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Generation of human and rabbit recombinant antibodies for the detection of Zearalenone by phage display antibody technology

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ABSTRACT

This article reports the identification, engineering and characterisation of recombinant single chain variable fragment (scFv) antibody against Zearalenone (ZEN), an oestrogenic mycotoxin, using phage display antibody technology. To increase the chance of obtaining clones that can bind to free toxin, the conjugated proteins of the target antigen, i.e. bovine serum albumin ZEN-BSA and ovalbumin ZEN-OVA, were switched during the bio-panning. One phage-displayed scFv clone specific to free ZEN, designated yZEN2A8, could be isolated. The gene encoding the yZEN2A8 scFv was sub-cloned into the pET-21d (+) and pKP300 delta III vectors to generate the recombinant scFv and scFv-AP antibody formats, respectively. After ELISA optimisation by checkerboard titration, the sensitivities of the recombinant yZEN2A8 scFv antibody and scFv-AP fusion were improved approx. 2 and 60 folds, respectively. Competitive ELISA indicated that the median inhibition concentration (IC_{50}) of recombinant yZEN2A8 scFv antibody and scFv-AP fusion after ELISA optimisation were 90 and 14 ng mL^{-1}, with a limit of detection (LOD) of 20 and 2 ng mL^{-1}, respectively. No cross-reactivity to other common mycotoxins was observed. Homology modelling illustrated specific binding of the recombinant antibody to ZEN and demonstrated the role of complementary determining regions (CDRs) of both the variable heavy and light chains in antibody-antigen interactions. Efficient application of scFv-AP for the detection of ZEN contamination in corns and wheat samples were investigated for the first time. The antibody in the form of scFv-AP can be used as a prototype for the development of a convenient reagent for the detection of ZEN contamination in various format, including biosensor-based.

1. Introduction

Zearalenone (ZEN) is a nonsteroidal oestrogenic mycotoxin produced by several species of fungi belonging to the genus *Fusarium* [1]. It is insoluble in water and heat-stable, persisting in foods and feeds prepared from contaminated grains [2]. ZEN is classified as a type-III carcinogenic agent [3], it plays a major role in inducing reproductive toxicological effects such as pre-copious puberty [4,5] and may act as a key factor in certain pregnancy disorders in humans [6,7]. When fed to animals, the compound causes hyperoestrogenism with symptoms such as enlargement of the uterus and nipples, vulvar swelling and infertility [8]. Therefore, establishing a sensitive and convenient method to detect ZEN is very important for food safety issue.

Currently, several methods have been utilised for the analysis of ZEN, such as HPLC, TLC, LC-MS/MS and immunoassay [4,9–13]. The advantages offered by immunoassays over more classical analytical techniques include rapidity and ease-of-use. Many types of immunoassays have been developed including immunosensors, quantum dot-based detection, fluorescence polarisation and enzyme-link immunosorbent assay (ELISA) [8]. ELISA technique is simple but requires high sensitivity and a specific antibody against target antigens for detection. Conventionally, mycotoxin or hapten-specific antibodies are produced from hyperimmunised animals or hybridoma cell lines. However, the supply of polyclonal antibodies is limited with batch-to-batch variation, while monoclonal antibody production is technically demanding and sometimes not straightforward enough [14]. Recombinant antibody has potential to complement or replace hybridoma technology for the production of monoclonal antibodies because the long-term cost for the production of antibody would be lower and the antibody could be adopted for various biosensor formats, allowing easy

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access to larger consumers. Even if there have been some reports on the generation of a recombinant antibody against ZEN [8,15–19], so far there have been no commercial test kit that uses recombinant antibodies for the detection of ZEN in the market. Phage display technology represents one of the most powerful tools for the selection of a high affinity reagent [20] and has been recognised as a valuable alternative way for the generation of antibody [21], with a desired specificity via molecular evolution method [22].

This study is the first report on the identification, engineering and characterisation of recombinant single chain variable fragment (scFv) antibodies against ZEN from the human phage display antibody library (Yamo I) [23], on the contrary to previous reports on the generation of recombinant anti-ZEN using hybridoma created from pre-immunised mice. The binding properties of different formats of the recombinant antibodies were evaluated. The scFv gene was cloned into the expression vector for expression as a soluble scFv antibody in an *E. coli* B strain, engineered to promote disulfide bond formation in the cytoplasm, and as alkaline phosphatase (AP) fusion protein (scFv-AP). Binding properties of these recombinant antibodies and their applications for mycotoxin ZEN contaminations in food and feed raw materials are reported.

2. Materials and methods

2.1. Materials and bacterial strains

Phage display scFv library from non-immunised human, Yamo I [23] and immunised rabbit library, Bozmix I.2 [24], were constructed in our laboratory. KM13 helper phage was propagated as described in the MRC phage display protocol. Standard Zearalenone; ZEN (CH-01-S5), ZEN conjugated with Bovine albumin serum; BSA (CJ-01-BSA), ovalbumin; OVA (CJ-01-OVA) and keyhole limpet hemocyanin; KLH (CJ-01-KLH) were prepared from *Fusarium* species (Aokin, Germany). Standard Aflatoxin B1 (AFB1), Ochratoxin A (OTA A), Fumonisins (FUM) and Deoxynivalenol (DON) were purchased from Aokin (Berlin, Germany). Colorimetric substrates, 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and *p*-Nitrophenyl phosphate disodium salt hexahydrate (*p*NPP) were obtained from Amresco (USA). Mouse *anti*-M13-HRP, His probe-HRP and Protein L-HRP were purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden), Thermo Fisher Scientific (USA) and Sigma (Germany), respectively. *E. coli* strains TG1TR (suppressor) and HB2151 (supE, non-suppressor) were obtained from MRC, Cambridge, UK. *E. coli* B strain, engineered to promote disulfide bond formation in the cytoplasm (SHuffle® T7 Express) was purchased from New England Biolabs (NEB, Massachusetts, USA).

