunotherapy, and affinity purification. The major difficulty related to generation of an antibody to haptens is to find the right conjugate, as the hapten is not intrinsically

K. Rangnoi · N. Jaruseranee · P. Pansri · M. Yamabhai (✉)
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, 111 University Avenue, Nakhon Ratchasima 30000, Thailand
e-mail: montarop@sut.ac.th; montarop@gmail.com

K. Rangnoi
e-mail: kuntaleerang@gmail.com

N. Jaruseranee
e-mail: nanthanitjaruseranee@gmail.com

P. Pansri
e-mail: potjamas@gmail.com

R. O'Kennedy
School of Biotechnology and National Centre for Sensor Research, Dublin City University, Dublin 9, Ireland
e-mail: richardokennedy@gmail.com

*Present Address:*
P. Pansri
Department of Molecular Biology, University of Aarhus, Science Park, Denmark

immunogenic [3]. Moreover, many antibodies that can bind very well to conjugated haptens, cannot recognize the soluble free form of the haptens, and this is required for diagnostic tests and certain therapeutic applications [4].

Aflatoxin $B_1$ or $AFB_1$ is an extremely potent and carcinogenic mycotoxin that is produced by *Aspergillus flavus* and *Aspergillus parasiticus*. These two strains of *Aspergillus* also produce other alflatoxins such as $AFB_2$, $AFG_1$, and $AFG_2$. However, they are not as abundant and toxic as $AFB_1$ [5]. Aflatoxin $M_1$ is a metabolite of $AFB_1$, which can be found in milk of animals that consume aflatoxin-contaminated feeds [6]. The structures of various aflatoxins are illustrated in Fig. 1. Aflatoxins are often found to contaminate agricultural products in Thailand and other countries in tropical and subtropical regions, where the surroundings are warm and humid, favoring the growth of aflatoxin-producing *Aspergillus* both during and post harvesting period [7–10]. AFB1 has been classified as Group 1 carcinogen by WHO-IARC (World Health Organization and International Agency for Research on Cancer) as it can lead to mutation in the gene that can result in the development of liver cancer [11]. Therefore, aflatoxin contamination is a serious problem and there is a very urgent need to develop a rapid, highly sensitive, reliable, and inexpensive assay to monitor agricultural products, to protect consumers and animals, and to meet regulatory requirements. There is also potential to use such antibodies in analyses of complex matrices where preliminary sample 'clean-up' and concentration is needed prior to analysis.

Many techniques have been developed to analyze aflatoxins such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), near infrared spectroscopy (NIR), and immunoassay [12]. Among these, immunological methods that use polyclonal or monoclonal antibody are appropriate and necessary for rapid, high-throughput, highly sensitive, and specific analysis.

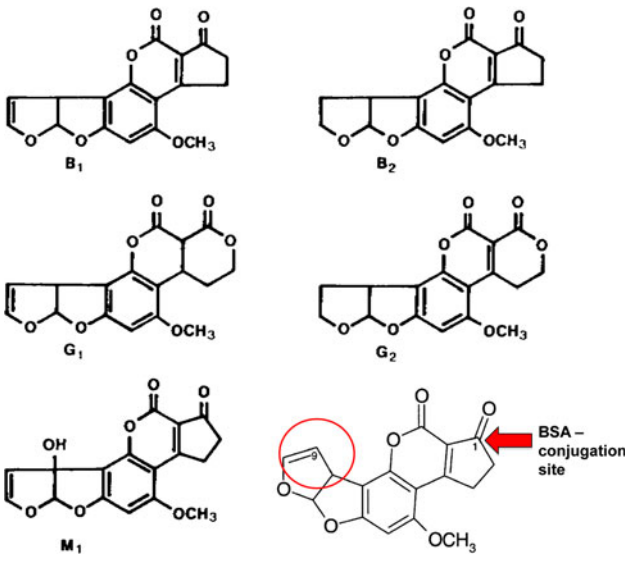


**Fig. 1** Structures of different types of aflatoxins. C1 position used for conjugation of $AFB_1$to BSA is indicated. The area around C9 where the scFv antibodies bind is highlighted with a circle. $AFM_1$ and $AFM_2$ are metabolic products of $AFB_1$ and $AFB_2$, respectively

This research reports the engineering and characterization of human single chain variable fragment (scFv) antibodies against $AFB_1$ from two human phage display antibody libraries. The first library is Yamo1, a compact human non-immunized phage display library generated from 140 healthy individuals in Northeastern Thailand [13], and the second library is Tomlinson I&J, a semi-synthetic library that is based on one framework but has different amino acids on 18 positions that are diverse in the primary repertoire. Binding properties of antibody selected from these two libraries were studied. In addition, selected scFv antibodies were further engineered to create scFv-alkaline phosphatase fusions (scFv-AP) and used as convenient one-step detection probes for competitive ELISA. The sensitivity and specificity of scFv and scFv-AP to different aflatoxins were determined.

## Materials and Methods

### Materials

Yamo 1, a human non-immunized scFv library, was constructed in our laboratory using B-lymphocytes from 140 healthy individuals in the Northeastern Thailand [13]. Tomlinson I&J, two similar semi-synthetic libraries, were kindly provided by the MRC, Cambridge. They are based on a single human framework and variation at 18 residues (H50, H52, H52a, H53, H55, H56, H58, H95, H96, H97, H98, L50, L53, L91, L92, L93, L94 and L96) that are responsible for antigen binding. KM13 helper phage was provided with the Tomlinson libraries and was propagated as described in the MRC phage display protocols. Aflatoxins B1 conjugated with BSA ($AFB_1$-BSA), and soluble aflatoxins B1, B2, G1, G2, and M1 were obtained from Sigma, Germany. *E. coli* TG1 and HB2151 were obtained from the MRC, Cambridge, UK, and used for cloning and amplification of phage, or production of soluble scFv fragments, respectively. The anti-M13/HRP detection kit was purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden), and Protein L peroxidase HRP was from Sigma. *E. coli* TG1 was used for cloning and expression of scFv-AP fusions.

