Development of immunoassays and immuno-affinity columns for the detection and isolation of mycotoxins

A thesis submitted for the degree of Ph.D.
by
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This thesis is dedicated to my lovely brother Ranjit Kumar and all the rest of my family members especially my daughters

Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Abstract

Mycotoxins are highly toxic in nature and are widely found in food and feed at levels above the permissible limits. The work described focuses on mycotoxin detection using a wide array of immunoassays with high sensitivity.

Polyclonal and two recombinant antibodies for specific detection of T-2 toxin, aflatoxin B_2 (AFB_2) and zearalenone (ZEN) respectively, were generated. Single chain antibody fragment (scFv) libraries were generated from spleenomic RNA of mice immunized with AFB_2 and ZEN conjugates. Polyclonal antibody (IgY) was isolated from eggs of T-2-KLH immunized chicken. Mono-specific IgY was isolated by affinity purification on T-2 toxin-immobilized amine-activated sepharose beads. Anti-AFB_2/ZEN scFvs and anti-T-2 IgY, highly specific to the target (with very low cross reactivity to other mycotoxins), were used to develop competitive ELISAs and SPR-based inhibition assays which were applied to relevant food matrices and had assay sensitivities below the EU regulatory action levels.

Novel separation and quantification techniques were developed using antibodies. A new method for the isolation of toxins was developed by covalent immobilization of highly specific antibody fragments onto silica particles, using an anti-AFB_1 Fab fragment and anti-AFB_2/ZEN scFvs. An ELISA assay, using covalently immobilized AFB_1 surfaces and an anti-AFB_1 Fab antibody fragment was optimised.

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Abbreviations

AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFM_2	Aflatoxin M_2
ATP	Adinosine triphosphate
AOAC	Association of Official Analytical Chemists
15-AcDON	15-acetyl deoxynivalenol
BSA	Bovine serum albumin
CE	Capillary electrophoresis
C_H	Constant heavy
C_L	Constant light
CDRs	Complementarity-Determining Regions
CMO	Carboxymethyloxime
CV	Coefficient of variance
DON	Deoxynivalenol
DAS	Diacetylscripenol
DCC	N,N′- Dicyclohexylcabodiimide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EU	European Union
ENN	Enniatins
ELISA	Enzyme-linked immunosorbent assay
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FAO	Food and Agricultural Organization of the United Nations
Fc region	Fragment, crystallisable region
Fab	Fragment, antigen binding
FP	Flourescence polarization
FITC	Fluorescein isothiocyanate
GC	Gas chromatography
HPLC	High performance liquid chromatography
HACCP	Hazard analysis and critical control points
HRP	Horseradish peroxidase

IARC	International Agency for Research on Cancer
IACs	Immuno-affinity columns
IC_{50}	Half maximal inhibitory concentration
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgE	Immunoglobulin E
IgY	Immunoglobulin Y
IgA	Immunoglobulin A
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KLH	Keyhole limpet hemocyanin
LC-MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MALDI-TOF	Matrix Assisted Laser Desorption Ionization - Time of Flight
MPL	Maximum permissible level
MON	Moniliformin
mRNA	Messenger ribonucleic acid
MIA	Magnetic immunoassay
M.Wt.'cut-off'	Molecular weight cut-off
NHS	*N*-hydroxysuccinimide
NIV	Nivelenol
OTA	Ochratoxin A
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
RNA	Ribonucleic acid
RIA	Radioimmunoassay
SOE	Splice overlap extension
scFv	Single-chain variable fragment
SPR	Surface plasmon resonance
TLC	Thin layer chromatography
TDI	Tolerable daily intake
UV	Ultraviolet
V_H	Variable heavy
V_L	Variable light
WHO/FAO	World Health Organization/Food and Agriculture

	Organization
ZEN	Zearalenone
α-ZOL	alpha-zearalenol
β-ZOL	beta-zearalenol
α-ZAL	alpha-zearalanol
β-ZAL	beta-zearalanol

Units

Å	Angstrom units
µg	Microgram
µL	Microlitre
cm	Centimetre
Da	Dalton
*k*Da	Kilodaltons
*k*b	Kilobase
L	Litre
M	Molar
mM	Millimolar
mL	Millilitre
min	Minute
ng	Nanogram
^{0}C	Degrees celcius
pfu	Plaque-forming units
rpm	Revolutions per minute
v/v	Volume per unit volume
w/v	Weight per unit volume
xg	Centrifugal acceleration
g	Gram

Publications and presentations

Publications

Soujanya Ratna Edupuganti, Om Prakash Edupuganti, Richard O'Kennedy, Eric Defrancq, Stéphanie Boullanger, Use of T-2 toxin-immobilized amine-activated beads as an efficient affinity purification matrix for the isolation of specific IgY, *Journal of Chromatography B*, 923-924 (2013), 98-101.

Soujanya Ratna Edupuganti, Om Prakash Edupuganti, Richard O'Kennedy, Biological and synthetic binders for immunoassay and sensor-based detection: generation and characterization of an anti-AFB_2 single-chain variable fragment (scFv), World Mycotoxin Journal, 3 (2013), 273-280.

Soujanya Ratna Edupuganti, Om Prakash Edupuganti, Stephen Hearty & Richard O'Kennedy, A highly stable, sensitive, regenerable and rapid immunoassay for detecting aflatoxin B_1 in corn incorporating covalent AFB_1 immobilization and a recombinant Fab antibody, *Talanta*, 115 (2013,) 329–335.

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Soujanya Ratna Edupuganti, Om Prakash Edupuganti, Richard O'Kennedy, Recombinant antibodies based immuno-affinity column for the simultaneous extraction of aflatoxin B_1, B_2 and zearalenone in foods (Manuscript under preparation).

Poster

Soujanya Ratna E, Om Prakash E, Richard O'Kennedy, Studies on the effect of mycotoxin conjugation method on hapten density and their immunopotential in murine models

Invention disclosure

Om Prakash Edupuganti, Richard O'Kennedy, Johanna Bruneau, **Soujanya Ratna Edupuganti**, Development of immuno-affinity columns for the isolation and analysis of mycotoxins using genetically engineered antibodies

Chapter 1
Introduction

1.0 Introduction

Mycotoxins are defined as low molecular weight, aromatic/aliphatic compounds produced by microfungi (Weidenborner, 2001) like *Aspergillus, Fusarium* and *Penicillium*. Mycotoxins are mainly found in tree nuts, peanuts and oil seeds, including corn and cottonseed. It is estimated that 25% of world food produce may be contaminated annually with mycotoxins (Hussein and Brasel, 2001). Despite wide-spread research that has been carried out for decades the mold infection is still a challenging issue (Munkvold, 2003).

The global prevalence of these toxins has attracted the attention of regulatory authorities such as the European Commission and the WHO/FAO, resulting in the implementation of regulations for maximum permitted levels (MPL's) in different food-stuffs, including cereals, baby foods and pasta (Table 1.1). The factors that govern these regulations include:

- the availability of toxicological data and occurrence.
- awareness of what foods are commonly contaminated.
- knowledge of possible sampling techniques that may be used.
- capacity of available analytical methods for detecting the toxins at the required levels.

To achieve the required levels of food quality the necessary analytical tests for contaminants are necessary. The research described in this thesis focused on generating antibodies and test systems for mycotoxin determination.

1.1 Mycotoxins, foods safety and economic impact

Mycotoxin contamination has a significant impact on the economy. The globalization of the food trade leads to the estimation of the economic losses based on two important factors. The first factor is the market and trade impact and the second is the health-related effects on both humans and livestock. To-date, there are no substantial data as to the economic cost of mycotoxin contamination in the EU, except for Hungary, where major wheat contamination took place in 1998 with an overall loss of about €100 million. The annual losses due to

mycotoxin contamination in U.S.A. are approximately $0.5 million - 1.5 billion from aflatoxins in peanuts, fumonisins in corn and deoxynivalenol in wheat, respectively (Robens and Cardwell, 2003). The major economic cost in developing countries is associated with effects on human health (Miller, 1998).

Significant outbreaks of aflatoxin poisoning in Kenya were due to the consumption of maize contaminated with AFB_1 (Lewis *et al.*, 2005). The trichothecene outbreak in the Kashmir Valley, India, was due to consumption of mold-infected wheat (Ramesh *et al.*, 1989). Therefore, in view of the devastating effects of mycotoxins and, also, to protect the end user (consumer) from contamination, the regulatory authorities are striving hard to make the necessary amendments to the existing permissible limits of mycotoxins to minimize exposure.

Table 1.1: *Maximum permissible levels of major mycotoxins in foods marketed in the EU (EU/ 165/2010)*

Toxin	EU maximum permissible levels (µg/kg)
Aflatoxins	
Groundnuts, oilnuts, hazelnuts, almonds and pistachio	15
Dried fruits	10
Cereals and cereal-based foods	4
Maize & rice	10
Chilli, pepper, nutmeg, ginger & turmeric	10
Zearalenone	
Cereals	10
Maize	350
Pastries, biscuits and breakfast cereals	50
Infant foods	20
T-2 & HT-2 toxins	
Cereals and cereal products	N/A

N/A indicates maximum permissible limits are not established

1.2 Classification and chemical properties of mycotoxins

Mycotoxins are toxic secondary metabolites and are classified based on the fungal genera producing them. The detailed chemical description and structural elucidation of the mycotoxins that are used in the present work are described in the following sections.

1.2.1 Mycotoxins of *Asperigillus* species

Asperigillus species are aerobic and, hence, are present in all 'oxygen-rich' environments. These species are the predominant producers of mycotoxins, consisting predominantly of aflatoxins.

1.2.1.1 Aflatoxins

Aflatoxins (M.Wt. ~ 320 Da) belong to a group of difuranocoumarins produced by *Aspergillus flavus*, *A. nomius* and *A. parasiticus*. Even though there are 20 different types of aflatoxins only AFB_1, B_2, G_1, G_2 are found in foodstuffs (http://www.micotoxinas.com.br/aflafacts.pdf). Other significant members such as AFM_1 and AFM_2 are hydroxyl metabolites of AFB_1 & B_2 and were isolated from bovine milk and less toxic than AFB_1. The aflatoxins have a coumarin ring and a ketone carbonyl group. The ketone group is linked at the C3 or C4 position of the coumarin ring. The carbonyl group plays a key role in the modification of the toxin without affecting its active properties (Asao *et al.*, 1965). The chemical structures of aflatoxins are shown in Figure 1.1.

Aflatoxins are crystalline compounds that can be isolated from contaminated matrices with highly polar solvents e.g. chloroform, methanol and dimethylsufoxide (DMSO). They are highly heat-stable and can withstand heating above 100^0C. They possess excellent storage stability when kept in the dark. These molecules contains a carbonyl group in a ring structure that is cross conjugated with αβ unsaturated lactone functions (Pavao *et al.*, 1995). This lactone ring makes them susceptible to alkaline hydrolysis, and processes involving ammonia or hypochlorite have been investigated as a means for their removal from food commodities.

Among the aflatoxins, AFB_1 is the most toxic and their order of toxicity is $B_1>G_1>B_2>G_2$ (http://www.icrisat.org/aflatoxin/aflatoxin.asp). These four types of aflatoxins are distinguished based on their fluorescence under UV and their separation patterns on TLC (Keller *et al.*, 2005). The fluorescence emissions under UV, as stated by O'Neil *et al.* (2001), were blue for AFB_1 & B_2, green for AFG_1, green-blue for AFG_2 and blue-voilet for AFM_1.

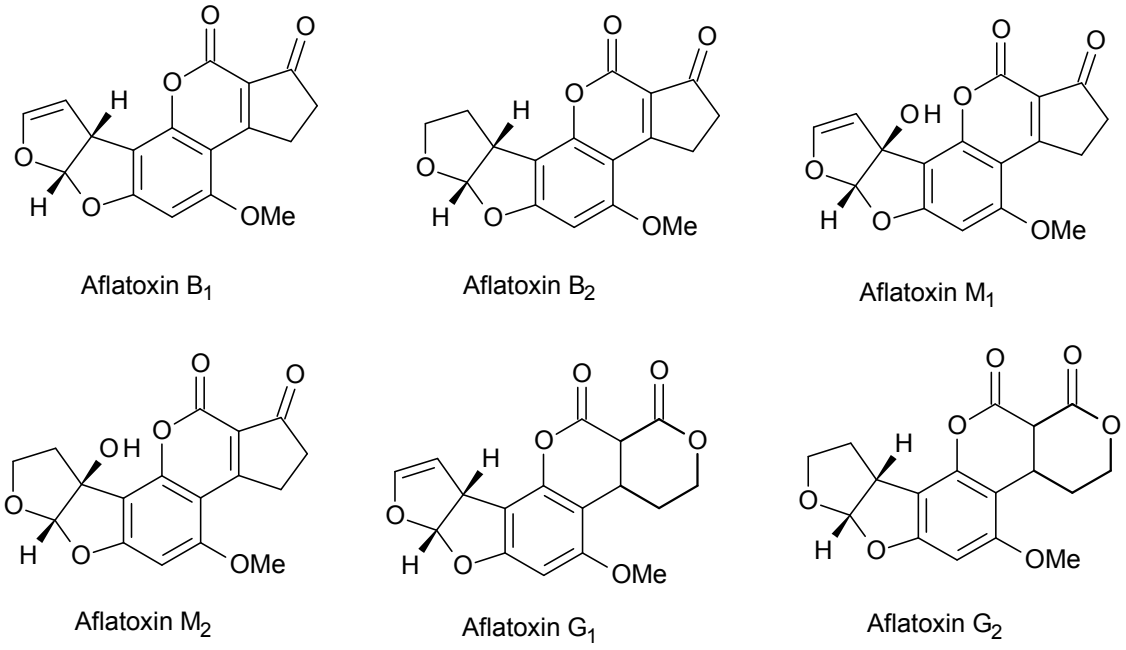


Figure 1.1: Structures of the different types of aflatoxins (adopted from Huang et al., 2010).

Aflatoxin contamination is mainly seen in food products like maize, peanuts, pecans, almonds, hazelnuts, brazil nuts, cheese, corn, pistachio nuts, almonds, spices and walnuts (Creppy, 2002). Aflatoxins are detected occasionally in eggs, milk, cheese, yogurt and meat products, due to the consumption of aflatoxin-contaminated feed (Garrido *et al.*, 2003) by hens or cattle. The food commodities with the highest risk of aflatoxin contamination are corn and peanuts due to the stability of aflatoxins in most associated food processing techniques. They are, however, unstable in processes such as those used in making tortillas that employ alkaline conditions or oxidizing steps.

1.2.2 Mycotoxins of *Fusarium* species

Fusarium species (spp.) are potent producers of an array of toxins and play an important role as plant pathogens. The most important toxins of this group that can affect human and animal health are zearalenone, fumonisin, patulin, ochratoxin, moniliformin, enniatins and trichothecenes such as T-2 toxin & deoxynivalenol (Bennett and Klich, 2003; Eva, 2007). These are considered the most hazardous due to the contamination of cereals, wheat, oats, corn, fruit and vegetables (Richard, 2007). Trichothecenes (M.Wt. ~ 250-550 Da) are a large group of sesquiterpene epoxides produced by the genera *Fusarium, Stachybotrys, Myrothecium, Trichodezma* and *Cephalosporium* (Ciegler, 1978). Trichothecenes (12,13-epoxy-trichothecenes) are subdivided into four groups, based on the substituted group at position 8 and the presence/absence of macrocyclic ring (Table 1.2).

Table 1.2: *Structural variations and trichothecene classification*

Type	Structural difference	Example
Type A	Ketone group at C8 position	T-2, HT-2, DAS
Type B	Carbonyl function at C8 position	DON, NIV, FUS-X & acetylated DON
Type C	Second epoxide group at C7, 8 or C9, 10	Crotocin
Type D	Macrocyclic ring system between C4 and C15 with two ester linkages	Satratoxin, Roridin & Verrucarin

The toxins belonging to trichothecene group are non-volatile and they are highly soluble in acetone, chloroform, dimethylsulfoxide (DMSO), ethanol, methanol and propylene glycol (Cole and Cox, 1981). They are highly stable compounds during storage/processing of food, and they do not degrade at high temperatures but can be inactivated through the application of 3-5% (w/v) sodium hypochlorite (Wannemacher *et al.*, 1989). Massive outbreaks of trichothecene contamination were reported in the 19th century in Soviet Union, Japan and China. All of them resulted from the contamination of cereal-based products with T-2 toxin

along with other trichothecene members. The toxicological effects of the first outbreak in Soviet Union led to alimentary toxic aleukia and the other outbreak in China was associatd with gastrointestinal disorders.

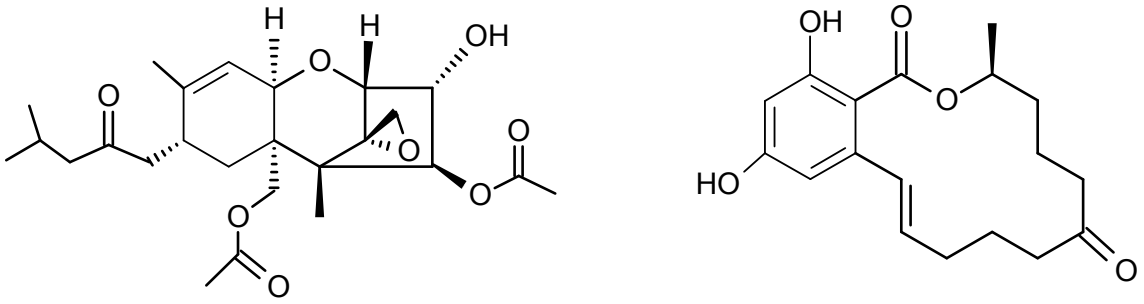
1.2.2.1 T-2 toxin

T-2 toxin (M.Wt. ~ 466.5 Da), a trichothecene A member is produced by *F. acuminatum, F. poae* and *F. sporotrichioides*. It is commonly found in cereal crops like wheat, maize, barley, oats & rye and processed grains e.g. in malt, beer and bread. T-2 toxin a non-volatile compound, is insoluble in water and petroleum ether, but is highly soluble in other solvents such as acetone, ethylacetate, chloroform, dimethyl sulphoxide, propylene glycol, ethanol and methanol (Betina, 1984). It is highly resistant to temperatures employed during various food processing techniques and UV light. Heating above 200^0C or treatment with base solutions such as sodium hypochlorite and sodium hydroxide solutions inactivates the toxin (Wannemacher and Wiener, 1997). The presence of a hydroxyl group at the C3 position is responsible for its biological activity (Figure 1.2). This toxin is metabolized to HT-2, which leads to the additive effects when both forms are present in food material simultaneously. The major structural difference between T-2 and HT-2 is based on the presence of an acetyl group in the C4 position.

1.2.2.2 Zearalenone

Zearalenone (M.Wt. ~ 318 Da) (Figure 1.2) is a resorcyclic acid lactone and naturally occurring estrogen produced by *Fusarium graminearum*. A double bond is present at the C12 position along with a ketone group. Zearalenone is a white crystalline solid that exhibits blue green fluorescence when excited at 360 nm and intense green flourescence when excited at 260 nm. It is soluble in benzene, acetonitrile, methanol, ethanol and acetone (Agag, 2004). ZEN is thermally stable and is found in maize, barley, oats, wheat, rice, sorghum and bread. The order

of their estrogen receptor (ER) binding affinity is α -ZOL > α -ZAL > β -ZAL > ZEN > β – ZOL (Shier *et al.*, 2001). The toxicity of the ZEN is reduced by ozone treatment, nutritionally inert sorbents and microorganisms (Zinedine *et al.*, 2007). ZEN is classified as a type-III carcinogenic agent (IARC, 2002).



T-2 toxin Zearalenone

Figure 1.2: Structures of the T-2 toxin and zearalenone (adopted from Bouaziz et al., 2009).

1.3 Effects, mode of action and mycotoxicosis

Mold infection is the cause of a wide range of human ailments and disabilities. During colonization, fungi will produce enzymes for the breakdown of protein, carbohydrates, nucleic acids and lipids, which are vital for fungal growth. The secondary metabolites produced by the molds during the process of colonization are called 'mycotoxins' and may induce toxicological effects in humans.

Mycotoxins are present in all the parts of the fungi including hyphae, mycelia, spores and the substrate on which they grow. Their presence in the substrate results in serious symptoms upon consumption by humans/animals, and leads to 'mycotoxicosis'. The associated symptoms may be acute or chronic, resulting in reduced growth and development, immunosuppression and tumor formation. Overexposure to mycotoxins causes symptoms such as fever, nausea, vomiting, diarrhoea, leukopeneia, lesions of skin and mucosa (Ellenhorn and Barceloux, 1988). The factors that increase the acute 'mycotoxicosis' in humans include limited availability of uncontaminated food, fungal development in crops and commodities induced by environmental

conditions, and lack of regulatory systems for mycotoxin monitoring and control. The toxicological effects of different classes of mycotoxins are described in Table 1.3.

Table 1.3: *Toxicological effects of some major mycotoxins*

Toxin	System affected	Toxicological symptoms
Aflatoxins	Circulatory system Immune system Nervous system Digestive system Gene expression	Haemorrhage, anaemia Immunosupression Nervous syndrome Motility, diarrhoea Teratogenic, carcinogenic and hepatotoxic effects
Trichothecenes	Circulatory system Immune system Digestive system Nervous system Dermal system	Haemorrhage Immunosupression Gastroenteritis, vomiting Restlesness and lack of reflexes Necrosis, oral and dermal lesions
Zearalenone	Digestive system Reproductive system Gene expression	Diarrhoea Enlargement of mammary glands, uterus hypertrophy, testicular atrophy Teratogenic effects

1.4 Mycotoxin risk management measures

Mycotoxins are extremely difficult to remove or eradicate. To minimize the amount of mycotoxin food contamination two types of strategies were followed, including 'in-field' (pre-harvest) and during storage (post-harvest) prevention strategies (Figure 1.3). The pre-harvest strategies involve gene level modification of plants or adoption of specific cultivation practices to minimize contamination. Post-harvest detoxification of contaminated grains will require physical, chemical or biological agents (Cole, 1989). The best approach should significantly prevent toxicity, be cost effective and should not create detrimental effects on animals.

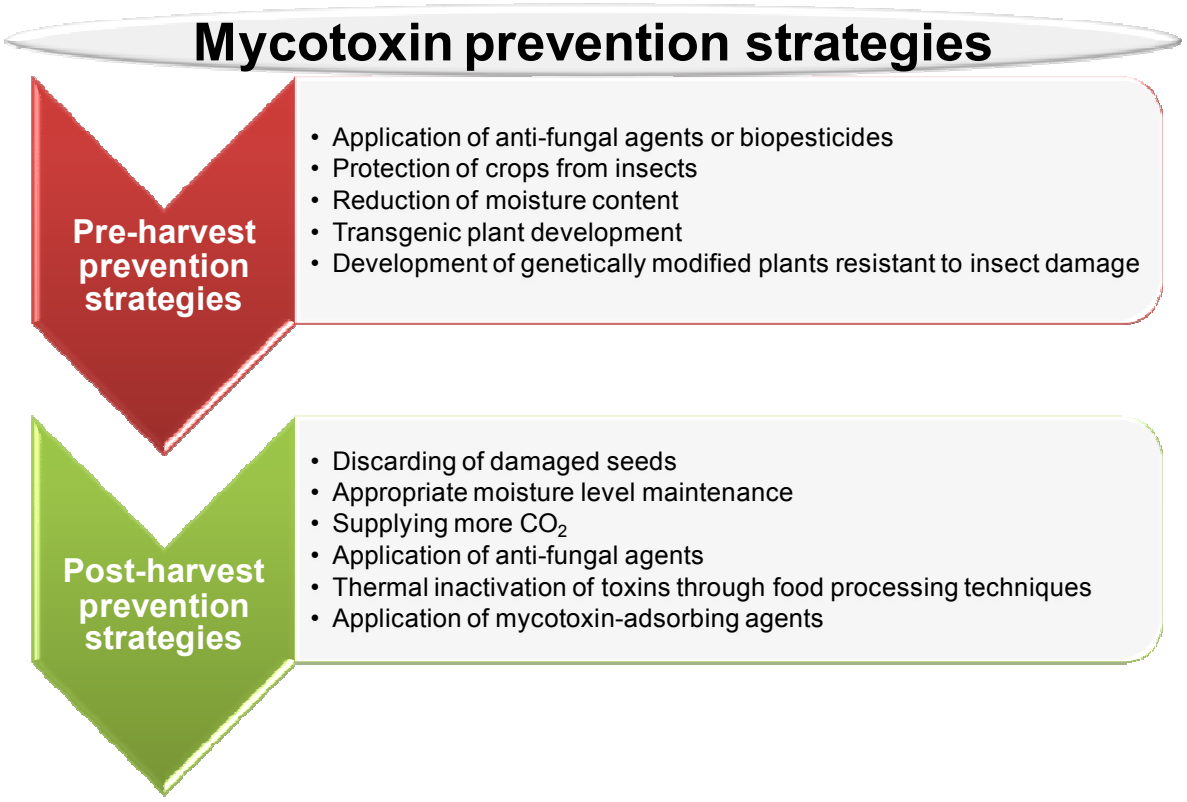


Figure 1.3: Prevention strategies for elimination of mycotoxins.

Major mycotoxins, such as aflatoxins, ochratoxin A, *Fusarium* toxins (trichothecenes like deoxynivalenol, nivalenol, T-2/HT-2 toxin, zearalenone and fumonisins), are potentially removable from feed by use of various adsorbing agents in the compound feed or by degradation with bio-transforming agents such as bacteria/fungi or enzymes. Some of the reported degrading agents were ammonia (Hoogenboom *et al.*, 2001) and formaldehyde plus ammonia (Awadalla *et al.*, 1991). The major disadvatanges of chemical detoxification is that such approaches are fungistatic but are not fungicidal and, in some cases, they may be bio-hazardous. Most of the adsorbent materials have not, as yet, received EU regulatory approval. Certain biological agents, e.g. microorganisms, are capable of biotransforming particular mycotoxins into less toxic metabolites. For example, yeast and yeast cell walls can bind mycotoxins thus facilitating removal of ochratoxin A. In recent studies it was shown that the removal of the epoxide group of trichothecenes by bacterial species of *Eubacterium* (Binder *et*

al., 2001) leads to detoxification. Alkaline treatement of fumonisins leads to hydrolyses, thus making them less toxic (Palencia *et al.*, 2003).

The adoption of the HACCP (hazard analysis and critical control points) system, in parallel to the development of prevention techniques were found to be effective in mycotoxin control (FAO, 2001). This system is used to identify and control hazards within the production, handling, storage and processing of the individual food produce. However, in developing countries these processes are not always economically feasible.

1.5 Mycotoxin detection methods

The presence of mycotoxins in only trace quantities requires the development of highly sensitive and reproducible techniques for their quantification. The diversity in chemical structures and the variations in the physio-chemical properties of mycotoxins led to the development of various approaches for mycotoxin detection. The reliability of any assay is determined by characteristics such as precision, accuracy, detectability, sensitivity and specificity (Hans *et al.*, 1986).

1.5.1 Analytical techniques for mycotoxin detection

Analytical techniques recognized by the AOAC frequently used for mycotoxin detection include TLC (Thin layer chromatography), HPLC (High performance liquid chromatography), GC (Gas chromatography) and LC-MS (Liquid chromatography-Mass spectrometry) (Turner *et al.*, 2009). The basic steps involved in any chemical analytical technique are sampling, extraction, clean-up, separation, detection, quantification and confirmation. Each of the individual steps involved in the development of analytical techniques will now be discussed.

1.5.1.1 Sample preparation or extraction methods

Due to the non-homogenous distribution of mycotoxins in a single crop or lot of product, it is necessary to use the proper sampling method to avoid sampling error. The choice of sampling method used is based on the structure of the mycotoxins to be determined (Stroka *et al.*, 2004). Sample preparation plays a vital role in the success of the analytical technique because the accuracy of the analysis is based on the quality of the sample. Mycotoxin analysis frequently relies on extract clean-up that is based on polarity of the individual analytes in organic solvents. Problems with sample preparation have been addressed by the selection of an extraction method that is designed to take into account the type of food or feed matrix (Wilkes and Sutherland, 1998). Various extraction methods used include: (a) liquid-liquid extraction, which is suitable for small-scale experiments and is based on the dissolution property of the toxin in either the organic or the aqueous phase of the solution. (b) supercritical-fluid extraction involves the usage of CO_2 for the extraction of the required compound but is not very feasible due to the high cost and also the need for sophisticated equipment (http://micotoxinas.com.br/boletim36.pdf) and (c) solid-phase extraction, where small columns are packed with silica gel, C-18 (Octadecylsilane), florisil and phenyl solid supports. This technique is used routinely for the isolation of toxins of interest from complex matrices.

1.5.1.2 Separation methods

After clean-up of samples, mycotoxins need to be separated from various impurities and interferences and then quantified by chromatographic techniques outlined below:

1.5.1.2.1 Thin layer chromatography (TLC)

TLC is both quantitative and semi-quantitative, is cost-effective and is able to separate complex mixtures. Mycotoxins are readily separated by TLC with a range of organic solvent mixtures, as the mobile phase, and are easily observed under UV or with visualization reagents. Silica gel TLC plates are widely used for the separation of aflatoxins (B_1, B_2, G_1, G_2) (Mehan

and Gowda, 1996). A number of improvements of conventional TLC such as two-dimensional and high-performance TLC have been applied to improve the separation of mixtures of mycotoxins. TLC was found to be cost-effective and suitable for the analysis of aflatoxins, zearalenone and ochratoxin, with detection limits of 1 ng/kg, 20-40 µg/kg and 0.3 µg/kg, respectively. Other mycotoxins have also been successfully analysed by TLC (Sokolović and Simpraga, 2006).

1.5.1.2.2 Gas chromatography (GC)

GC is a separation technique employed for the analysis of volatile compounds, in which the vaporized volatile compounds are separated and fractionated as a consequence of partition between a mobile gaseous phase and stationary phase held in the column. The volatilised sample will be carried through the column by an inert carrier gas such as helium or nitrogen. The components are separated based on their partition ratio. Concentration equilibration occurs repeatedly between the solid, stationary and the mobile phases. The level of adsorption or partition for each component causes differences in the retention time for each component within the column. Since mycotoxins are non-volatile in nature they are derivatized into their volatile trimethylysilyl forms through treatment with either trifluoroacetic acid or heptafluorobutyric acid anhydride, prior to analysis by GC.

Various examples of the application of GC techniques for mycotoxin detection exist. The analysis and isolation of 13 different types of trichothecene members using GC was studied extensively using the derivatization of the hydroxyl group present on these molecules. The detection limits achieved with trichothecenes ranged from 50-200 µg/kg (Croteau *et al.*, 1994). GC along with mass spectrometry was employed for the detection of zearalenone in cereals (Tanaka *et al.*, 2000). Trichothecene detection in beer samples was evaluated using GC, with a flame ionization detector, and had a detection range of 8-25 µg/kg (Schothorst and Jekel, 2003). Simultaneous detection of type A and B trichothecenes was developed by Eke *et al.*

(2004) using GC coupled with flame ionization in semolina, with a detection range of 300-470 µg/kg.

1.5.1.2.3 High performance liquid chromatography (HPLC)

The principle for HPLC involves using high pressure to pass the sample through a pre-packed column with sorbant (stationary phase) and subsequent addition of a mobile phase consisting of solvents of different polarity. Due to the differences in the polarity of the individual components of the analyte they will partition to different degrees between the solid and mobile phases. The component with least interaction will be eluted first followed by the others. HPLC is commonly used for analysis of fumonisin, aflatoxins, zearalenone and ochratoxin A (Coker, 2000). HPLC-MS/MS is employed for the detection of mycotoxins and their metabolites (Spanjer *et al.*, 2008). In mycotoxin analysis by HPLC, UV and fluorescence are commonly used methods of detection, which rely on the presence of a chromophore in the toxin moiety. Derivatization is performed for compounds which are not fluorescent by nature. Due to the polar nature of mycotoxins they can be readily separated on reverse-phase HPLC leading to the emergence of a diverse array of methods (Bunaciu, 2010). The detection limits achieved with HPLC with a fluorescence detector are 0.025 ng/g for AFB_1 and AFG_1, 0.012 ng/g for AFB_2 and AFG_2, 0.2 ng/g for OTA, 1.5 ng/g for ZEN, 6.2 ng/g for FB_1, FB_3 and HT-2 toxin, 9.4 ng/g for FB_2 and T-2 toxin, and 18.7 ng/g for DON. In addition, the limits of quantification (LOQ) ranges from 0.04 ng/g for AFB_2 and AFG_2 and 62 ng/g for DON (Soleimany *et al.*, 2011). Ochratoxin A (0.035-0.689 ng/g) and AFM_1 (0.025 ng/g) were detected in infant formula milk using HPLC with fluorescence detection (Meucci *et al.*, 2010).