2.2. Biopanning of phage display scFv library against ZEN

For both non-immunised human Yamo I and immunised rabbit libraries, each round of biopanning was performed with an alternate selection strategy through the antigen conjugated with OVA and BSA, at a concentration of 20 and 15 μg mL^{-1} in phosphate buffer saline (PBS), respectively. The antigens were incubated at 4 °C overnight, followed by blocking with 2% (w/v) skim milk in wells of immune 96 micro well™ plate (Nunc, Denmark). After three washings with PBS, 100 μL of phage library (10^{11} pfu μL^{-1}) was added and incubated at room temperature for 1 h with shaking and standing on a bench for additional 1 h. For comparison, a biopanning method using single conjugate (200 μg mL^{-1} of ZEN-BSA in PBS) was performed. After incubation with the phage libraries, the unbound phages were removed by washing for 10 to 20 times with 0.05% (v/v) of Tween-20 in PBS (PBST), followed by 10 times with PBS, for each round of biopanning. The bound phages were eluted with 1 μg mL^{-1} trypsin buffer, followed by 0.2 M glycine-HCl, pH 2.0. The eluted phage was used to infect *E. coli* TG1 to obtain individual phage clones as previously described [25]. Biopanning was performed from 2 to 4 rounds.

2.3. Monoclonal phage ELISA

A single phage-infected bacterial colony from each round of biopanning was picked randomly and cultured in a 96-deep-well plate before super-infection with KM13 helper phage. After culturing for 16-18 h at 30 °C, phage supernatants were collected and subjected to ELISA to identify phage displaying monoclonal anti-ZEN scFv. Phage ELISA was performed using 2 μg $well^{-1}$ of ZEN-KLH in PBS as a target and 1% (w/v) BSA and 1% (w/v) KLH as negative controls, 150 μL of phage supernatant was added into each well and the bound phage displaying scFv was detected with horseradish peroxidase (HRP)-conjugated *anti*-M13 antibody (1:5000). The colour reaction was developed with ABTS reagent (Ameresco, USA). The reaction was quantified by measuring an absorbance at 405 nm.

2.4. Production of soluble scFv antibody

To confirm the binding of selected soluble scFv antibody, the *E. coli* non-suppressor strain, HB2151, was infected with selected phage as previously described [25]. Briefly, the infected *E. coli* HB2151 were cultured in 2xYT medium supplemented with 100 μg mL^{-1} of ampicillin and 0.1% (w/v) glucose at 30 °C with 250 rpm shaking until the OD_{600} of the culture reached 0.9. Then, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the cells were incubated on a shaker at 30 °C overnight. Supernatants containing extracellular soluble scFv antibodies were separated from the cell pellets by centrifugation at 4000 rpm for 15 min. The binding of secreted soluble scFv antibody to ZEN could be detected by scFv ELISA as previously described without further concentration [25].

2.5. DNA sequencing and molecular docking

Plasmids from positive phage clones were extracted using a DNA miniprep kit (Qiagen, Germany) and the DNA sequences were determined by automated DNA sequencing (Macrogen, Korea), using primers pMOD5': 5′ CAG GAA ACA GCT ATG ACC 3′, and pMOD3': 5′ CCC TCA TAG TTA GCG TAA CG 3'. The DNA sequence was analysed with IgBLAST and the complementarity determining regions (CDRs) 1, 2, 3 were identified with IMGT software. Homology modelling of the three-dimensional (3D) structures of yZEN2A8 scFv was generated from the amino acid sequences using the SWISS-MODEL website. The server chose the template by sequence identity analysis. After that, the sequence was processed by the server for modelling. Models were visualised with the program PyMOL (www.pymol.org). To understand the molecular interaction between ZEN and the scFv antibody, GOLD software was used to analyse antigen-antibody docking. A ZEN molecule (PubChem accession no. 5281576) was obtained from PubChem compound database (www.pubchem.ncbi.nlm.nih.gov). All scoring functions that were available for GOLD at the time of the study (ChemPLP, GoldScore, ChemScore, and Astex Statistical Potential (ASP)) were tested in separate runs. The active site for docking was defined according to the homology model of scFv at the position of NZ-atom of Lys175 within 6 Å radius. The best scored solution (ChemPLP) was considered and viewed in Discovery studio 2016 (BIOVIA, San Diego).

2.6. Cloning and expression of scFv antibody against ZEN

For larger scale production of soluble scFv antibody, the gene encoding scFv antibody clone yZEN2A8 was sub-cloned into pET-21d (+) vector (New England Biolabs, NEB, USA) by cutting the yZEN2A8 scFv DNA insert from the phagemid vector (pMOD) [23], located between the *Nco* I and *Not* I sites, using corresponding restriction enzymes and sub-cloned into corresponding site of the pET21d + expression vector, to generate pET21d+/yZEN2A8 plasmids. The integrity of the construct was confirmed by automated DNA sequencing (Macrogen,

Korea), using universal primer, i.e., T7 promotor and T7 terminator.

To express yZEN2A8 scFv, pET21d+/yZEN2A8 plasmid was transformed into *E. coli* SHuffle® T7 Express. After that, a single colony containing the pET21d+/yZEN2A8 plasmid was inoculated into 5 mL of LB media containing 100 μg mL^{-1} of ampicillin and cultured at 30 °C overnight. Four mL of overnight culture was inoculated into 400 mL of LB medium containing 100 μg mL^{-1} of ampicillin. Cells were cultured at 30 °C until the OD_{600} reach 0.9, before induction with 0.4 mM IPTG and further incubated at 25 °C for 16 h. This expression condition was an optimised condition for the expression of yZEN2A8 scFv, according to the standard guideline of *E. coli* SHuffle® T7 Express strain datasheet (New England Biolabs, NEB, USA).

To harvest and purify the recombinant antibody, the cell pellets were harvested by centrifugation at 8000 rpm for 10 min, then re-suspended in 20 mL of ice-cold lysis buffer (20 mM sodium phosphate, 500 mM NaCl and 20 mM imidazole, pH 7.4) containing 1 mg mL^{-1} of lysozyme. Cells were disrupted by intermittent sonication for 5 min on ice using 30s pulse and 30s break for cooling, followed by centrifugation at 4 °C for 30 min at 10,000 × *g*. The retained soluble fractions were further processed for protein purification. The supernatant was applied to the Ni-NTA column pre-equilibrated with the binding buffer (20 mM sodium phosphate, 500 mM NaCl and 20 mM imidazole, pH 7.4). The soluble scFv was eluted with 250 mM imidazole in an elution buffer (pH 7.4). Fractions containing yZEN2A8 scFv were pooled and exchanged by dialysis with PBS buffer at 4 °C. The samples were collected and kept at 4 °C. The soluble fraction and purity of the samples were assessed by SDS-PAGE. The binding affinities of soluble scFv antibodies were determined by scFv ELISA as described in section 2.7.