### Biopanning

The selection was performed on Nunc Maxisorp immuno tubes (Nunc, Denmark). Various concentrations of

aflatoxin B1-BSA conjugate, in 100 µl phosphate buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$, pH 7.4) were used. The amounts of $AFB_1$-BSA conjugate used for the first, second, and third rounds of selection were 50 µg, 15 µg, and 5 µg per tube (4 ml), respectively. After overnight incubation (16–18 h) at 4 °C, the immune tubes were washed three times with PBS and blocked with PBS supplemented with 4% (w/v) skimmed milk (4%, w/v, MPBS) for 2 h at room temperature. After removal of the blocking solution, the tubes were washed three times with PBS. The libraries were pre-incubated with 4% (w/v) MPBS and 1% (w/v) BSA for 30 min at room temperature. The libraries were then added into the immuno-tubes and incubated at room temperature for 2 h. Unbound phage was washed away three times with PBS supplemented with 0.05% (v/v) Tween 20 (PBST) and three times with PBS. For the Tomlinson library, bound phage was eluted by adding 500 µl of 1 µg/ml trypsin buffer (in PBS) and incubated at room temperature for 20 min with rotation. For the Yamo1 library, an additional acid elution step was carried out after treatment with trypsin. Five hundred µl of 50 mM glycine–HCl, pH 2.0 was added into the tube and immediately after incubation for 10 min, 500 µl of neutralization buffer (200 mM $NaHPO_4$, pH 7.5) was added. The eluted phage was recovered by infecting 350 µl of exponentially growing *E. coli* TG1 and by incubation at 37 °C for 30 min without shaking. Infected cells were subjected to 10-fold-serial dilution ($10^2$–$10^6$) and spread on TYE (10 g tryptone, 5 g yeast extract, 8 g NaCl and 15 g bacto-agar in 1 L) agar plates supplemented with 100 µg/ml ampicillin and 1% (w/v) glucose. The agar plates (inverted) were incubated overnight at 37 °C.

For the Yamo1 library, one round of selection was sufficient to obtain specific binding phage, whereas for Tomlinson libraries, two more rounds of selection were performed. To continue with the next round of selection, 1 ml of 2 × YT (16 g tryptone, 10 g yeast extract and 5 g NaCl in 1 l) media was added on agar plates containing a lawn of infected bacteria, and the cells were loosened with a glass spreader. The scraped cells were kept as a 15% (v/v) glycerol stock at −70 °C, and 10 µl of scraped bacteria were added into 10 ml of 2 × YT supplemented with 100 µg/ml ampicillin and 1% (w/v) glucose, and incubated at 37 °C with shaking until the $OD_{600}$ was 0.4 (approximately 2 h). After this procedure, phage were was rescued by super-infecting the cells with $5 \times 10^{10}$ helper phage KM13 and incubated at 37 °C, without shaking, for 30 min. Afterward, the culture media was exchanged by centrifugation at 4,000 rpm at 4 °C for 15 min, the supernatant was removed, and the pelleted bacteria were resuspended in 5 ml of 2 × YT containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 0.1% (w/v) glucose. Later, it was incubated at 30 °C with shaking overnight. On the following day, the overnight culture was centrifuged at 4,000 rpm and 4 °C for 15 min. Phage was precipitated by adding 1 ml of PEG/NaCl (20% (v/v) polyethylene glycol 6000 in 2.5 M NaCl) into 4 ml of the supernatant and kept on ice for 1 h, and later centrifuged at 4,000 rpm, at 4 °C for 30 min. The supernatant was removed and the pellet was resuspended in 100 µl PBS for the next round of selection.

## Individual Phage Rescue

Individual phage-infected colonies were randomly picked from the TYE plate and grown in wells of a 96-well plate (Nunc, Denmark) containing 100 µl 2 × YT plus 100 µg/ml ampicillin and 1% (w/v) glucose. After overnight incubation at 37 °C, small inocula (5 µl) from each well were transferred to a second 96-well plate containing 200 µl of 2 × YT plus 100 µg/ml ampicillin and 1% (w/v) glucose. The first plate was kept as master stock by adding glycerol to a final concentration of 20% (v/v) and kept at −20 °C. The second plate was incubated with shaking at 37 °C for 2 h, and later phage was rescued by adding $10^{10}$ helper phage to each well. Following this they were then incubated at 37 °C for 1 h before centrifugation of the plate at 4,000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 200 µl of 2 × YT containing 100 µg/ml ampicillin and 50 µg/ml kanamycin, and cultured at 30 °C overnight (20 h) with shaking (250 rpm). The overnight culture was spun at 4,000 rpm for 10 min, and 50 µl of the supernatant-containing phage was used in monoclonal phage ELISA.

## Phage ELISA

The wells of 96 MicroWell™ plates (Nunc, Denmark) were coated with 5 µg of aflatoxin B1-BSA in PBS buffer, or 1% (w/v) bovine serum albumin (BSA) in 100 µl PBS (control). After incubation at 4 °C overnight, the plates were washed three times with PBS. The wells were then blocked with 4% (w/v) MPBS for 2 h at room temperature. The wells were re-washed three times with PBS. Fifty µl of phage supernatant and 50 µl of 4% (w/v) MPBS were added to each well and incubated at room temperature for 2 h. Unbound phage was removed by washing three times with PBST and 3 times with PBS. Subsequently, 100 µl of HRP-labeled anti-M13 (1:5000 dilution in 2% (w/v) MPBS) was added into each well. After incubation for an additional 1 h at room temperature, the wells were washed again, as described previously, and 100 µl of substrate solutions (TMB or ABTS, Sigma) were added into each well and incubated at 37 °C for 30 min. The resulting absorbance was read at 450 nm for TMB or 405 nm for ABTS.