1.5.1.2.4 Capillary electrophoresis (CE)

This is based on the separation of components depending on their mass to charge ratio in an electric field. CE is performed in smaller volumes of aqueous buffer and this method can eliminate the usage of large amounts of organic solvents that will be required for other

chromatographic techniques. Most of mycotoxins, such as aflatoxins, moniliformin, ochratoxin A, deoxynivalenol, fumonisins and zearalenone were separated successfully by CE (Galaverna *et al.*, 2008). Capillary electrophoresis was used for the detection of MON in maize with a limit of detection of 0.1 µg/g (Maragos, 2004) and ZEN with a limit of detection of 0.0084 mg/L (Zeng *et al.*, 2003). This technique was adopted for the detection of aflatoxin B_1 by Maragos and Greer (1997). The CE method may suffer from problems with analytical impurities and smaller sample injection volumes for analysis, which might limit the usage of this technique for mycotoxin analysis (Bohs *et al.*, 1995; Otieno and Mwongela, 2008).

1.5.1.3 Separation and identification

1.5.1.3.1 Chromatography with tandem MS (LC-MS/MS)

In the LC-MS method, the physical separation capabilities of liquid chromatography are coupled with the mass analysis capabilities of mass spectrometry. This technique is now very widely used as it allows multi-toxin analysis (up to 31 types) in a single experiment. This technique was used for the simultaneous detection of multi-toxins including trichothecences, aflatoxins, fumonisins, ochratoxin A, zearalenone, beauverin and sterigmatocyst in sweet pepper with a detection range of 0.32 µg/kg-42.48 µg/kg (Monbaliu *et al.*, 2009). Type A and B trichothecenes were also analyzed by LC-MS/MS in maize with a detection limit of 0.3-3.8 µg/kg (Berthiller *et al.*, 2005). This technique was adopted for the determination of 39 mycotoxins in wheat and maize (Sulyok *et al.*, 2006). Mycotoxins are negatively and positively charged chemically diverse molecules, and these can be analyzed easily on positive or negative modes of mass spectrometry. Increased high throughput detection and identification make this technique highly versatile. The major advantage of this method is the detection of mycotoxin metabolites, which cannot be analysed by chromatographic techniques and also the sensitivity of this technique falls within the permissible limit of contamination set by the regulatory

authorities. The high cost involved for the analysis using this method makes the adoption of this technique unsuitable for certain types of analysis e.g. low cost rapid qualitative tests.

1.5.1.4 Limitations of current analytical techniques

- The lack of ease of automation of TLC led to development of other chromatographic techniques.
- The need for derivatization of the non-volatile mycotoxins for performing GC is a major limiting factor for the application of this technique to detect mycotoxins.
- Although chromatographic techniques are feasible they are expensive, time-consuming and require trained personnel for handling the sophisticated equipment involved.
- Almost all of these techniques require a sample 'clean-up' procedure, which might not generate a representative sample lot for analysis.
- Most of the mycotoxins undergo conversion to their metabolites, which are termed masked mycotoxins either due to plant enzyme activity or the degradation of the toxin during processing techniques. This may cause issues for analysis.
- Formation of masked mycotoxins, like DON 3-glucoside (Berthiller *et al.*, 2009), leads to the false experimental results due to the inability of the technique to detect the 'hidden toxin'.
- Due to the highly polar nature of these metabolites they are easily lost during extraction and sample clean-up procedures.
- There are no set commercial standards for some masked mycotoxins.

To overcome the aforementioned limitations of the analytical techniques, the application of immunoassays was suggested as they are sensitive and the results are highly comparable with analytical techniques such as HPLC, GC and Mass spectrometry.

1.5.2 Novel mycotoxin binders for assays

A wide array of mycotoxin binding ligands such as synthetic polymers (Appell *et al.*, 2008; Maragos, 2009) and recombinant antibodies were developed in the recent past for incorporation into mycotoxin assays. Key roles played by antibodies include isolation of pure mycotoxins using immuno-affinity columns (IACs) and their incorporation into immunoassays based on the interaction of the antibody with the toxin. The limitations on the usage of antibodies are due to the fact that they are suseptable to denaturation and proteolytic degradation, the use of animals for immunization and also, the instability of the antibodies in organic solvents. This has driven researchers to use some alternatives that can overcome these limitations, e.g. polymers (e.g. molecular imprinted polymers) and modified natural materials (e.g. single chain antibody fragments). The following sections focus mainly on the generation of different types of recombinant antibodies.

1.5.2.1 Antibodies

Antibodies are globular proteins termed immunoglobulins. These are the key bio-recognition elements of the immune system. Structurally, full-length antibodies, such as immunoglobulin G (IgG) (Figure 1.4), possess amino and carboxyl-termini and are comprised of four polypeptide chains, namely, the variable heavy (V_H), variable light (V_L), constant heavy (C_H) and constant light (C_L) domains, which are interconnected via disulphide bonds. Natural antibodies are glycoproteins, possessing carbohydrate side chains attached to their polypeptide backbones at the antibody constant region. Interactions with antigenic determinants are promoted via Complementarity-Determining Regions (CDRs), which reside in the variable domains of the immunoglobulin and vary from antibody to antibody.

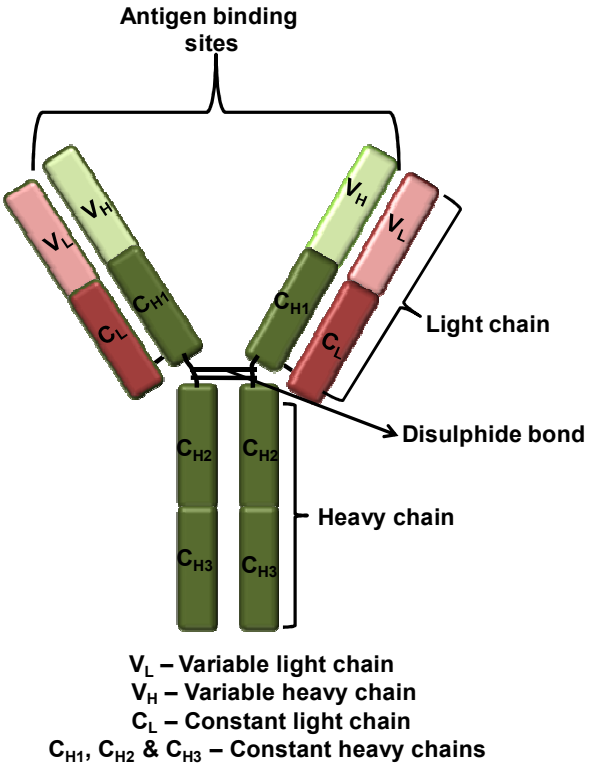


Figure 1.4: Structure of intact IgG molecule. *IgG (molecular weight ~ 150 kDa) consists of four peptide chains, which include two variable heavy (V_H) and light chains (V_L) (Eremin et al., 2002) that are covalently linked together by disulfide bonds. The two arms of the IgG molecule are arranged to form a 'Y' shaped moiety with two antigen (binding) sites on either arm of the molecule.*

The other types of immunoglobulins (Figure 1.5) include IgM, IgE, IgD & IgA. IgM, which comprises 13% of the serum antibodies, is pentameric with 10 epitope binding sites and exhibits a half-life of 5 days. It is the first immunoglobulin produced in the immune response. IgA forms approximately 6% of the serum antibodies and has two sub-classes namely $IgA_{1\ \&\ 2}$. It is dimeric with 4 epitope binding sites, with a half-life of 6 days. The Fc portion of this class of antibody contributes to the ability of mucous to trap microbes. IgD, which is monomeric, forms approximately 0.2% of total serum antibodies. It plays a vital role in the generation of self-reactive autoantibodies by the B-cells. IgE is monomeric, forms approximately 0.002% of the total serum antibodies, and plays a major role in mediating many allergic reactions.

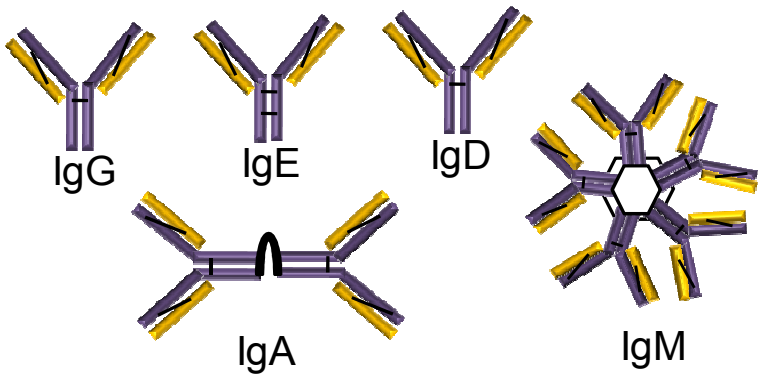


Figure 1.5: Structure of different classes of antibodies. *Antibodies may be monomeric (IgG, IgD and IgE), dimeric (IgA) or pentameric (IgM).*

1.5.2.2 Production of different types of antibodies

Antibody preparations are classified into three types based on the production technique employed (Figure 1.6). These are polyclonal, monoclonal and recombinant forms. They may vary in their affinity, avidity, sensitivity and cross reactivity. For the production of antibodies against mycotoxins, first of all mycotoxins are covalently linked to carrier proteins like bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) or ovalbumin, since they are of low molecular weight and, therefore, cannot directly generate an immune response in the host used for immunization (i.e., hapten). It is a well established fact that the specificity and sensitivity of antibodies produced post-immunization are directly related to the quality of the immunogen. The availability of the functional groups like sulfhydryl, amine, carboxyl, etc., on mycotoxins plays a crucial role in efficient conjugation of the toxin to the protein carrier. The proper orientation of the hapten also plays a major role in the generation of antibodies that specifically recognize the antigen. Hapten density on the conjugate is also important and it influences the immune response directed toward the newly created antigenic determinant. After appropriate hapten-conjugate synthesis, the next step is to select a host for immunization. From the literature available on the generation of antibodies (Table 1.4) for mycotoxins, murine and leporine species are commonly choosen hosts. The route of immunization selected for conjugate administration may be intra-peritoneal, subcutaneous or intravenous. After boosting

the host several times with the antigen-conjugate and a suitable adjuvant such as Freund's, the immune cells of the host will be trigerred for the generation of antibodies against the target.

Table 1.4: *Literature based major anti-mycotoxin antibodies and their detection limits*

Toxin	Reported antibodies & host	Sensitivity	Reference
Deoxynivalenol	Monoclonal [murine]	18 ng/mL	(Maragos and McCornmick, 2000)
	Recombinant scFv [murine]	N/A	(Choi *et al.*, 2004)
T-2 and HT-2 Toxin	Monoclonal [murine]	20 ng/mL	(Ueda and Akiwa, 2007)
	Polyclonal [leporine]	0.12 ng/mL	(Wang *et al.*, 2010)
Zearalenone	Recombinant scFv [murine]	14 ng/mL	(Yuang *et al.*, 1997)
	Monoclonal [murine]	0.8 ng/mL	(Burmistrova *et al.*, 2009)
	Monclonal [murrine]	0.3 ng/mL	(Teshima *et al.*, 1990)
Fumonisins	Polyclonal [murine]	100 ng/mL	(Azcona-Olivera *et al.*, 1992)
	Polyclonal [leporine]	0.45 ng/mL	(Yu and Chu, 1996)
	Monoclonal [murine]	1000-5000 ng/mL	(Fukuda *et al.*, 1994)
	Recombinant scFv [naïve library]	N/A	(Lauer *et al.*, 2005)
Aflatoxin B_1	Monoclonal [murine]	0.006 ng/mL	(Devi *et al.*, 1999)
	Polyclonal [leporine]	15.8 pg/mL	(Gathumbi *et al.*, 2001)
	Recombinant Fab [murine]	14.8 ng/mL	(Stapleton, 2007)
Aflatoxin B_2	Monoclonal [murine]	50 pg/mL	(Kenneth *et al.*, 1988)

1.5.2.2.1 Polyclonal antibodies

Polyclonal antibodies are a pool of immunoglobulin molecules secreted by the host immune system in response to an antigen. This pool of antibodies possess different immunoglobulins with different 'epitope-recognizing' capabilties (Scholler *et al.*, 2010). Isolation of specific antibodies from the pool of immunoglobulins is an important step in polyclonal antibody production. Conventional ammonium sulfate precipitation (Ahmadzadeh *et al.*, 2011) has certain limitations e.g. yield and purity. This is often replaced by affinity purification with

protein A or G (Yan and Huang, 2000) (Figure 1.6(b)). With advances in chromatographic techniques, in conjunction with the conjugation chemistry, various other techniques such as purification on antigen-immobilized beads were introduced. While polyclonal antibodies are relatively easy to produce, a disadvantage is that a variety of different specificities exist with varying affinities for the desired analyte. In addition, it is not possible to re-create the identical binding properties once a given batch of antibody is completely used up.

1.5.2.2.2 Monoclonal antibodies

Monoclonal antibodies were first reported by George Kohler and Cesar Milstein in 1975 (Kohler and Milstein, 1975; Akshaya *et al.*, 2008). This technique uses B-cells that specifically produce the required antibodies to the target antigen. These antibodies have identical epitope binding specifications (Alisa, 1984). The production of monoclonal antibodies (Figure 1.6(c)) starts with immunization of the selected host with the specific antigen. When a suitable serum titre is obtained, the spleen is removed and antibody-producing B-cells are harvested (generally from the spleen). These cells are subsequently fused to myeloma cells. Selection of the hybridomas is carried out in selective medium followed by testing the specificity of the antibodies produced towards the antigen of interest (Tomita and Tsumoto, 2010).

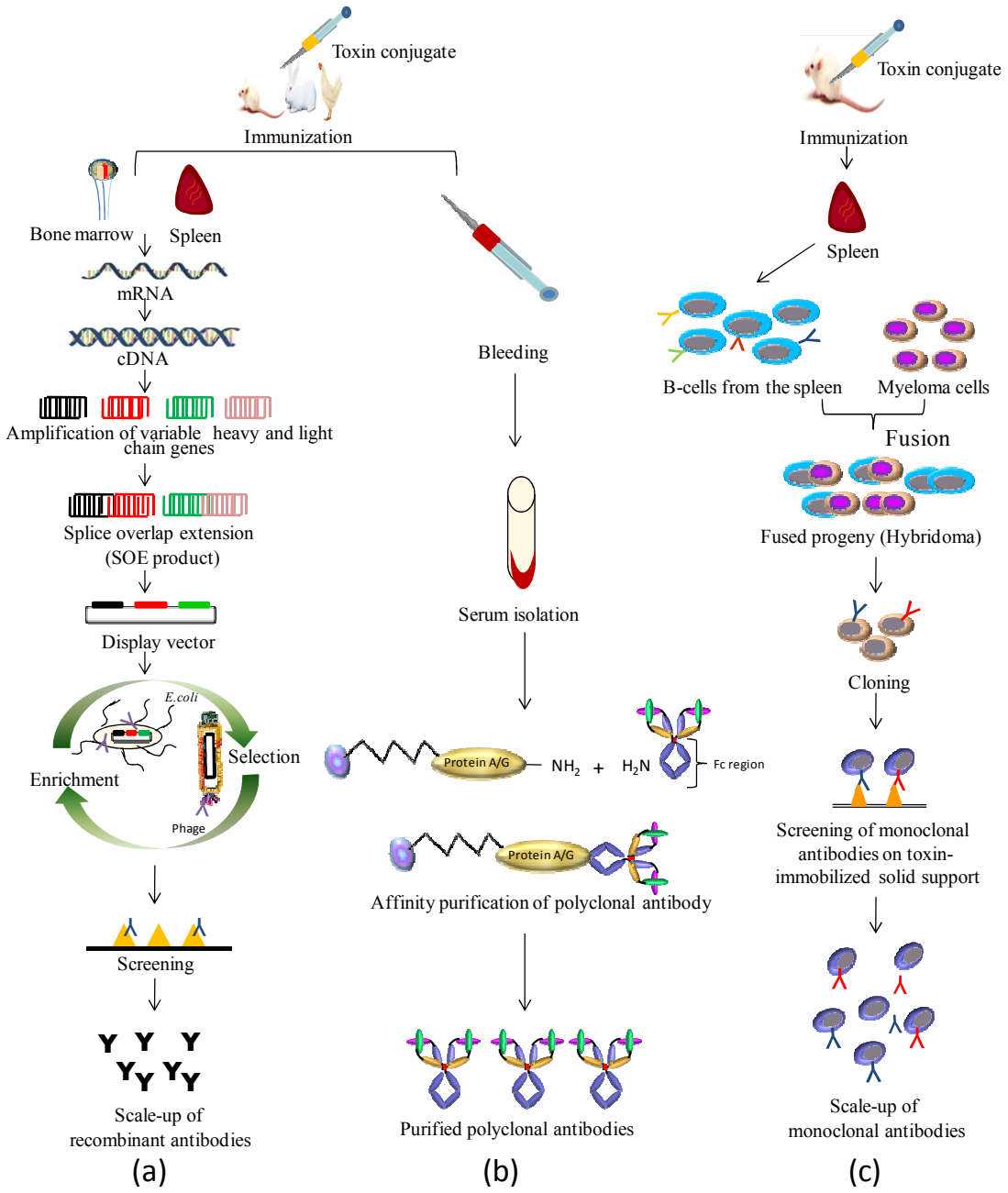


Figure 1.6: Schematic representation of the production of recombinant, polyclonal and monoclonal antibodies. *Immunization of the hosts is the initial step for the generation of any antibodies. For the production of recombinant antibodies (a) RNA will be isolated from the spleen or bone marrow followed by the synthesis of cDNA. The variable heavy and light chain genes will be amplified and the SOE product made and incorporated into the display vector. This is then electroporated into E. coli followed by the amplification of the genetic material through the usage of bacteriophage and subsequent screening of the antibody. Polyclonal antibody production (b) involves the isolation of the serum from the blood obtained from the immune host followed by affinity purification. Monoclonal antibody production (c) involves the isolation B-cells and their fusion with myeloma cells leading to the production of hybridoma. These are selectively enriched by screening on the antigen-immobilized surface ultimately leading to the production of the required specific antibodies.*

1.5.2.2.3 Recombinant antibodies

Recombinant antibody generation (Figure 1.6(a)) offers a valuable alternative to polyclonal and monoclonal antibodies. Recombinant antibody libraries can be classified based on two criteria. One criteria refers to the library's source of derivation i.e., whether the library is derived from a naïve or an immunized source with or without semi-synthetic or synthetic sequences (Brichita *et al.*, 2005). The other criterion of classification is mainly based on the display system employed, which may be phage display (the most common method), bacterial display, yeast display, mammalian display or ribosomal display.

1.5.2.2.3.1 Naïve or single-pot libraries

Naïve or single-pot libraries are built through PCR amplification of IgM or IgG variable regions and subsequent cloning into vectors for screening. These libraries exhibit large antibody diversity of up to 10^{11} (Sblattero and Bradbury, 2000). Naïve libraries are non-immune, aimed at a variety of targets through synthetic or semi-synthetic assembling of the genes (Vaughan *et al.*, 1996). The first successful generation of an antibody against aflatoxin B_1 using naïve libraries was reported by Moghaddam *et al.* (2001). Later on successful generation of anti-AFB_1 scFv from a human naïve library was reported by Rangnoi *et al.* (2011).

The advantages assosciated with the utilization of naïve libraries include:

- High antibody diversity, thus increasing the chances of obtaining the antibody for the desired target.
- Avoids the need for immunization and reduces the usage of animals for the purpose of library generation.
- A very short time for implementation.
- An increased opportunity of obtaining high affinity antibodies using these libraries.

The limitations assosciated with these libraries include:

- Major chances of formation of inclusion bodies leading to expression-related problems.
- Multiple electroporation steps required for generation of diverse libraries.
- Availability of limited information about the contents of naïve libraries.

To circumvent these limitation posed by the naïve libraries synthetic and semi-synthetic antibody libraries were developed.

1.5.2.2.3.2 Synthetic & semi- synthetic antibody repoitre libraries

In synthetic library generation, randomized sequences will be incorporated into CDR regions to bring about complete or partial degeneracy. The synthetic diversity bypasses the natural biases and redundancies (Winter and Milstein, 1991). The semi-synthetic library construction involves the incorporation of randomized sequences in one or more CDR regions through PCR reactions. The overall advantage of the utilization of these libraries over the naïve libraries is that the variability and overall diversity of the library can be controlled.

1.5.2.2.3.3 Immune libraries

Immune libraries are generated from hosts, where the host was injected with the target antigen. In general, the size of the immune library is quite low, which can be due to the development of selective antibodies recognizing the target (Abi-Ghanem *et al.*, 2008). The quality of RNA originally isolated from lymphoid organs, coupled with the nature of the host-generated response, are key contributory factors in producing a diverse library.

Immune library construction starts with the amplification of antibody-encoding genes from harvested lymphoid organs (e.g. spleen and bone marrow) using PCR. This process is simplified by the availability of primers for the amplification of variable heavy and variable light genes from a selection of different host species (Barbas *et al.*, 2001). Once cloned into a suitable expression vector, the construct is introduced into *E. coli*, typically by electroporation, and the resultant library is harvested (Conroy *et al.*, 2009). The affinity of the antibodies can be

improved through affinity maturation using molecular biological approaches such as site-directed mutagenesis, random mutagenesis or chain shuffling (Lou and Marks, 2010).

Various types of antibody fragments may be produced, including the single chain variable fragment (scFv) and the fragment antigen binding (Fab) entity (Figure 1.7). The scFv fragment is composed of variable heavy and light chain domains of the immunoglobulin, which are covalently linked by an flexible linker with approximately 15 amino acids. The flexible nature of linker allows the proper folding of the antigen combining sites. The linker is rich in glycine (aids in the proper folding) and serine and threonine (enchances solubility). These scFv antibody fragments can be purified using protein L, which can bind to the variable region of the kappa light chain. The assembly of scFv libraries requires fewer PCR amplification cycles, and multimerisation of antibody fragments may be advantageous in improving avidity, which can be particularly useful when detecting structurally-related analogues. Furthermore, yields of scFv produced by induced cultures of *Escherichia coli* are often significantly higher than for those seen for Fab antibody fragments. Fab molecules, in contrast, have higher stability and do not multimerise (O'Kennedy *et al.*, 2010). Papain digestion of the whole immunoglobulin molecule or monoclonal antibodies results in Fab and Fc fragments. The alternative way for the generation of Fab is recombinant library construction through utilizing primers and PCR reactions. The Fab fraction consists of a single antigen combining site with one variable domain of heavy and light chain each.

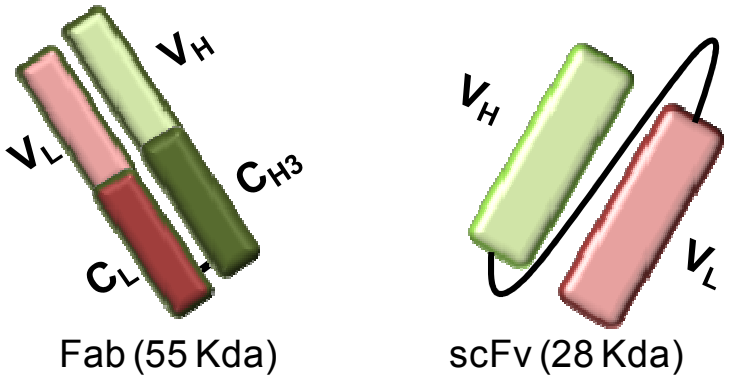


Figure 1.7: Recombinant antibody formats. *The scFv and Fab antibody fragments are shown. The scFv consists of heavy (V_H) and light (V_L) chain that were connected through linker from the N-terminus of V_H to the C- terminus of V_L and vice-versa. scFv's are relatively small and are easily expressed in E. coli. The Fab consists of constant and variable domains of the heavy and light chains.*

For the development of the aforementioned recombinant antibodies, phage display is frequently employed, due to its efficient antibody generation capability. This technique was first described by George P. Smith in 1985 (Smith, 1985). This has proven to be a powerful tool for recombinant antibody selection (Willats, 2002; Yau *et al.*, 2003). The display of antibodies on the phage surface allows affinity selection by the antigen (Petrenko and Vodyanoy, 2003). The development of molecular methods for the expression and engineering of recombinant antibodies led to the development of a new era in the antibody generation (Maynard and Georgiou, 2000; Ahmad *et al.*, 2012). The most prominant phage display technology used is based on the M13 display system.

1.5.2.2.4 Structure of filamentous phage

A bacteriophage is a virus that infects the bacterium by attaching to the surface. This is followed by the injection of the viral DNA into the host bacteria. The term bacteriophage is derived from two words i.e. bacteria and a Greek word 'Phagin' meaning 'to eat'. These phage were discovered by Frederick Twort and Felix d'Herelle. The four most commonly used types of bacteriophages in molecular biology applications include the T-series of bacteriophages, temperate bacteriophages, small DNA bacteriophages (spherical phage and filamentous phage)

and RNA bacteriophages. Among these different types of phages, filamentous phage are widely used for phage display applications. A detailed description of the structure of a filamentous phage follows.

All bacteriophage makes use of the host cell replication machinery to encode and replicate their genes. A filamentous phage is a rod shaped, single-stranded DNA (ssDNA) virus that mainly infects gram-negative bacteria (Figure 1.8). A typical phage particle is approximately 6.2 nm in diameter and 930 nm in length. This phage coat is composed of one major (pVIII comprising 50 amino acids) and four minor hydrophobic (pIII, pVI, pVII & pIX) proteins. The F-pilus of the *E. coli* host attaches with the phage through the N-terminal of the pIII. The stages in the phage life-cycle include infection, replication of the viral genome, assembly of new viral particles and release of progeny particles into the host cells. The phage genome is transferred into the host's cytoplasm and is converted into DNA, which serves as template for the expression of phage genes.

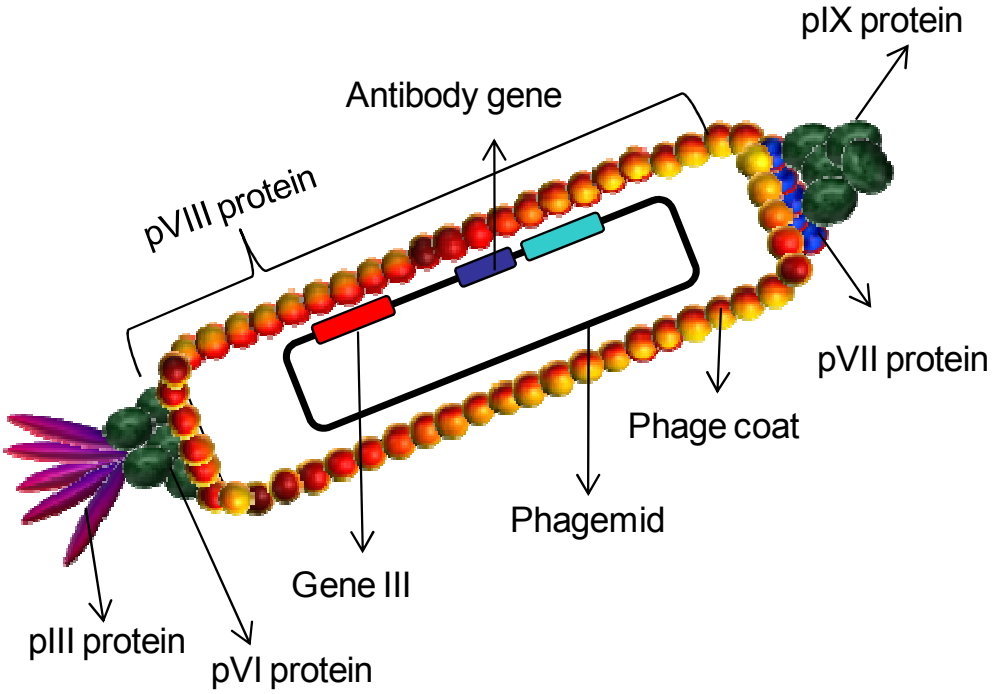


Figure 1.8: Pictorial depiction of the M13 bacteriophage. *The bacteriophage is typically cylindrical in shape with a phage coat composed of five different types of proteins. The pIII protein plays a vital role for antibody attachement to the phage. The major part of the phage coat is composed of pVIII proteins with minor proteins (pVI, pVII & pIX) on either ends of the cylindrical structure.*

1.5.2.2.5 Antibody phage display

The physical linkage of the encoding genotype to the phenotype *in-vitro* forms the basis for all display techniques (Leemhuis *et al.*, 2005). The similarities in all the different display techniques include (a) generation of genotypic diversity and (b) combining genotype to the phenotype (http://micotoxinas.com.br/boletim36.pdf) clonal selection (http://ec.europa.eu/food/ fs/sc/scf/ out74_ en.pdf) amplification of the selected clones.

The *in-vitro* binding process used for selection of the desired antibody is called "panning" (Figure 1.9). Here, the antigen of interest is immobilized on a solid support like nitrocellulose (Hawlisch *et al.*, 2001), magnetic beads (Moghaddam *et al.*, 2003), plastic surfaces of polystyrene tubes (Hust *et al.*, 2002) or 96 well microtitre plates (Kobayashi *et al.*, 2005). The phage are then incubated with the antigen on the solid support. The weakly bound and unbound phage particles are removed through stringent washing. Specifically bound phage displaying antibodies will remain and may be eluted either through trypsinization or pH shift. They are then reamplified through infecting into *E. coli* cells. For the production of new antibody phage the phagemid-bearing *E. coli* will be supplemented with helper phage for quicker amplification of the required antibody gene.

The various strategies that might be employed for increasing the number of high-affinity binders include:

a. decreasing the antigen concentration during each round of panning (Strachan *et al.*, 2002).
b. use of free hapten for competitive elution, and
c. adoption of substractive panning technique in which the phage will be pre-incubated with the carrier protein to eliminate non-specific binding.

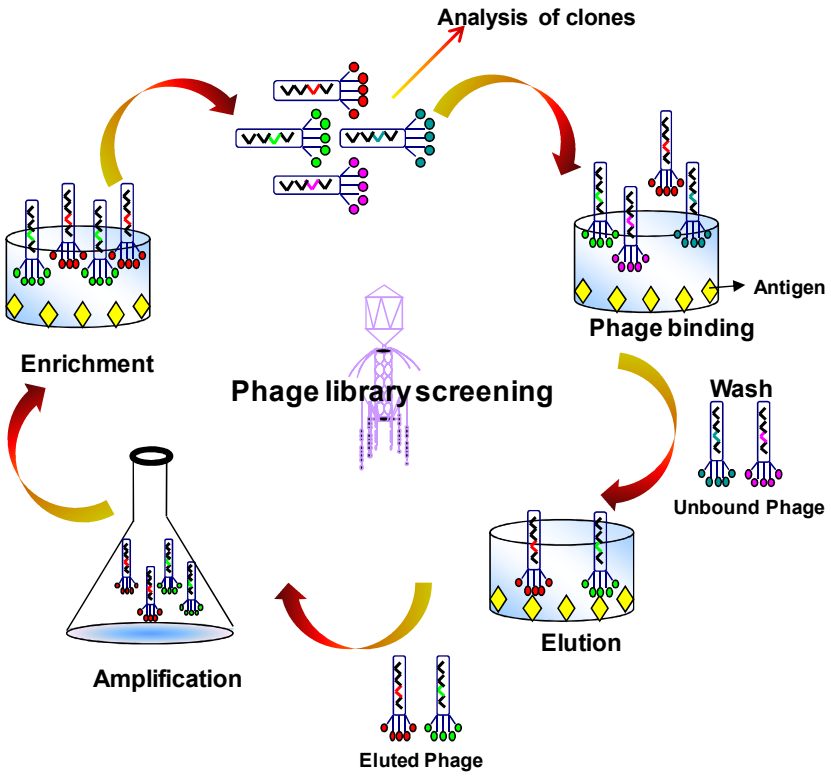


Figure 1.9: Representation of the steps involved in the biopanning process for enrichment and selection of recombinant antibody fragments. *The phage with the relevant target-specific antibody, are bound to the immobilized antigen. The unbound phage will be washed away leading to the selective enrichment and amplification of the required antibody gene. The enriched phage are then analysed to assess their capability to recognize the antigen moiety.*

The recombinant antibodies obtained after performing phage display will then be solubly expressed. Despite the availability of a huge number of vectors for antibody expression the gram negative *E. coli* is frequently choosen (Karu *et al.*, 1995). The presence of the Lac promoter in the expression vector allows high level expression upon the addition of IPTG (De Bellis and Schwartz, 1990). The various factors that might alter the yield include nutrient enrichment media used, temperature and IPTG induction time and quantity. The antibody expression will be followed by the determination of the reactivity of the antibody to the specified target through incorporating into a wide array of immunoassays that are specifically designed for the efficient detection of mycotoxins. The sections below give a detailed insight into some of the immunoassays that are developed to-date.