2.7. scFv ELISA

The immune 96 micro well™ plate (Nunc, Denmark) was coated with 2 μg $well^{-1}$ of ZEN-BSA, ZEN-OVA and ZEN-KLH; 1% (w/v) BSA, 1% (w/v) KLH and 2% (w/v) skim milk were used as negative controls. Detection of the bound scFv antibody was determined with 1:5000 dilution of His-Prop conjugated HRP catalog number 15165, Thermo Fisher Scientific (Rockford, lL, USA). The colour of the reaction was developed with ABTS reagent and an absorbance was measured at 405 nm.

2.8. Generation of scFv-AP (alkaline phosphatase) fusions

To generate yZEN2A8 scFv-AP fusion, the DNA fragment encoding yZEN2A8 scFv was sub-cloned into the pKP300 ΔIII vector [26]. The scFv-AP was expressed and analysed as previously described [25]. The scFv-AP from crude culture supernatant or cell lysate can be used directly for ELISA, no further purification or concentration is required for the assay.

2.9. Competitive ELISA

Competitive ELISA, using purified scFv, was performed to determine the sensitivity and specificity of the recombinant antibodies (scFv and scFv-AP). In addition, this method can be used to estimate the binding affinity of an antibody by measuring the binding constant (IC_{50}) [25]. Two μg of ZEN-BSA, ZEN-OVA and ZEN-KLH were coated on an ELISA plate. The procedure was conducted as described in normal ELISA except that scFv and scFv-AP antibodies were pre-incubated with various concentration of soluble ZEN, ranging from 0.0 to 5000.0 ng ml^{-1} before adding into wells of ELISA plate, previously coated with 2 μg of ZEN-BSA, ZEN-OVA and ZEN-KLH as previously described [25]. The binding constant or IC_{50} value was determined as the concentration of ZEN that led to 50% inhibition of the binding signal (A/A0 = 0.5).

2.10. ELISA optimisation

The optimum amount of ZEN-BSA, ZEN-OVA and ZEN-KLH required for coating onto wells of the microtiter plate, and the best working concentration of the recombinant yZEN2A8 scFv and scFv-AP were determined by checkerboard titration. In the titration procedure, various concentrations of ZEN-BSA, ZEN-OVA and ZEN-KLH, ranging from 0.01 to 2 μg $well^{-1}$ were diluted with PBS. The recombinant yZEN2A8 scFv antibody and scFv-AP fusion were 2-folded serially diluted from 1:10 to 1:1280 in PBST or TBST for scFv and scFv-AP, respectively. A set of experimental parameters including the concentration of ZEN-BSA, ZEN-OVA and ZEN-KLH and the dilution of anti-ZEN antibody was optimised in parallel. The optimal condition was used to determine IC_{50} value of anti-ZEN antibodies against free ZEN by competitive ELISA as described in section 2.10.

2.11. Cross reactivity of scFv-AP fusion by competitive ELISA

To test the specific binding of recombinant yZEN2A8 scFv-AP, competitive ELISA was performed against various concentrations of free AFB_1 (Alfatoxin B_1), OTA (Ochratoxin), FUM (Fumonisin) and DON (Deoxynivalenol). Stock solutions of AFB1, OTA, FUM and DON were diluted with TBST. The appropriate dilution of yZEN2A8 scFv-AP antibody were pre-incubated with different concentrations of soluble AFB1, OTA, FUM and DON, ranging from 4 to 5000 ng mL^{-1}. The competitive ELISA was done as described in section 2.10.

2.12. Detection of ZEN with contaminated corn and wheat samples

To investigate whether recombinant yZEN2A8 scFv-AP antibody could be used to detect ZEN contamination in agricultural or cereal products. Two grams of non-contaminated corns (MT-C-ND) and wheat (Z-WD-ND) were suspended in 10 mL of 70% (v/v) methanol, PBST or TBST. After that, the suspensions were mixed by vortex for 5 min, followed by the centrifugation at 4000 rpm for 5 min at 4 °C and filtered through a Whatman #1 filter. For extraction using 70% (v/v) methanol, the extracted samples were further diluted in PBST or TBST at a ratio of 1:3 and 1:5 for scFv or scFv-AP, respectively. Spiked experiments were performed by adding various concentration of ZEN (3000, 1000, 500, 300, 100, 50, 30 and 10 ng mL^{-1}) into the extracts of the reference mycotoxin-free samples. Then, 50 μL aliquots of the extract samples were mixed with an equal volume of the appropriate dilution of antibody before adding into wells of the microtiter plate previously coated with ZEN-OVA. An optimised amount of yZEN2A8 scFv-AP and immobilised targets, as determined from section 2.11, were used in the assay. The experiments were performed in triplicate.

In addition, reference samples of ZEN-contaminated corn at 454.2 ng mL^{-1} (Z-C-323) and wheat at 1085.5 ng mL^{-1} (Z-W-3306) were also used for the analysis. The procedures for the extraction of these samples and the assay were the same as described above.

3. Results and discussion

Affinity selection (Biopanning) of anti-ZEN antibodies from phage display scFv libraries.

Biopannings were performed against ZEN, with and without alternating the conjugated proteins, using non-immunised human and immunised rabbit phage-displayed scFv antibody libraries, as described in section 2.1. The biopanning results are shown in Tables 1 and 2 for human and rabbit scFv libraries, respectively. Enrichment of ZEN-specific phage after each round of affinity selection, as indicated by increasing numbers of phage clones obtained from each round of biopanning and the numbers of positive phage clones, could be observed only from method II, which involved switching of the conjugated proteins. These results confirmed previous observation that obtaining specific antibody to free hapten is often very difficult; therefore, various

Table 1
Biopanning results using non-immunised human library (Yamo I).

Rounds	Method I* (200 μg of ZEN conjugated BSA)	Method II* (Switching of conjugated protein)	Number of positive clones	
			Method I	Method II
1	1.18×10^3	5.88×10^2	–	3/96[a]
2	5.7×10^2	$2.08 \times 10^{4\#}$	–	16/96[a#]
3	$4.04 \times 10^{4\#}$	–	44/96[a]	–

*Number of phage clones obtained after each round of biopanning are reported.
#Enrichment of bound phage is indicated by an increase in the numbers of phage clones after each round of biopanning, and the number of positive phage clones tested by ELISA.
[a] The number of positive clones/the number of tested clones analysed by phage ELISA. The positive clones were defined as clones with an ELISA signal at least two-fold greater than the negative control. These clones were picked randomly from each round of biopanning.