### Expression and Purification of scFv Antibody in Microtiter Plates

*Escherichia coli* non-suppressor strain HB2151, which reads the amber codon as a stop signal, thereby solubly expressing the antibody fragments, was infected with individual phage clones to express soluble scFvs. Five µl of individual phage particles from the master glycerol stock were mixed with 150 µl of exponentially growing *E.coli* HB2151 ($OD_{600} = 0.4$), and incubated at 37 °C for 30 min, without shaking. the solution. Infected bacteria were then streaked onto TYE agar plates containing 100 µg/ml ampicillin and 1% (w/v) glucose. The plates were incubated at 37 °C overnight. On the next day, single colonies were picked and cultured in the wells of a 96-well plate containing 100 µl of 2 × YT plus 100 µg/ml ampicillin and 1% (w/v) glucose.The culture was incubated with shaking at 37 °C overnight. On the next day, 8 µl of culture was transferred to another 96 deep-well plate containing 800 µl of 2 × YT plus 100 µg/ml ampicillin and 0.1% (w/v) glucose, and incubated with shaking at 37 °C until the $OD_{600}$ reached 0.9. Later, 100 µl of 2 × YT containing 100 µg/ml ampicillin and 9 mM isopropyl-$\beta$-d-thiogalactopyranoside (IPTG)was added to a final concentration of 1 mM IPTG. The incubation was continued at 30 °C with shaking overnight (18–20 h). The secreted antibody could be found in the supernatant. The first plate was kept as a master stock by adding glycerol to a final concentration of 15% (v/v) and was then stored at −70 °C.

### Large-Scale Production of Soluble scFv

Production of soluble scFv antibodies was carried out as previously described [13]. In some cases, the crude scFv products were purified by immobilized metal affinity chromatography (IMAC) according to the manufacturer's protocol (Qiagen, Germany)The resins were washed twice with washing buffer 1 (20 mM Tris; pH 8.0, 150 mM NaCl, 5 mM imidazole) and washing buffer 2 containing 20 mM imidazole before scFv fragments were collected with elution buffer (20 mM Tris; pH 8.0, 150 mM NaCl, 250 mM imidazole).

### ScFv Antibody ELISA

The procedure was similar to the phage ELISA method described previously, except that 50 µl of culture supernatant containing the scFv antibody and 50 µl 3% (w/v) BSA were added into each of the wells instead of individual phage clones. Bound scFv was detected by addition of 100 µl of peroxidase-labeled Protein L (1:5000 dilution in 3% (w/v) BSA) using TMB as substrate.

### Cloning and Expression of scFv-AP Fusion

The scFv gene from the phagemid vector was cut with *Nco* I and *Xho* I restriction enzymes and subcloned into pKP300ΔIII, an alkaline phosphatase fusion vector (Kay. B.K. unpublished). The integrity of the constructs was confirmed by automated DNA sequencing. To prepare scFv-alkaline phosphatase (scFv-AP) fusions, a single colony of *E. coli* TG1 harboring recombinant plasmid was inoculated into 10 ml of low phos media (3.75 g ammonium sulphate, 0.71 g sodium citrate dehydrate, 1.07 g KCl, 5.36 g Yeast extract, 5.36 g Hy-case SF casein hydrolysate, 7 mM $MgSO_4$, 14 mM glucose per liter, pH 7.3 (adjusted by addition of KOH)). The culture was allowed to grow for 20 h before the culture supernatant containing the secreted scFv-AP fusion product was collected and kept at 4 °C for no more than 1 week. No further purification is required for the assay.

### scFv-AP ELISA

Five µg of aflatoxin B1-BSA or 1% (w/v) BSA (control) in 100 µl PBS were immobilized into wells of an immuno 96 Wells™ plate. The plate was washed three times with Tris-buffered saline (TBS; 25 mM Tris–HCl, pH 7.5, 140 mM NaCl, 3 mM KCl) supplemented with 0.05% (v/v) Tween 20 (TBST) and blocked with 3% (w/v) BSA in TBS. After incubation for 1 h at room temperature, the wells were washed 5 times with TBST. Later, 50 µl of scFv-AP supernatant and 50 µl of 3% (w/v) BSA were added into each well and incubated at room temperature for 2 h. The plate was then washed five times with TBST. Subsequently, 100 µl *p*-Nitrophenyl phosphate substrate (pNPP; SIGMA, USA) was added to each well. After incubation for 20 min at room temperature, absorbance was measured at 405 nm.

### Competitive ELISA

Competitive or inhibition ELISA was performed as described in the normal ELISA methods, except that optimal amount of the phage particle, scFv or scFv-AP were pre-incubated with increasing amount of soluble $AFB_1$ ranging from 0.019 to 5.0 µg/ml before adding into previously coated, blocked, and washed wells of ELISA plates. For every assay, appropriate dilutions of scFv or scFv-AP that showed a linear relationship by direct ELISA (Fig. 3), was used.

### Determination of Cross-Reactivity

Selected scFv or scFv-AP antibodies were assayed for binding against a range of soluble aflatoxins; $B_1$, $B_2$, $G_1$,

$G_2$, and $M_1$. Stock solutions of aflatoxins were prepared in 100% (v/v) methanol and diluted using TBST containing 5% (v/v) methanol. The assays were performed following the competitive ELISA protocol, as described above, for each of the aflatoxins tested.

## Results

### Bio-Panning of Aflatoxin-Specific scFv Antibodies

A simple bio-panning procedure was carried out to select phage that displays specific antibody to soluble $AFB_1$ from two human phage display scFv libraries. The libraries were pre-incubated with 3% (w/v) BSA to minimize the possibility of non-specific binding to BSA since the $AFB_1$-BSA conjugate was used as an affinity selection target. For semi-synthetic libraries (Tomlinson I & J), three rounds of bio-panning was performed according to the manufacturer's protocol, whereas for a compact non-immunized Yamo1 library, we have previously reported that only one round of bio-panning was sufficient to obtain specific phage [13]. A summary of bio-panning results is listed in Table 1. Enrichment of $AFB_1$-specific phage after each round of affinity selection was observed for the Tomlinson libraries. After three rounds of affinity selection, $2.2 \times 10^5$ and $2 \times 10^6$ phage clones could be obtained from Tomlinson I and J, respectively. For Yamo1 library, $2.5 \times 10^2$ phage clones could be obtained after one round of selection. Confirmation by ELISA revealed that 71, 32, and 4 phage clones from Tomlinson I, J, and Yamo1, respectively, were authentic binders to $AFB_1$-BSA. The frequency of specific binders from Tomlinson and Yamo1 libraries was the same after one round of selection; however, none of the clones from round one of Tomlinson biopanning could bind to soluble $AFB_1$ in the next step.