1.5.3 Immuno-techniques for mycotoxin detection

Immunoassays are sensitive analytical techniques that utilize the unique properties of antibodies. The principle of immunoassay was first suggested by Rosalyn Yalow and Salmon Berson in 1959 (Wu, 2006). Selections of different quantitative immunoassay formats are available for toxin detection, and these include indirect, competitive and sandwich immunoassays (Figure 1.10). Exemplification of different types of immuoassays adopted so far for the detection of mycotoxins are depicted in Table 1.4.

Table 1.5: *Examples of some of the reported immunoassays for mycotoxins*

Immunoassay	Toxin	Matrix & assay sensitivity	Reference
Fluorescence polarization	Aflatoxins	Maize, sorghum, peanut, popcorn [28 ng/mL]	(Nasir and Jolley, 2002)
	Deoxynivalenol	Wheat & maize [12 ng/mL]	(Maragos and Plattner, 2002)
	Ochratoxin A	Barley [3 ng/mL]	(Won-Bo Shim *et al.*, 2004)
	Zearalenone	Corn [137 µg/kg]	(Chun *et al.*, 2009)
Enzyme-linked immunoassays	Aflatoxin M_1	Milk and milk products [3 ng/L]	(Guan *et al.*, 2011)
	Ochratoxin A	French wines [400 ng/L]	(Radoi *et al.*, 2009)
	Fumonisin B_2	Maize [6.09 µg/L]	(Song *et al.*, 2009)
	Fumonisin B_1	Cereals [7.6 ng/g]	(Barna-Vetro *et al.*, 2000)
	Zearalenone	Cereals [1.13 µg/L]	(Thongrussamee *et al.*, 2008)
Lateral flow	Aflatoxins	Maize [1 µg/kg]	(Anfossi *et al.*, 2011)
	Fumonisins	Maize [120 µg/L]	(Anfossi *et al.*, 2010)
	T-2 toxin	Wheat & oats [100 µg/kg]	(Molinelli *et al.*, 2008)

Dipstick	T-2 toxin	Wheat [0.25 ng/mL]	(De Saeger and Van Peteghem, 1996)
	Aflatoxin B_2	Penauts, hazelnuts, pistachio & almonds [0.9 ng/mL]	(Tang *et al.*, 2009)
	Aflatoxin B_1	Rice, wheat, chillies & almonds [0.1 ng/mL]	(Liao and Li, 2010)
Biosensors	Aflatoxin B_1	Corn [60 pg/mL]	(Piermarini *et al.*, 2007)
	Ochratoxin A	Cereals [4-100 ng/g]	(Ngundi *et al.*, 2005)
Immuno-affintiy Columns	Nivalenol & Deoxynivalenol	Wheat [0.2 mg/kg]	(Kadota *et al.*, 2010)

1.5.3.1 Indirect immunoassay

An indirect assay is performed using the conjugated or unconjugated antigen immobilized on solid surfaces (e.g. microtitre plates). In this type of assay the antigen-antibody complex formation is detected through the use of labels present on one of the reactants. The general labels include radioisotopes, enzymes, flurophores and luminescent substances. In this format, the primary antibody is added and binds to the immobilized antigen as depicted in Figure 1.10. The secondary labelled antibody is added with a suitable label for visualization. The major advantages and limitations with this types of assay format include:

Advantages

1. Commercial availability of wide range of labelled secondary antibodies makes the employment of this technique more versatile.
2. Signal amplification can be achieved.
3. Different visualization substrates can be used based on the labelling agent in the secondary antibody (e.g. alkaline phosphatase, horseradish peroxidase etc.).

Limitations

1. Potential cross reactivity of the primary antibody with the secondary antibody resulting in a non-specific signal.
2. Problems with non-specific binding of antibodies to surfaces.

3. Need for effective blocking mechanisms to minimize non-specific binding.

1.5.3.2 Competitive assay format

Competitive assay formats are of two types, namely: direct and indirect. In the direct format, antibody is immobilized, whereas in indirect antigen is immobilized on the solid surface. Figure 1.10 shows the various steps involved in a competitive indirect assay format. Antigen immobilization is followed by the addition of antibody that was either pre or directly incubated with the antigen to be determined. Labelled secondary antibody is then added. Visualization is brought about by addition of substrate (e.g. in the case of enzyme labels). In this format the absorbance values decrease as the analyte concentration increases i.e. the amount of antigen and the signal are inversely related.

1.5.3.3 Sandwich assay format

In the sandwich assay format development, two distinct antibodies that could simultaneously bind to the analyte (antigen) will be utilized. One of the two antibodies will be labelled. The other antibody will be first immobilized on the solid surface followed by the addition of antigen. Later labelled antibody is added to form a sandwich. The amount of colour generated by the label is directly proportional to the quantity of analyte present. The sensitivity of this assay is governed by two major factors: the amount of captured antibody on solid surface and the avidity of the two antibodies towards the antigen. For a sandwich assay a given antigen must have atleast two epitopes that are available for binding antibodies. Ideally, this should be affected allowing the first antibody to capture the antigen whilst the second labelled antibody binds to the other epitope.

Advantages

1. These assays are highly sensitive.

Limitations

1. Sandwich assay is particularly suitable for macromolecules. This assay is not suitable for lower molecular weight compounds, such as mycotoxins, due to the presence of only a single epitope.

2. The large size of an antibody relative to that of the toxin inherently prevents a second antibody from binding due to steric hindrance (Frederick *et al.*, 2009).

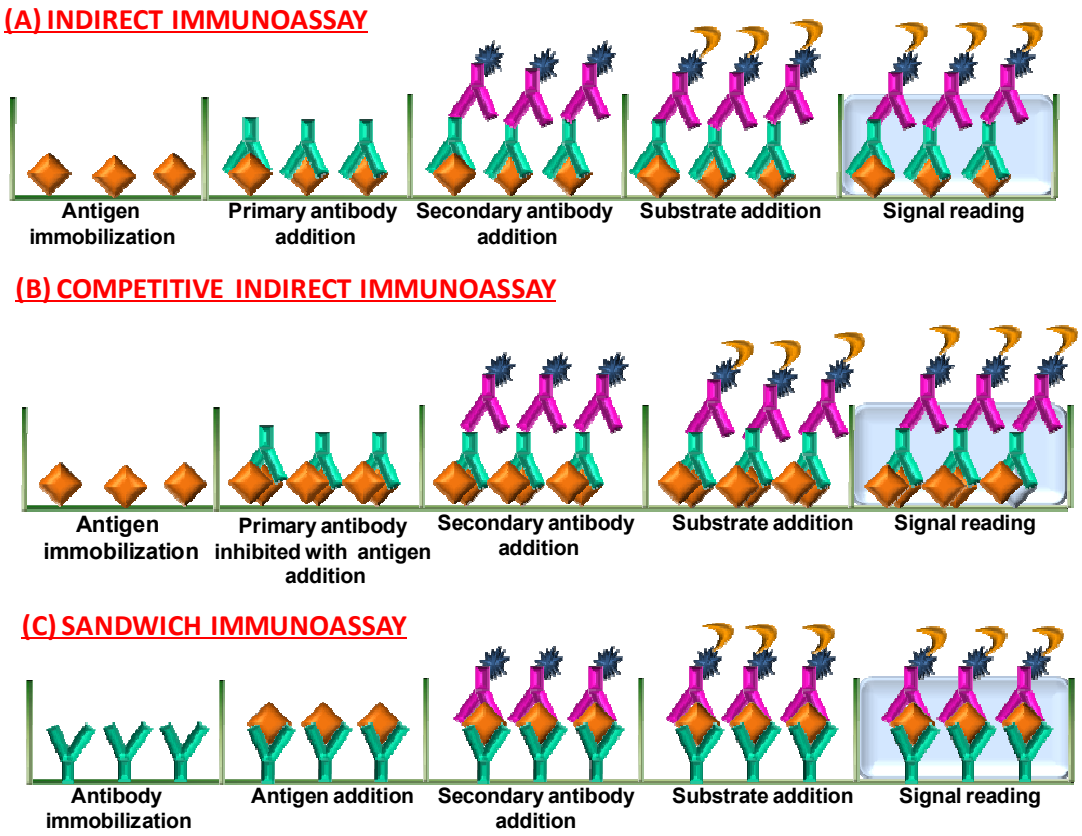


Figure 1.10: Pictorial representation of the different types of immunoassay formats. *Figure (A) represents the indirect immunoassay, where the antigen is immobilized on the solid support, followed by the addition of varying dilutions of antibody. Figure (B) indicates the competitive indirect immunoassay format in which the antibody that was pre-incubated with antigen will be used instead of antibody alone. Figure (C) shows the sandwich immunoassay format, in which two different antibodies will be used. In all the above mentioned immunoassay formats the addition of primary antibody will be followed by the addition of suitable secondary antibody with a label, followed by the detection of the chromophore reaction with a visualizing agent.*

1.5.4 Enzyme immunoassays

Enzyme-based immunoassays, such as ELISA, were developed to overcome the difficulties associated with RIAs, and also to provide an analytical method with improved sensitivity.

1.5.4.1 Stages in ELISA

For the performance of ELISA, antigen is immobilized on the solid support. After antigen immobilization the antibody is added leading to the formation of antigen-antibody complex, which is detected using a secondary antibody. To reduce non-specific binding of the antibodies, washing steps are performed with mild detergents like PBS and PBST. The visualization is brought about through the addition of substrate, which is converted to a product whose absorbance is read using a plate reader.

1.5.4.2 Factors affecting the ELISA assay performance

The various physiochemical factors that might have influence on the performance of an ELISA include: pH shift, ionic strength, organic solvents, incubation time, type of blocking solution used, quality of the reagents used for the assay and the presence of detergents. Organic solvents like methanol, which are frequently used for dissolving haptens, show a significant impact on overall assay performance. The characteristics of the antibody, as represented by its sensitivity and cross reactivity, also play a major role in ELISA performance. The immobilization strategy employed for incorporating antigen onto the solid support is another important factor.

1.5.4.3 Strategies to improve ELISA assay performance

To improve the overall assay performace the following strategies can be followed:

- Employing a well-designed and optimized antigen immobilization strategy to increase the number of antigens available on the surface.
- Selection of an appropriate antibody with high affinity towards the antigen.
- Selection of optimal buffers for dilution and washing steps.

- Minimizing buffer pH shifts.
- Reduction in the amount of organic solvents for dissolving the antigens in the assay.
- Optimization of the incubation times of the primary and secondary antibodies.
- Optimizing the dilutions of both primary and secondary antibodies.

1.5.4.4 Hapten immobilization strategies onto the solid support

The antigen is immobilized (either covalently or non-covalently) onto a solid support such as glass slides, a gold surface or polystyrene microtitre plates (Vidal *et al.*, 2009). Non-covalent approaches, e.g. adsorption, for coating hapten-protein conjugates onto solid surfaces were frequently used (Shan *et al.,* 2002). Drawbacks associated with non-covalent immobilization of haptens include the limited availability of haptens on the solid support surface for antibody binding (Wortberg *et al.*, 1996). In addition, the synthesis of hapten-protein conjugates with reproducible hapten density may be problematic. Decreased sensitivity of immunoassays with hapten-protein conjugate-coated plates in the presence of organic solvents such as methanol (Sittampalam *et al.*, 1996) and pH changes (Liu *et al.*, 2009) were also reported. Covalent immobilization of antigens onto solid supports, may enhance stability and sensitivity of assays (Dixit *et al.*, 2011), since the covalently attached antigen is less affected by factors such as pH, ionic strength, substrate, solvent and temperature (Willner and Katz, 2000).

1.5.5 Fluorescence-based immunoassays

Fluorescence is the emission of energy from fluorophore upon absorbing radiation (Cui *et.al.,* 2012). Fluorophores are chemical entities that fluoresce e.g. rhodamine, FITC or fluorescein. Fluorescence-based assays are often significantly more sensitive than conventional spectrophotometric assays. The sensitivity of these assays is affected mainly by interfereces caused by impurities and instability of fluorophores.

1.5.5.1 The MBio assay platform

This platform (Figure 1.11) is based on the use of mutlimode planar wave guide technology. Briefly, laser light is directed into a waveguide substrate where total internal reflection takes place. The plastic wave guide is activated to make it amine- reactive. In this microarray assay format toxin conjugates or proteins are printed prior to assembly into the disposable cartridge. They are subsequently blocked with a protein-based blocking agent, spin-dried, and then coated with a sugar-based stabilizer for storage. The fluid is driven through the cartridge by capillary action and hydrostatic pressure. Low autofluorescent plastic is used in the construction of the cartridge. The assay was performed by passing a fluorescently-labelled antibody over the antigen that was immobilized on the membrane of the MBio cartridges, followed by fluorescence intensity measurment. The assay results are processed using the MBio Diagnostics image processing software for measuring the fluorescence signal intensity.

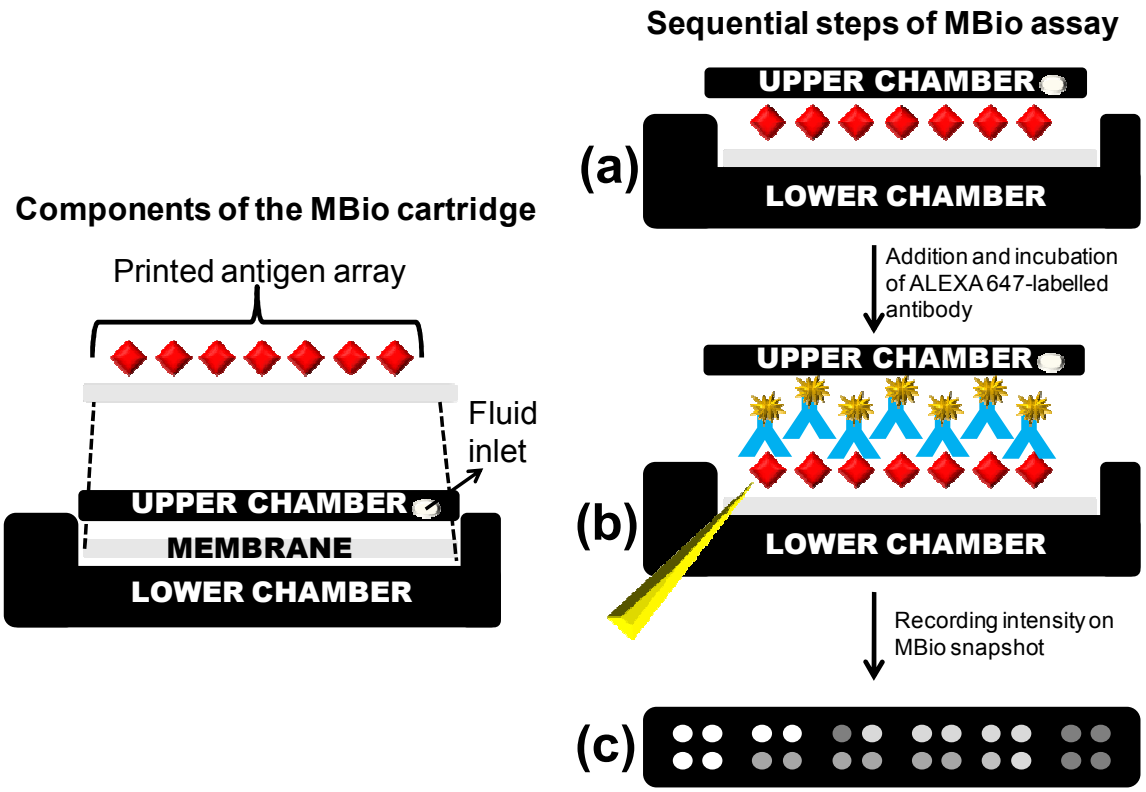


Figure 1.11: Schematic representation of the MBio diagnostic platform. *The major components of this system include the lower, upper chamber with a middle membrane (plastic waveguide), on which the antigens were imprinted. The upper chamber contains a fluid inlet. The sequential steps involved in the MBio assay setup after assembling the antigen immobilized plastic waveguide into the cartridge are (a) addition of primary antibody with a fluorescent label (b) incubation for 10-20 mins and reading the fluoresecence intensity using the MBio snapshot. The fluorescence intensities corresponding to different antigen-immobilized spots were recorded and image will be generated using the normalized values of the fluorescence reading attained (c).*

1.5.6 Immunosensors (biosensors)

A biosensor (Figure 1.12) is an analytical device that combines a biological ligand with a physical detector. It consists of three components, namely, a biological element (e.g. enzyme or antibody), a transducter for the conversion of the signal generated upon the interaction of analyte with the ligand into a measurable signal and a sensor output device that is capable of processing the signal and presenting it in an user-friendly format. Surface plasmon resonance (SPR) is an optical phenomenon, that occurs as a result of total internal reflection of light at a metal film-liquid interface. The major factors that may have significant impact on the quality of the biosensor-based assay are the binding capability and molecular weight of the analyte used. Due to their low molecular weight, a competitive assay is commonly used for mycotoxin detection in SPR-based systems.

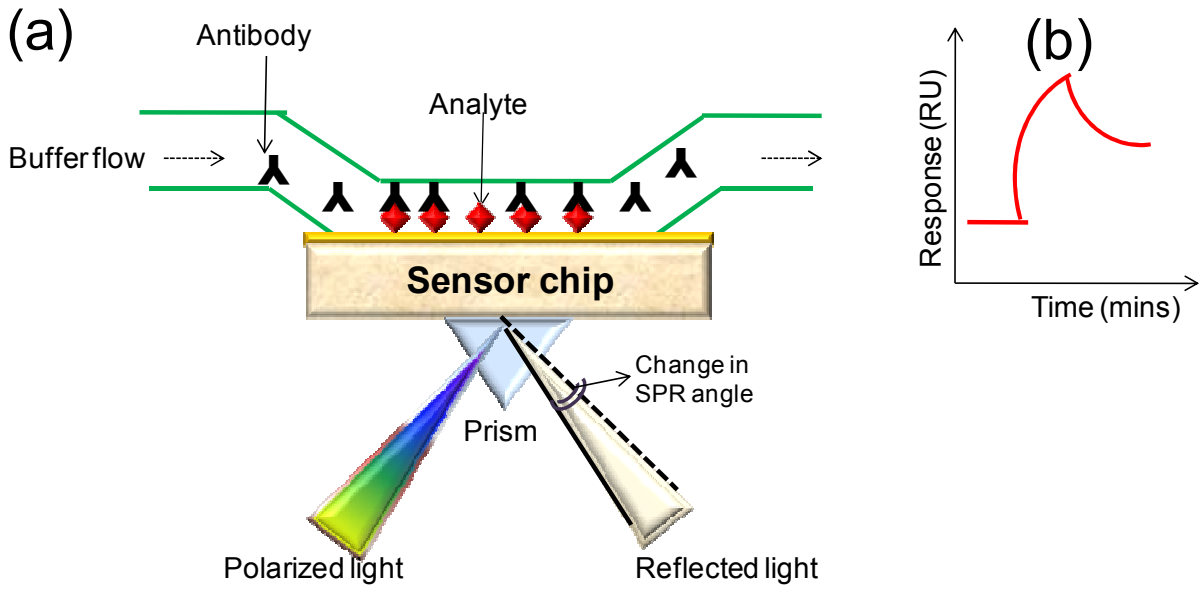


Figure 1.12: Schematic representation of the detection employed in a Biacore SPR-based assay. *Figure (a) indicates the specific change in the refractive index upon the passage of antibody over the analyte-immobilized surface. Figure (b) indicates a typical sensogram generated through measuring the shift in the reflected light and is presented as the change in measured response (RU) over time (mins).*

1.5.7 Immunochromatographic assays

Immunochromatographic assays utilize the principles of immunoassays combined with chromatographic techniques. They may be both qualitative and quantitative in nature. The advantages offered include 'user-friendliness', storage stability, rapid and 'on-site' determination and results that are highly comparable with the ELISA-based assays. The various practical considerations and steps involved in the immuno-affinity columns that was developed in the present work are described below.

1.5.7.1 Immuno-affinity columns

Immuno-affinity columns for use in mycotoxin isolation were designed for rapid, effective and 'one-step' purification of mycotoxins from complex matrices with extremely low limits of detection (e.g. determination of toxins in baby food). The major steps in the preparation of immuno-affinity columns include: choosing the immunosorbant, selection of the appropriate covalent linkage strategy to be followed for antibody immobilization, sample loading, washing, elution of the analyte and regeneration of the column for further use. Different types of solid supports that can be used as immunosorbant materials include polysaccharides, silica-based supports and synthetic polymers.

1.5.7.1.1 Key requirements for immuno-affinity column design

- The porosity of the immunosorbant plays a major role in maintaining the stability of the matrix. Porosity and stability are inversely proportional i.e., as the porosity increases the stability of the immuno-affinity column (immunosorbant) may frequently decrease.
- The immunosorbant should have functional groups on its surface that can be activated through the appropriate linker to allow the covalent immobilization of the antibody onto its surface.
- The avidity and specificity of the antibody used for immobilization is vital.

- Judicious selection of the activation and coupling chemistries employed play a major role in the development and practical success of immuno-affinity columns.

1.5.7.1.2 Steps involved in the operation of immuno-affinity columns

- *Loading the column:* The capacity of the column is governed by three factors i.e, the nature of the sorbant, the coupling capacity of the sorbant and also the quality of the antibody. The quality of the antibody attained is dependent on the type of conjugate and conjugation chemistry employed for the generation of the immunogen originally used for the pupose of raising an immune reponse in the animal host used for immunization. Other factors that also play a significant role in the performance of the column include the antibody immobilization procedure employed, the availability of antigen binding sites and also the 'flow-rate' used. The higher the 'flow-rate' the lower will be the time available for antigen-antibody interaction, which could lead to decreased column performance. However, it is also possible to incubate the analyte matrix with the sorbant for longer periods.
- *Column washing:* This step is performed to remove unbound and non-specifically bound substances from the column matrix. The lower the non-specific binding that occurs with the sorbant used the less stringent the washing required.
- *Elution of the analyte:* Elution of the analyte may be achieved through:
    - Usage of organic solvents leads to reduction in the K_a value of the antibody.
    - Altering the ionic strength may also decrease the K_a. This type of strategy is very rarely used.
    - Application of a chaotropic agent is another way of achieving elution, but it is also infrequently used.
- *Regeneration of the column:* To enable the column to be re-used a regeneration cycle is mandatory. In general the column will be washed with either PBS or water.

- *Storage of the column:* After regenerating the column can be stored in PBS with sodium azide (for preservation) at 4^0C.

Immuno-affinity columns (IACs) can provide excellent recovery of the analyte, due to the highly specific nature of the antibodies. In the present work, scFvs specific to AFB_2 and ZEN were developed and incorporated into IAC using conjugation chemistry. In addition, a Fab against AFB_1 was similarly included.

1.6 Thesis outline and aims

The purpose of this research (Figure 1.13) was to develop antibody-based assays for the specific detection of four major mycotoxins i.e., AFB_1, AFB_2, ZEN and T-2 toxin and to determine their potential for toxin infected food samples analysis. AFB_1 is highly toxic and hence there is a need to develop novel assays for its detection. AFB_2 is highly resistant to alkaline conditions normally employed during food processing techniques leading to their presence in processed foods, coupled with its conversion (metabolic oxidation) to AFB_1, led to the development of antibodies that could recognize AFB_2. Zearalenone is another mycotoxin of interest because of its higher prevalence in cereals. Most of the antibodies developed to date were highly cross reactive with structural analogs of ZEN especially α-Zearalenol and β-Zearalenol, which are the bye-products of biotranformation of ZEN. These components are not widely present in foods. Hence there is a need to develop antibodies that detect the parent moiety i.e. ZEN. Development of an antibody that could recognize structural analogs would be of interest for clinical sample analysis. T-2 toxin is a major contaminant of many agricultural commodities and causes various disorders in humans and in liverstock. Thus, polyclonal antibodies were developed for purification and isolation of T-2 specific antibodies from egg yolks.

Even though cross reactive antibodies are desirable in many cases (e.g. to isolate total levels of mycotoxins from food), the logic behind the generation and selection of very highly specific antibodies (without any crossreactivity) was to develop highly targeted immunoassays.

The production and characterization of scFv antibodies for AFB_2 and ZEN were described in chapter 3 & 4, respectively. The scFvs were characterized by ELISAs (indirect and competitive ELISA), SDS-PAGE/western blotting and SPR-based inhibition assays for the detection of AFB_1 and ZEN. Cross reactivity studies and the application of scFvs for the detection of AFB_2 in spiked almond extract and ZEN in sorghum reference materials was also performed.

The application of a polyclonal anti-T-2 IgY for the development of Biacore SPR-based inhibition assay was presented in chapter 5. Polyclonal IgY was isolated from eggs from a hyperimmune chicken and subjected to affinity purification followed by the ELISA, FPLC and MALDI-TOF analysis. This IgY was then used in an SPR-based inhibition assay.

Finally, in chapter 6, assay formats were developed for the isolation and detection of the aforementioned toxins using recombinant scFvs (for AFB_2 & ZEN) or Fab (for AFB_1) and polyclonal IgY (for T-2 toxin). Immuno-affinity columns were developed for specific isolation of AFB_1, B_2 & ZEN. The intention of developing an MBio assay capable of simultaneous detection of AFB_1, B_2, ZEN and T-2 toxins, using fluorescence-based detection, was not successful due to the unavailability of the requested MBio cartridges. In addition, an ELISA-based assay was developed for specific detection of AFB_1, where the AFB_1 was covalently immobilized on microtitre plates.

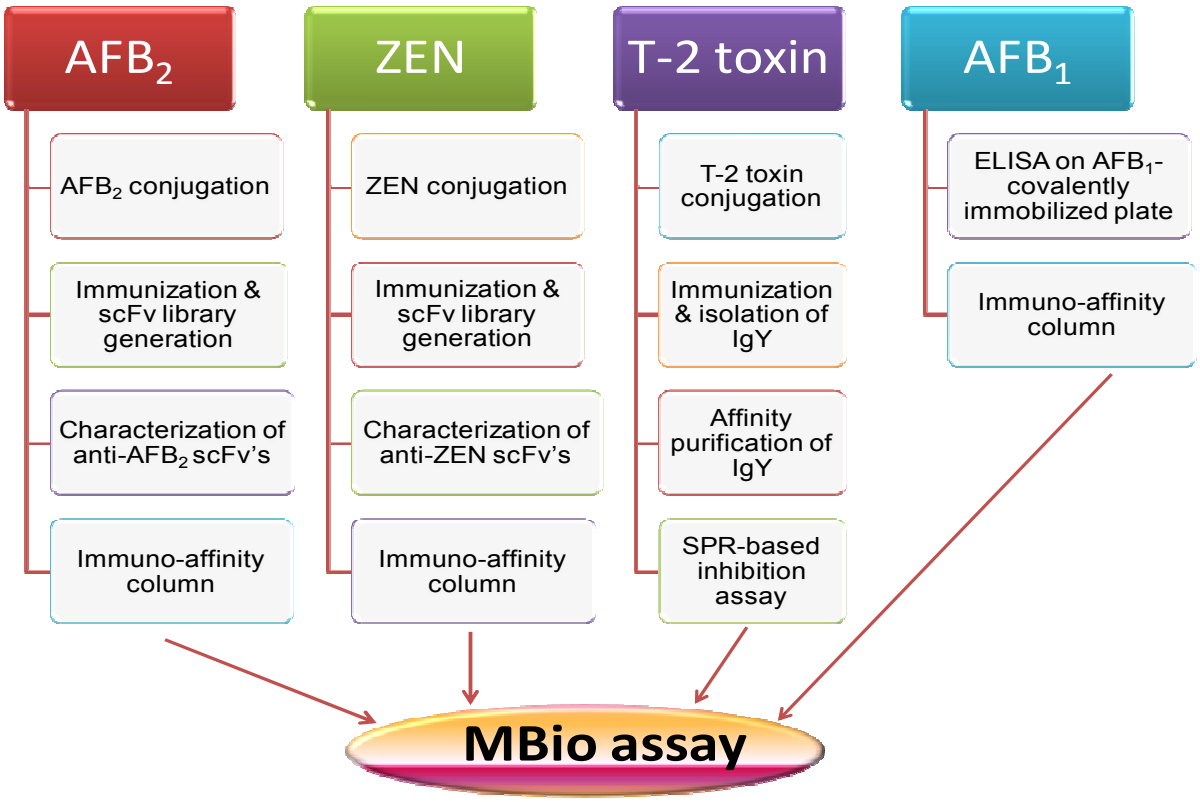


Figure 1.13: Layout of the thesis. *This thesis has four major chapters excluding the introduction and materials and methods chapters. Recombinant antibodies were developed and incorporated into an immuno-affinity column format for the isolation of AFB_2 and ZEN. An anti-T-2 toxin IgY was isolated with its subsequent incorporation into SPR-based inhibition. An immuno-affinity column was developed using anti-AFB_1 Fab antibody. A microtitre plate-based assay using an AFB_1 covalent immobilizaton approach was also developed. Thereafter MBio platform, which employs a fluorescence assay principle in conjunction with laser-activated waveguide technology was developed for the simultaneous detection of AFB_1, B_2, ZEN and T-2 toxins.*

Chapter 2
Materials and Methods

2. Materials and methods

2.1 Materials

The materials used for this study were described under the following headings: reagents, equipment, composition of culture media, bacterial strains, bacteriophages, buffers, commercial kits and antibodies.

2.1.1 Reagents

Reagent	Supplier
Bacteriological agar, tryptone and yeast extract	Cruinn Diagnostics Ltd., Hume Centre, Parkwest Business Park, Nangor Road, Dublin 12, Ireland.
T4 DNA ligase, helper phage and restriction enzymes (S*fi*I, S*ac*I, Antarctic Phosphatase, *XboI, XhoI*)	ISIS Ltd., Unit 1 & 2, Ballywaltrim Business Centre, Boghall Road, Bray, Co. Wicklow, Ireland.
PCR primers	Eurofins MWG Operon, 318 Worple Road, Raynes Park, London SW20 8QU, U.K.
Page Ruler^{TM} Prestained protein ladder	Fermentas, Sheriff House, Sheriff Hutton Industrial Park, York Y060 6RZ, U.K.
Superscript system	Bio-sciences, 3 Charlemont Terrace, Crofton Road, Dun Laoghaire, Dublin, Ireland.
1 Kb plus DNA ladder	Sigma Aldrich, Spruce street, St. Louis M063103, U.S.A.

2.1.2 Equipment

Equipment	Manufacturer
Applied Biosystems Veriti 96 well Thermal Cycler (MODEL: 9902)	Life Technologies Corporation, 5791 Van Allen Way, PO Box 6482, Carlsbad, California 92008, U.S.A.
Nanodrop™ ND-1000	NanoDrop Technologies, Inc., 3411, Silverside Rd 100BC, Wilmington DE19810-4803, U.S.A.
Gene Pulser Xcell ™ electroporation system	Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Drive, Hercules, California 94547, U.S.A.
ORBI-safe TS Netwise orbital shaking incubator	Sanyo Europe Ltd., 18 Colonial way, Walford WD24 4PT, U.K.
Trans-Blot® Semi-Dry Transfer Cell	Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Drive, Hercules, California 94547, U.S.A.
Roller mixer SRT1	Sciencelab, Inc., 14025, Smith Rd. Houston, Texas 77396, U.S.A.
Safire II plate reader	Tecan Group Ltd., Seestrasse 103, Mannedorf CH-8708, Switzerland.
Vibra Cell™ sonicator	Sonics and Materials Inc., 53 Church Hill Road, Newtown CT 06470-1614, U.S.A.
AKTA explorer FPLC	GE Healthcare Life Sciences, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, U.K.
Shimadzu LC system HPLC	Shimadzu Europa GmbH., Albert-Hahn-Str. 6-10, Duisburg 47269, Germany.
ESI-Mass spectrometer	Bruker Daltonics, Banner line, Coventry CV 4 9GH, U.K.
Speedvac apparatus (Model: DNA 23050-A00)	GeneVac Ltd, IP SWICH, England.

pH meter (Orion 3 star)	Medical Supply Company Ltd., Damastown, Mulhuddart, Dublin 15, Ireland.
IKA® MTS 2/4 digital plate shaker	Lennox, John F. Kennedy Drive, Naas Road, Dublin 12, Ireland.
Water bath (Y6 model)	Grant instruments (Cambridge) Ltd., 29 Station Road, Shepreth, Royston, Herts SG8 6PZ, U.K.
Biacore ® 3000 machine	GE Healthcare Biosciences Ltd., Uppsala SE-751 84, Sweden.
MBio array system	Mbio Diagnostics Inc., 5603 Arapahoe Avenue, Suite 1, Boulder, CO, 80303 U.S.A.