Table 2
Biopanning results using the immunised rabbit library (Bozmix I.2).

Rounds	Method I* (200 μg of ZEN conjugated BSA)	Method II* (Switching of conjugated protein)	Number of positive clones	
			Method I	Method II
1	2.30×10^2	6.04×10^2	–	16/96[a]
2	480	$1.57 \times 10^{3\#}$	13/96[a]	4/80[a]
3	–	$1.34 \times 10^{5\#}$	–	–
4	–	2.98×10^3	–	30/96[a#]

*Number of phage clones obtained after each round of biopanning are reported.
#Enrichment of bound phage is indicated by an increase in the numbers of phage clones after each round of biopanning, and the number of positive phage clones tested by ELISA.
[a] The number of positive clones/the number of tested clones analysed by phage ELISA. The positive clones were defined as clones with an ELISA signal at least two-fold greater than the negative control. These clones were picked randomly from each round of biopanning.

strategies must be employed [27] and one of the effective methods demonstrated in this report is to switch the conjugated protein during the biopanning procedure.

The binding of selected phage clones obtained from both libraries was confirmed by phage ELISA against ZEN conjugated with different proteins, i.e. BSA, OVA and KLH as shown in Fig. 1. A total of 2 positive phage clones from immunised rabbit library were isolated, these are clones RC12 and RD4. In addition, 8 positive clones from the non-immunised human scFv library were isolated. There are clones YA1, YA7, YA9, YE11, YB1, HYG9, HYH10 and YA8. The reason why more phage clones were obtained from the non-immunised human antibody library could be because the size of human scFv antibody is slightly larger and it is generally difficult to produce rabbit scFv from the *E. coli* expression system [24,28].

3.1. Characterisation of soluble scFv produced in E. coli HB2151

To confirm the binding of free scFv antibodies, ten individual phage clones from both libraries obtained from the biopanning in section 3.1 were used to infect *E. coli* HB2151. In the non-suppressor strain of *E. coli* HB2151, the amber stop codon between scFv gene and gIII of phage coat protein was recognised as a stop codon, consequently soluble scFv fused with 6xHis and Myc tag were produced and secreted into the culture supernatants. Confirmation of specific binding by ELISA indicated that only scFv clones YA8, YB1 and HYH10 isolated from the human library could bind specifically to ZEN conjugated proteins (Fig. 2). None of the scFv clones from the rabbit library could bind to the target. These results confirmed previous biopanning results in section 3.1 and previous report that rabbit scFv is hard to obtain [29].

3.2. Amino acid sequence analysis and molecular docking of selected scFv clones

Three scFv clones which are bona fide binders, i.e., YA8, YB1 and HYH10 (Fig. 2) were chosen for DNA sequence analysis and it was revealed that all of them were identical with the amino acid sequence as indicated in Fig. 3D. Therefore, the isolated scFv antibody from this study was re-designated as yZEN2A8 (patent application submitted). Amino acid sequence analysis indicated that the yZEN2A8 scFv contained a variable heavy chain (VH) from family 5, which was derived from germ line V5-51*01 and a variable light chain (VL) from family λ, which was derived from germ line V1-44*01. The reason why the three positives clones that were obtained from human scFv phage display library showed the same sequence could be because the biopanning were performed for three rounds and the library has a compact size. Consequently, only the phage that displayed the anti-ZEN scFv that could grow well were selected and these are phage that display the same scFv antibody. However, even if this library is relatively small, but it was constructed from healthy people in North-eastern Thailand. It is highly possible that some of the healthy volunteers who have donated their blood for the construction of Yamo I library had previously exposed to ZEN, which is prevalent in the region, consequently a specific scFv antibody against ZEN could be obtained from this compact size library.

To further understand the nature of this scFv antibody, homology modelling of its 3D structure with the ligand was generated based on template; PDB code: 4BUH, at the resolution of 1.30 Å (Fig. 3). The percentage of sequence identity with the template was 80.08. This scFv antibody with a 15-amino acid long peptide, $(GGGGS)_3$, that linked the VH and VL domains was composed of antiparallel folded β-sheets connected by loops, displaying the characteristic variable regions of immunoglobulin folding. A favourable binding surface is formed and the CDRs of both the heavy and light chains are involved in antibody-antigen interactions as show in Fig. 3A–C. The interactions of scFv antibody with ZEN include two hydrogen bonds, one between the carboxyl of ZEN and Arg103 of CDRH3, and a second one between the other carboxyl of ZEN and Lys175 of CDRL3. In addition, hydrophobic interactions involving the Val111 of CDRH3 and Trp235 of CDRL3 were also observed. In the binding pocket of yZEN2A8 scFv, a deep cavity is highly complementary to the hapten ZEN (Fig. 3C). As indicated in Fig. 3A, both the heavy (green) and light (blue) chains contributed to antigen binding.

In summary, homology modelling indicated that the yZEN2A8 scFv antibody can form an appropriate cavity to embed the entire ZEN molecule and a steady-state complex could be formed through the interactions of hydrogen bonds and hydrophobic interactions. This information is valuable for further improvement of the antibody properties by a combination of site-directed mutagenesis, chain-shuffling and phage display technology [27,30].