### Specific Binding of scFv Antibody to Soluble AFB1

For diagnostic or therapeutic purposes, it is necessary to obtain antibody that can bind to free (soluble) $AFB_1$, and thus we performed competitive ELISAs to identify these scFv-secreting clones. Soluble scFv fragments were first generated by infecting selected phage clones into *E. coli* HB2151, which reads the amber stop codon (TAG) as a stop signal, allowing secretion of soluble scFv into the periplasm, upon induction with IPTG. From the selected phage clones tested, 19, 2, and 3 clones from Tomlinson I, J, and Yamo1, respectively, could be successfully induced for expression, and from these soluble scFv clones, only 3 and 1 clone from Tomlinson I, and Yamo1, respectively, showed specific competitive binding to soluble $AFB_1$ by competitive ELISA. Amino acid sequence analysis of selected scFv clones revealed that the three clones that were isolated from Tomlinson I (TomI-F6, TomI-H2, and TomI-E9) have an identical DNA sequence, thus there was one scFv fragment from each Tomlinson I and Yamo1 library that could be selected by our bio-panning procedure. These two clones (TomI-F6 and YM1-C3) were used for further study. Amino acid sequence analyses of these two scFv fragments are shown in Fig. 2. The TomI-F6 scFv antibody was based on a single human framework for VH (IGHV3-23*05, DP47) and V$\kappa$ (IGKVID-39*01, DPK9), which belong to subgroup VH3 and VK1, respectively. The positions of amino acid different from the germ lines are located in CDR2 and CDR3 regions of the VH and VL, respectively. The second scFv antibody, YM1-C3 was obtained from Yamo I. The VH and VL fragments were derived from germlines IGHVI-3*01 (DP25) and IGKVID-39*01 (DPK9), which belong to subgroup VH1 and VK1, respectively. There is no amino acid different from the germline gene in VH fragment, whereas there are four amino acid differences from germline gene in the framework region 1, 2, and 3 of VL fragment.

**Table 1** Overview of selection of antibodies against aflatoxin B1-BSA

| Results from each step | Tomlinson I | Tomlinson J | YAMO 1 |
|---|---|---|---|
| Number of clone after 1st panning | $3.2 \times 10^3$ | $4 \times 10^2$ | $2.5 \times 10^2$ |
| Number of clone after 2nd panning | $1 \times 10^5$ | $1.4 \times 10^5$ | – |
| Number of clone after 3rd panning | $2.2 \times 10^5$ | $2 \times 10^6$ | – |
| Number of positive phage clones after 1st round from ELISA | 6/96[a] | 8/96[a] | 4/56[a] |
| Number of positive phage clones after 3rd round from ELISA | 71/96[a] | 32/96[a] | – |
| Number of expressed soluble scFv | 19/62[a] | 2/34[a] | 3/4[a] |
| Number of scFv fragments that can bind soluble Aflatoxin $B_1$ | 3/8[a] | 0 | 1/3[a] |
| Number of different scFv clones | 1 | 0 | 1 |

[a] The number of positive clones/the number of tested clones

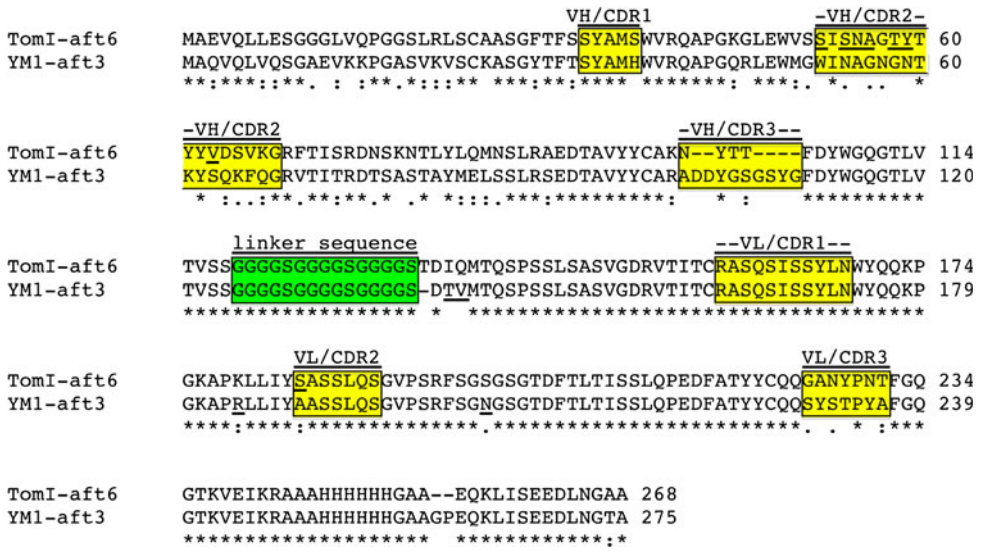


**Fig. 2** Amino acids sequence analysis The amino acid sequence of the whole scFv antibody fragments of TomI-F6 (TomI-aft6, *upper panel*) and YM1-C3 (YM1-aft3, *lower panel*) are shown. The GC-rich sequence that links $V_H$ and $V_L$ segments is indicated. The three complementarity determining regions (CDRs) are shown. The *underlined letters* are amino acids that are different from the germ line. Structure analysis was carried out based on IgBLAST software (http://www.ncbi.nlm.nih.gov/igblast/) and reconfirmed by DNA-PLOT from V BASE. (http://vbase.mrc-cpe.cam.ac.uk/). Sequence alignment of the two scFv antibodies was done using CLUSTALW 2.1 (http://www.ebi.ac.uk/Tools/msa/clustalw2/)

## Cloning and Characterization of scFv-Alkaline Phosphatase (scFv-AP) Fusions

To create a convenient one-step detection reagent for diagnostic purposes, we cloned the TomI-F6 and Yamo1-C3 scFv DNA fragments into an alkaline phosphatase (AP) expression vector. The scFv-AP fusions could be induced for expression in *E. coli* and secretion into the periplasmic space. Culture supernatant can then be used directly in ELISA as illustrated in Fig. 3. The signal was excellent and the background was low. Directed ELISA using $AFB_1$-BSA conjugated as a target revealed that the optical density using YM1-C3-AP fusion at 405 nm was 3-fold higher than that of the TomI-F6-AP. This result suggested that scFv-AP could be used as a convenient reagent for detecting soluble AFB1 by competitive ELISA.