2.1.3 Bacterial culture media composition

In this section, different types of media (and their composition) used for growing bacterial strains are given.

Media	Composition	
Superbroth medium	Tryptone	30 g/L
	Yeast extract	20 g/L
	MOPS	10 g/L
Super optical catabolite medium (SOC medium)	Tryptone	20 g/L
	Yeast extract	5 g/L
	5 M NaCl	0.5 g/L
	1 M KCl	2.5 mL
	1 M $MgCl_2$	10 mL
	1 M $MgSO_4$	10 mL
	1 M Glucose	20 mL
2x Tryptone Yeast extract medium (2xTY)	Tryptone	16 g/L
	Yeast extract	10 g/L
	NaCl	5 g/L
Luria broth agar (LB agar)	Tryptone	10 g/L
	Yeast extract	5 g/L
	NaCl	10 g/L
	Agar	15 g/L
100X 505 media	Glycerol	50% (v/v)
	Glucose	50% (w/v)
Terrific broth (TB broth)	Tryptone	12 g/L
	Yeast extract	24 g/L
	Glycerol	4 mL
	KH_2PO_4	2.31 g/L
	K_2HPO_4	12.5 g/L

2.1.4 Bacterial strains & bacteriophages

Different types of bacterial strains used for expressing recombinant antibody fragments and bacteriophages, used for the effective enrichment of the required antibody fragments by phage display, are listed below:

Bacterial strains
***E. coli* TOP10 F ′ strain:** {*lac*I^{q}, Tn*10*(Tet^{R})} *mcr*A Δ(*mrr-hsd* RMS-*mcr*BC) ϕ80*lac*Z ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^{R}) *end*A1 *nup*G
***E. coli* XL1-Blue strain:** recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac *[F′* proAB lac I^{q}ZΔM15 *Tn*10 (*Tet*^{R})*]*
Bacteriophage (Helper phage)
M13K07

2.1.5 Antibiotic and synthetic analog of lactose (IPTG) stocks

The antibiotic and synthetic analog of lactose (IPTG) stocks described were used for the supplementation of nutrient media and to induce expression of cloned gene.

Antibiotic	**Stock**
Tetracycline	5 mg/mL of ethanol
Carbenicillin	100 mg/mL of 150 mM PBS (pH 7.4)
Kanamycin	50 mg/mL of 150 mM PBS (pH 7.4)
0.5 M Isopropyl β-D-1-thiogalactopyranoside (IPTG)	1.19 g/10 mL of distilled water

2.1.6 Buffer composition

The chemical compositions of the various buffers used in this study are listed below:

Buffers	Composition	
1 L of Phosphate buffered saline (PBS)	0.15 M NaCl	8 g
(150 mM, pH 7.4)	2.5 mM KCl	0.2 g
	10 mM Na_2HPO_4	1.44 g
	18 mM KH_2PO_4	0.24 g
The constituents were dissolved, the pH adjusted and the final volume made up to 1L.		
1 L of Phosphate buffered saline Tween (PBST)	1 L of PBS (pH 7.4)	
(150 mM, pH 7.4)	0.05% (v/v) Tween 20	
2% (w/v) milk Marvel PBST	200 mg of milk Marvel	
	10 mL PBST	
2% (w/v) milk Marvel PBS	200 mg of milk Marvel	
	10 mL of 150 mM PBS (pH 7.4)	
4% (w/v) milk Marvel PBS	400 mg of milk Marvel	
	10 mL of 150 mM PBS (pH 7.4)	
1 L of HEPES buffered saline (HBS, pH 7.4)	HEPES	2.38 g
	NaCl	8.77 g
	EDTA	1.27 g
	Tween	0.5 mL
The constituents were dissolved, the pH adjusted and the final volume made up to 1L.		

20 mL of MES NaCl (pH 7.4)	0.1 M MES	0.39 g
	0.9% (w/v) NaCl	0.18 g

The constituents were dissolved, the pH adjusted and the final volume made up to 20 mL.

20 mL of 1 M Tris (pH 9.0)	Tris-base	2.42 g

The constituents were dissolved, the pH adjusted and the final volume made up to 20 mL.

100 mM of Glycine-HCl (pH 3.0)	Glycine	1.11 g

The constituents were dissolved, the pH adjusted and the final volume made up to 100 mL.

20 mL of 1 M NaCl	NaCl	1.17 g
	Water	20 mL
100 mL of 2 M NaOH	NaOH	8 g
	Water	100 mL
200 mL of 8 M Urea, 2% (w/v) sodium dodecyl sulphate with 2% (v/v) mercaptoethanol	Urea	96 g
	2% (w/v) sodium dodecyl sulphate	4 g
	2% (v/v) mercaptoethanol	3.6 g
	Water	200 mL
500 mL of 20 mM Tris-HCl (pH 7.5), 0.1% (v/v) Tween 20 and 500 mM NaCl	20 mM Tris-HCl	0.6 g
	0.1% (v/v) Tween 20	200 µL
	500 mM NaCl	5.85 g

The constituents were dissolved, the pH adjusted and the final volume made up to 500 mL.

2.1.7 SDS-PAGE and western blotting reagents

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) involves the principle of separation of proteins based on their molecular mass upon electrophoretic migration towards anode in a polyacrylamide matrix. The gel matrix is formed from polyacrylamide chains, which are cross linked with N,N′-methylene bisacrylamide. The proteins are denatured under the presence of SDS leading to linearization. The protein will migrate through the gel towards the anode. The proteins are stained using Coomassie blue dye for visualization. The gel composition, buffer compositions, staining and destaining reagents for SDS-PAGE are given in the following list:

Separating gel	**Amounts/6 mL gel**
1 M Tris-HCl (pH 8.8)	1.5 ml
30% (w/v) Acrylagel	2.5 ml
2% (w/v) Bis-acrylagel	1.0 ml
10% (w/v) Sodium dodecyl sulphate (SDS)	30 µL
10% (w/v) Ammonium persulphate (APS)	30 µL
TEMED (N,N,N',N'- tetramethylethylenediamine)	6 µL
Water	934 µL
Stacking gel	**Amounts/2.5 mL gel**
1 M Tris HCl (pH 6.8)	300 µL
30% (w/v) Acrylagel	375 µL
2% (w/v) Bis-acrylagel	150 µL
10% (w/v) Sodium dodecyl sulphate (SDS)	24 µL
10% (w/v) Ammonium persulphate (APS)	24 µL
TEMED	2.5 µL
Water	1.74 µL
Composition of SDS buffers **10 X electrophoresis buffer**	**Amounts**
50 mM Tris	30 g
196 mM Glycine	144 g
0.1% (w/v) SDS	10 g

Water	1 L
Loading buffer	**Amounts**
0.5 M Tris	2.5 mL
Glycerol	2 mL
2-mercaptoethanol	0.5 mL
20% (w/v) SDS	2.5 mL
Bromophenol blue	20 ppm
Water	2.5 mL
1 L Coomassie stain dye	**Amounts**
Coomassie blue R-250	2 g
Methanol	450 mL
Acetic acid	100 mL
Water	450 mL
1 L Coomassie destain	**Amounts**
Acetic acid	100 mL
Methanol	250 mL
Water	650 mL
Western blot	
1L Transfer buffer	**Amounts**
Glycine	14.4 g
Tris base	3.0 g
Water	800 mL
Methanol	200 mL

2.1.8 Commercial kits used for these studies

Kits	Supplier
Superscript III reverse transcriptase kit (for cDNA synthesis from RNA)	Invitrogen Corporation, 5791 Van Allen Way, Carlsbad, CA 92008, U.S.A.
Nucleospin Extract II (for DNA purification from PCR products)	Fischer Scientific, Suite 3, Plaza 212, Blanchardstown Corporate Park 2, Ballycoolin, Dublin 15, Ireland.
Nucleospin Plasmid (for plasmid purification)	Fischer Scientific, Suite 3, Plaza 212, Blanchardstown, Corporate Park 2, Ballycoolin, Dublin 15, Ireland.
Eggcellent IgY purification kit (for IgY isolation from egg yolk)	Fisher Scientific, Suite 3 Plaza 212, Blanchardstown Corporate Park 2, Ballycoolin, Dublin 15, Ireland.

2.1.9 Commercial antibodies

Antibody	Supplier
HRP-labelled mouse anti-M13 monoclonal antibody	GE Healthcare Bio-Sciences AB, Uppsala SE-751 84, Sweden.
HRP-labelled rat anti-HA monoclonal Antibody	Roche Diagnostics, Grenzacherstrasse 124, Basel 4070, Switzerland.
AP-labelled goat anti-mouse IgG	Sigma Aldrich, Spruce Street, St. Louis M063103, U.S.A.
HRP-labelled rabbit anti-chicken Ig	Sigma Aldrich, Spruce Street, St. Louis M063103, U.S.A.

2.2 Methods

2.2.1 Synthesis of mycotoxin conjugates

The mycotoxin conjugates mentioned below were synthesized and characterized by Dr. E. Om Prakash. The Matrix assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF) analysis and fluorescence estimation data were presented in appendix (Appendix III & IV).

2.2.1.1 AFB_2-BSA and KLH

2.2.1.1.1 Preparation of Aflatoxin B_2–carboxymethoxylamine hemihydrochloride (AFB_2-CMO):

Carboxylmethoxylamine hydrochloride (15 mg) was added to a stirred solution of aflatoxin B_2 (2 mg) in 600 µL of ethanol with 1 N NaOH (70 µL). After 10 mins stirring at room temperature, the solution was refluxed for 4 hrs at 80^0C. The solvent was evaporated on a speed vac. The residue was dissolved in 300 µL of water and the pH was adjusted to 2.0. The aqueous mixture was extracted with ethylacetate and spotted onto a 20 x 20 cm (L X W) 250 µm thick silica gel (particle size 17 µm, pore size 60 Å) coated TLC plate with fluorescent indicator (254 nm). Preparative TLC plate was developed under UV light in chloroform:methanol (9:1) plus 1% (v/v) acetic acid. The AFB_2-CMO derivative was removed from the TLC plates through

scratching the upper silica layer and re-dissolved in chloroform. The mixture was centrifuged for 5 mins to remove silica gel residues before proceeding to next step.

2.2.1.1.2 Preparation of NHS ester of AFB_2-CMO

Freshly prepared DCC (50 µL from 40 mg/mL) and NHS (40 µL from 40 mg/mL) in dioxane were prepared and added to above AFB_2-CMO in dioxane (500 µL) solution. The reaction mixture was stirred overnight at room temperature. Fine needles of the dicyclohexylurea bye-product were then observed and the mixture was centrifuged (2,109 xg) for 5 mins to remove them. The formation of the NHS ester was confirmed by TLC in chloroform and methanol (7:3) solvent system.

2.2.1.1.3 Preparation of AFB_2-protein conjugates

Stock solutions of KLH (8 mg) and BSA (6 mg) were prepared by dissolving in coupling buffer (800 µL of 0.1 M $NaHCO_3$, pH 9.5). AFB_2-CMO-NHS ester solution (300 µL) was then added dropwise to an ice-cold solution of each respective protein and kept at room temperature for another 2 hrs with continuous magnetic stirring. The reaction mixture was dialyzed (buffer exchanged) with 150 mM PBS (pH 7.4) using a Vivaspin column (M.Wt. 'cut-off': 5 *k*Da). After buffer exchange, the mixture was collected and resuspended in PBS to make up 1 mg/mL final concentration of AFB_2-BSA/KLH conjugate. This conjugate was confirmed by indirect ELISA using monoclonal anti-AFB_2 antibody obtained commercially from Abcam.

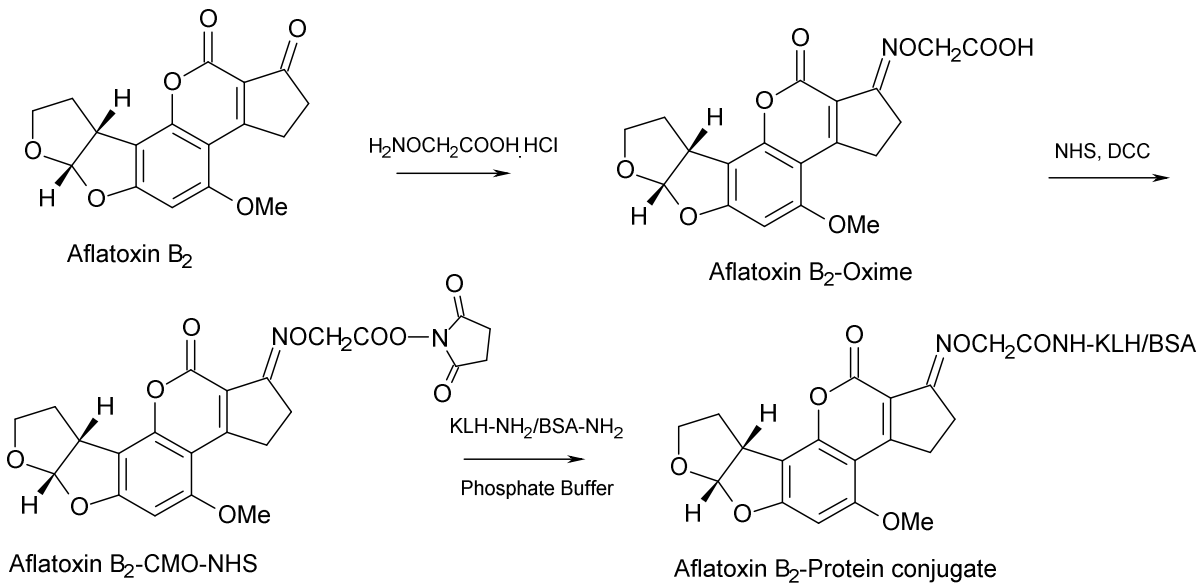


Figure 2.1: Schematic representation of AFB_2 conjugate synthesis.

2.2.1.2 ZEN-BSA and KLH

2.2.1.2.1 Preparation of Zearalenone-carboxymethoxylamine hemihydrochloride (ZEN-CMO)

Zearalenone (5 g) was dissolved in 300 µL of anhydrous pyridine. Then, 10 mg of carboxymethoxylamine hydrochloride were added. The mixture was stirred for 24 hrs at room temperature under argon. The solvent was evaporated on a speed vac apparatus. The residue was dissolved in 500 µL of water and extracted with toluene (500 µL X 5) spotted onto silica gel TLC plate with fluorescent indicator (254 nm). Preparative TLC plate (20 X 20 cm) was developed with the solvent system toluene:ethylacetate:formic acid (5: 4: 1 (v/v)) under UV light to visualize the spots. The ZEN-CMO derivative was then removed from the TLC plates by scratching the silica surface and re-dissolved in chloroform and centrifuged for 5 mins to remove silica gel residues.

2.2.1.2.2 Preparation of NHS ester of ZEN-CMO

Freshly prepared DCC (50 µL from 40 mg/mL of dioxane) and NHS (40 µL from 40 mg/mL of dioxane) solutions were prepared and added to 5 mg of ZEN-CMO in 500 µL of dioxane. The

reaction mixture was stirred overnight at room temperature. Fine needles of the dicyclohexylurea by-product were then observed and the mixture was centrifuged (2,109 xg) for 5 mins to remove them. The formation of NHS ester was confirmed by TLC in chloroform and methanol (7:3).

2.2.1.2.3 Preparation of ZEN-protein conjugates

Stock solutions of KLH (8 mg) and BSA (6 mg) were prepared by dissolving in coupling buffer (800 µL of 0.1 M $NaHCO_3$, pH 9.5). ZEN-CMO-NHS ester solution (300 µL) was then added to an ice-cold solution of each respective protein and kept at room temperature for another 2 hrs with continuous stirring. After the reaction, the mixture was dialyzed (buffer exchanged) with 150 mM PBS (pH 7.4) using a Vivaspin column (M.Wt. 'cut-off': 5 *k*Da). The buffer exchange was followed by the confirmation of the conjugate formation through indirect ELISA assay using anti-ZEN serum.

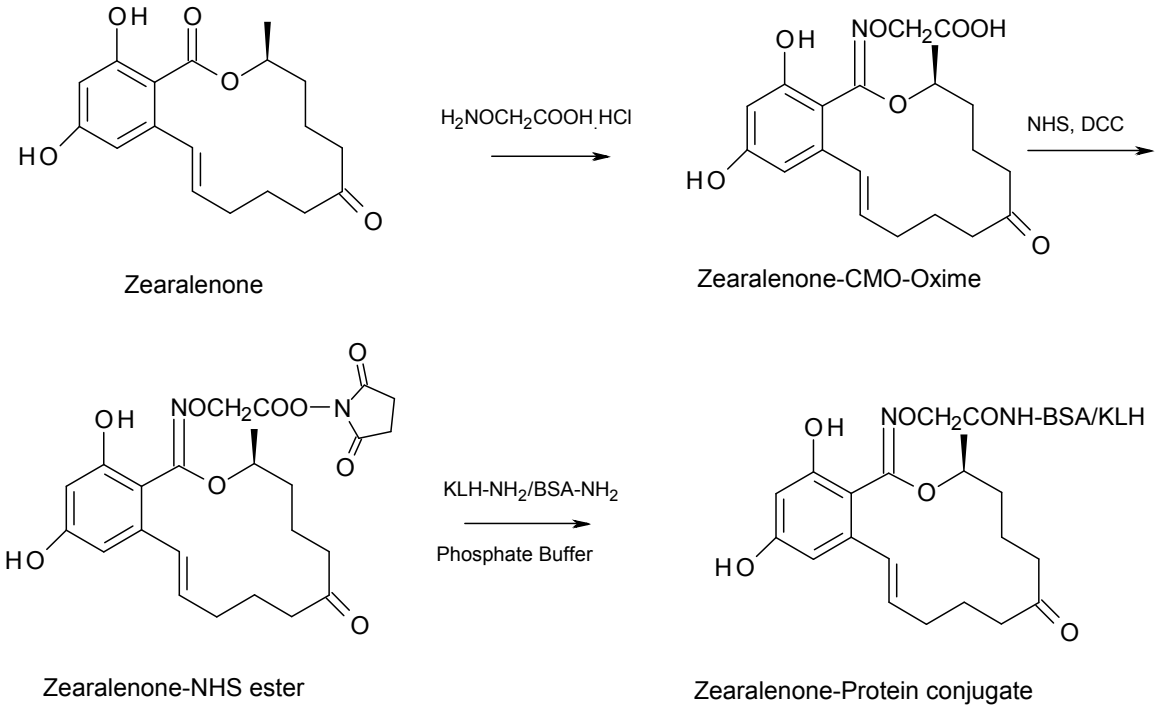


Figure 2.2: Schematic representation of ZEN conjugate synthesis.

2.2.1.3 T-2-BSA and KLH

T-2 (5 mg) was dissolved in dimethyl sulfoxide (100 µL), and 2.5 mg of carbonyldiimidazole were added. The mixture was stirred for 15 mins at room temperature in the dark. The mixture was split into half and added drop-wise to an ice-cold solution of either BSA (15 mg) or KLH (15 mg) dissolved in 1 mL of 0.1 M Na_2CO_3 (pH 9.6) solution, in individual vials. The reaction mixture was stirred at 4^0C overnight followed by stirring at room temperature for 1 hr. The mixture was then dialyzed (buffer exchanged) with 150 mM PBS (pH 7.4) using a Vivaspin column (M.Wt. 'cut-off': 5 *k*Da). This was followed by the performance of indirect ELISA assay for confirmation of conjugate formation.

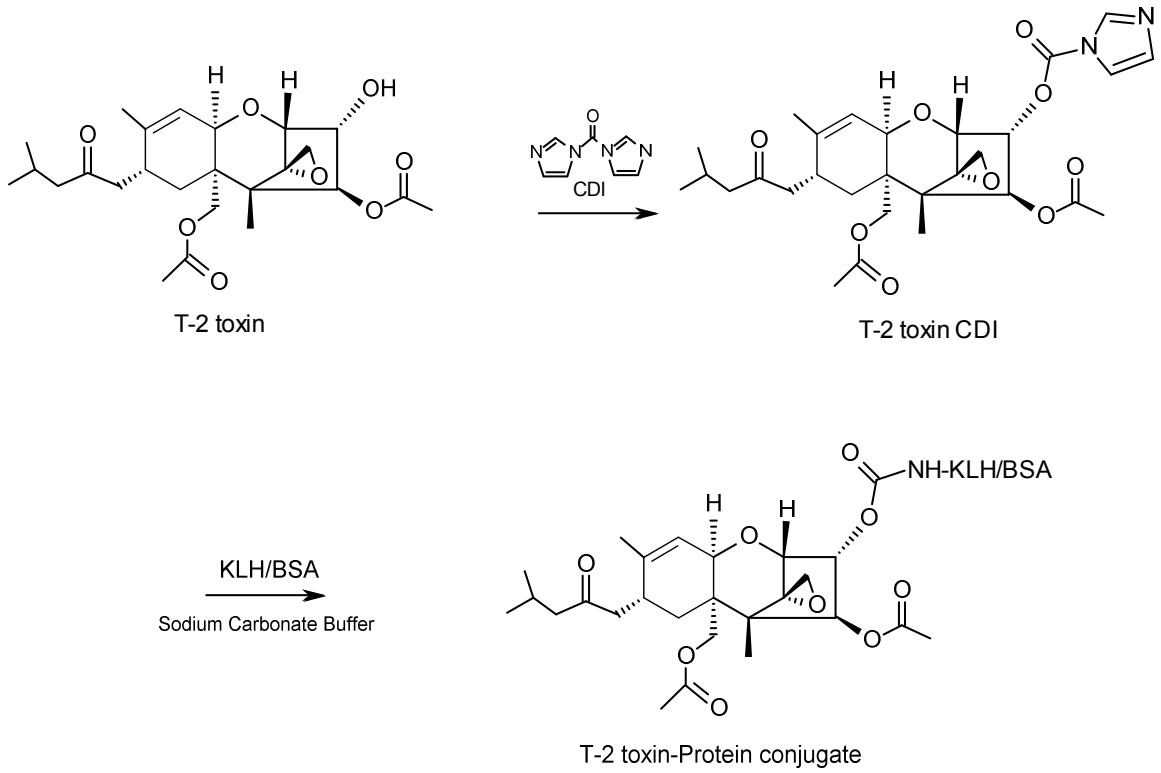


Figure 2.3: T-2 toxin conjugate synthesis by CDI method.

2.2.1.4 Mycotoxin conjugate confirmation

2.2.1.4.1 MALDI-TOF analysis of hapten conjugates

The mass analysis of hapten conjugates (Appendix IV) was done with a MALDI-TOF mass spectrometer (Bruker Autoflex MALDI-TOF Mass Spectrometer). The matrix (sinapinic acid solution) was prepared at a concentration of 15 mg/mL in acetonitrile. All the above mentioned

hapten protein conjugates were dialyzed (buffer exchanged) with distilled water using a Vivaspin column (M.Wt. 'cut.off': 5 *k*Da) to a final conjugate concentration of 2.0 mg/mL. The hapten-protein conjugate sample and matrix solution were mixed in equal amounts (1 µL each) with 0.1% (v/v) formic acid and added onto a silicon MALDI-TOF chip. The samples were allowed to dry in a vaccum desiccator and were then used for mass analysis. The data were acquired in the linear mode at 20 kV and analyzed using the Compass software suite.

2.2.1.4.2 Fluorescence assay

The AFB_2 and ZEN conjugates were confirmed by fluorescence intensity measurements (Appendix III). Fluorescence of 150 mM PBS (pH 7.4) and BSA/KLH were also read at the same wavelengths, which were treated as background values. A higher fluorescence reading was recorded when either AFB_2 or ZEN conjugates were used, when compared to the background values, which clearly indicates the presence of fluorescent compounds (AFB_2 and ZEN).

2.2.1.4.3 2, 4, 6-Trinitrobenzene sulfonic acid (TNBS) assay

The hapten densities of both BSA and KLH conjugates were determined through estimating the number of amino groups present on the protein before and after hapten conjugation by TNBS assay. The percentage amino groups consumed was estimated by the difference in absorbance of control (BSA/KLH) and conjugate read at 335 nm. This result will give the approximate number of amino groups used for conjugation (where BSA has 60 and KLH has 1430 available number of amino groups). A higher density of haptens on KLH when compared to BSA was noticed (Appendix III), which is due to the availability of more lysine NH_2 groups that are capable of reacting with carboxylic groups of hapten.

2.2.2 Immunization and immune response determination in Balb/c mice boosted with mycotoxin conjugates

2.2.2.1 Immunization schedule

This work was carried out under the license number B100/4202. Utmost care was taken to minimize the pain and suffering to any animals used. For the isolation of highly specific antibodies, a widely diverse library coupled with an efficient selection procedure is required. The initial step for the construction of an immune library is the proper immunization schedule with an antigen capable of eliciting immune response in the animal models. A high titer value indicates the presence of abundant amount of RNA that was enriched with the antigen binding immunoglobulin genes. Sets of three female Balb/c mice, aged 5-8 weeks, were immunized for the first time with 200 µL/mouse of 100 µg/mL of BSA or KLH conjugates of AFB_2 and ZEN, individually, which were synthesized following the procedure that was stated in section 2.2.1, along with Freund's complete adjuvant in the ratio of 1:1. The conjugate-adjuvant emulsion was made through vortexing the eppendorf tube with both the conjugate and adjuvant for one hour. Further immunizations (boosters) were carried out with the same amount (200 µL/mouse of 100 µg/mL of conjugate) of immunogen along with Fruend's incomplete adjuvant in the ratio of 1:1. Pre-immunization bleeds were collected through tail bleeding and the serum titre was checked after administering the immunogen for the third time. The immunization schedule is depicted in the Table 2.1.

Table 2.1: *Immunization schedule of female Balb/c mice*

Immunogen	Quantity/mouse	Injection interval
AFB_2-KLH/BSA	20 µg/200 µL	3 weeks
ZEN-KLH/BSA	20 µg/200 µL	3 weeks

2.2.2.2 Immune response determination of mice to mycotoxin conjugates (Indirect ELISA)

A 96-well Nunc Maxisorb™ microtitre plate was coated overnight with 100 µL of 5 µg/mL of individual mycotoxin conjugates (4 rows) as determined by checkerboard ELISA, BSA (2 rows) and KLH (2 rows). The plate was then blocked with 4% (w/v) milk Marvel in 150 mM PBS (pH 7.4) for 2 hrs at 37^0C. Serial dilutions (1:100 to 1:512,000) of both pre-immunization and post-immunization sera were made in 2% (w/v) milk Marvel PBST and 100 µL of each were added in duplicate to the individual wells of the microtitre plate. Following one hour incubation, the plate was washed three times with both PBST & PBS. Then, 100 µL of anti-mouse IgG AP-labelled goat secondary antibody (1:1,000 dilution) were added and incubated for an hour at 37^0C. The plates were again washed thrice with PBST & PBS after decanting the solution. Following the addition of 100 µL of pNPP substrate, the plates were incubated for 30 mins at 37^0C. The absorbances were then read on a Tecan Saffire plate reader at 405 nm.

2.2.3 Generation of murine scFv library for AFB_2

2.2.3.1 Serum titre determination

Four out of the 8 rows for a 96 well Nunc microtitre plate were coated overnight at 4^0C, with 5 µg/mL of AFB_2-BSA conjugate. Two rows were coated with 5 µg/mL BSA in 150 mM PBS (pH 7.4) and the remaining two rows with 5 µg/mL KLH in 150 mM PBS (pH 7.4). An indirect ELISA procedure described in section 2.2.2.2 was performed.

2.2.3.2 Isolation of RNA from the spleen

The mouse with the highest immune response (titre value 1:512,000) was sacrificed for the isolation of RNA. In this process, the laminar hood was cleaned with denatured alcohol (IMS) and sprayed with RNA Zap to kill any RNases that might destroy the RNA present in the spleen. The mouse was euthanized with tetraflourane, followed by neck dislocation. The spleen was then carefully and quickly transferred into a 50 mL 'RNase-free' tube with TRIZOL

reagent, which aids in maintaining the structural intergrity of the RNA. Efficient homogenization is critical in reducing the effects of nucleases present in the spleen, hence, homogenization was carried out using a handheld device. The homogenized spleen was kept static for 15 mins at room temperature in a laminar hood to aid in the settlement of the solid fraction to the bottom of the tube. The mixture was then centrifuged for 10 mins at 2,109 xg at 4^0C in an Eppendorf centrifuge (model: 5810R). This process aids in the separation of solid particles leaving behind the solution that possess the genetic material i.e., RNA in the supernatant. Then, 2 mL of chloroform was added, mixed gently and incubated for 15 mins at room temperature. Through the addition of chloroform, the proteins were denatured and became soluble in the organic phase while the nucleic acids remained in the aqueous layer. The mixture was then centrifuged at 17,500 xg for 20 mins at 4^0C in an Eppendorf centrifuge (model: 5810R) using the rotor model No. F-34-6-38, which led to the formation of three layers i.e., lower organic, middle fatty and the upper aqueous layers. Due to the presence of nucleic acids in the top aqueous layer, it was collected into a separate tube and 5 mL of isopropanol was added, then mixed and kept at room temperature for 10 mins. The use of isopropanol with NH_4 cations removed the alcohol-soluble salts and also caused to the aggregation of the RNA for the formation of a fine pellet upon centrifugation of the solution at 4^0C for 25 mins at 17,500 xg. After centrifugation, the supernatant was removed carefully without disturbing the pellet. Then 10 mL of 75% (v/v) ice cold ethanol was added to the pellet for dehydrating the RNA and, thus, leading to its subsequent precipitation. The eppendorf tube with the pellet and 75% (v/v) ice-cold ethanol in 25% (v/v) molecular grade water, was centrifuged for 20 mins at 4^0C at 17,500 xg in an Eppendorf centrifuge (model: 5810R) using the rotor model No. F-34-6-38. The supernatant was discarded leaving behind the pellet, which was air dried and re-suspended in 250 µL of molecular grade water. The amount of RNA thus obtained was quantified on the Nanodrop™ and the final amount of RNA attained was calculated using the formula nano drop reading (ng/µL) X total volume.

2.2.3.3 Reverse transcription of cDNA from RNA

The process of synthesis of cDNA from the RNA template by means of the enzyme reverse transcriptase is termed reverse transcription. For the performance of reverse transcription, all the reagents except the enzymes, were thawed on ice. The PCR reaction was carried out by placing the master mixture 1 (composition described in the table below), into 8 PCR tubes each containing a final volume of 25 µL. Denaturation of secondary structure of RNA was performed by placing mixture 1 at 65^0C for 5 mins. Then 25 µL of the master mixture 2 (composition given in table mentioned below) were added to the master mixture 1 with denatured RNA. The reaction mixture was incubated at 50^0C for 50 mins, during which the first cDNA strand was synthesized. The reaction was terminated by heating the mixture at 85^0C for 5 mins. During these two steps of PCR reaction annealing of the Oligo-(dt) to the RNA template with a poly (A) tail at the 3′ end was performed. Finally, 5 µL of RNase™ H was added to individual tubes and incubated for 20 mins at 37^0C. This step ensured the removal of residual RNA, which might effect the final quality of the cDNA. The composition of the two types of mixtures and the PCR conditions are outlined below:

Mixture 1	
RNA	15 µL (5 µg/rxn)
Oligo-(dt)20	20 µL (0.5 µg/rxn)
dNTPs	20 µL (1 mM)
Water (DEPC)	145 µL (N/A)

Mixture 2	
10X RT Buffer	40 µL (2X)
$MgCl_2$	80 µL (2.5 mM)
DTT	40 µL (20 mM)
RNaseOUT	20 µL (1 U/µL)
Superscript III RT	20 µL (200 U/µL)

PCR conditions

The PCR reaction was carried out on Applied Biosystems 96 verticell machine.