3.3. Production and evaluation of anti-ZEN scFv and scFv-AP antibodies

While *E. coli* HB2151 is commonly used as a convenient host for the production of scFv antibody during the screening process; for larger scale production, expression of scFv from pET-based expression vector is more effective. The pET-21d(+)/yZEN2A8 plasmid, encoding 6xHis tagged scFv was expressed using *E. coli* B, which was engineered to promote disulfide bond formation in the cytoplasm by constitutively expression of disulfide bond isomerase DsbC in the bacterial chromosome in addition to trxB/gor mutations [31]. The cytoplasmic DsbC can also act as a chaperone that can assist the folding of proteins that do not require disulfide bond formation [32]. Hence proper disulfide bond formation and correct folding of yZEN2A8 scFv could be expected [33], allowing better binding performance of the scFv antibody by competitive ELISA as demonstrated in Fig. 4 (red and green lines). The size of purified scFv was approx. 29 kDa on SDS-PAGE (Fig. S1A). The binding

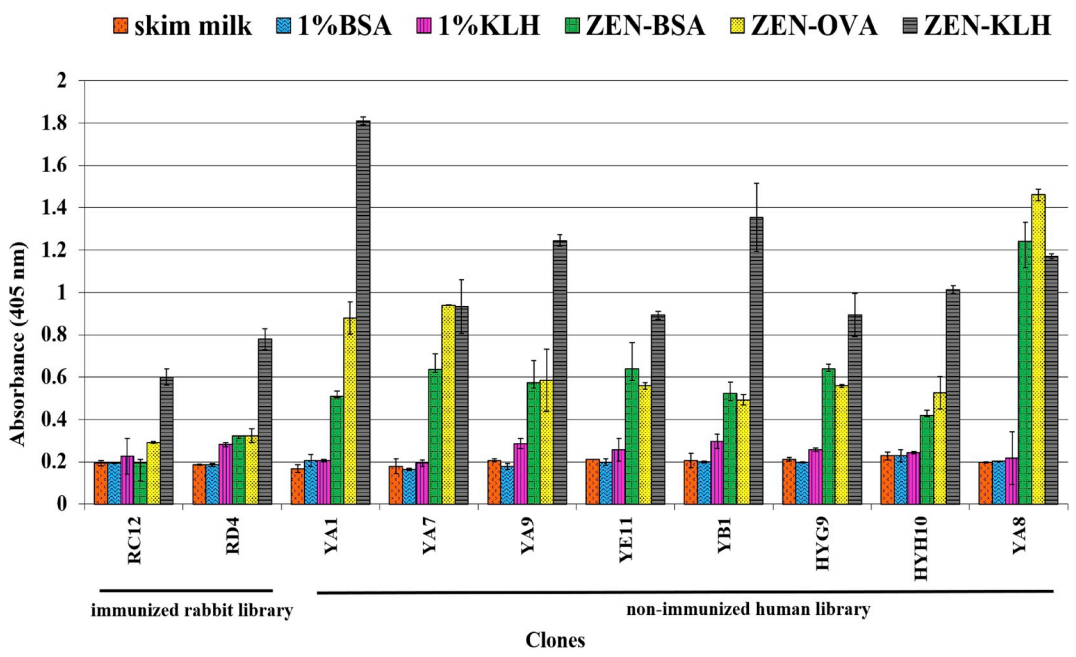

Fig. 1. Binding property of rabbit and human phage-displayed- scFv antibodies to ZEN. ZEN-BSA, ZEN-OVA and ZEN-KLH were immobilised on wells of microtiter plate. Negative controls are skim milk, 1% BSA and 1%KLH. Bound phage was detected with *anti*-M13-HRP conjugate. The data is expressed as average of triplicate measurement of absorbance at 405 nm with standard deviations.

of purified yZEN2A8 scFv to ZEN as shown by indirect ELISA against ZEN conjugated with various proteins (ZEN-BSA, ZEN-OVA and ZEN-KLH) are illustrated in Fig. S1B.

In addition to soluble scFv, an alkaline phosphatase (AP) fusion of antibody (yZEN2A8 scFv-AP) was created because this format can be used as a convenient one-step detection probe in ELISA [34]. Moreover, previous investigations have shown that the binding sensitive of scFv-AP against hapten is higher than those of free scFv format, due to the avidity effect [35]. The size of scFv-AP fusions was around 79 kDa (Fig. S1A). The application of yZEN2A8 scFv-AP as a one-step detection probe for the identification of ZEN conjugated to various proteins (ZEN-BSA, ZEN-OVA and ZEN-KLH) was demonstrated by direct ELISA (Fig. S1B). Competitive ELISA using scFv-AP as a one-step detection probe is demonstrated in Fig. 4 (blue line).

3.4. ELISA optimisation

To improve the binding property of the recombinant antibody against ZEN, the condition of ELISA was optimised by checkerboard titration to obtain the optimal targets and antibody concentrations for competitive ELISA. Various concentration of ZEN conjugated proteins (ZEN-BSA, ZEN-OVA and ZEN-KLH), ranging from 0.5, 0.1, 0.05, 0.02 and 0.01 μg well^{-1} were immobilised on wells of ELISA plates and incubated with serial dilutions of recombinant yZEN2A8 scFv and scFv-

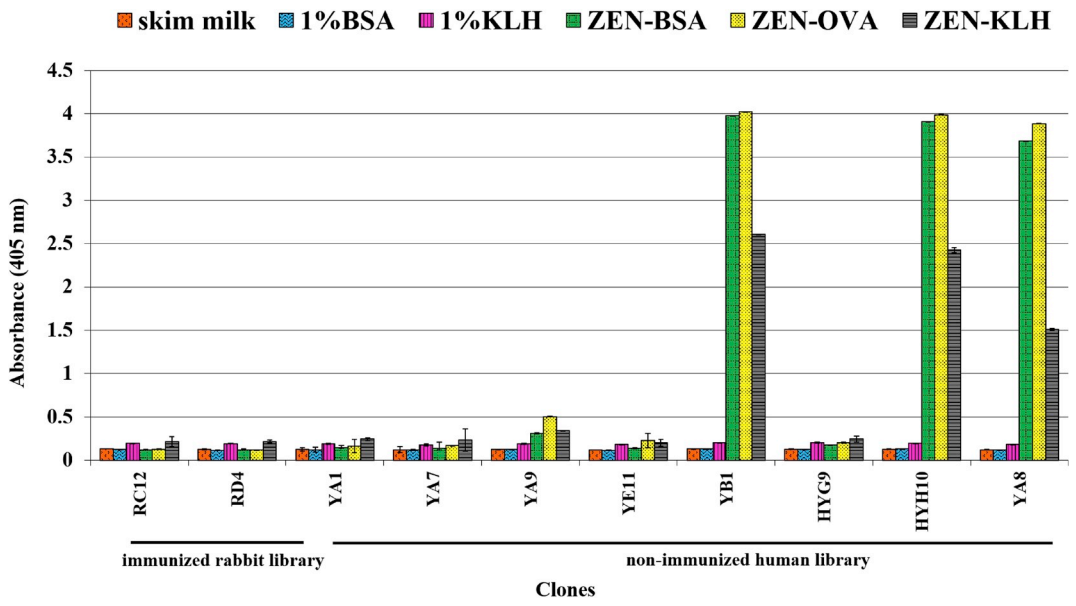

Fig. 2. Binding property of rabbit and human soluble scFv antibodies to ZEN. ZEN-BSA, ZEN-OVA and ZEN-KLH were immobilised on wells of microtiter plate. Negative controls are skim milk, 1% BSA and 1%KLH. Bound scFv were detected with His Probe-HRP. The data is expressed as average of triplicate measurement of absorbance at 405 nm with standard deviations.