To compare the binding properties of soluble scFv and scFv-AP, competitive ELISA was performed on the two forms of the YM1-C3 antibody. Various concentrations of free $AFB_1$ were pre-incubated with appropriate dilutions of scFv or scFv-AP before adding into well of an ELISA plate coated with the 3–5 μg of AFB1-BSA conjugate. Bound scFv or scFv-AP was detected by use of a protein-L-peroxidase, followed by TMB substrate, or pNP substrate, as appropriate. The $IC_{50}$ values (Fig. 4), being the concentration of soluble $AFB_1$ that results in 50% inhibition, for the scFv and scFv-AP fusion, were 0.12 and 0.04 μg/ml, respectively (Fig. 4). This result indicated that scFv-AP fusion has 3-fold higher sensitivity than soluble scFv form. Therefore, the scFv-AP fusion format was subsequently used to characterize the binding properties of the two scFv antibodies in the next step.

## YM1-C3 scFv-AP has Higher Binding Sensitivity than TomI-F6 scFv-AP

Competitive or inhibition ELISA was successfully used as an indirect method to estimate the binding affinity of an antibody [14]. In addition, it has also been used to determine binding constant ($IC_{50}$) of modular binding domain-AP fusions to peptide [15]. Thus, we performed competitive ELISA to estimate the binding affinity and compare the binding sensitivity of the two scFv fragments that were isolated from two different phage libraries, as shown in Fig. 5. Various concentrations of free $AFB_1$ wwere pre-incubated with the appropriate dilution of scFv-AP fusions and the ratio of OD of bound scFv-AP over the OD in an absence of free $AFB_1$ was plotted against the log of concentration of free $AFB_1$. The $IC_{50}$ values for YM1-C3 and TomI-F6 were 0.034 and 0.14 μg/ml, respectively, suggesting that the binding sensitivity of YM1-C3 is approximately 4 fold higher than that of TomI-F6. The useful assay range for YM1-C3 and TomI-F6 was between 0.007–0.2 and 0.04–1.0 μg/ml.

## Cross-Reactivity to Various Aflatoxins

To evaluate the binding specificity of the selected scFv antibody, we compared $IC_{50}$ values derived from inhibition ELISA of structurally related aflatoxins, i.e., B2, G1, G2,

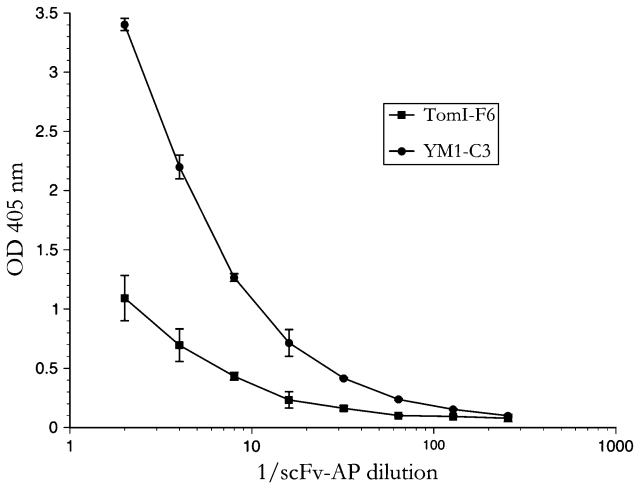


**Fig. 3** Direct ELISA of scFv-AP antibody fusions. Various 2-fold dilutions of culture supernatant containing scFv-AP fusions from ½ to 1/256 were assayed on an ELISA plate coated with $AFB_1$-BSA. Bound antibodies were detected by colorimetric assay with pNPP (p-Nitrophenyl Phospate) as substrate. The average $OD_{405}$ values and standard errors are shown. Antibody dilutions of 1/10 (TomI-F6) and 1/20 (YM1-C3), which was in the linear range of the curve, were chosen for competitive ELISA

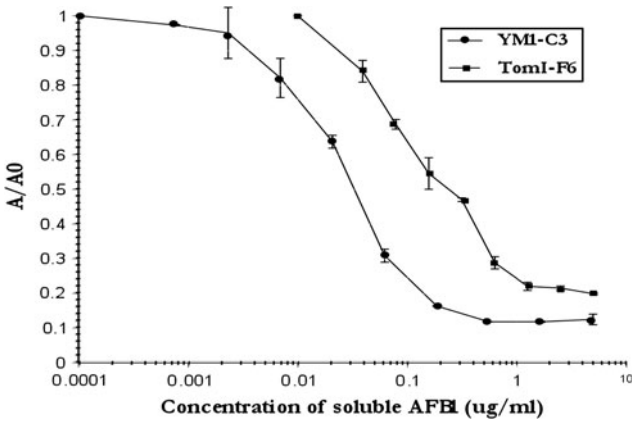


**Fig. 5** Comparison of binding properties of TomI-F6 and YM1-C3 scFv-AP antibodies. Various concentration of soluble $AFB_1$ from 5.0 μg/ml to 0.762 ng/ml were incubated with two scFv-AP (TomI-F6, YM1-C3) antibodies at 37 °C for 30 min before addition to wells of Immuno 96 MicroWell™ plates, coated with 5 μg $AFB_1$-BSA. The plates were washed five times with TBS-T after a 1 h incubation. Bound antibodies were demonstrated by colorimetric detection using the AP substrate, pNPP. The average absorbance at 405 nm and S.Ds are shown. Normalized absorbance values (expressed as $A/A_0$) were plotted against the logarithm of $AFB_1$ concentration. The $IC_{50}$ values for the TomI-F6-AP and YM1-C3-AP were found to be 0.14 and 0.034 μg/ml, respectively