Stages	PCR temperature	Time
1	65^0C	5 mins
Added 25 µL of mixture 2 after keeping on ice for 1 min **(PAUSE)**		
2	50^0C	50 mins
3	85^0C	5 mins
Added 5 µL of RNase H **(PAUSE)**		
4	37^0C	20 mins
5	4^0C	∞

2.2.3.4 Amplification of variable heavy and light chains

Following the reverse transcription of RNA to cDNA the V_λ, V_k and V_H genes were amplified separately on a PCR machine. In this process the conditions were first optimized using a range of $MgCl_2$ concentrations (1, 1.5, 2 & 3 mM). The optimization was followed by scale-up of the product through performing 20X reactions. Ethanol precipitation of the amplified heavy and light chain genes was carried out through the addition of 100% (v/v) ice cold ethanol (2X volume of either V_λ/ V_k/V_H), $1/10^{th}$ volume of 3 M sodium acetate and 2 µL of glycogen. The low dielectric point of ethanol when compared to water makes the usage of ethanol more feasible for precipitating DNA. Three molar sodium acetate aids in neutralizing the charges on the sugar phosphate backbone, thus making it far less hydrophilic. Glycogen was used as a carrier for better recovery of the DNA present in lower concentrations, upon overnight incubation. The ethanol-precipitated PCR products were then resolved on 1% (w/v) agarose gel. The DNA present in the gel was purified using Nucleospin Extract II kit.

The primers used for the amplification of heavy and light chains along with the PCR mixture composition and the PCR conditions were:

Murine scFv primers

V_{λ} primers

Forward primers

5′ GGG CCC AGG CGG CCG AGC TCG ATG CTG TTG TGA CTG TTG TGA CTC AGG AAT C 3′

Reverse primers (Long linker)

5′ GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA GCC TAG GAC AGT CAG TTT GG 3′

V_H primers

Forward primers

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAG GTC ATC GAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAC TTG GAA GTG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTC CAA CTV CAG CAR CC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTT ACT CTR AAA GWG TST G 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAR STT GAG GAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTR AAG CTT CTC GAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG CAR CTT GTT GAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAG CTG ATG GAR TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG CAM CTG GTG GAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG CAG SKG GTG GAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AWG YTG GTG GAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG AAS STG GTG GAA TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTC CAC GTG AAG CAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTY CAR CTG CAG CAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTY CAG CTB CAG CAR TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTC CAR CTG CAA CAR TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTG CAG CTG AAG SAS TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTB CAG CTB CAG CAG TC 3′

5′ GGT GGT TCC TCT AGA TCT TCC CTC GAG GTR MAG CTT CAG GAG TC 3′

5′ GGA AGA TCT AGA GGA ACC ACC TTT CAG CTC CAG CTT GGT CCC 3′

Reverse primers

5′ CCT GGC CGG CCT GGC CAC TAG TGA CAT TTG GGA AGG ACT GAC TCT C 3′

5′ CCT GGC CGG CCT GGC CAC TAG TGA CAG ATG GGG CTG TTG TTG T 3′

5′ CTG GCC GGC CTG GCC ACT AGT GAC AGA TGG GGS TGT YGT TTT GGC 3′

V_K primers

Forward primers

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTT TGC TGA CTC AGT C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGA TGA CAC AAC C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGA TGA CCC AGW T 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGA TAA CYC AGG A 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGA TGA CBC AGK C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYR TTK TGA TGA CCC ARA C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTS TRA TGA CCC ART C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG WGC TSA CCC AAT C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TTC TCA WCC AGT C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TYC AGA TGA CAC AGA C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTC AGA TGA YDC AGT C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTM AGA TRA MCC AGT C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TRA TGA CMC AGT C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGY TRA CAC AGT C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TGM TMA CTC AGT C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TTG TTC TCW CCC AGT C 3′

5′ GGG CCC AGG CGG CCG AGC TCG AYA TCC AGC TGA CTC AGC C 3′

Reverse primers (Long linker)

5′ GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TTT KAT TTC CAG YTT GGT CCC 3′

5′ GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TTT TAT TTC CAA CTT TGT CCC 3′

5′ GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TTT CAG CTC CAG CTT GGT CCC 3′

PCR mixture composition	Amount in 50 µL final volume
cDNA	0.5 µL
Sense primers	0.5 µL
Reverse primers	0.5 µL
5X PCR buffer	10 µL
2.5 dNTP	1 µL
$MgCl_2$	2, 3, 4 or 6 µL
Molecular grade water	35.24, 34.25, 33.25 or 31.25 µL
Tag polymerase	0.25 µL

PCR condition Stages	Temperature	Time	
Initialization	95^0C	5 mins	
Denaturation	94^0C	15 sec	30 cycles
Annealing	56^0C	30 sec	
Elongation	72^0C	90 sec	
Final elongation	72^0C	10 mins	
	4^0C	∞	

2.2.3.5 Nucleospin Extract II purification of variable heavy and light chains

The amplified heavy and light chain products were run on 1% (w/v) agarose gel at 100 V in Bio-Rad gel chamber using 1X TAE as the running buffer. The bands corresponding to the 386-440 bp, for the V_H chain and 375-402 bp, for the V_L chain were cut out and placed in weighed 2 mL eppendorf tubes. The weight of the gel was determined and twice the volume of the binding buffer with the chaotropic agent (for easy dissolution of the DNA present in the gel) was added and incubated at 50^0C for 10 mins in a water bath. After the gel was dissolved completely, the solution was transferred onto silica columns provided with the Nucleospin Extract II system to enable the binding of the DNA to the silica particles present in the column. The column with the dissolved gel was centrifuged at 11,498 xg for 1 min at 4^0C in Hermle Z233 MK-2 centrifuge. The solution in the lower collection tube was discarded. This was followed by 'clean-up' of the column with NT3 wash buffer, which is an ethanol-based solution that will aid in the removal of unnecessary salts and macromolecules from the column. The washing step was performed through the addition of 700 µL of wash buffer and the column was centrifuged for 1 min at 11,498 xg at 4^0C followed by a another spin (without any solution in the column) of the column for 2 mins at 11,498 xg to remove any traces of wash buffer. For the efficient elution of DNA, the elution buffer, composed of 5 mM of Tris/HCl (pH 8.5), was pre-heated to 70^0C and was added to the column and incubated at room temperature for 1 min. This was followed by centrifugation for 1 min at 11,498 xg at 4^0C. The final quantification of pure DNA was determined on a Nanodrop™ at 260 nm.

2.2.3.6 Splice by overlap extension (SOE)

The purified V_H and V_L antibody fragments were connected via glycine-serine linker using an SOE-PCR. The following primers were utilized for the performance of splice by overlap extension using a Platinum® Taq DNA Polymerase High Fidelity for efficient proof reading of the proper base pairs.

SOE primers
Forward primers
5′ GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC 3′
Reverse primers
5′ GAG GAG GAG GAG GAG GAG CCT GGC CGG CCT GGC CAC TAG TG 3′

The PCR reaction was carried out on an Applied Biosystems PCR machine under the following conditions:

PCR condition Stages	Temperature	Time	
1	94^0C	2 mins	
2	94^0C	30 sec	30 cycles
3	56^0C	30 sec	
4	72^0C	120 sec	
5	72^0C	10 mins	
6	4^0C	∞	

2.2.3.7 S*fi*I restriction digestion of the SOE product

The purified SOE fragment was used for S*fi*I restriction digestion at the sites 5′ GGCCNNNNNGGCC 3′ and 3′ CCGGNNNNNCCGG 5′. The reaction mixture was composed of 10 µg of purified overlap PCR product, 360 units of S*fi*I (36 units per µg of DNA) and 20 µL of 10X reaction buffer. The digestion was carried out at 50^0C for 5 hrs on an heating block. Later, the digested SOE product was ethanol-precipitated overnight. Purification of the digested insert (~700 bp) was carried by running on a 1% (w/v) agarose gel and through subsequent

purification of the DNA using a Nucleospin Extract II system. The composition of the S*fi*I digestion mixture is described in the following table:

Components	SOE (k)	SOE (ʎ)
SOE	240 µL	240 µL
S*fi*I	10 µL	12 µL
100X BSA	3 µL	3 µL
NEB II buffer	30 µL	30 µL
Molecular grade water	17 µL	15 µL
TOTAL	300 µL	300 µL

2.2.3.8 Restriction digestion of pComb 3XSS

The plasmid with human influenza hemagglutinin (HA) tag was first confirmed through restriction digestion with *Sfi*I and S*ac*I restriction enzymes. For this a small-scale reaction was carried out with S*fi*I (50^0C for 4 hrs) and S*ac*I (37^0C for 4 hrs) along with the vector (pComb 3XSS). This was followed by running the samples on a 0.5% (w/v) agarose gel. The recognition sites for S*ac*I were 5′ GAGCTC 3′ and 3′ CTCGAG 5′. The vector was linearized when S*ac*I digestion was performed. Restriction digestion with S*fi*I leads to the segregation of Stuffer fragments present between the two S*fi*I recognizing sites. The vector used for ligation was restriction digested only with S*fi*I enzyme.

Large-scale digestion of the vector used was performed using S*fi*I enzyme. The reaction mixture for the S*fi*I restriction digestion of pComb 3XSS consisted of 20 µg of vector DNA, 120 units of S*fi*I (6 units per µg of plasmid DNA) and 20 µL of 10X reaction buffer. The S*fi*I digestion of the vector was carried out for 4 hrs at 50^0C. Then, the mixture was treated with X*ho*I and X*ba*I for 1 hr at 37^0C for the further breakdown of the Stuffer fragments. Later Antarctic phosphatase was added and incubated at 37^0C for 15 mins for the prevention of self-ligation of the vector through removing the phosphate group from the 5′ end. The enzyme was then deactivated by placing at 65^0C for 5 mins. The digested vector was ethanol-precipitated

overnight. Purification of digested vector, which is ~3,400 bp, was performed by running the ethanol-precipitated mix over an 0.5% (w/v) agarose gel, followed by the purification of the spliced DNA embedded gel using Nucleospin Extract II system.

The composition of the restriction digestion mixture is as follows:

Components	Amount/100 µL final volume
Vector (pComb 3XSS)	20 µL
NEB II buffer	10 µL
100 X BSA	1 µL
S*fi*I	9 µL
Molecular grade water	60 µL

2.2.3.9 Ligation of SOE product into pComb 3XSS

The digested vector was then ligated into the S*fi*I cut SOE using T4 DNA ligase. The vector and SOE were in the ratio of 3:1 and the reaction mixture was kept static at room temperature (18^0C) for 18 hrs and the enzyme was deactivated through heating the mixture at 90^0C for 10 mins over a heating block. The components of the ligation mix are shown in the table below:

Components	SOE (k)	SOE (ƙ)
5 X ligase buffer	20.0 µL	20.0 µL
Digested SOE	46.97 µL	7.15 µL
Digested pComb 3XSS	11.55 µL	11.55 µL
T4 DNA Ligase	0.5 µL	0.5 µL
Molecular grade water	20.98 µL	160.80 µL
Final Volume	200 µL	200 µL

The ligation mixture was then subjected to ethanol precipitation using $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2), twice the volume of 100% (v/v) ice cold ethanol and 2 µL of glycogen. The ethanol-precipitated ligation mixture was left overnight at -20^0C and it was then centrifuged at 18,625 xg for 30 mins at 4^0C in Hermle Z233 MK-2 centrifuge. The supernatant

was discarded leaving behind the pellet. The pellet was washed with 70% (v/v) ice cold ethanol. The washing procedure was done in such a way that the pellet remained undissolved in the ethanol solution. This washing step was followed by the centrifugation of the solution at 18,625 xg for 20 mins at 4^0C in Hermle Z233 MK-2 model centrifuge. Again, the supernatant was discarded and the pellet was air dried and dissolved in 15 µL of molecular grade water. This DNA mixture was then transformed into the XL-1 Blue electrocompetent cells using a Gene Pulser electroporation unit.

2.2.3.10 Transformation of the ligation mixture into XL-1 Blue electrocompetent cells

Initially the commercially obtained XL-1 Blue electrocompetent cells (Agilent Technologies) were thawed for 10 mins on ice. The parameters for the electroporation were set on the machine to 25 µF, 1.25 kV and gene pulse controller at 200 Ω. Following thawing of the competent cells, 5 µL of the ligation mixture were added to the cells, mixed, tapped twice, and left on ice for 1 min. Then the mixture was transferred into an 0.2 cm electroporation cuvette and placed in the shockpad. The mixture was pulsed and immediately 1 mL of fresh pre-warmed SOC medium was added. The culture was transferred into a 50 mL tube followed by thorough cleaning of the cuvette with another 1 ml of the SOC medium. The cells were rescued at 37^0C with shaking at 220 rpm for 1 hr. Culture (20 µL) was diluted in fresh SB medium to make dilutions from 10^1 to 10^{10}. A 100 µL volume from each of these dilutions was plated out, using the spread plate technique, on the individual LB agar plates with 100 µg/mL of carbenicillin and 1% (v/v) glucose. The agar plates were incubated in upright position overnight and on the following day the colonies were counted and the library size was determined using the formula.

$$\text{Library size} = \frac{\text{No. of colonies X culture volume X total ligation volume}}{\text{Plating volume X ligation volume}}$$

To the rest of the culture 10 mL of pre-warmed SB medium with 6 µL of 100 mg/mL of cabenicillin and 30 µL of 5 mg/mL tetracycline were added and incubated for another hour at

37^0C. Then another 9 µL of 100 mg/mL carbenicillin was added and shaken for one more hour at 37^0C. M13K07 (900 µL) was added along with 570 µL of pre-warmed SB medium supplemented with carbenicillin and tetracycline. The culture was grown for 2 hrs at 37^0C. Kanamycin (840 µL of 50 mg/mL stock) was added and the culture was grown overnight at 37^0C. This will inititate the biopanning process for the selective screening of the scFv-expressing clones.

2.2.3.11 Selective enrichment of anti-AFB_2 clones through biopanning

The aforementioned (section 2.2.3.10) culture, that was infected with M13K07 helper phage (2 X 10^{11} pfu/mL), was selectively screened on immunotubes coated with AFB_2-BSA conjugate. For this, the overnight grown culture was transferred into 250 mL Sorval tubes and centrifuged at 5,858 xg for 20 mins in Sorvall RC5B plus centrifuge. The pellet was discarded and the phage particles, expressing the scFv gene present in the supernatant, were precipitated on ice for 30 mins with 4% (w/v) polyethylene glycol (PEG) and 3% (w/v) sodium chloride (NaCl). After half an hour the supernatant was centrifuged without breaks in Sorvall RC5B plus centrifuge at 13,182 xg for 20 mins. The precipitated phage particles were re-suspended in 2 mL of 1 mg/mL of BSA in 150 mM PBS (pH 7.4), following discarding of the supernatant and subsequent drying of the phage, by placing the Sorval tube with phage in an inverted position for 10 mins. In the meanwhile, the immunotube that was coated with AFB_2-BSA conjugate overnight at 4^0C was blocked for 2 hrs at room temperature on a roller with 1 mL of 4% (w/v) milk Marvel 150 mM PBS (pH 7.4). After blocking, the immunotube was washed three times with PBST & PBS and then 1 mL of phage, that had been resuspended in 1 mg/mL of BSA in 150 mM PBS (pH 7.4) was added. The phage were incubated in the milk Marvel blocked immunotube for 2 hrs at room temperature with continuous rolling. The unbound phage were removed through washing three times with PBST & PBS. The bound phage were eluted

through trypsinization. For this 10 mg/mL of trypsin dissolved in 150 mM PBS (pH 7.4) was added to the immunotube and incubated for 30 mins at 37^0C.

Eluted phage were added to 2 mL of XL-1 Blue *E. coli* culture, which had reached the mid-exponential growth phase (OD 0.4 @ 600 nm) and left static at room temperature for 15 mins. Then, 6 mL of pre-warmed SB medium, with 12 µL of 5 mg/mL tetracycline and 1.6 µL of 100 mg/mL of carbenicillin were added. After incubating the culture for 1 hr at 37^0C, another 2.4 µL of 100 mg/mL of carbenicillin was added and allowed to incubate at 37^0C for another hour. Then, 600 µL of M13K07 helper phage were added. The culture was then transferred into a conical flask with 200 mL of pre-warmed SB medium with 368 µL of tetracycline (5 mg/mL) and 92 µL of 100 mg/mL of carbenicillin. Following 2 hrs of culture growth at 37^0C, 280 µL of 50 mg/mL of kanamycin were added and the culture allowed to grow overnight. The changes that were made during different rounds of panning are outlined below:

Rounds of panning	Amount of AFB_2-BSA coated	Number of washes	Culture volumes
Round 1	250 µg	3X PBST & PBS	200 mL
Round 2	100 µg	3X PBST & PBS	100 mL
Round 3	50 µg	6X PBST & PBS	100 mL
Round 4	25 µg	9X PBST & PBS	100 mL
Round 5	5 µg	12X PBST & PBS	100 mL

2.2.3.12 Polyclonal phage ELISA

A 96-well microtitre plate was incubated with 100 µL/well of 5 µg/mL of AFB_2-BSA in 150 mM PBS (pH 7.4) overnight at 4^0C. The solution was decanted and the plate was washed once with 150 mM PBS (pH 7.4). The plate was then blocked for an hour with 200 µL of 4% (w/v) milk Marvel PBS. Immediately after blocking, the plate was washed three times with PBST & PBS and 100 µL of phage inputs from the five rounds of panning along with the pre-pan (diluted 1:2 in 2% (w/v) milk Marvel PBST) were added to three different wells (triplicates) of the microtitre plate. The plate was then incubated for 1 hr at 37^0C followed by three times

washes with PBST & PBS. Next, 100 µL/well of 1:1,000 dilution of anti-M13 HRP-labelled rat secondary antibody was added, followed by incubation for 1 hr at 37^0C. The plate was washed thrice with PBST & PBS and the antigen-antibody complex was detected through the addition of 100 µL of TMB substrate. After 20 mins at room temperature the substrate color development was stopped though the addition of 50 µL of 1 M HCl. The absorbance was read on a Tecan Saffire plate reader at 450 nm.

2.2.3.13 Soluble expression of scFv in Top 10F′ cells

Soluble expression of the clones was initiated through infecting the phage from the fifth round of panning into E. *coli* Top 10F′ cells (Stratagene, U.S.A.) at mid-logarithmic phase (OD_{600nm}= 0.6). For this SB medium (5 mL) with 10 µL of 5 mg/mL tetracycline was inoculated with 5 µL of Top 10F′ cells and grown overnight at 37^0C. The overnight culture of Top 10F′ was then sub-cultured and was grown until the OD_{600nm} reached 0.6. Then 20 µL of phage from the fifth round of panning were added to the Top 10F′ cells and incubated at room temperature for 15 mins while static. Serial dilutions from 10^1 to 10^8 were made using SB medium and the culture was plated out on LB agar plates with 100 µg/mL of carbenicillin and 1% (v/v) glucose. The agar plates were incubated overnight at 37^0C to allow growth of colonies.

2.2.3.14 Monoclonal ELISA of the solubly expressed anti-AFB_2-expressing scFvs

Individual colonies (384 in total) were picked from the agar plate that was incubated overnight (2.2.3.13) and inoculated into 96 wells of a sterile Nunc plate containing 200 µL of SB medium with 100 µg/mL of carbenicillin and 505 medium. After overnight incubation at 37^0C, 20% (v/v) glycerol was added to the culture and it was stored at -80^0C. Prior to adidition of glycerol, 20 µL of culture were taken and added to another 96 well Nunc plate with 200 µL of SB medium. The culture was allowed to grow at 37^0C while shaking at 220 rpm until the OD_{600nm} reached 0.6 and 1 mM of IPTG was added and incubated overnight at 30^0C while shaking at 220 rpm. Separate 96 well plates were coated overnight with 5 µg/mL of AFB_2-BSA at 4^0C.

The following morning the conjugate-coated microtitre plates were blocked for 1 hr with 200 µL/well of 4% (w/v) milk Marvel 150 mM PBS (pH 7.4). Meanwhile, the culture plates were freeze-thawed thrice for the lysis and release of the proteins into the periplasmic layer. After freeze-thawing, the plates were centrifuged in an Eppendorf centrifuge (model: 5810R) at 2,754 xg for 20 mins and 100 µL of the supernatant (recombinant scFv's with HA tag) were added to the individual wells of the already blocked microtitre plates and incubated for 1 hr at 37^0C. The incubation step was followed by the washing of the plates three times with PBST & PBS. Then 100 µL/well of 1:1,000 dilution of anti-HA HRP-labelled rat secondary antibody in 2% (w/v) Milk Marvel PBST was added and incubated for 1 hr at 37^0C. The plates were washed thrice with PBST & PBS and 100 µL/well of TMB substrate added. The plates were left at room temperature for 20 mins for the color reaction to take place. The chromophore reaction was then stopped with addition of 50 µL of 1 M HCl and the absorbance was read at 450 nm on a Tecan Saffire plate reader.

2.2.3.15 Titration and competitive inhibition ELISA of randomly selected positive AFB_2 binding clones

Out of 384 clones (section 2.2.3.14), 10 positive clones whose absorbance was high (absorbance above 3 @ O.D. 450 nm), were selected and titre determined by indirect ELISA. The 10 clones were grown up overnight in 2 mL SB medium with 1 µL of 100 mg/mL of carbenicillin. The following morning these were sub-cultured into another 10 mL of SB medium with 5 µL of 100 mg/mL of carbenicillin. They were allowed to grow until the OD_{600nm} reached 0.6 and 20 µL of 0.5 M IPTG was added in order to promote the expression of the desired antibody. After IPTG induction they were grown at 30^0C overnight. Next day the 10 mL cultures were subjected to freeze-thawing followed by centrifugation in an Eppendorf centrifuge model No. 5810R at 2,754 xg for 20 mins at 4^0C.

Meanwhile, the microtitre plates that were coated overnight with 100 µL/well of 5 µg/mL of AFB_2-BSA were blocked for an hour using 200 µL/well of 4% (w/v) milk Marvel 150 mM PBS (pH 7.4). After blocking, the plates were washed thrice with PBST & PBS and 100 µL/well of serially diluted (1:10 to 1:5,120) scFv lysates were added to the individual wells of the microtitre plates (in duplicate). The rest of the ELISA procedure was the same as that described in section 2.2.3.12. A graph was plotted to determine the titre value for individual scFvs. From the titration curve the dilution of antibody to be used for competitive ELISA was determined.

The competitive inhibition ELISA was performed through inhibiting 1:4 dilution of the supernatant in 2% (w/v) Milk Marvel PBST with 20 µg/mL of free AFB_2 in methanol and blocked for one hour at 37^0C with 200 µL of 4% (w/v) milk Marvel 150 mM PBS (pH 7.4). Doubling dilutions of AFB_2 were made from a concentration of 20 µg/mL down to 0.039 µg/mL. Equal amounts of lysate, and antigen of varying concentrations (0.039-20 µg/mL), were incubated for 30 mins at 37^0C. The blocked plates were washed thrice with PBST & PBS followed by addition of 100 µL/well of inhibited antigen-antibody mixture. Following incubation for 1 hr at 37^0C, the plates were washed thrice with PBST & PBS and then 100 µL of 1:1,000 dilution of secondary antibody (anti-HA HRP-labelled rat mAb) were added to the individual wells. The plate was incubated for another hour at 37^0C. The washing step, as described earlier, was repeated and 100 µL/well of TMB substrate was added and incubated for 20 mins at room temperature for the color development. The reaction was stopped with 50 µL/well of 1 M HCl. The absorbances were read on a Tecan Saffire plate reader at 450 nm. An inhibition curve was plotted after calculation of the A/A_0 value (A= absorbance of the antigen antibody complex & A_0= absorbance value of 1:4 dilution of antibody).

2.2.3.16 Cross reactivity based ranking of anti-AFB_2 scFvs

Cross reactivity studies were performed by competitive ELISA (section 2.2.3.15) using varying concentrations (0.039-20 µg/mL) of AFB_1, G_1, G_2 and M_1. Calibration curves were generated from the data and cross reactivity percentage was calculated through considering IC_{50} value of AFB_2 as 100% and measured using the formula

$$\text{Percentage cross reactivity} = \frac{IC_{50} \text{ of scFvs in presence of aflatoxins X 100}}{IC_{50} \text{ of scFvs in presence of } AFB_2}$$

2.2.3.17 Large-scale expression, IMAC purification and analysis of scFvs-E9 and H12

SB medium (10 mL) with 5 µL of 100 mg/mL of carbenicillin was inoculated with 20 µL of scFvs stocks and grown overnight at 37^0C. The following morning 1 mL of culture was inoculated into 500 mL SB culture with 250 µL of 100 mg/mL of carbenicillin and grown at 37^0C with shaking (220 rpm) until the OD_{600nm} reached approximately 0.6, after which 500 µL of 1 M IPTG were added and the culture grown overnight at 30^0C. The overnight culture was then transferred into 250 mL sorval tubes (Nalgene) and centrifuged at 2,754 xg for 30 mins at 4^0C. The bacterial pellets were resuspended in 20 mL of lysis buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 10 mM imidazole, pH 8.0) after discarding the supernatants. This suspension was sonicated for 5 mins with 10 sec pulses at amplitude 60 using Sonicator. The cell debris was removed by centrifugation of the sonicated mixtures at 18,514 xg for 30 mins at 4^0C. The lysate supernatants were collected into a fresh tube. Then 2 mL of Nickel-Nt resin were packed in two columns, which were equilibrated with 10 mL of loading buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 10 mM imidazole, pH 8.0). The lysates were added to the equilibrated columns, followed by washing the columns with 5 mL of washing buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 30 mM imidazole, pH 8.0). The bound scFvs were eluted using 6 mL of buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 250 mM imidazole, pH 8.0). The eluates were dialyzed against (buffer exchanged with) 1X PBS using 5,000 M. Wt. 'cut-off' Vivaspin columns

(Sartorious Stedium, Germany). The scFv concentrations were then quantified on a Nanodrop™ spectrophotometer.

2.2.3.17.1 Competitive inhibition ELISA for scFvs-E9 and -H12

Competitive inhibition ELISA procedure described in section 2.2.3.15 was performed using the scFvs-E9 and -H12. Due to the higher sensitivity of scFv-E9 further analyses were carried out using this scFv.

2.2.3.17.2 SDS-PAGE and western blotting of scFv-E9

The anti-AFB_2 scFv-E9 after immuno-affinity purification (IMAC) was run on SDS-PAGE gel, whose composition was described in section 2.1.7. The scFv-E9 was dissolved in loading dye in the ratio of 1:3. The gel was run at 100 V for 1 hr 30 mins and stained 1 hr on roller shaker at room temperature using Coomassie blue and subsequently destained for 1 hr at room temperature using destaining solution. Another gel was run as described above and the protein was transferred onto the nitrocellulose membrane and blocked with 4% (w/v) milk Marvel PBS for 1 hr at room temperature. The blocked nitrocellulose membrane was probed with 1/5,000 dilution of anti-HA-HRP labelled secondary antibody, after which the blot was developed using TMB solution.

2.2.3.18 CM5 chip immobilization and competitive inhibition assay development

The immobilization of AFB_2 onto the CM5 chip surface was done on bench. The volumes of solutions used were 30 µL unless otherwise stated. Briefly, a CM5 chip was primed with HBS-EP buffer. The surface was activated with 1:1 mixture 0.4 M EDC and 0.1 M NHS in water for 30 mins, after which the buffer was removed using a tissue paper. An amine (Jeffamine, in 50 mM carbonate buffer (pH 8.5)) was applied to the surface for 1 hr followed by the blockage or deactivation of unreacted sites with 1 M ethanolamine, pH 8.5 for 30 mins. The reactive AFB_2-CMO-NHS synthesized following the procedure described in section 2.2.1.1. was allowed to

react with amine activated chip overnight at room temperature, followed by washing and storage at 4^0C for future use.

2.2.3.19 Binding and kinetic analysis of the scFv-E9

Kinetic analysis of the scFv-E9 (without any cross reactivity and highest displacement) was carried out after subjecting to purification on IMAC. Initially varying concentrations of scFv were analyzed by SPR to attain response units of 200 to 300 RU. The scFv-E9 was injected at concentrations of 43.7-350 nM, randomly onto the AFB_2 immobilized CM5 chip for 2 mins (flow rate of 10 µL/min), and dissociation was monitored for 10 mins. Regeneration of the surface was carried out, after 10 mins of dissociation with 30 mM NaOH for 30 sec twice (flow rate 30 µL/min). The data were evaluated on BIAevaluation software. The equilibrium dissociation constant (K_D) was calculated through fitting the data in 1:1 Langmuir binding model.

2.2.3.20 Post extraction spiking of almond samples

Almond sample was extracted using 80% (v/v) methanol (Whitaker *et al.,* 2006), followed by centrifugation for 30 mins at 2108 xg. The supernatant was filtered using Whatsman grade 1 filter paper. Five millilitres of almond extract were evaporated to dryness in a rotary evaporator and resuspended in 5 mL of HBS buffer. To each mL of almond sample in HBS buffer varying concentration of AFB_2 (0.15-2.5 µg/mL) were added and recovery was measured through fluorescence estimation.

2.2.3.21 SPR-based inhibition assay condition and validation

Anti-AFB_2 scFv-E9 (1/100 dilution) in HBS buffer was added to decreasing concentrations of AFB_2 (0.9-1,000 ng/mL) in the ratio of 1:1. This mixture was incubated for 30 mins at 37^0C. The samples were then randomly passed over the AFB_2 immobilized sensor chip in triplicate. Likewise, spiked almonds samples were also mixed with antibody (1:1 spiked sample/antibody)

and injected (flow rate 15 µL) onto the surface of the sensor chip, with a contact time of 90 sec. The surface was regenerated with 30 mM NaOH for 30 sec twice (flow rate 30 µL/min). The limit of detection (LOD) of the assay was determined from the mean of limit of blank (n=20) plus 3 times standard deviation of blank samples. The precision of the assay was measured through performing inter-day (within runs) and intra-day (between runs) of the assay and represented as % CV (coefficient variance).

2.2.4 Generation of anti-Zearalenone murine scFv antibodies

Zearalenone (M.Wt. 318.36 g mol^{-1}) was first conjugated to carrier proteins (BSA and KLH) following the procedure given in section 2.2.1.2. The ZEN-KLH conjugate was used as an immunogen in the presence of Freund's adjuvant to generate immune responses in mice. The immune response was determined by indirect ELISA.

2.2.4.1 Serum titre determination

Four out of the 8 rows for a 96 well Nunc microtitre plate were coated overnight at 4^0C, with 5 µg/mL of ZEN-BSA conjugate. Two rows were coated with 5 µg/mL BSA in PBS and the remaining two rows with 5 µg/mL KLH in 150 mM PBS (pH 7.4). An indirect ELISA (section 2.2.2.2) was performed.

2.2.4.2 RNA isolation and cDNA synthesis from mice spleen enriched with anti-ZEN immunoglobulins

The procedure described in section 2.2.3.2 was followed for the isolation of RNA. Synthesis of the first strand of DNA i.e., cDNA through the reverse transcription was carried out empoying the procedure outlined in section 2.2.3.3.

2.2.4.3 Recombinant anti-ZEN scFv library

2.2.4.3.1 Amplification of antibody variable heavy and light chains using pComb 3X series primers

A long linker library in which the V_L is linked to V_H with an 18 amino acid linker was generated using the set of primers described in section 2.2.3.4 following the procedure outlined by Barbas *et al.* (2001). The PCR conditions for the amplification of V_λ, V_k and V_H were optimized using a $MgCl_2$ gradient. Using the appropriate amount of $MgCl_2$ the variable heavy and light chain genes were amplified and resolved on a 1% (w/v) agarose gel with Sybersafe. The required bands were cut out and purified using Nucleospin Extract II, following the procedure outlined in section 2.2.3.5.

2.2.4.3.2 Splice by overlap extension (SOE) PCR

The ~750 bp amplicon SOE product was synthesized after performing a set of optimizations using a $MgSO_4$ gradient. The following PCR mixture was used for overlapping of the heavy and light chain genes.