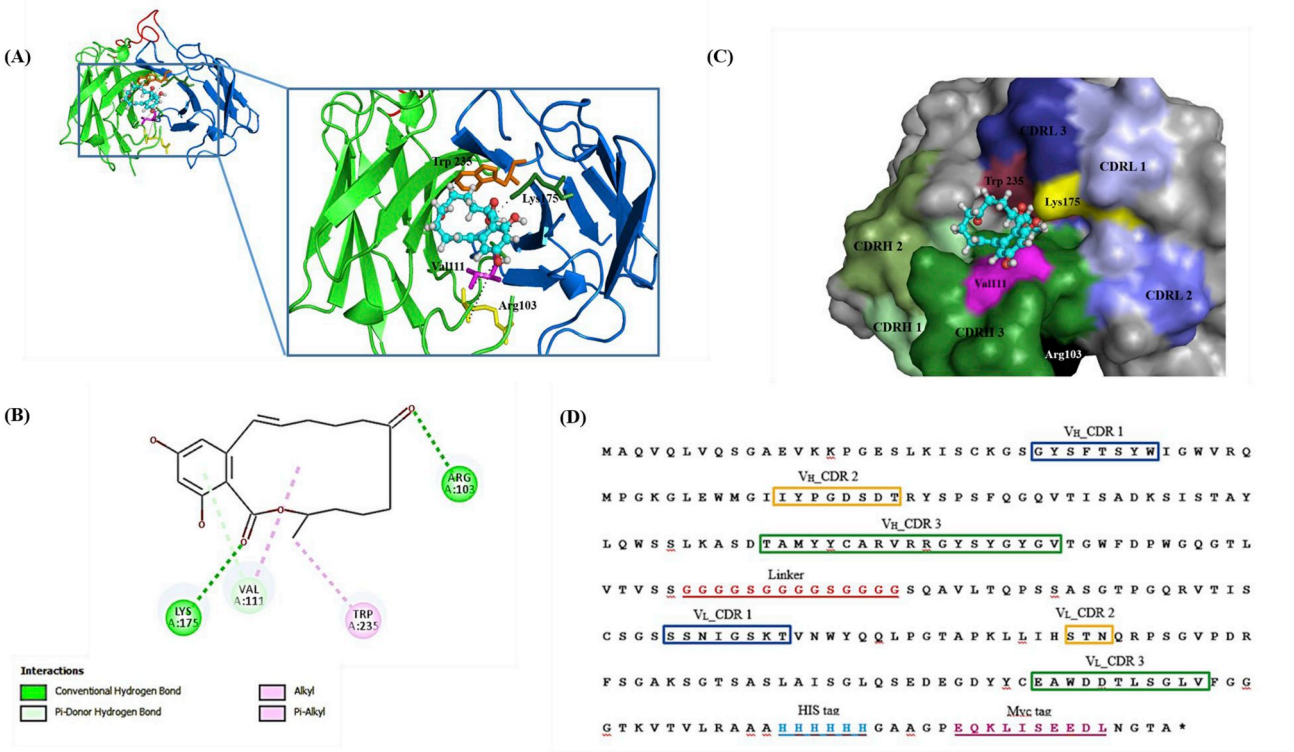

Fig. 3. Molecular docking model of yZEN2A8 scFv with ZEN and close-up views of the antibody and hapten interaction. The backbones of the heavy and light chains are shown in green and blue, respectively (A). Two-dimensional diagram of ligand (ZEN) interaction to yZEN2A8 scFv is depicted in panel B. Amino acid residues involved in interactions with the ligand are displayed as capped sticks. Panel C illustrates the binding pocket of the scFv antibody with ZEN. Amino acid sequence of the yZEN2A8 clone labelled with different regions are shown in panel D. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

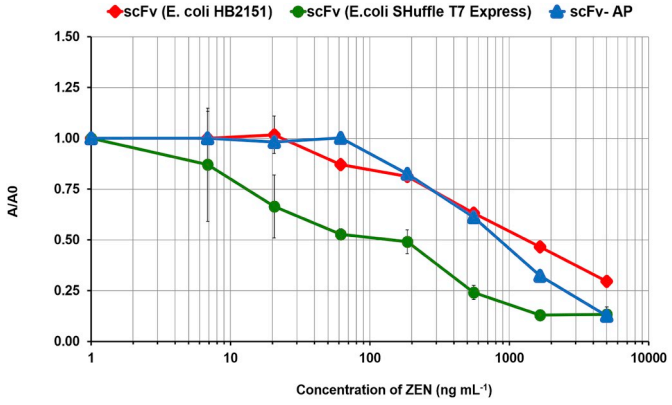

Fig. 4. Competitive ELISA of yZEN2A8 scFv and scFv-AP against ZEN-KLH. The binding properties of yZEN2A8 scFv, produced from *E. coli* HB2151 (red line) or *E. coli* SHuffle® T7 Express (green line); and yZEN2A8 scFv-AP fusion (blue line) were compared. The half-maximal inhibitory concentration (IC_{50}) value of the yZEN2A8 scFv antibodies produced from *E. coli* SHuffle® T7 Express and HB2151 were 200 ng mL^{-1}, and 1500 ng mL^{-1}, with a linear range from 20 and 100 to 1000 and 5000 ng mL^{-1}, respectively. For yZEN2A8 scFv-AP, the IC_{50} was 800 ng mL^{-1}, with a linear range from 100 to 5000 ng mL^{-1}. The data is expressed as average of triplicate measurement of absorbance at 405 nm with standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

AP. The checkerboard titration results are shown in Fig. S2 A–F. The conditions that led to the binding signals in the middle of the log-phase of the binding curves are considered optimal. A summary of optimised conditions when using various ZEN conjugates for both scFv and scFv-AP are shown in Table 3. The optimal conditions for different targets were different because the number of ZEN molecules conjugated to

Table 3
Optimised concentrations of immobilised ZEN conjugated proteins and anti-ZEN yZEN2A8 scFv and scFv-AP obtained from a checkerboard titration.