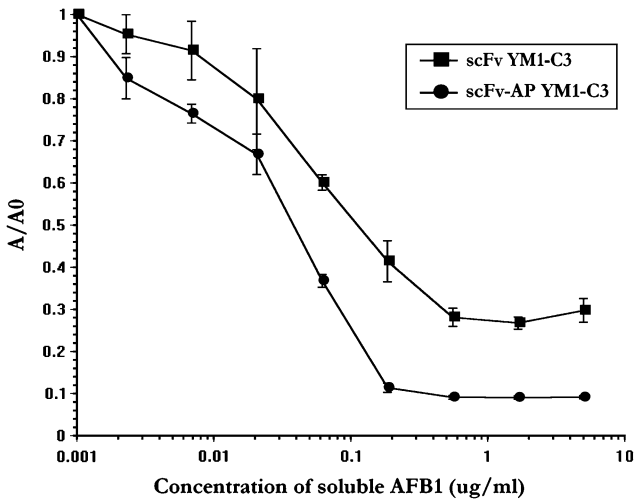


**Fig. 4** Comparison of binding properties of soluble scFv and scFv-AP antibody. Two formats of YM1-C3 scFv antibody were compared by competitive ELSIA. Optimal dilutions of soluble scFv or scFv-AP were incubated with equal volumes of various amounts of free $AFB_1$ in TBST containing 5% (v/v) methanol to reach a final concentration ranging from 2.3 ng to 5,000 ng/ml. The $IC_{50}$ values for the scFv YM1-C3, and YM1-C3-AP were found to be 0.12 and 0.04 μg/ml, respectively. The scFv-AP fusions showed improved binding sensitivity of approximately 3 fold better than the soluble scFv format

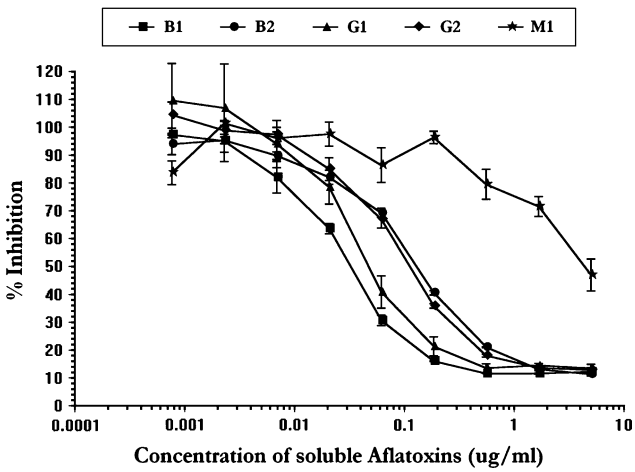


**Fig. 6** Cross-reactivity against different aflatoxins. Competitive ELISA of YM1-C3 scFv-AP antibody with various structurally related aflatoxins ($AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $AFM_1$) is demonstrated. Results were plotted as the mean percent inhibition vs. concentration of soluble aflatoxins. Percent inhibition of aflatoxin antibody was calculated from this type of competitive ELISA

and M1 as demonstrated for YM1-C3 scFv-AP in Fig. 6. Standards of each potential cross-reactive aflatoxins, ranging from 2.3 to 5,000 ng/ml in TBST containing 5% (v/v) methanol, were mixed with an equal volume of scFv-AP antibody at the optimal concentration, before incubation on wells of an ELISA plate that were coated with $AFB_1$-BSA conjugates. The cross-reactivity potential was approximated as the percent inhibition according to the equation: % inhibition = [(OD sample/OD control)] × 100. The percentage cross-reactivity determined at $IC_{50}$ (%CR50) was expressed as 50% inhibitory concentration of aflatoxin B1 divided by the 50% inhibitory

**Table 2** Percentage cross-reactivity values of the TomI-F6 and YM1-C3 scFv-AP antibody to various structurally related aflatoxins

| Aflatoxins | TomI-F6 | | YM1-C3 | |
|---|---|---|---|---|
| | $IC_{50}$ (ug/ml) | $CR_{50}$ (%) | $IC_{50}$ (ug/ml) | $CR_{50}$ (%) |
| $B_1$ | 0.22 | 100 | 0.035 | 100 |
| $B_2$ | 1.3 | 16.92 | 0.13 | 26.92 |
| $G_1$ | 0.32 | 68.75 | 0.05 | 70 |
| $G_2$ | 1.6 | 13.75 | 0.12 | 29.17 |
| $M_1$ | 1.7 | 12.94 | 4 | 0.88 |

The cross-reactivity potential was approximated at the $IC_{50}$ value, which was estimated at 50% $A/A_0$

concentration of other aflatoxins and multiplied by 100. Percentage cross-reactivity values of the TomI-F6 ad YM1-C3 scFv-AP antibodies to various potential cross-reactive aflatoxins are reported in Table 2. Both scFv-AP antibodies showed a high degree of cross-reactivity, especially to AFG1 > AFB2 > AFG2 > M1 (in decreasing order). The highest levels of cross-reactivity with aflatoxin G1 were 68.75% and 70% for TomI-F6 and YM1-C3, respectively. There is no significant difference in percent cross-reactivity against $AFB_2$, $AFG_2$, and $AFM_1$ for TomI-F6, whereas for YM1-C3, percent cross-reactivity to $AFB_2$ and $AFG_2$ were similar, i.e., 27 and 30%, respectively. However, YM1-C3 showed very low cross-reactivity (0.9%) to $AFM_1$, which is a metabolite form of aflatoxin $B_1$ found in milk. These results are in accordance with those obtained from using soluble scFv forms (data not shown).

## Discussion

Phage display is a powerful method to generate human monoclonal antibodies. This research reports the advantage of using a phage display antibody library generated from a non-immunized population that had previously experienced high exposure to certain antigens of interest, for the selection of a specific and sensitive antibody. In addition, we also demonstrated that the alkaline phosphatase fusion of scFv antibody (scFv-AP) is an attractive format for the detection of hapten by inhibition ELISA. Since the antibody is human, its potential application encompasses therapeutic purposes, such as the treatment of acute toxicity or drug addiction [16, 17].

Since obtaining specific antibody to free hapten is often very difficult with either naïve libraries or libraries derived from immunized animals, many strategies have been developed to increase the chance of selecting specific antibodies from a phage display antibody library. These include modification of the elution step by competing with free hapten [18], alternative hapten-carrier conjugates [6], selection from a focused library that favors binding haptens (cavity library) [19], or selection from an immunized library [20]. However, in this study, we were able to isolate specific antibody by using a simple bio-panning procedure. The discrepancy of affinity selection results from various publications could be due to differences in the libraries used. Biopanning from Yamo1, a human non-immunized library that is generated from a large population, including those that are highly exposed to mycotoxin, could increase the likelihood of obtaining highly specific antibody. Therefore, our results suggested that some of these Thai people, whose B-cells are the source of library, have mounted an immune response against this toxin. Indeed, there have been two previous reports indicating antibody activity against aflatoxin B1 in serum from individuals in Kenya who experience high exposure to AFB1 [21], and in Danish workers who are laboring in animal-feed processing plants [22]. Therefore, this finding should be applicable for other antigens such as other mycotoxins, pesticides, and viruses, to which certain populations are commonly exposed.