PCR mixture composition	Amount in 50 µL final volume	Conc. in 50 µL volume
V_H	0.8 µL	100 ng/rxn
V_k	2 µL	100 ng/rxn
dNTP	1 µL	0.2 mM
Hi fi buffer	5 µL	1X
SOE forward primer	0.5 µL	60 pM
SOE reverse primer	0.5 µL	60 pM
$MgSO_4$	1, 1.5, 2 or 4 µL	1, 1.5, 2, 4 mM
Molecular grade water	39, 38.5, 38 or 36 µL	N/A
Platinum tag	0.2 µL	5 U/ µL

The PCR reaction was carried out on Applied Biosystems machine using the following conditions:

PCR condition			
Stages	**Temperature**	**Time**	
1	94^0C	5 mins	
2	94^0C	30 sec	30 cycles
3	56^0C	30 sec	
4	72^0C	120 sec	
5	72^0C	10 mins	
6	4^0C	∞	

2.2.4.3.3 S*fi*I restriction digestion and ligation of the purified SOE and pComb 3XSS vector

Both the SOE product and vector were restriction digested with S*fi*I. This aids in the unidirectional cloning of the scFv (SOE) into the vector. The digestion of both SOE and vector was carried out at 50^0C for 5 hrs on a heating block. The digested SOE product was ethanol-precipitated at -20^0C. The vector was treated with X*ho*I and X*ba*I to further digest the Stuffer fragment. Finally, the vector was treated with antarctic phosphatase in order to prevent re-ligation of vector prior to the performance of the ligation of SOE with the vector. The completely digested vector was then ethanol-precipitated. The composition of the reaction mixtures are given below:

Components	SOE(k)	SOE(ʎ)
SOE	240 µL	240 µL
S*fi*I	10 µL	12 µL
100X BSA	3 µL	3 µL
NEB II buffer	30 µL	30 µL
Molecular grade water	17 µL	15 µL

Components	Amount in 100 µL volume
Vector	20 µL
NEB II buffer	10 µL
100X BSA	1 µL
S*fi*I	9 µL
Molecular grade water	60 µL

The ethanol-precipitated mixtures of both the SOE and the vector were resolved on 0.5% (w/v) agarose gel and purified following the procedure described in section 2.2.3.5. The restricted digested SOE was then ligated into pComb 3XSS vector overnight using T4 DNA ligase. The composition of the ligation mixture is outlined below:

Components	SOE (k)	SOE (λ)	Conc. in 200 µL volume
5 X ligase buffer	20 µL	20 µL	2X
Digested SOE	46.97 µL	7.15 µL	1.4 µg/rxn
Digested pComb 3XSS	11.55 µL	11.55 µL	2.4 µg/rxn
T4 DNA Ligase	0.5 µL	0.5 µL	800 U/µL
Molecular grade water	120.98 µL	160.80 µL	N/A
TOTAL	200 µL	200 µL	N/A

After ligating the SOE with the vector the ligase enzyme was deactivated through heat treatment at 60^0C for 15 mins. The ligation mixture was then subjected to ethanol precipitation overnight at -20^0C using $1/10^{th}$ volume of 3 M sodium acetate, twice the volume of 100% (v/v) ice-cold ethanol and 2 µL of glycogen. The ethanol precipitation was followed by the centrifugation of the mixture in Hermle Z233 MK-2 centrifuge at 18,625 xg for 30 mins followed by the washing of the pellet with 70% (v/v) ethanol. The pellet was air dried and then dissolved in 16 µL of molecular grade water.

2.2.4.3.4 Transformation of scFv gene into *E. coli* by electroporation

Commercially available XL-1 Blue electrocompetent cells (Agilent technologies) were used for transforming the ligated scFv vector construct using the Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, U.S.A.) unit. The electrocompetent cells (100 µL) were thawed on ice for 5 mins. The ligation mixture (4 µL) was added to each of four thawed vials of the electrocompetent cells. This was then transferred into an ice-cold 0.2 cm electroporation cuvette (Bio-Rad Laboratories, U.S.A.). The cuvette was tapped for the cells to settle down and was pulsed using 25 µF, 1.25 kV and the Pulse Controller at 200 Ω after placing the cuvette in

the shock pad. Then, the cuvette was washed twice with 1 mL of pre-warmed SOC. The re-suspended cells were transferred into a 50 mL sterile universal container containing 2 mL of SOC medium, and incubated for 1 hr at 37^0C while shaking at 220 rpm, to rescue the library. A set of dilutions 10^1 to 10^{10} was made using the rescued library to determine the library size. They were plated out on LB agar supplemented with 100 µg/mL of carbenicillin and 1% (v/v) glucose to determine the library size. In parallel, XL-1 Blue cells (negative control) alone were plated out. The plates were incubated overnight at 37^0C. The rescued library was used for performing biopanning.

2.2.4.4 Selective enrichment and screening of scFv-expressing clones

To 2 mL of the rescued library (section 2.2.4.3.4) 10 mL of pre-warmed SB broth enriched with 3 µL of 100 mg/mL carbenicillin and 30 µL of 5 mg/mL tetracycline were added. This culture was incubated for one hour at 37^0C, while shaking at 220 rpm. Further supplementation of the medium with 4.5 µL of 100 mg/mL of carbenicillin was followed by incubation for 1 hr at 37^0C. M13KO7 helper phage (900 µL) was added and the final culture volume was made up to 600 mL through addition of 588 mL of SB medium supplemented with 450 µL of 100 mg/mL carbenicillin and 1800 µL of 5 mg/mL tetracycline. The culture was grown for 2 hrs at 37^0C followed by supplementation with 840 µL of 50 mg/mL of kanamycin and then grown overnight at 37^0C.

2.2.4.4.1 Phage precipitation

The overnight 'grown-up' culture was transferred into 250 mL Sorval tubes (Nalgene) and centrifuged at 2,603 xg for 20 mins in Sorval RC5B plus centrifuge. The bacterial cell pellet was discarded and the phage in the supernatant was precipitated through the addition of 4% (w/v) polyethylene glycol (PEG) and 3% (w/v) sodium chloride (NaCl) on ice bath for 30 mins. The precipitated phage were pelleted by centrifugation for 20 mins at 13,182 xg in Sorval

RC5B plus centrifuge. The phage pellet was air dried and re-suspended in 2 mL of a 1 mg/mL solution of BSA in 150 mM PBS (pH 7.4).

2.2.4.4.2 Biopanning for selective enrichment of scFv-expressing clones

Six rounds of panning were performed on immunotubes that were coated overnight at 4^0C with ZEN-BSA conjugate. The immunotubes were blocked with 1 mL of 4% (w/v) milk Marvel in 150 mM PBS (pH 7.4) for 2 hrs followed by washing thrice with PBST & PBS. The precipitated phage in BSA (section 2.2.4.4.1) were added to the immunotube and incubated for 1 hr at room temperature followed by washing to remove the unbound phage. Bound phage particles were removed from the immunotube through the addition and susbsequent incubation with 10 mg/mL of trypsin for 30 mins at 37^0C. The trypsinized phage mixture was now added to the 2 mL of XL-1 Blue culture whose OD_{600nm} is 0.4 and was left static at room temperature for 15 mins. To this, 2 mL of XL1-Blue phage culture, 8 mL of SB medium, supplemented with 12 µL of 5 mg/mL of tetracycline, and 1.6 µL of 100 mg/mL of carbenicillin were added and incubation for 1 hr at 37^0C followed. Then the culture was again supplemented with another 2.4 µL of 100 mg/mL of carbenicillin. The culture was grown for 1 hr and M13K07 phage (4×10^{11} pfu) was added along with 200 mL of SB medium. After 2 hrs of incubation at 37^0C, 280 µL of 50 mg/mL of kanamycin was added and the culture was allowed to grow overnight at 37^0C. The determination of the output and input titre from each of the round was done through plating out a set of dilutions from the inputs and outputs every day. The variations in the conditions during each round of panning are shown in the table below:

Rounds of panning	ZEN-BSA coating concentration	Number of washes	Culture volumes
Round 1	250 µg	3X PBST & PBS	200 mL
Round 2	100 µg	3X PBST & PBS	100 mL
Round 3	50 µg	6X PBST & PBS	100 mL
Round 4	25 µg	9X PBST & PBS	100 mL
Round 5	5 µg	12X PBST & PBS	100 mL
Round 6	2.5 µg	12X PBST & PBS	100 mL

2.2.4.5 Polyclonal phage ELISA and colony pick PCR for ZEN

The microtitre plate was coated with 5 µg/mL of ZEN-BSA conjugate, BSA and 4% (w/v) milk Marvel. The ELISA procedure used was as in section 2.2.3.12.

To ensure that the scFv insert was present in the colonies, six colonies were picked from the output plates from round 5 of the panning. A colony pick PCR reaction was carried out using the primers for the amplification of the appropriate insert (SOE) present in the individually picked colonies. The composition of the reaction mixture is given below:

Composition	Amount in 50 µL reaction	Amount in control reaction
Water	35.75 µL	35.75 µL
Forward primers	0.5 µL	0.5 µL
Reverse primers	0.5 µL	0.5 µL
5 X PCR buffer	10 µL	10 µL
dNTP	1 µL	1 µL
Colony	1	N/A
$MgCl_2$	2 µL	2 µL
Tag polymerase	0.25 µL	0.25 µL

The PCR reaction was carried out on an Applied Biosystems PCR machine using the following PCR condition:

PCR condition Stages	Temperature	Time	
1	94^0C	3 mins	
2	94^0C	30 sec	30 cycles
3	56^0C	30 sec	
4	72^0C	120 sec	
5	72^0C	10 mins	
6	4^0C	∞	

2.2.4.6 Soluble expression of clones expressing scFv that binds to ZEN

The phage (10 µL) from rounds 5 & 6 was infected into Top 10F′ cells whose OD_{600nm} reached the mid-exponential phase (0.6) in individual vials. The mixture was left static at room temperature for 15 mins. A set of doubling dilutions were made and 100 µL of the culture from each dilution were plated out on separate LB agar plates with 100 µg/mL carbenicillin and supplemented with 1% (v/v) glucose. The plates were incubated overnight at 37^0C. To individual wells of a sterile 96 well microtitre plate 200 µL of SB medium with 100 µg/mL of carbenicillin and 505 medium (0.5% (v/v) glycerol & 0.05% (w/v) glucose) were added. Individual colonies from the agar plates were inoculated into the 96 wells of the microtitre plate with SB medium. The culture was allowed to grow overnight at 37^0C. A aliquot 20 µL of culture was sub-cultured into a fresh microtitre plate with SB medium. To the rest of the overnight grown culture (master plate) 20% (v/v) glycerol was added and stored at -80^0C. The sub-cultured plate was incubated at 37^0C until the OD_{600nm} reached 0.6. Twenty µL of 1 M of IPTG was then added to the individual wells in order to promote the process of protein expression. After IPTG addition the culture was grown at 30^0C overnight, while shaking at 220 rpm.

2.2.4.7 Monoclonal ELISA for anti-ZEN antibody secreting clones

The culture plates that were induced with IPTG (section 2.2.4.6) were freeze-thawed three times followed by centrifugation at 2,754 xg for 20 mins (Eppendorf centrifuge, 5810R, A4-62 rotor). The ELISA was performed on a microtitre plate coated with 5 µg/mL of ZEN-BSA conjugate overnight at 4^0C. The conjugate-coated ELISA plates were blocked with 200 µL/well of milk Marvel PBS at 37^0C for one hour. The blocked plates were washed thrice with both PBS and PBST. Then 100 µL of supernatant from the freeze-thawed culture plates were added to the individual wells of the microtitre plates. A detailed description of the ELISA procedure was given in section 2.2.3.14.

2.2.4.8 Master plate construction and scale-up of the positive clones binding to ZEN

Twenty positive binders were selected based on the signal obtained in the monoclonal ELISA performed using the solubly expressed clones. These 20 positive clones were grown in an microtitre plate, with 200 µL of SB medium with 100 µg/mL of carbenicillin, overnight at 37^0C with shaking at 220 rpm. Clones were then sub-cultured in a fresh 200 µL, 1 mL and 10 mL of SB medium with 100 µg/mL of carbenicillin. The sub-cultures were allowed to grow at 37^0C till they reached the mid-exponential phase (OD_{600nm}= 0.6). One mM of IPTG was then added to the culture and it was allowed to grow overnight at 30^0C while shaking at 220 rpm. On the following morning the cultures were freeze-thawed thrice followed by centrifugation at 2,754 xg for 20 mins (Eppendorf centrifuge, model 5810R, A4-62 rotor). ELISA was performed on the supernatant to determine the level of expression and specificity of the expressed antibodies towards ZEN.

2.2.4.9 Titration and inhibition assay of the ZEN-positive clones

The supernatant obtained from the 10 mL culture was used for the determination of the titre value of the expressed positive clones. The microtitre plate was coated overnight at 4^0C with 5 µg/mL ZEN-BSA conjugate. The ZEN-BSA-coated microtitre plate was blocked with 200

µL/well of milk Marvel PBS for 1 hr at 37^0C followed by washing thrice with PBS and PBST. Varying dilutions of cell lysate from 1:2 to 1:256 were made by doubling dilutions. One hundred µL of these dilutions were added to the individual wells of a milk Marvel-blocked plate. The titration was performed following the procedure described in section 2.2.3.14.

A competitive inhibition assay was then performed to determine the reactivity of the clones towards ZEN. A 50 µL of 1:2 dilution of the antibody was used to inhibit 50 µL of 0.039-20 µg/mL of ZEN toxin. The protocol followed for performance of the inhibition assay is described in section 2.2.3.15.

2.2.4.10 Large-scale expression, purification and analysis of scFv-P2A2

For the expression of scFv-P2A2 a single colony was incubated overnight at 37^0C with shaking (220 rpm) in 5 mL of Terrific Broth and 50 µg/mL of carbenicillin. The following morning 1 mL of this culture was used to inoculate 500 mL of fresh Terrific Broth supplemented with 50 µg/mL of carbenicillin and grown at 37^0C with shaking (200 rpm) until the OD_{600nm}= 0.6. Then 1 mM of IPTG was added and incubated overnight at 30^0C with shaking (220 rpm). The cultures were spun down at 2,108 xg for 30 mins at 4^0C and the pellet was resuspended in lysis buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 10 mM imidazole, pH 8.0) and subjected to sonication. The sonication mixture was subjected to centrifugation at 2,108 xg for 30 mins followed by the addition of the supernatant to already packed Nickel Nt resin (2 mL) equilibrated with 10 mL of loading buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 10 mM imidazole, pH 8.0). After completely passing the supernatant through the column, it was washed with 20 mL of washing buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 30 mM imidazole, pH 8.0), followed by elution of bound protein using 6 mL of elution buffer (50 mM $Na_2H_2PO_4$, 300 mM NaCl and 250 mM imidazole, pH 8.0). The eluted protein was dialyzed (buffer exchanged) with PBS in a Vivaspin column (M. Wt. 'cut-off': 5 *k*Da). The amount of protein was estimated on Nanodrop™ spectrophotometer.

2.2.4.10.1 Competitive inhibition assay

Competitive inhibition ELISA procedure described in section 2.2.3.15 was performed with an exception of the usage of 1/800 dilution of scFv in the presence of 0.004-5 µg/mL of ZEN.

2.2.4.10.2 Cross reactivity of the scFv-P2A2

Cross reactivity studies were performed using β & α- Zearalenol at concentration 0.004-5 µg/mL following the competitive inhibition assay described in section 2.2.3.15.

2.2.4.10.3 SDS-PAGE and western blotting of scFv-P2A2

The gel compositions were described in section 2.1.7. The gel was run at 100 V for 1 hr 30 mins. It was then stained for 1 hr on roller shaker at room temperature using Coomassie blue and subsequently destained for 1 hr at room temperature using destaining solution.

2.2.4.11 Immobilization of ZEN onto CM5 sensor chip

Immobilization of ZEN on the surface of CM5 sensor chip was done following the EDC-NHS method (Jonsson *et al.,* 1991) with a few additional steps (e.g., addition of an amine). The ZEN-CMO-NHS synthesis procedure described above (section 2.2.1.2) was used for the generation of ZEN derivative to be immobilized on the CM5 sensor chip. The immobilization of ZEN was carried out under static condition and the volume of reagents used in each of the steps was 30 µL unless otherwise stated. Initially the sensor chip was primed with HBS-EP buffer. Then 1:1 mixture of 0.4 M EDC/0.1 M NHS was applied to the surface for 30 mins. An amine (Jeffamine in 50 mM carbamate buffer (pH 8.5)) was added and allowed to incubate for 1 hr at room temperature. Like in other CM5 immobilization procedures, 1 M ethanolamine was used to block the unreacted sites for 30 mins. The ZEN-derivative (section 2.2.1.2.2) was allowed to react with the amine activated surface overnight at room temperature in dark. The sensor chip was washed with water and stored at 4^0C for future use.

2.2.4.12 Binding and kinetic analysis of scFv-P2A2 utilizing ZEN immobilized CM5 chip

For the determination of affinity constants of scFv-P2A2 varying concentration (1.25-10 mM) of it were passed over the ZEN-immobilized sensor chip with a flow rate of 10 µL/min for 3 mins followed by the dissociation phase of 10 mins using kinetic analysis tool in Biacore™ 3000.

2.2.4.13 Extraction of ZEN from sorghum

Sorghum was extracted using modified procedure developed by Bennett *et al.* (1984). To 50 g of sorghum 250 mL of methanol and water mixture (1:1) were added and stirred continuously for 10 mins followed by centrifugation at 2108 xg for 5 mins at 4^0C. The supernatant was taken into another tube followed by the addition of another 250 mL of methanol and water mixture (1:1). This suspension was again centrifuged for 5 mins at 2108 xg. The two supernatants were pooled together, filtered and evaporated to dryness through rotary evaporation. The residue was re-suspended in 50 mL acetonitrile. The recovery efficiency of extraction procedure was evaluated through fluorescence estimation.

2.2.4.14 SPR-based inhibition assay and its validation

To assess the binding ability of P2A2 scFv to ZEN in solution, an inhibition assay was performed by passing the mixture containing equal volumes of scFv-P2A2 (1/100 dilution) and varying concentration of ZEN (0.0039-1 µg/mL). The flow rate was kept at 10 µL/min with a total contact time of 60 sec. Regeneration of chip surface was performed twice using 20 mM NaOH in between the successive steps. A standard curve was constructed using the sorghum samples. The accuracy of measurement was evaluated through inter-day (n=3) and intra-day (n=5) analyses. The final working concentration of ZEN was 0.0039-1 µg/mL. The limit of blank (LOB) was determined by passing equal volumes of 1/100 dilution of antibody and sorghum extract that was free from ZEN contamination (n=20).

2.2.5 Isolation and purification of IgY raised against T-2 toxin

2.2.5.1 Immunization of chicken

A leghorn chicken was immunized with 1 mL of 200 µg/mL of T-2-KLH-Fruend's complete adjuvant for the initial boost followed by three subsequent boosts with Fruend's incomplete adjuvant in the ratio of 1:1, with a time interval of 3 weeks between each boost. After the third boost the chicken was bled and the serum isolated. A bleed was taken from the same chicken prior to immunization. The antibody response of the chicken for T-2 toxin was determined by an indirect ELISA.

2.2.5.2 Determination of serum antibody titre (indirect ELISA) to T-2 toxin

A microtitre plate was coated overnight with 5 µg/mL of either T-2-BSA conjugate in PBS (4 rows), BSA in PBS (2 rows) or KLH in PBS (2 rows). The titre was determined (as in section 2.2.2.2.) with the exceptions that the anti-chicken HRP-labelled rabbit secondary antibody was used instead of anti-mouse AP-labelled goat secondary antibody, TMB in place of pNPP as substrate and the absorbance measured at 450 nm on Tecan Saffire plate reader.

2.2.5.3 Isolation of IgY from egg yolks

Eggs were collected from the immunized chicken after the third and fourth boost and stored at 4^0C. The isolation of IgY was performed using the 'Eggcellent' chicken IgY purification kit. This kit consists of delipidating agent and IgY precipitation reagent. The egg shell was broken and the egg white along with the yolk were placed in an yolk separator provided with the IgY purification kit. The egg white was drained completely into a waste beaker. The yolk left in the yolk separator, was washed with distilled water. The washing of the yolk was followed by rolling the yolk on a piece of tissue to ensure removal of any adhering materials to the yolk surface. The egg yolk sac was pierced at one point to drain off egg yolk leaving behind the yolk sac. The yolk was collected in a weighed beaker. The beaker was weighed once again to determine the amount of yolk (12.5 g) obtained. Assuming 1 mL of yolk = 1 g as per the

instructions given in the kit, five times the yolk volume of delipidating agent (62.5 mL) provided was added to the yolk. The resulting solution was mixed gently and left to incubate at 4^0C for 2 hrs. The mixture was centrifuged at 4^0C (10,000 xg for 15 mins). The supernatant was decanted and the volume was measured in a volumetric cylinder. Equal volumes of IgY precipitating reagent were added to the supernatant. It was stirred gently and left static at 4^0C for 2 hrs. The mixture was centrifuged for 15 mins at 10,000 xg at 4^0C. The pellet was dissolved in 150 mM PBS (pH 7.4), whose volume corresponds to the original yolk volume.

2.2.5.4 Purification of IgY using T-2-immobilized amine-activated beads

2.2.5.4.1 Immobilization of T-2 on amine-activated beads

T-2 (5 mg) toxin was dissolved in 200 µL of DMSO and 5 mg of carbonyldiimidazole (CDI) was added and stirred for 1 hr at room temperature. The amine-activated beads were pre-packed in a column and equilibrated with 10 mL of coupling buffer (0.1 M MES, 0.9% (w/v) NaCl, pH 4.7). The buffer was drained off and the T-2-CDI with 0.5 mL of coupling buffer was added and mixed on a roller for 2 hrs at room temperature and then at 4^0C overnight. The following day, the column was washed with 4 mL of wash buffer (1 M NaCl). The washing step was followed by equilibration of the column with 150 mM PBS (pH 7.4) containing 0.05% (w/v) sodium azide.

2.2.5.4.2 Purification of IgY using T-2-immobilized column

The T-2 toxin immobilized beads in the column were equilibrated with 6 mL of 150 mM PBS (pH 7.4). The PBS used for equilibration was drained off and 5 mL of the crude IgY (section 2.2.5.3) solution was then added to the equilibrated column and incubated at room temperature for 1 hr. After incubation the column was washed with sample buffer (4 mL X 3). The bound IgY was eluted from the column using 8 mL of 100 mM glycine-HCl in water (pH 3.0). Each 1 mL eluted fraction of IgY collected was neutralized with 50 µL of 1 M Tris (pH 9.0).

2.2.5.5 IgY analysis

2.2.5.5.1 Indirect and competitive inhibition ELISA using anti-T-2 IgY

A 96 well Nunc microtitre plate was coated overnight at 4^0C with 5 µg/mL of T-2-BSA conjugate. The plate was washed once with 150 mM PBS (pH 7.4), followed by blocking of the microtitre plate with 200 µL of 4% (w/v) milk Marvel PBS for 1 hr. Out of the six rows that were coated with T-2-BSA conjugate, three rows were used for the determination of the titration value of crude IgY. The remaining three rows were used for the titre determination of purified IgY. A dilution range from 1:100 to 1:512,000 of both the crude and purified IgY were made and 100 µL of these were added to the individual wells of the microtitre plate. The addition of IgY was followed by the incubation of the plate at 37^0C for 1 hr. Then 100 µL of 1:1,000 dilution of anti-chicken HRP-labelled rabbit antibody in 2% (w/v) milk Marvel PBST were added and incubated at 37^0C for 1 hr. Washing of the plates was performed three times with PBST and PBS. TMB substrate solution (100 µL) was added and incubated at room temperature for 20 mins followed by stopping of the reaction with the addition of 50 µL of 1 M HCl. Absorbances were read at 450 nm on Tecan Saffire plate reader.

2.2.5.5.2 Cross reactivity of anti-T-2 toxin IgY

Cross reactivity of both affinity-purified and unpurified IgY were subjected to competitive ELISA. The ELISA procedure is similar to that outlined in section 2.5, with one exception i.e., the usage of 1/2,500 dilution of antibody that was pre-incubated with known concentrations (0.007-1 µg/mL) of antigens (T-2, HT-2, Deoxynivalenol, Zearalenone and Fumonisin B_1). The absorbance IgY in presence of antigen was divided by the absorbance of IgY alone (A/A_0). A semi-log plot was generated with A/A_0 values on Y-axis versus log concentration of antigen on X-axis.

2.2.5.5.3 FPLC analysis of IgY

IgY purity was analyzed by gel filtration chromatography. The Sephacryl S-200 HR column, (45 mL bed volume) was equilibrated with 10 mM HEPES containing 118 mM NaCl (pH 7.3) buffer at a flow rate of 1 mL/min for a duration of 180 mins using an AKTA explorer 100 (GE Healthcare, UK). Monitoring of absorbance at 280 nm revealed the presence of IgY, which was confirmed by running different molecular weight markers, under similar conditions, for calibration.

2.2.5.5.4 MALDI-TOF analysis of IgY

The mass analysis of IgY was performed by MALDI-TOF mass spectrometry (Bruker Analytical Systems) in University of Joseph Fourier by Prof. Eric Defrancq. The matrix (sinapinic acid solution) was prepared at a concentration of 15 mg/mL in acetonitrile. IgY was dialyzed with distilled water using Viva spin column (M. Wt. 'cut-off': 30 *k*Da) and IgY was made to a final concentration of 1.0 mg/mL. The IgY sample and matrix solution were mixed in equal amounts (1 µL each) with 0.1% (v/v) trifluro acetic acid and added on to the chip. The samples were allowed to dry at in a vaccum desiccator and then used for mass analysis. The data were acquired in the linear mode at 30 kV and analyzed using the Compass software suite (MALDI software).

2.2.5.5.5 SDS-PAGE analysis of IgY

The IgY sample was run on an SDS-PAGE gel under reducing and non-reducing conditions, before and after purification, on T-2 toxin-immobilized sepharose beads. For non-reducing conditions the sample was boiled with the loading dye in the absence of DTT, which is the reducing agent. A detailed description of the conditions for SDS-PAGE analysis was given in section 2.2.3.17.2.

2.2.5.6 Immobilization of T-2 toxin on CM5 sensor chip

Immobilization of T-2 toxin on a CM5 chip was performed manually on the bench by first equilibrating the chip with HEPES buffer (50 µL) at room temperature for 15 mins. The chip was then surface-activated by incubating at room temperature with 1:1 mixture of 0.4 M EDC/ 0.1 N NHS for 30 mins, and washed carefully with de-ionized water. Then, 20% (v/v) of Jeffamine, in 50 mM carbonate buffer (pH 8.5), was applied to the chip and incubated for 1 hr at room temperature and the chip was then washed once with de-ionized water. The un-reacted amine sites on the chip were blocked with 1 M ethanolamine (pH 8.5) for 30 mins by incubation at room temperature and then washed once with de-ionised water. The T-2-carbamate was diluted in 1:1 ratio with 10 mM of sodium bicarbonate solution (pH 8.0) and incubated on the chip for 24 hrs at room temperature.

2.2.5.7 SPR-based inhibition assay and its validation

The CM5 chip immobilized with T-2 toxin, was docked into the Biacore machine. This was followed by the injection of 1/2,500 dilution of anti-T-2 IgY. Non-specific antibody (anti-AFB_1 Fab antibody) was used to confirm that the binding occurred only between the T-2 toxin and antibodies raised against that toxin. A 1/2,500 dilution of the anti-T-2 IgY was incubated with equal volumes of known amounts of T-2 toxin (ranging from 0.007-1 µg/mL) and passed over the sensor chip at a flow rate of 15 µL/min for 90 sec. The chip was regenerated with 15 µL of 40 mM NaOH and 30 mM HCl after each cycle for 30 sec at a flow rate of 30 µL/min. Response of the IgY in the presence of varying concentrations of T-2 toxin (R) was divided by the responses of IgY in the absence of T-2 toxin (R_0) and four parameter logistic curve was fitted with R/R_0 value on Y-axis versus log concentration of T-2 toxin on X-axis.

Wheat samples (10 g) were ground and extracted with 30 mL ethanol/water 85:15 (v/v) by constant stirring on a magnetic stirrer for 30 mins. The extract was filtered through Whatman Grade 1 filter paper. The filtrate was evaporated to dryness in a rotary evaporator and dissolved

in 10 mL HBS buffer (pH 7.4), after which known amounts of T-2 toxins (0.007-1 µg/mL) were added and an SPR-based inhibition assay, as described above was performed. The LOD (limit of blank (LOB) + 3 S.D. of LOB) and LOQ (LOB + 10 S.D. of LOB) of the assay were determined.

2.2.6 Assay format development

2.2.6.1 Covalent immobilization of AFB_1

2.2.6.1.1 Passivation and amine functionalization of microtitre plate surfaces

Individual wells of the microtitre plate were first cleaned three times with 250 µL of distilled water. The wells were incubated with 250 µL of freshly prepared piranha solution (1:3 H_2O_2:conc.H_2SO_4) for 1 hr with constant shaking at 37^0C. The plate was then washed three times with 250 µL distilled water. Then 250 µL of 5% (v/v) APTES in distilled water was added to each well and incubated at 60^0C for 1 hr. The amine-functionalized wells were washed three times with 250 µL of 30% (v/v) ethanol in water and once with PBS and then with distilled water to remove excess APTES from the wells. The plates were dried at 80^0C for 1 hr.

2.2.6.1.2 Synthesis of AFB_1-CMO-NHS ester

AFB_1 (1 mg) was dissolved in 250 µL of ethanol and 50 µL of 1 M NaOH was added. The mixture was then stirred for 15 mins at room temperature after the addition of carboxylhydroxylamine hydrochloride (10 mg). The mixture was refluxed in an oil bath for 3 hrs, with constant stirring, followed by overnight stirring at room temperature. The solvent was evaporated in a Speed Vac apparatus. The residue was dissolved in 300 µL of water. The pH of the aqueous solution was adjusted to 9.1 using 0.5 M NaOH and then the un-reacted AFB_1 was extracted with 500 µL of ethylacetate. The pH of aqueous layer was adjusted to 2.0 using 6 M HCl, followed by extraction with 500 µL of ethylacetate to obtain AFB_1-CMO (aflatoxin B_1-carboxymethyl oxime). The solvent was evaporated in a Speed Vac apparatus and the product was confirmed by TLC performed on silica gel plates (10 X 20 cm) with a fluorescent indicator

(254 nm) developed in methanol:dichloromethane (7:3). AFB_1-CMO (1 mg) was dissolved in 200 µL of dioxane. DCC (1.5 equivalents) and NHS (1.5 equivalents) were added and the reaction mixture was stirred overnight at room temperature. The precipitated dicyclohexyl urea was then removed by centrifugation at 18,625 xg for 10 mins at room temperature in an Hermle Z233 MK-2 centrifuge with a 220.87.V05 rotor. The presence of the NHS ester was confirmed by TLC using silica gel plates with a fluorescent indicator (254 nm) developed in methanol:dichloromethane (7:3; by volume).

2.2.6.1.3 Immobilization of the AFB_1-CMO-NHS ester onto microtitre plates

The NHS ester in 1, 4-dioxane was dissolved in 1 M sodium hydrogen carbonate buffer (pH 9.4). To determine the optimal concentration of AFB_1-CMO-NHS ester for efficient signal generation, a checker board ELISA was performed by varying the NHS ester concentration (25, 50, 100 and 200 µg/mL). Indirect ELISA assays were performed. Optimization of the covalent attachment of the ester on amine-modified plates was performed by overnight incubation of the individual wells with 100 µL of ester at three temperatures, namely 4^0C, 37^0C and room temperature. Competitive assays were performed using plates incubated at the three temperatures. The IC_{50} value, which is half the maximal inhibitory value of the antibody in the presence of free antigen, was determined.

2.2.6.1.4 Electrospray ionization (ESI) mass analysis

Cleavage of the AFB_1-CMO-NH-$(CH_2)_3$-Si-O-polystyrene from the wells of the microtitre plate covalently-immobilized with AFB_1 was performed through incubating the wells with 1 M TBAF in H_2O. The cleaved TBAF product was purified using a C18 reverse phase column. The elution of the sample was performed using a mixture (1:1; by volume) of acetonitrile and water. The purified sample was dried in a Speed Vac apparatus and was dissolved in LC/MS grade acetonitrile and water in the ratio of 1:1 (v/v). The sample (100 µL) was injected into the ESI

mass spectrometer and analyzed in positive mode with a range from 300-600 Da to determine the molecular mass of the cleaved product.