Recombinant antibody	ZEN conjugated proteins	Concentration per well (µg/well)	antibodies dilution
scFv antibody	ZEN-BSA	0.1	1:40
	ZEN-OVA	0.1	1:40
	ZEN-KLH	0.5	1:40
scFv-AP fusion	ZEN-BSA	0.05	1:40
	ZEN-OVA	0.05	1:40
	ZEN-KLH	0.1	1:100

different proteins are not equal [18].

After ELISA optimisation by checkerboard titration, competitive ELISA was performed again using the best conditions for each immobilised target as demonstrated in Fig. 5. A summary of the IC_{50} value after ELISA optimisation is shown in Table 4. The OVA-conjugates seemed to be the most appropriate target when compared with ZEN-BSA and ZEN-KLH for immobilisation. This could be because the molecular weight of OVA is the smallest; hence, the number of conjugated ZEN molecules on OVA was the lowest, when compare with the number of conjugated ZEN molecules on BSA and KLH, which were 35 and 94, respectively [18]. Therefore, the binding of antibody to ZEN-OVA could be inhibited at a lower concentration of free ZEN in the competitive ELISA. When using ZEN-OVA, the IC_{50} of recombinant yZEN2A8 scFv antibody and scFv-AP fusion were 90 and 14 ng mL^{-1}, with a limit of detection (LOD) of 20 and 2, ng mL^{-1}, respectively. The sensitivities were improved from 2 to 60 folds for yZEN2A8 scFv and scFv-AP formats, when compared with the result before ELISA optimisation, respectively. These results confirmed previous observation that alkaline phosphatase fusion of scFv antibody (scFv-AP) is more effective for the

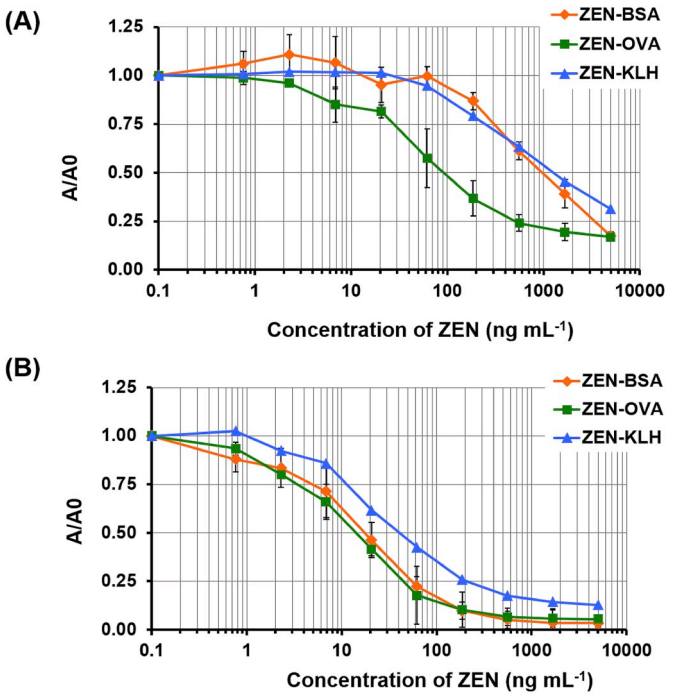

Fig. 5. Competitive ELISA after optimisation. Inhibition curves detected by using yZEN2A8 scFv followed by HisProbe-HRP (A) or scFv-AP as one-step detection probe (B) are shown. Three types of ZEN conjugates were compared, i.e., ZEN-BSA (orange/diamond line), ZEN-OVA (green/square line), and ZEN-KLH (blue/triangle line). The data is expressed as average of triplicate measurement of absorbance at 405 nm with standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

detection of hapten by competitive ELISA [25]. The results also underscore the important of ELISA optimisation to obtain appropriate dilution of detecting antibodies and amount of immobilised target for more efficient analysis of free toxin [36]. The IC_{50} shown here are comparable [8,15] or better [16,18] than previous reports using scFv from hybridoma in a competitive ELISA format. Improvement of sensitivity had been achieved by using surface plasmon resonance-based assay [18].

3.5. Cross-reactivity of scFv-AP fusion

Binding specificity of identified anti-ZEN antibody in this study was determined by competitive ELISA using yZEN2A8 scFv-AP and various concentrations of different free toxins, i.e. Aflatoxin B1 (AFB1), Ochratoxin A (OTA A), Fumonisins (FUM) and Deoxynivalenol (DON) as competitors. As demonstrated in Fig. 6, the binding of yZEN2A8 scFv-AP fusion to ZEN-conjugated to KLH was only reduced with increasing concentrations of ZEN but not soluble AFB1, OTA, FUM and

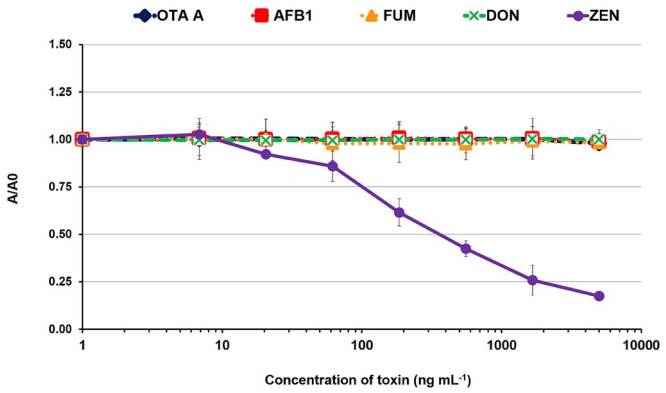

Fig. 6. Cross-reactivity analysis. Competitive ELISA were performed using yZEN2A8 scFv-AP in the presences of various concentrations of non-related prevalence mycotoxins as indicated. The data is expressed as average of triplicate measurement of absorbance at 405 nm with standard deviations.

Table 5
Competitive ELISA results of spiked corn and wheat extracts, detected with yZEN2A8 scFv and scFv-AP.