In general, the main reason for performing only a single round of biopanning is to maximize the diversity of binders selected. Increasing the number of panning rounds affects the outcome with regard to the number of weaker binders. The stronger binders usually overgrow and dominate in the final output of panning when multiple panning rounds are employed. Therefore the weaker binders, which may express unique characteristics, e.g., specificity for potentially relevant antigens or cross-reactivity to related toxins, are often lost during repeated panning rounds. To address this issue, only one round of panning was used in this study. Moreover, the Yamo library used was generated by using the KM13 helper phage, which could help in reducing the background of non-displaying antibody phage during biopanning [23]. Consequently, bona fide binders could be obtained after a single round of panning. Our result was a 'proof-of-principle' to demonstrate that the library was indeed capable of generating anti-hapten antibodies, and that the population used to make the library would have had exposure to the toxin. In addition, another advantage of obtaining antibody from a single round of panning is that the isolated antibody may be able to cross react with other related toxins (aflatoxin G1, B2, G2) with similar structures more so than antibodies from the TomI library obtained after three rounds of panning. While it is possible to obtain the highest affinity and most specific scFvs by performing more rounds of biopanning, the affinity, stability, or specificity of the isolated antibody from a single round of panning can also be further improved by affinity maturation [4].

The scFv format has many advantages over other antibody formats, i.e., it is more efficient for display on phage

coats [24], it may pass through the blood–brain-barrier [25], and the library is more stable [26]. However, one key disadvantage of this format is the formation of dimers or higher order multimers [27]. This complicates standard affinity measurement by systems such as surface plasmon resonance (SPR) analysis. The formation of multimeric antibody molecules depends on the amino acid sequence of individual clones and the linker length, and can be unpredictable. Indeed, both of the two scFv antibodies described in this study did form several multimers as observed by SDS-PAGE analysis (data not shown). Purified monomeric scFv antibodies isolated by gel filtration chromatography could re-aggregate when left in solution.

Analysis of binding sensitivity by competitive ELISA revealed that the $IC_{50}$ of YM1-C3 was approximately 0.04 μg/ml or 40 ppb, whereas, the $IC_{50}$ of TomI-F6 was approximately 0.14 μg/ml or 140 ppb. These values are in the same range as previously published clones that were isolated from pre-immunized or naïve libraries [6, 18, 28]. The detection limit of YM1-C3 was 0.007 μg/ml (7 ppb). In several regions where aflatoxin contamination is prevalent, the acceptable limits of aflatoxin contamination in agricultural products are 20 ppb [29], thus, this YM1-C3-AP fusion could be applicable for analytical purposes.

Determination of cross-reactivity to structurally related aflatoxin (B2, G1, G2, and M1) revealed that YM1-C3 scFv could cross react with $AFG_1$ (70%), with slight cross-reactivity (~30%) with $AFB_2$ and $AFG_2$, but showed almost no binding to $AFM_1$. Judging from the structure of these aflatoxins (Fig. 1), it seems that the antibody interacts near the C9 position. These data are in accordance with previous reports that also used $AFB_1$-BSA conjugate as a target for affinity selection [18, 28], confirming that the type of conjugates used could dictate the outcome of affinity selection results. A wide specificity of antibody could be very useful in the following cases: (a) extraction of toxins from complex food or environmental matrices for analysis or in the purification of expensive food ingredients, (b) application as an antidote in food to sequester the toxin or to reduce potential toxicity, and (c) broad specificity toxin detection e.g., on an array.

One important outcome from this study is that we have demonstrated that the isolated antibodies could be further engineered to create a scFv-alkaline phosphatase fusion (scFv-AP) protein and this could be used as a convenient one-step detection probe for competitive ELISA. Bacterial alkaline phosphatase (BAP) is a homodimeric enzyme and fusions of various proteins or peptides to this enzyme have been previously demonstrated to be useful reagents for various detection formats [30]. Our results indicated that the sensitivity of scFv-AP fusion was approximately 3-fold better than the soluble scFv form and 20-fold better than the phage-displayed format [13]. This result is in accordance with previous reports that demonstrate higher specificity and sensitivity of peptide-AP fusion when compared to free biotinylated peptides or peptides that display on phage [31]. It is possible that the increased affinity is due to a higher proportion of the secreted AP-fused scFv being present in a correctly folded—and therefore active format than in the case of soluble scFv. The AP-fused proteins are likely to be dimeric, because AP itself is a homodimer, hence the binding avidity is increased. Ease of engineering, expression, and purification makes scFv-AP fusion antibodies attractive for large-scale production for the diagnostics industry.

Although the $IC_{50}$ or detection limit of the antibody in this study is comparable to other antibodies isolated from a naïve library, it is approximately ten times less sensitive than antibodies from some immunized mouse libraries [12]. However, it is important to note that this antibody is 100% human and it is not possible to directly immunize humans with toxins for ethical and safety reasons. Nevertheless, the sensitivity of these antibodies can be further improved by various methods of affinity maturation [4].

## Conclusions

Our results demonstrate the value of phage display antibody technology to engineer human recombinant antibodies for carcinogenic haptens such as aflatoxins. This knowledge should be applicable to a host of other haptens or antigens to which human populations may have been exposed. The library and approach used should be advantageous over other human library-based systems.

**Acknowledgments** This research was supported by National Research Council of Thailand (NRCT), Duo-Thailand Fellowship 2008, and Suranaree University of Technology. We would like to thank Jenny Fitzgerald for valuable advice.