2.2.6.1.5 Fluorescence assay for the determination of the covalently immobilized AFB_1

Individual wells of the microtitre plate were incubated with 150 µL of 1 M tetrabutylammonium fluoride (TBAF) in H_2O for 60 mins at room temperature. A similar incubation procedure was performed with APTES-modified, unmodified and passively adsorbed (AFB_1-BSA conjugate-coated) wells as negative controls. A set volume of TBAF solution was removed from the wells and added to the individual wells of a fresh F96 Nunc black maxisorb microtitre plate. The fluorescence intensities were measured at 360/440 nm (Excitation/Emission) against standard AFB_1 concentrations (2.5-25 µg/mL of AFB_1). The loading value of AFB_1 was estimated using a calibration curve.

2.2.6.1.6 Indirect and competitive inhibition assay

These two types of ELISAs were performed in accordance with the procedure outlined in section 2.2.3.14 except that 5 µg/mL of AFB_1-BSA was used to coat the plate and AFB_1 (0.0048-5 µg/mL concentration) was used for inhibition.

2.2.6.1.7 Extraction of AFB_1 from corn samples and validation of competitive inhibition assay performed on covalently immobilized microtitre plates

Reference corn material (TR-A100) (10 g) was extracted for 30 mins with 40 mL of 85% (v/v) methanol in the presence of 500 mg of celite filter aid and 500 mg of sodium chloride. The extract was centrifuged at 2108 xg for 30 mins at 4^0C and filtered using Whatman filter paper. The solvent was evaporated in a rotary evaporator and the residue was diluted in 40% (v/v) methanol in water. The concentration of AFB_1 in the crude extract was quantified by fluorescence estimation at 360/440 nm (Excitation/Emission) using standard calibration curve (slope equation $y = 361.7x$ ($R^2 = 0.99$)) with known amounts of AFB_1 (10-320 ng/mL) diluted

in 40% (v/v) methanol in water. Similarly 10 samples of corn (each 10 g) were extracted. The corn extract was used to perform competitive ELISA on AFB_1 covalently immobilized microtitre plates (section 2.2.6.1.6).

2.2.6.2 Immuno-affinity column for the detection of AFB_1, AFB_2 and ZEN

2.2.6.2.1 Preparation of Immuno-column

To confirm the covalent immobilization using allylated chemistry, the CC grade/Wakosil silica for covalent immobilization of protein (BSA and AFB_1 antibody) was chosen. Protein was attached to silica through the cysteamine and disuccinic ester of sebacic acid linkers. The attachment of linkers to the silica was confirmed by NHS ester and amino groups on the surface of silica. Protein was estimated using the BCA assay. The various stages involved are as shown in Figure 2.4.

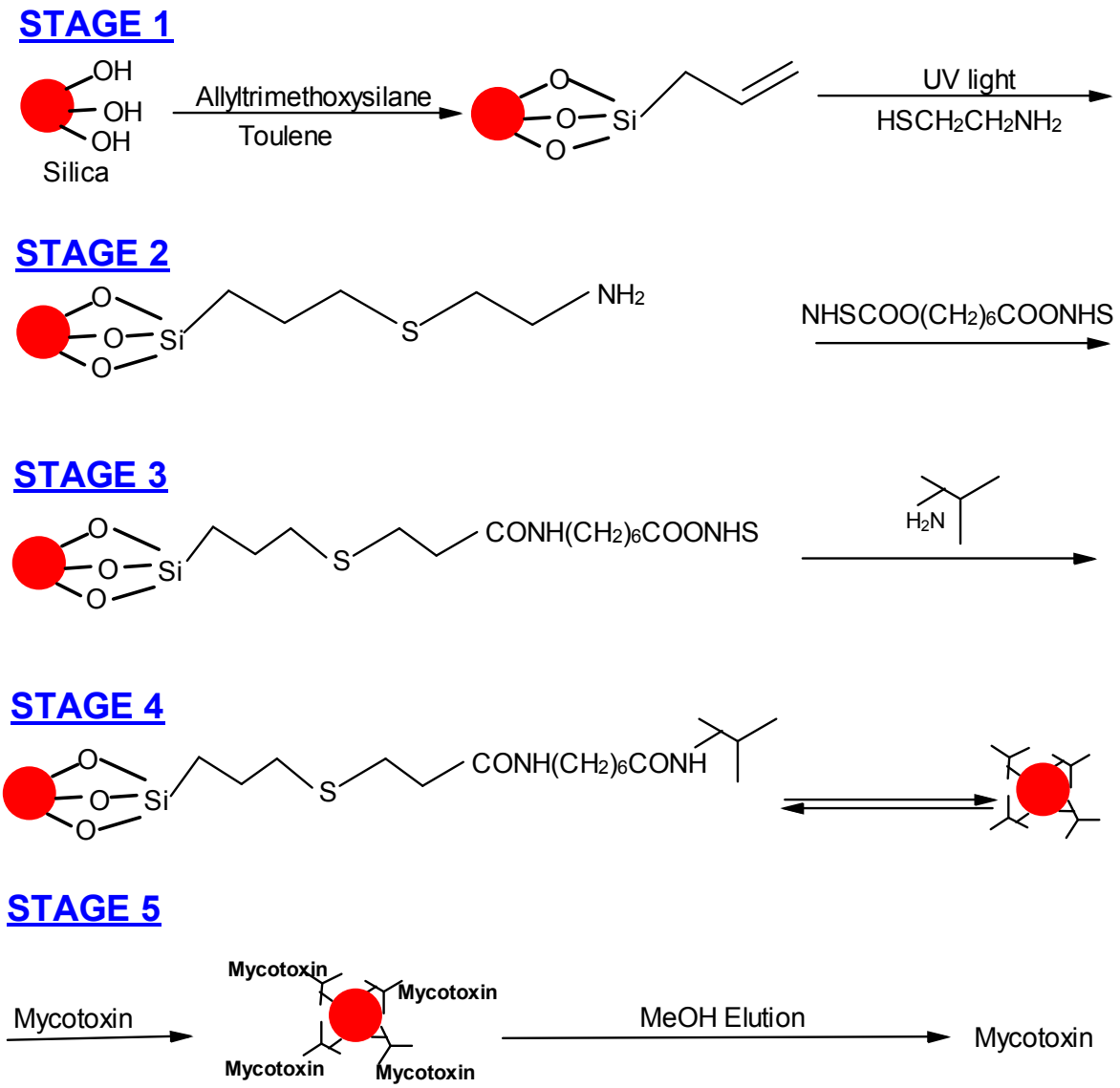


Figure 2.4: Schematic representation of the stages involved in the immobilization of recombinant antibodies onto the Wakosil silica and assessing the function of the column.

2.2.6.2.2 Immobilization of allyloxy silane (Stage I)

2.2.6.2.2.1 Passivation of silica

Sixty mL of 15% (v/v) HCl were added to 7 g of silica in a 250 mL single neck round bottom flask. The mixture was sonicated for 3 hrs at room temperature. After sonication another 80 mL of 15% (v/v) HCl was added to the reaction mixture and this was refluxed overnight at 130^0C with gentle stirring. The reaction mixture was filtered on a sintered crucible followed by four washes with 20 mL of distilled water. The silica was dried overnight at 100^0C.

2.2.6.2.2.2 Allylation of silica

Azeotropic distillation of silica (5 g) was performed with toluene (60 mL) for 30 mins and then silica was filtered on a sintered crucible. Toluene (60 mL) and 5 mL of allyloxysilane were added to the dried silica. The reaction mixture was refluxed at 120^0C for 24 hrs with stirring. The reaction mixture was filtered on a sintered crucible and then washed with toluene (3 X 30 mL), THF (3 X 30 mL) and acetone (2 X 20 mL).

2.2.6.2.3 Immobilization of cysteamine (Stage II)

Sixty mL of methanol and 1 g of cysteamine were added to 5 g of silica in a 150 mL round bottom flask. This mixture was exposed to UV illumination (λ 300 nm) for 24 hrs. The silica was filtered on a sintered crucible, and then washed with methanol (3 X 20 mL) and acetone (1 X 20 mL). The silica was dried in an oven at 80^0C for 30 mins. Amine estimation was performed using the ninhydrin assay with 4 N sodium acetate and $SnCl_2$ against the standard cysteamine solution.

Amine assay procedure: Ninety mg of ninhydrin was dissolved in 3.4 mL of ethylene glycol. To this, 112.5 µL of $SnCl_2$ solution (12 mg/120 µL) and 1.125 mL of 4 N sodium acetate were added. One hundred µL of the assay solution was added to 25 µL of each sample, control and standard. All samples were assayed in triplicate. The mixture was heated to 90^0C for 15 mins then cooled to room temperature and the absorbance was read at 575 nm on a Tecan Saffire plate reader.

2.2.6.2.4 Immobilization of sebacic acid (Stage III)

Aminated silica (500 mg) was transferred into a 10 mL round bottom flask and then 3 mL of DMF and 100 mg of sebacic acid were added. The reaction mixture was stirred overnight at room temperature and then filtered on a sintered crucible. The silica was washed with DMF (2 X 5 mL), methanol (1 X 5 mL) and acetone (1 X 5 mL). A control experiment was carried out using 500 mg of unmodified silica, which was incubated with sebacic acid in DMF overnight

followed by filtration and washing with DMF (2 X 5 mL), methanol (1 X 5 mL) and acetone (1 X 5 mL). The attachment of sebacic acid was confirmed by NHS ester assay against N-hydroxysuccinamide as standard.

NHS ester assay procedure: Two mL of 0.067 M phosphate buffer (pH 7.4) were added to the modified silica (20 mg). The reaction mixture was stirred for 3 hrs followed by filtration and the absorbance was read at 260 nm on a Tecan Saffire plate reader to estimate the number of carboxyl functional groups.

2.2.6.2.5 BSA immobilization (Stage IV)

NHS-modified silica (Stage III) (200 mg) was placed in a 10 mL round bottom flask and three mL of phosphate buffer (0.067 M, pH 7.6) and 20 mg of BSA were then added. The reaction mixture was stirred for 4 hrs at room temperature. The silica was filtered on a sintered crucible and then washed with phosphate buffer (3 X 4 mL). Two hundred µL of acetone was added for drying. An estimation of bound protein was determined by a BCA assay using BSA as the standard. BSA was incubated with unmodified silica as a control.

Assay Procedure: BCA assay reagent was prepared as per the Pierce kit instructions (Reagent A 10 mL + Reagent B 0.2 mL). The standard concentrations of BSA and silica (control, incubated & conjugated) were prepared in 200 µL of BCA reagent. The reaction mixture was incubated at 37^0C for 30 mins followed by cooling to room temperature and the absorbance was read at 562 nm.

2.2.6.2.6 Anti-AFB_1, B_2 & ZEN antibody immobilization (Stage IV)

We observed a lower loading value for protein adsorption on CC grade silica. Therefore, Wakosil grade silica was selected instead of CC grade silica to improve the loading of protein. This silica is less porous than CC grade silica and is commercially available. The same process was repeated for steps 2.2.6.2.2 to 2.2.6.2.5 using anti-AFB_1, B_2 & ZEN recombinant

antibodies instead of BSA. The amount of bound antibody on silica was estimated by the BCA assay.

2.2.6.2.7 Elution protocol for immuno-column (Stage V)

In order to check the toxins binding capacity to the immuno-affinity column, first recombinant antibody coupled silica beads (200 mg) were added to the individual columns. Ten micrograms of AFB_1, B_2 and ZEN in 500 µL of PBS were added to the specific columns. The reaction mixtures were stirred for 2 hrs at room temperature followed by washing with PBS and PBST. The fluorescence intensity of these washes was measured at 360/420 nm (Emission/Excitation) for AFB_1 or B_2 and 236/440 nm (Emission/Excitation) for ZEN. Washes were repeated until a constant reading was observed. Once the fluorescence reading was stabilized, the toxins bound to the silica were eluted with 1 mL of methanol. The control experiment was performed with unmodified silica, which was incubated with toxins and then eluted with methanol.

2.2.6.3 MBio assay for the simultaneous detection of AFB_1, B_2, ZEN and T-2 toxins

2.2.6.3.1 Imprinting of cartridges with conjugates of AFB_1, B_2, ZEN and T-2 toxins

The imprinting of mycotoxin conjugates (50 µg/mL) were performed using a Bio-Dot AD3200 robotic arrayer equipped with a Bio-Jet print head dispensing 28-nanoliter droplets by Precision photonics with 22 dots per row in duplicates.

2.2.6.3.2 Alexa-647 labelling of recombinant antibodies

2.2.6.3.2.1 Preparation of NHS ester of 4-formylbenzoic acid

4-formylbenzoic acid (10 mg) was dissolved in 200 µL of DMSO and then NHS (0.8 mg) and DCC (1.4 mg) were added. The reaction mixture was stirred for overnight at room temperature. The solvent was evaporated in a rotary evaporator (Buchi, Germany). The crude residue was loaded onto column (CH_2Cl_2:MeOH; 7:3) to obtain the NHS ester in 50% yield.

2.2.6.3.2.2 Preparation of Alexa 647 labelled antibody

Recombinant antibody (2 mg/mL, 100 µL) in 1X PBS (pH 7.4) was added to an above NHS ester (0.5 mg) and then stirred at room temperature for 2 hrs followed by dialyzing (buffer exchanging) with sodium acetate (150 mM, pH 6.0) using desalting column (Thermo Pierce, 0.5 mL). This was followed by addition of Alexa-647 aminooxyacetamide (10 µL, 1 mg/mL in DMF) and incubated for 1 hr. The mixture was dialyzed (buffer exchanged) with 1X PBS using Vivaspin column (M. Wt 'cut-off': 5 *k*Da).

2.2.6.3.3 Direct binding assay

Direct binding assay was perfromed by the additon of 1/100 dilution of Alexa-647 labelled anti-AFB_1, B_2, ZEN and T-2 antibodies in 4% (w/v) milk Marvel to the MBio cartridge that was inprinted with AFB_1, B_2, ZEN and T-2 BSA conjugates and incubated at room temperature for 20 mins. The flourescence intensity was recorded on an MBio snapshot machine.

2.3 Data analysis

1. The IC_{50} value was calculated using the four parametric logistic function

   Fifty = (Top+Baseline)/2

   Y = Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*HillSlope + log((Top-Bottom)/(Fifty-Bottom)-1)))

2. Limit of detection (LOD)

   LOD = LOB + 3 S.D. of LOB, where LOB is the limit of blank

3. Limit of quantification (LOQ)

   LOQ = LOB + 10 S.D. of LOB, where LOB is the limit of blank

4. Conversion units

   1 ppb = ng/mL = µg/kg

Chapter 3
Generation of murine anti-AFB_2 recombinant scFv antibodies

3.1 Introduction

Aflatoxins are major food contaminants produced by *Aspergillus* species. AFB_2 is toxic and although reports reveal the presence of AFB_2 along with AFB_1 (Juan *et al.*, 2008), this issue has not been extensively studied. To-date, the antibodies generated for the detection of AFB_2 were mainly monoclonal. These monoclonal antibodies exhibit a wide range of cross reactivity with other types of aflatoxins (Hastings *et al.*, 1988; Cervino *et al.*, 2008; Rieger *et al.*, 2009). An attempt was made by Kalinichenko *et al.* (2010) to isolate an AFB_2-specific antibody, through the generation of anti-AFB_2 Fab from the parent monoclonal antibody. This resulted in a Fab antibody with similar cross reactivity characteristics as the parent monoclonal antibody.

cDNA libraries are a true representation of the mRNA pool and these libraries facilitate the selection of highly specific antibody fragments with diverse affinities (Moroney and Pluckhun, 2005). Hence recombinant approaches (Figure 3.1) were chosen for the generation of a specific and high sensitivity scFv to AFB_2 for use in the development of an immuno-affinity column.

Mice were immunized with AFB_2-KLH, and the spleen used for the isolation of RNA and, subsequently, the antibody phage-display library was constructed to produce soluble scFv antibodies. The library generated was subjected to panning against AFB_2-BSA, which yielded a number of AFB_2-positive binders. The cross reactivity of the scFvs was investigated and anti-AFB_2-specific clones with no cross reactivity to other aflatoxin variants were obtained. A scFv-E9, with an IC_{50} value of 8 ng/mL was selected and a SPR-based inhibition assay developed and applied for quantitative analysis of AFB_2 in spiked almond samples.

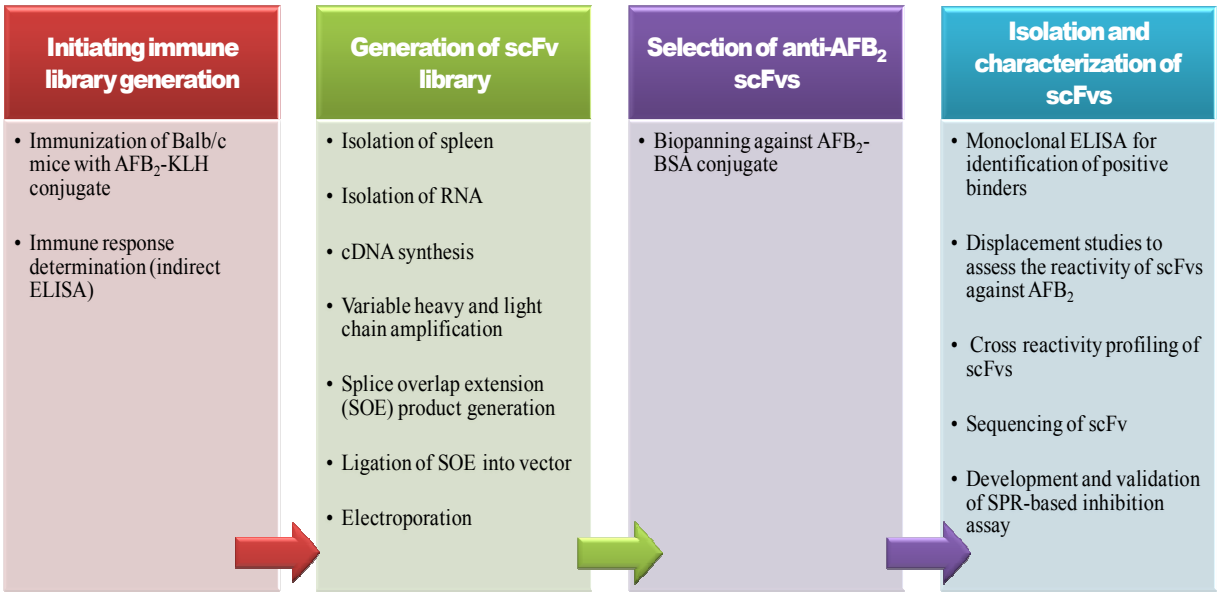


Figure 3.1: Approach employed for the generation of anti-AFB_2 scFvs. *The AFB_2 library was generated from Balb/c mice immunized with a AFB_2-KLH conjugate. Spleenomic RNA from a hyper-immune mouse was used to synthesize cDNA. Variable heavy and light chains were amplified followed by SOE product generation and subsequent ligation and electroporation steps. The next stage was the selection of anti-AFB_2 scFvs through biopanning, which utilizes phage for efficient propagation of antibody-encoding genes. Finally the analytical potential of scFvs was tested through competitive ELISA and SPR-based inhibition assays.*

3.2 Results

3.2.1 Immune response to AFB_2-KLH conjugate

The immunization schedule, as outlined in section 2.2.2.1, was followed to elicit an immune response in Balb/c mice immunized with AFB_2-KLH. The serum titre was determined after the third boost following the protocol described in section 2.2.3.1. With an indirect ELISA (Figure 3.2), the serum titre (1/512,000) was deemed sufficient to utilize spleenomic RNA for the generation of a recombinant antibody library following a final intravenous boost.

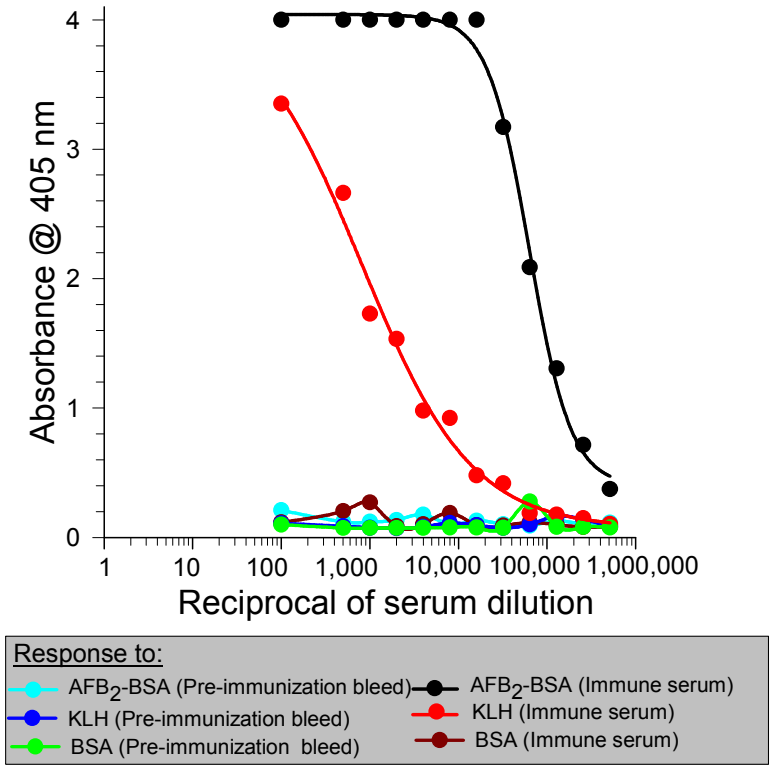


Figure 3.2: Serum titre of a AFB_2-KLH immunized mouse determined by indirect ELISA against AFB_2-BSA*. Doubling dilution of serum was added to the AFB_2-BSA-coated microtitre plate and probed with anti-mouse-alkaline phosphatase-labelled secondary antibody (1/1,000). Finally, visualization was brought about through the addition of pNPP substrate. The final titre recorded against AFB_2-BSA was in excess of 1/512,000.*

3.2.2 Reombinant scFv antibody library generation for AFB_2

3.2.2.1 RNA isolation and cDNA yields

A spleen homogenized in TRIZOL reagent was used for the extraction of RNA using chloroform. The total yield of RNA was 11.4 µg/50 µL. RNA (4.5 ng) was used for the generation of cDNA (yield; 11.9 ng/µL), from which heavy and light chains were amplified (Figure 3.3).

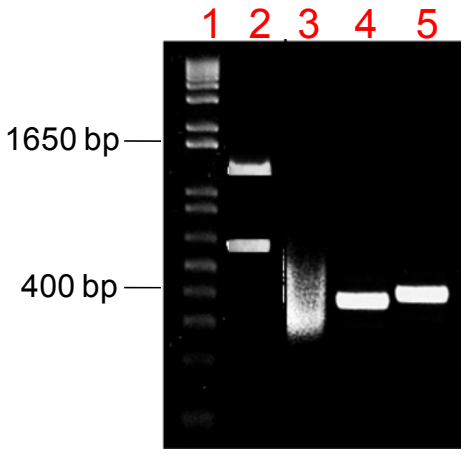


Figure 3.3: Agarose gel showing the RNA, cDNA and amplified heavy and light chains. *cDNA was synthesized from RNA by reverse transcription using Superscript III Kit. From the template cDNA variable heavy (V_H) and light (V_L) were amplified using the primers specific for mice and PCR products were run on 1% (w/v) agarose gel. The representation of the various lanes is as follows: lane 1: 1 Kb plus DNA ladder; lane 2: RNA; lane 3: cDNA; lane 4: V_L and lane 5: V_H.*

3.2.2.2 Optimization of buffer concentration

The PCR reaction was optimized by varying the amount of $MgCl_2$ (for heavy and light chain amplification) and $MgSO_4$ (for SOE), as Mg^{++} is an essential cofactor in the DNA polymerisation.

3.2.2.2.1 Amplification of variable heavy and light chains

For the amplification of variable heavy and light chain genes GoTaq® DNA polymerase was used. $MgCl_2$ concentrations of 1, 1.5, 2 & 3 mM were used to determine the optimal concentration of $MgCl_2$, for better amplification of the gene. A negative PCR reaction without the template cDNA was also performed. The PCR products were resolved on 1% (w/v) agarose gel. The presence of bands at approximately 330 bp (Figure 3.4), indicated amplification of variable heavy and light chains.

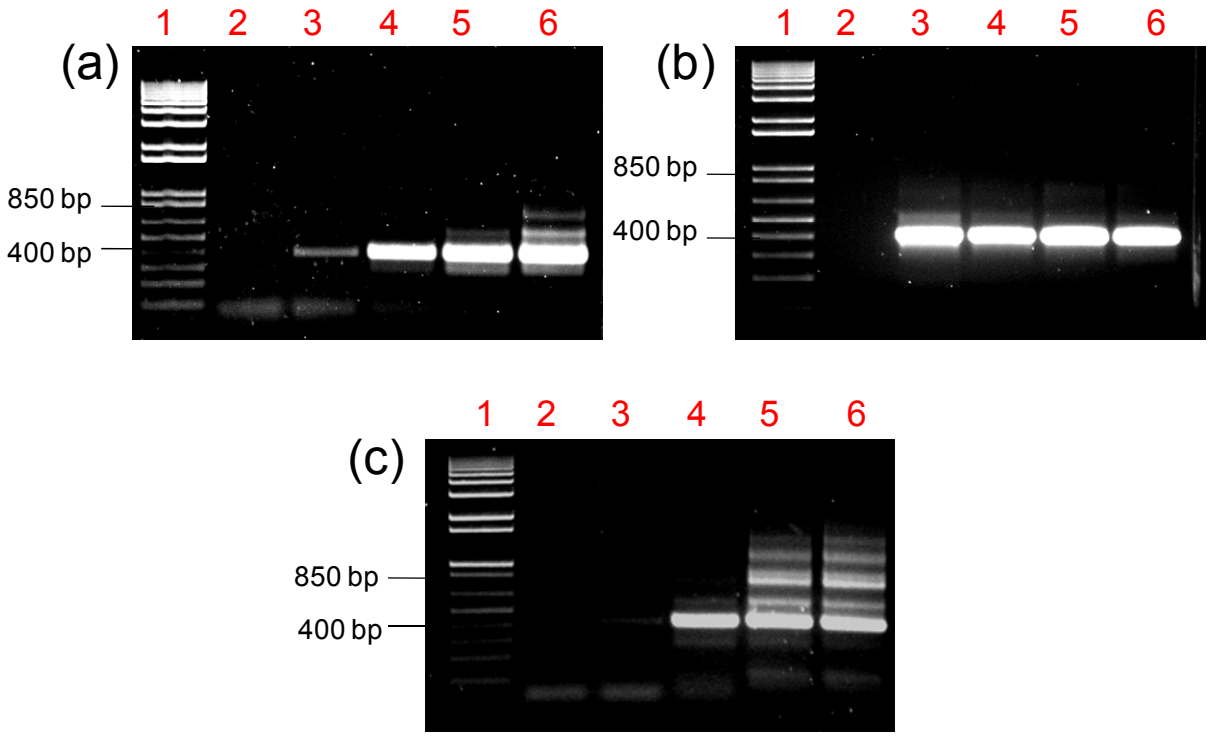


Figure 3.4: Optimization of magnesium concentration for amplification of V_k (a), V_λ (b) and V_H (c). *The PCR reaction mixture is composed of 0.5 µL of cDNA, 100 pmol of mouse primers, 5x PCR buffer, 200 µM dNTPs, 0.25 µL of GoTaq® DNA Polymerase and 1-3 mM of $MgCl_2$. The PCR amplification conditions were as follows: initialization for 5 mins at 94^0C; 30 cycles of denaturation; annealing and elongation for 15, 30, 90 sec at 94^0, 56^0 and 72^0C, respectively; followed by the final elongation step for 10 mins at 72^0C. The representation of the various lanes is as follows: lane 1: 1 Kb plus DNA ladder; lane 2: negative PCR reaction; lanes 3-6: PCR products amplified using four different concentrations of $MgCl_2$ (1, 1.5, 2 & 3 mM).*

The lack of band in the second lane of Figure 3.4 indicates lack of non-specific amplification (negative PCR reaction). $MgCl_2$ (1.5 mM) was used to perform large-scale amplification (20X) reactions of the variable heavy and light chains. Immediately after scale-up the PCR mixtures were ethanol-precipitated and gel-purified using a Nucleospin Extract II kit. The final yields were 27.3 µg (V_H), 34.2 µg (V_λ) and 12.4 µg (V_k) per 220 µL, as determined on the Nanodrop^{TM} ($A_{260\ nm}$).

3.2.2.2.2 Splice overlap extension (SOE)

The amplified heavy and light chain genes were linked together using a Platinum® Taq DNA polymerase Hi Fidelity, in the presence of the SOE primers and dNTPs. $MgSO_4$ concentrations of 1, 1.5, 2 and 4 mM were used to attain a PCR product of approximately 750 bp (Figure 3.5). Scale up of SOE was performed using 4 mM of SOE. The total SOE yields were 13.5 µg/ 240 µL (SOE (k)) and 11 µg/ 240 µL (SOE (ʎ)).

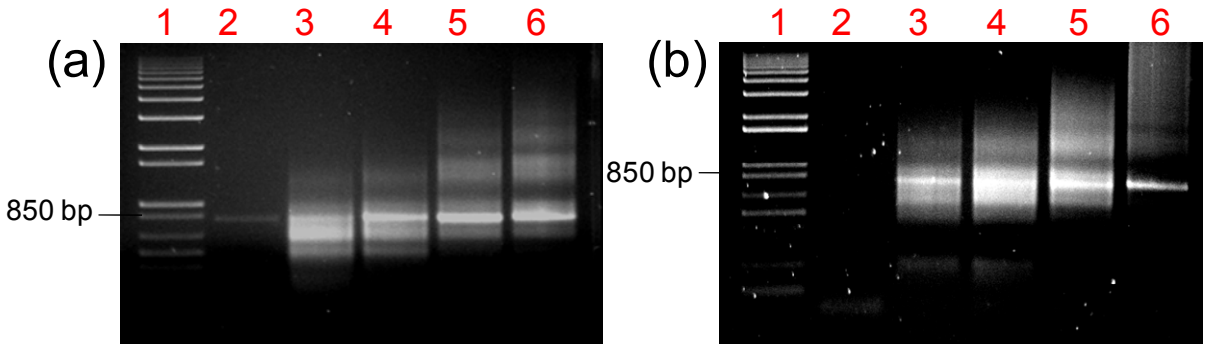


Figure 3.5: Optimization of $MgSO_4$ concentration for SOE(k) (a) and SOE(ʎ) (b). *The PCR reaction mixture is composed of 100 ng of V_L and V_H, 100 pmol of CSC-F and CSC-B, 10x PCR buffer, 200 µM dNTPs, 0.2 µL of Platinum® Taq DNA polymerase and 1-4 mM of $MgSO_4$. The PCR amplification conditions were as follows: initialization for 5 mins at 94^0C; 30 cycles of denaturation; annealing and elongation for 30, 30, 120 sec at 94^0, 56^0 and 72^0C, respectively; followed by the final elongation step for 10 mins at 72^0C. The representation of the various lanes is as follows: lane 1: 1 Kb plus DNA ladder; lane 2: negative PCR reaction; lanes 3-6: SOE obtained using four different concentrations of $MgSO_4$ (1, 1.5, 2 & 4 mM).*

3.2.2.2.3 Restriction digestion and ligation of SOE and plasmid

The plasmid was digested with S*fi*I, X*baI* and X*hoI*, which cuts the vector into three fragments consisting of the digested PCR product (1500 bp), the digested vector (3400 bp) and the stuffer fragment (1600 bp) (Figure 3.6). The SOE (SOE (k) 9.4 ng & SOE (ʎ) 11 ng) was digested with S*fi*I alone (Figure 3.6). Both the restriction digested plasmid and SOE (k & ʎ) were ethanol-precipitated and gel purified using a Nucleospin Extract II kit. The total yields of plasmid, SOE (k) and SOE (ʎ) after restriction digestion were 14.5 µg, 2.6 µg and 7.8 µg, respectively as determined on the Nanodrop^{TM} ($A_{260\ nm}$). The SOE and plasmid (700 ng:1400 ng) were ligated together using T4 DNA ligase for 18 hrs at 27^0C.