Antibody format	Samples	Buffers	IC_{50} value (ng mL^{-1})
scFv	Corn	70 (1:3 v/v) % MeOH	-
		70 (1:5 v/v) % MeOH	-
		PBST	1000
		TBST	–
	Wheat	70 (1:3 v/v) % MeOH	-
		70 (1:5 v/v) % MeOH	-
		PBST	-
		TBST	-
scFv-AP	Corn	70 (1:3 v/v) % MeOH	500
		70 (1:5 v/v) % MeOH	400
		PBST	300
		TBST	1000
	Wheat	70 (1:3 v/v) % MeOH	1000
		70 (1:5 v/v) % MeOH	800
		PBST	200
		TBST	700

DON, which are frequently present together with ZEN in agricultural samples, even when the concentrations were as high as 5000 ng mL^{-1}. These results indicated that the recombinant yZEN2A8 scFv-AP fusion can be used to detect ZEN, with high specificity. Notably, the results from this assay was performed without ELISA optimisation.

3.6. Assay validation

To confirm the applicability of recombinant yZEN2A8 scFv and scFv-AP for the analysis of ZEN contamination in real samples, corn and wheat reference materials were used in two types of experiments. In the first investigation, mycotoxin-free corn and wheat were extracted by

Table 4
IC_{50} values of clone yZEN2A8 scFv and scFv-AP before and after ELISA optimisation.

Form	Target(ug/well)	IC_{50}(ng/ml)	LOD (ng/ml)
scFv (*E. coli* HB2151)	2 µg of ZEN-KLH	1300	60
scFv (*E. coli* SHuffle® T7 Express)	2 µg of ZEN-KLH	190	20
scFv-AP	2 µg of ZEN-KLH	850	200
After ELISA optimisation			
scFv	0.1 µg of ZEN-BSA	900	200
	0.1 µg of ZEN-OVA	90	20
	0.5 µg of ZEN-KLH	1200	100
scFv-AP	0.1 µg of ZEN-BSA	18	9
	0.05 µg of ZEN-OVA	14	2
	0.05 µg of ZEN-KLH	40	10

Table 6
Competitive ELISA of contaminated samples using various extraction methods and detected with either scFv or scFv-AP.

Samples	Buffers	scFv antibody (% detected)	scFv-AP fusion (% detected)
Corn (454.2 ng mL^{-1})	70 (1:3 v/v)% MeOH Inter-assay (n = 6)	66.7%	85.7%
	70 (1:5 v/v)% MeOH Inter-assay (n = 6)	66.7%	63.3%
	PBST Inter-assay (n = 6)	83.3%	66.7%
	TBST Inter-assay (n = 4)	0%	33.3%
Wheat (1085.5 ng mL^{-1})	70 (1:3 v/v)% MeOH Inter-assay (n = 6)	50%	57.1%
	70 (1:5 v/v)% MeOH Inter-assay (n = 6)	66.7%	100%
	PBST Inter-assay (n = 6)	83.3%	100%
	TBST Inter-assay (n = 4)	0%	66.7%

different methods, i.e. 70% methanol, PBST and TBST. After that, the extracts were spiked with various concentrations of soluble ZEN (3000, 1000, 500, 300, 100, 50, 30 and 10 ng mL^{-1}). Extracts with methanol were further diluted 1:3 or 1:5 before spiking as described in section 2.13. The IC_{50} values obtained from the spiked corn and wheat using different extraction methods and detected with scFv or scFv-AP are summarised in Table 5. The anti-ZEN in an scFv-AP format clearly outperformed scFv. The best buffer for extraction was PBST, showing IC_{50} values of 300 ng mL^{-1} for corn extract and 200 ng mL^{-1} for wheat extract as shown in Fig. S3. These IC_{50} values ranged between 10 and 15 folds higher than when using PBST buffer in standard competitive ELISA, due to matrix interference from the presence of organic solvents solutions, lipids, vitamins or proteins in the sample as previously described [37,38]. The results also indicated that a variety of matrix from various samples could affect the sensitivity of the assay differently.

In the second experiment, agricultural samples with a known amount of ZEN contamination were used in the study. Reference corn and wheat samples contaminated with 454.2 and 1085.5 ng mL^{-1} of ZEN, respectively, which were available for purchase, were used in the study. Only qualitative detection for the presence of the toxins as judged by the reduction of the ELISA signal could be performed. A summary of qualitative analysis results is shown in Table 6. Four extraction methods were compared using both scFv and scFv-AP formats. While both scFv and scFv-AP could be used to detect the toxins, scFv-AP performance was far better as previously observed. The results showed that the most suitable extraction buffers for corn and wheat are 70% methanol (1:3 dilution) and PBST, respectively. Since wheat samples were contaminated with a higher concentration, the analysis was more accurate. The performance of this recombinant antibody complied with the standard of feed samples according to Thailand's regulation, of which the maximum allowance of contaminated corn and cereal in animal feed were set at 3000 or 2000 ng mL^{-1}, respectively (Standard Criteria for Animal Feed Quality Control Act, Thailand 2017). However, the limitation for general foods were set around 30–1000 ng mL^{-1} [39,40].

In conclusion, Phage display antibody technology was successfully employed for the generation of a prototype of recombinant antibody, yZEN2A8 scFv-AP, for specific detection of zearalenone in animal feeds, within the limit of Thailand's regulation. This yZEN2A8 recombinant scFv antibody could be used as a prototype for further improvement to comply with CODEX standard. This can be done by 1) affinity maturation of the recombinant antibody to increase the affinity [35], 2) increasing antibody stability for use in hot and humid climates, 3) optimisation of the extraction protocol, 4) bioprocess optimisation for large-scale production and 5) further engineering of the recombinant antibody to be used with biosensors, coupling with nanoparticles, or convert to a structure which can be used in a lateral flow assay format. Optimisation of the extraction protocol involves the clean-up of the extracted buffer to remove co-extracted materials that often interfere with the determination of target analyses will also lead to higher sensitivity of the assay [19]. Improvement of recombinant antibody can be done by various methods of antibody engineering [27]. These improvements will also allow quantitative measurement of the level of toxin contamination. The detection format can be in the form of rapid and simple test kit or biosensor-based, for commercialisation as point-of-care diagnostic of food, feed, and agricultural products in the fields. This is the first report that the recombinant antibody in an scFv-AP format, generated from human phage display antibody library, is highly efficient format for the detection of mycotoxin ZEN. Moreover, since this antibody has a human origin, it could be used for therapeutic purposes, such as in the case of acute toxicity of the toxin.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2019.04.034.

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