## References

1. Shreder, K. (2000). Synthetic haptens as probes of antibody response and immunorecognition. *Methods, 20*, 372–379.
2. Singh, M. K., Srivastava, S., Raghava, G. P., & Varshney, G. C. (2006). HaptenDB: A comprehensive database of haptens, carrier proteins and anti-hapten antibodies. *Bioinformatics, 22*, 253–255.
3. Janeway, C. A., Travers, P., Walport, M., & Shlomchik, M. (2005). *Immunobiology* (6th ed.). New York: Garland Science Publishing.
4. Sheedy, C., MacKenzie, C. R., & Hall, J. C. (2007). Isolation and affinity maturation of hapten-specific antibodies. *Biotechnology Advances, 25*, 333–352.
5. Wogan, G. N. (1966). Chemical nature and biological effects of the aflatoxins. *Bacteriological Reviews, 30*, 460.
6. Daly, S., Dillon, P., Manning, B., Dunne, L., Killard, A., & O'Kennedy, R. (2002). Production and characterization of murine single chain Fv antibodies to aflatoxin B1 derived from a

pre-immunized antibody phage display library system. *Food and Agricultural Immunology, 14*, 255–274.
7. Ehrlich, K. C., Kobbeman, K., Montalbano, B. G., & Cotty, P. J. (2007). Aflatoxin-producing Aspergillus species from Thailand. *International Journal of Food Microbiology, 114*, 153–159.
8. Ruangwises, S., & Ruangwises, N. (2009). Occurrence of aflatoxin M1 in pasteurized milk of the School Milk Project in Thailand. *Journal of Food Protection, 72*, 1761–1763.
9. Tassaneeyakul, W., Razzazi-Fazeli, E., Porasuphatana, S., & Bohm, J. (2004). Contamination of aflatoxins in herbal medicinal products in Thailand. *Mycopathologia, 158*, 239–244.
10. Waenlor, W., & Wiwanitkit, V. (2003). Aflatoxin contamination of food and food products in Thailand: an overview. *Southeast Asian Journal of Tropical Medicine and Public Health, 34*(Suppl 2), 184–190.
11. RC, I. A. (1993). IARC monographs on the evaluation of carcinogenic risks to humans: Some naturally occurring substances food items and constituents heterocyclic aromatic amines and mycotoxins. *IARC Monographs, 56*, 245.
12. Li, P., Zhang, Q., & Zhang, W. (2009). Immunoassays for aflatoxins. *Trends in Analytical Chemistry, 28*, 1115–1126.
13. Pansri, P., Jaruseranee, N., Rangnoi, K., Kristensen, P., & Yamabhai, M. (2009). A compact phage display human scFv library for selection of antibodies to a wide variety of antigens. *BMC Biotechnology, 9*, 6.
14. Friguet, B., Chaffotte, A. F., Djavadi-Ohaniance, L., & Goldberg, M. E. (1985). Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *Journal of Immunological Methods, 77*, 305–319.
15. Yamabhai, M., Hoffman, N. G., Hardison, N. L., McPherson, P. S., Castagnoli, L., Cesareni, G., et al. (1998). Intersectin, a novel adaptor protein with two Eps15 homology and five Src homology 3 domains. *The Journal of Biological Chemistry, 273*, 31401–31407.
16. Eddleston, M., & Persson, H. (2003). Acute plant poisoning and antitoxin antibodies. *Journal of Toxicology: Clinical Toxicology, 41*, 309–315.
17. Peterson, E. C., Gunnell, M., Che, Y., Goforth, R. L., Carroll, F. I., Henry, R., et al. (2007). Using hapten design to discover therapeutic monoclonal antibodies for treating methamphetamine abuse. *Journal of Pharmacology and Experimental Therapeutics, 322*, 30–39.
18. Moghaddam, A., Løbersli, I., Gebhardt, K., Braunagel, M., & Marvik, O. J. (2001). Selection and characterisation of recombinant single-chain antibodies to the hapten Aflatoxin-B1 from naive recombinant antibody libraries, *Journal of Immunological Methods, 254*, 169–181.
19. Persson, H., Lantto, J., & Ohlin, M. (2006). A focused antibody library for improved hapten recognition. *Journal of Molecular Biology, 357*, 607–620.
20. Kalinichenko, A., Toporova, V., Panina, A., Aliev, T., Kryukova, E., Shemchukova, O., et al. (2010). Development and characterization of antibodies against aflatoxins. *Russain Journal of Bioorganic Chemistry, 36*, 114–123.
21. Autrup, H., Seremet, T., & Wakhisi, J. (1990). Evidence for human antibodies that recognize an aflatoxin epitope in groups with high and low exposure to aflatoxins. *Archives of Environmental Health, 45*, 31–34.
22. Autrup, J. L., Schmidt, J., & Autrup, H. (1993). Exposure to aflatoxin B1 in animal-feed production plant workers. *Environmental Health Perspectives, 99*, 195–197.
23. Kristensen, P., & Winter, G. (1998). Proteolytic selection for protein folding using filamentous bacteriophages. *Folding and Design, 3*, 321–328.
24. Sheets, M. D., Amersdorfer, P., Finnern, R., Sargent, P., Lindquist, E., Schier, R., et al. (1998). Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. *Proceedings of the National Academy of Sciences USA, 95*, 6157–6162.
25. Kasturirangan, S., & Sierks, M. (2010). Targeted hydrolysis of beta-amyloid with engineered antibody fragment. *Current Alzheimer Research, 7*, 214–222.
26. Hoogenboom, H. R., & Chames, P. (2000). Natural and designer binding sites made by phage display technology. *Immunology Today, 21*, 371–378.
27. Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., et al. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO Journal, 13*, 3245–3260.
28. Yang, L., Ding, H., Gu, Z., Zhao, J., Chen, H., Tian, F., et al. (2009). Selection of single chain fragment variables with direct coating of aflatoxin B1 to enzyme-linked immunosorbent assay plates. *Journal of Agriculture and Food Chemistry, 57*, 8927–8932.
29. Shephard, G. S. (2008). Risk assessment of aflatoxins in food in Africa. *Food Additives & Contaminants Part A: Chemistry, Analysis, Control, Exposure & Risk Assessment, 25*, 1246–1256.
30. Yamabhai, M. (2008). BAP-fusion: A versatile molecular probe for biotechnology research. In F. W. Richter (Ed.), *Biotechnology: Research, technology and applications* (pp. 327–345). Hauppauge, NY: NOVA Science Publishers, Inc.
31. Yamabhai, M., & Kay, B. K. (1997). Examining the specificity of Src homology 3 domain–ligand interactions with alkaline phosphatase fusion proteins. *Analytical Biochemistry, 247*, 143–151.