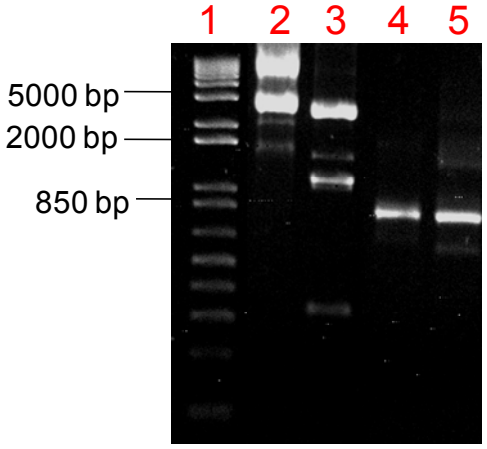


Figure 3.6: Agarose gel image showing the restriction digestion pattern of SOE and plasmid. *The reaction mixture is composed of 10 µg of SOE, 360 units of SfiI, 20 µL of 10x reaction buffer and 3 µL of 100x BSA. Restriction digestion of vector was carried out using 20 µg of vector, 120 of SfiI, 20 µL of 10x reaction buffer and 1 µL of 100x BSA. Both SOE and vector were SfiI digested for 5 hrs at 50^0C, after which the vector was further treated with XhoI, XbaI and Antarctic phosphatase. The representation of the various lanes is as follows: lane 1: 1 Kb plus DNA ladder; lane 2: plasmid; lane 3: SfiI, XbaI and XhoI restriction digested plasmid; lane 4: SOE and lane 5: SfiI restriction digested SOE.*

3.2.3 Biopanning of recombinant AFB_2 library

3.2.3.1 Selection of AFB_2-specific phage particles

The ligation mixture was electroporated into XL1-Blue electrocompetent cells. An scFv antibody library of 2.19 X 10^7 cfu/mL was obtained. This was subsequently panned on AFB_2-BSA (250, 125, 50, 25 and 5 µg/mL in 1X PBS) coated surfaces of immunotubes. After each successive round of panning the stringency of washes were increased. Input and output phage titres after each round of panning were determined (Table 3.1) using the formula:

$$\frac{\text{No. of colonies X dilution factor}}{\text{plated volume}}$$

Table 3.1: *Inputs and outputs from different rounds of panning*

Round	Input titre (cfu/mL)	Output titre (cfu/mL)
Round 1	1 X 10^{12}	9 X 10^7
Round 2	3 X 10^{11}	2 X 10^6
Round 3	1 X 10^{11}	0.8 X 10^4
Round 4	1 X 10^{12}	3 X 10^4
Round 5	2 X 10^{12}	1 X 10^5

3.2.3.2 Polyclonal phage ELISA indicating AFB_2-specific phage enrichment

Rescued phage particles, obtained after each round of panning, were analyzed for specific AFB_2-BSA (antigen) binding activity through polyclonal phage ELISA (Figure 3.7). An increase in the specific signal was evident from the phage collected after subjecting to fifth round of panning against AFB_2-BSA. Minimal background signal was obtained when phage was tested against BSA and milk Marvel (negative controls). The pool of phage posses different recombinant scFv fragments with varying affinities towards the target. These were infected into Top 10F′ cells for soluble expression.

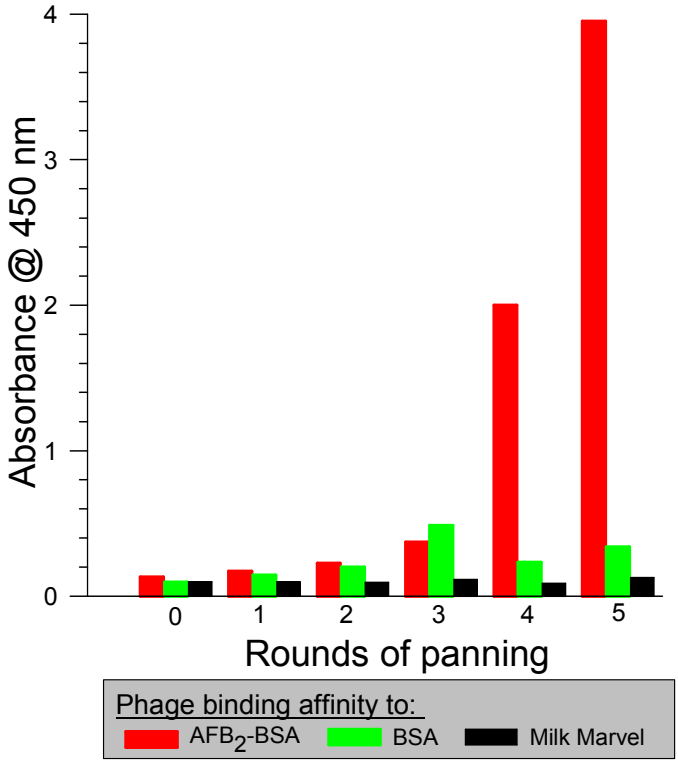


Figure 3.7: Polyclonal phage ELISA of the AFB_2-panned library against AFB_2-BSA. *Each of the analytes (AFB_2-BSA, BSA & milk Marvel) were coated on a microtitre plate (in triplicate). The phage (1/10 dilution) from different rounds of panning were added and probed with HRP-labelled mouse anti-M13 monoclonal antibody. Visualization with TMB solution was followed by absorbance reading. Significant enrichment of phage from fifth round of panning was noticed, which indicates the presence of more number of AFB_2-positive binders.*

3.2.3.3 Monoclonal ELISA for AFB_2 positive binders

Three hundred and eighty four individual colonies were picked, grown up and induced with IPTG. Each clone was tested on three different plates that were coated with either AFB_2-BSA, BSA (negative control) and KLH (negative control). The scFv-producing clones were found to be highly specific to AFB_2 with about 95% positive signal (Figure 3.8). No significant binding of the scFv-producing clones to BSA (Figure 3.9) or KLH (Figure 3.10) was recorded.

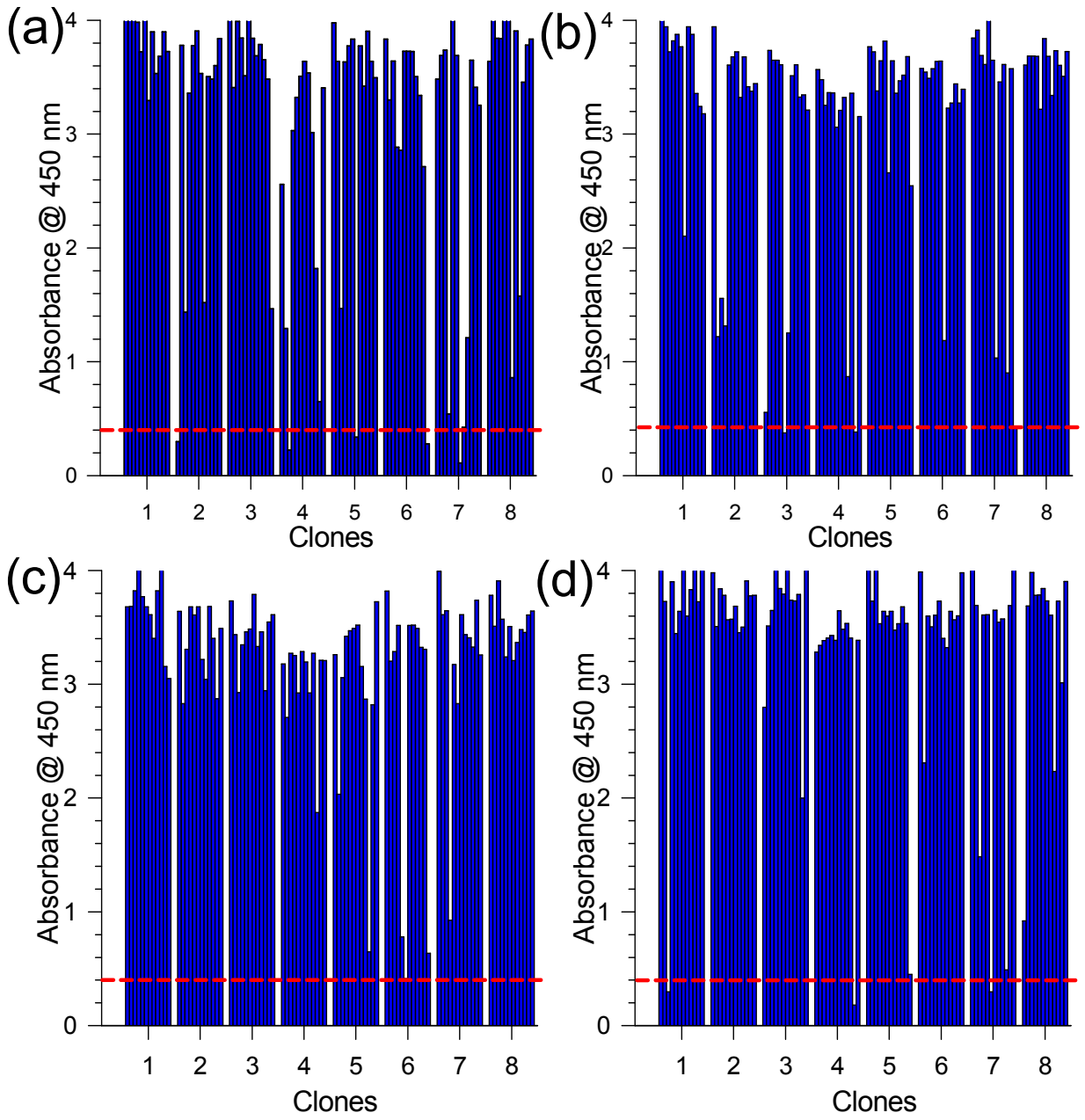


Figure 3.8: Graphical representation of monoclonal ELISA performed on anti-AFB_2 scFv-producing clones against AFB_2-BSA-coated microtitre plates. *The ELISA was performed using colonies picked from the fifth round of panning. A 1/2 dilution of supernatants were added to AFB_2-BSA coated and blocked plate. HRP-labelled rat anti-HA monoclonal (1/1,000) antibody was added followed by the detection using TMB solution. Figures a, b, c and d correspond to plates 1, 2, 3 and 4, respectively. The red line is the reference value, above which the clones were selected as AFB_2-positive binders.*

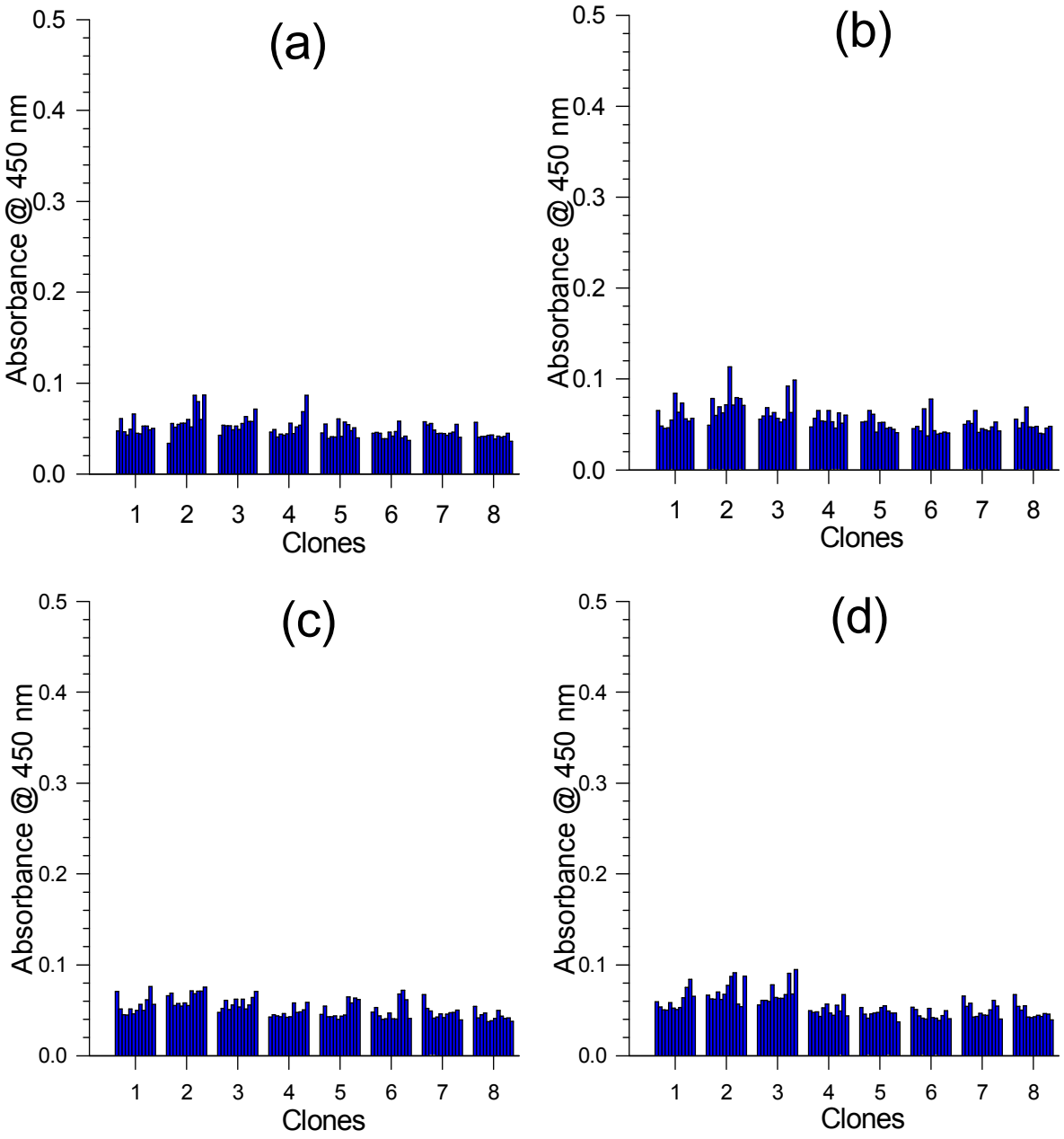


Figure 3.9: Graphical representation of monoclonal ELISA performed using anti-AFB_2 scFv-producing clones against BSA-coated microtitre plates. *The supernatants (1/2 dilution) were added to 5 µg/mL BSA coated and 4% (w/v) milk Marvel blocked plates. A 1/1,000 dilution of secondary antibody (HRP-labelled rat anti-HA monoclonal antibody) was added and visualization brought through addition of TMB. The lower absorbance (OD_{450nm}<0.2) recorded clearly indicate lack of binding of the scFvs to BSA. Figures a, b, c and d correspond to plates 1, 2, 3 and 4, respectively.*

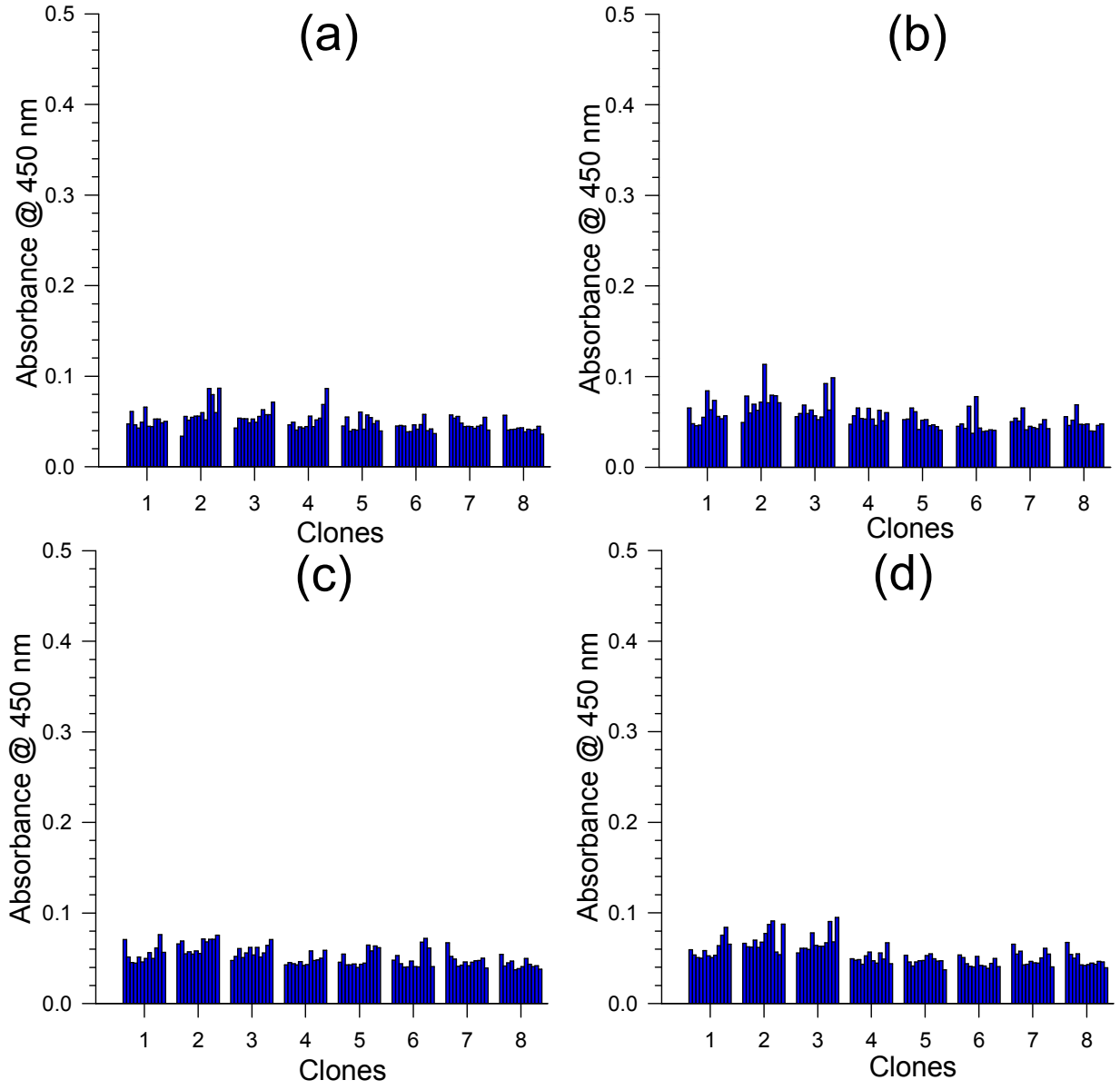


Figure 3.10: Graphical representation of monoclonal ELISA performed with anti-AFB_2 producing-scFv clones against KLH-coated microtitre plates. *ELISA plate was coated with 5 µg/mL of KLH and blocked with 4% (w/v) milk Marvel in PBS (pH 7.2). Diluted (1/2) supernatants were added to the wells. HRP-labelled rat anti-HA monoclonal antibody was used to detect binding of clones to KLH. The results (absorbance < 0.2 at OD_{450nm}) clearly indicate lack of binding of the scFvs to KLH. Figures a, b, c and d correspond to plates 1, 2, 3 and 4, respectively.*

3.2.3.4 Selection of anti-AFB_2 scFvs

Ten representative clones were picked from the positive clones and their AFB_2 displacement was measured using a 1:1 mixture of 1/10 dilution of antibody and 20 µg/mL of AFB_2. The results indicate that out of the ten anti-AFB_2 scFvs only seven were able to recognize AFB_2. Further cross reactivity studies and DNA finger printing analysis of the seven anti-AFB_2 scFvs were carried out.

3.2.3.4.1 Cross reactivity studies

The cross reactivity of the seven anti-AFB_2 scFvs to AFB_1, G_1, G_2 and M_1 was determined employing the procedure described in section 2.2.3.16. The IC_{50} value is defined as the point where the 50% reduction in the signal (in the presence of analyte) is noticed in comparison to the signal obtained in the presence of no analyte. The percentage cross reactivity was calculated was by taking the IC_{50} value of AFB_2 as 100%. The calculation was done using the formula

$$\% \text{ cross reactivity} = \frac{IC_{50} \text{ value of aflatoxins}}{IC_{50} \text{ value of } AFB_2} \times 100$$

From the % cross reactivity data (Table 3.2) it was found that two scFvs (E9 and H12) were specifically recognizing AFB_2 without exhibiting any cross reactivity to other aflatoxin congeners (AFB_1, G_1, G_2 & M_1). To further confirm the diversity of the clones, they were subjected to restriction digestion.

Table 3.2: *Cross reactivity of seven anti-AFB_2 scFvs. The cells in the table containing a dash indicates that the anti-AFB_2 scFv does not cross react with aflatoxin congeners*

Clone	% Cross reactivity				
	AFB_1	AFB_2	AFG_1	AFG_2	AFM_1
A8	85	100	19	67	58
D1	79	100	23	31	33
H8	77	100	82	81	67
H12	-	100	-	-	-
E9	-	100	-	-	-
A2	89	100	84	81	79
A1	86	100	45	49	84

3.2.3.4.2 DNA finger printing of anti-AFB_2 scFvs

Plasmids were isolated from all the seven scFv-expressing clones and splice overlap extension product (SOE) was amplified using specific primers. The digestion pattern of the SOE with an enzyme *Bst*N1 was studied (Figure 3.11). Three types of digestion patterns were noticed, thus indicating the presence of three different types of scFvs. The digestion pattern of the two scFvs (E9 & H12) were similar. Later on these two scFvs (E9 & H12) were expressed in 500 mL culture volumes. The cells were lyzed through sonication, followed by the subsequent IMAC purification and buffer exchange processes.

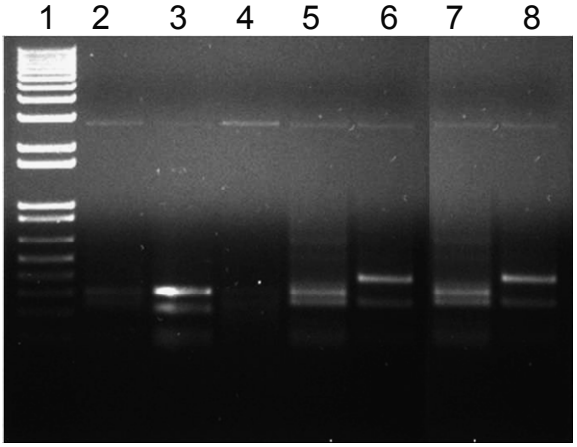


Figure 3.11: DNA finger printing profile of the seven scFvs recognizing AFB_2. *The plasmids were isolated and SOE was amplified from all the clones and they were digested with BstN1 for 3 hrs at 50^0C, after which they are resolved on 1% (w/v) agarose gel. The representation of the various lanes is as follows: lane 1: 1 Kb plus DNA ladder and lanes 2-8: digestion pattern of scFvs- A1, A8, H8, D1, E9, A2 and H12 .*

3.2.3.5 Competitive inhibition ELISA for scFv-E9 & H12

Competitive inhibition assay procedure outlined in section 2.2.3.15 was used to determine the IC_{50} values of the E9 and H12 clones. From Figure 3.12 it is apparent that the IC_{50} value of the scFv-H12 is higher than scFv-E9. The IC_{50}, limit of detection and limit of quantification for scFv-E9 were 85, 3 & 14 ng/mL, respectively.

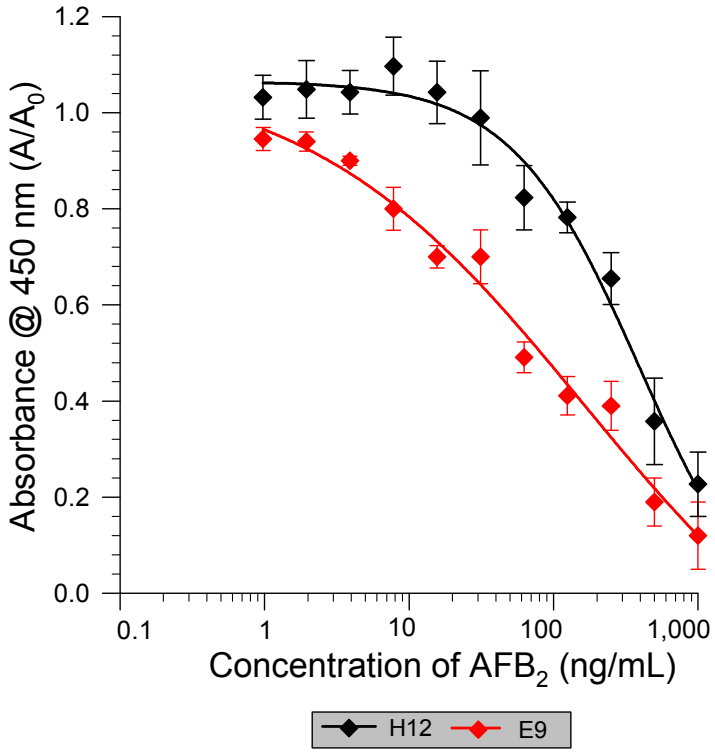


Figure 3.12: Competitive inhibition curves of scFv-E9 and H12. *The scFvs (E9 & H12) were expressed in 500 mL culture and IMAC purified and incubated with varying concentrations of AFB_2. The AFB_2-antibody mixture was then added to the ELISA plate that was coated with 5 µg/mL of AFB_2-BSA and blocked with 4% (w/v) milk Marvel PBS. The IC_{50} value of scFv-E9 and H12 were recorded to be 85 ± 0.05 and 375 ± 0.12 ng/mL, respectively.*

3.2.3.6 Intra- and inter-day competitive inhibition ELISA using scFv-E9

Purified scFv-E9 was used to develop an inhibition assay for the detection of AFB_2 as described in section 2.2.3.15. The assay was performed using eleven AFB_2 standards (0.9-1,000 ng/mL), assayed thrice on the same day and repeated over five days. Figure 3.13 represents the intra- and inter-day inhibition standard cruve using SigmaPlot software. The percentage coefficients of variance, which indicates the precision of the both intra- and inter-day assays were tabulated (Table 3.3). The inter-day coefficient of variance of the assay was between 1.3 and 10.1%.

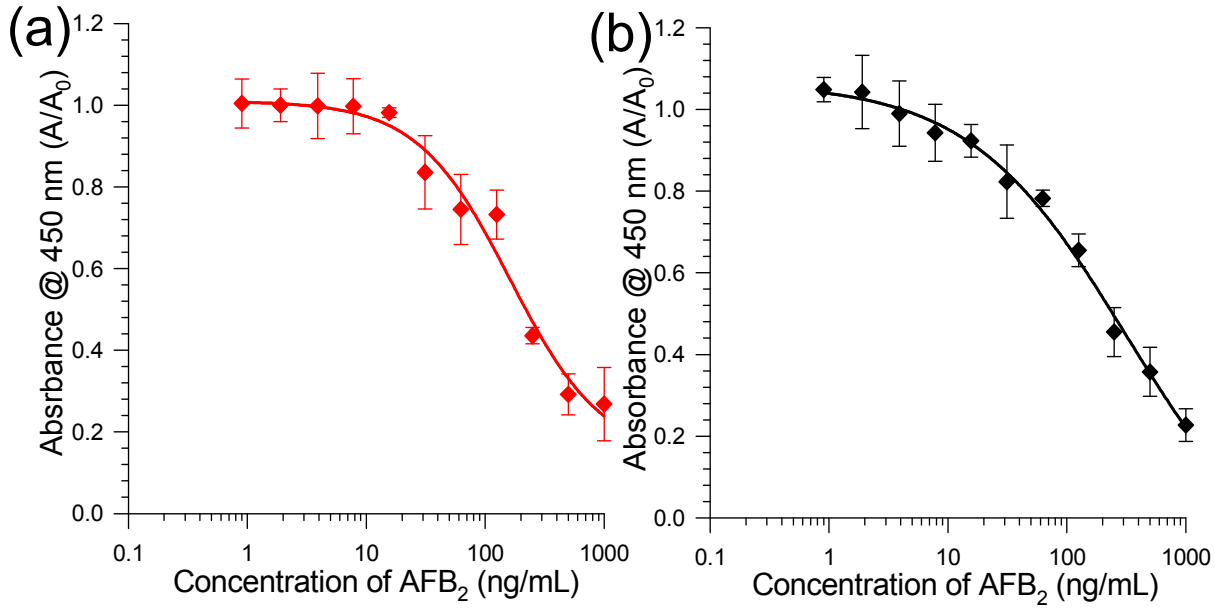


Figure 3.13: Intra- (a) and inter- (b) day competitive inhibition assays with scFv-E9. *A 5 µg/mL AFB_2-BSA coating concentration was used with a 1/2,500 dilution of anti-AFB_2 scFv that was incubated with AFB_2 (0.9-1000 ng/mL). For intra-day (a) studies the S.D. was computed from 3 validation run on the same day and inter-day (b) S.D. was determined over 5 validation runs on 5 separate days. A 4-parameter logistic curve was fitted to the data set.*

Table 3.3: *Intra- and inter-day coefficient of variance for the determination of free AFB_2 using scFv-E9. Three sets of eleven standards were assayed three times on one day and five times on five different days and CVs were calculated.*

AFB_2 concentration (ng/mL)	Intra-day assay				Inter-day assay			
	Back-calculated concentration (ng/mL)	A/A_0	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	A/A_0	% CVs	% Accuracies
1000	1033	0.2681	3.72	103.3	998	0.2270	7.2	99.8
500	477	0.2918	2.34	95.4	500	0.3576	3.4	100
250	247.75	0.4356	2.86	99.1	235.5	0.4549	8.6	94.2
125	145.87	0.7321	3.12	116.7	127.5	0.6549	1.3	102
62.5	57	0.7448	2.5	91.2	64.12	0.7823	5.3	102.6
31.2	27.41	0.8355	1.7	87.8	30.51	0.8233	7.2	97.8
15.6	16.63	0.9815	2.01	106.6	15.6	0.9234	5.6	100
7.8	7.93	0.9973	1.5	101.7	7.64	0.9425	10.1	97.9
3.9	3.9	0.9980	5.15	100	3.86	0.9894	5.9	98.9
1.9	1.89	1.0000	5.46	99.5	1.95	1.0425	5.4	102.6
0.9	0.91	1.0040	4.11	101.1	0.9	1.0487	6.1	100

3.2.3.7 SDS-PAGE and western blot analysis of scFv-E9

The purity of anti-AFB_2 scFv-E9 after subjecting to IMAC purification was analyzed by SDS-PAGE and western blotting using anti-HA HRP-labelled rat monoclonal antibody as described in section 2.2.3.17.2. A single band at approximately 27 *k*Da was visible, which represents the scFv (Figure 3.14).

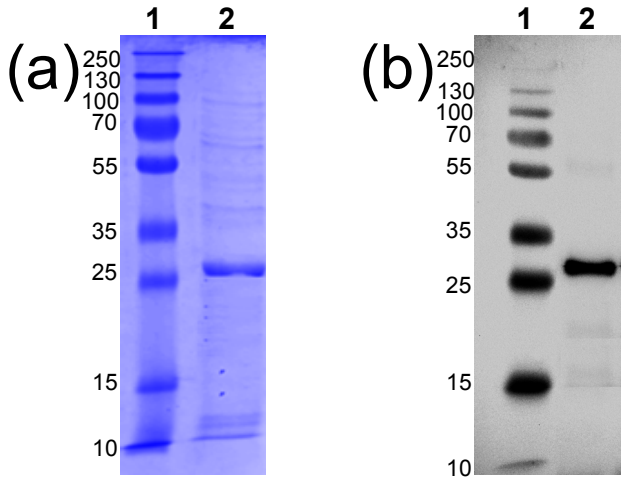


Figure 3.14: SDS-PAGE (a) and western blotting (b) of scFv-E9 after subjecting to IMAC purification. *A 12.5% SDS gel was run for 1.5 hrs at 100 V and the gel was stained with Commassie dye solution (Commassie blue R-250 (2 g), methanol (450 mL), acetic acid (100 mL) and water (450 mL) and destained overnight at room temperature with mixture containing acetic acid, methanol and water in the ratio of 2:5:13. For western blotting the SDS-PAGE gel was transferred onto the nitrocellulose membrane for 15 mins at 15 V and the membrane was blocked with 4% (w/v) milk Marvel for 1 hr. This was probed with 1/5,000 dilution of anti-HA HRP-labelled rat monoclonal antibody, followed by visualization of band using TMB substrate. The representation of the various lanes is as follows: lane 1: Fermentas Pageruler plus marker and lane 2: IMAC purified E9 clone. The purified E9 clone revealed the presence of scFv band at approximately 27 kDa.*

3.2.3.8 Sequence of scFv-E9

Plasmid DNA containing anti-AFB_2 scFv-E9 & D1 inserts were send to Source Biosciences Ltd. for sequencing. The linear nucleotide sequences were subsequently translated (using Expasy translate tools) into amino acid sequences and presented in Figure 3.15.

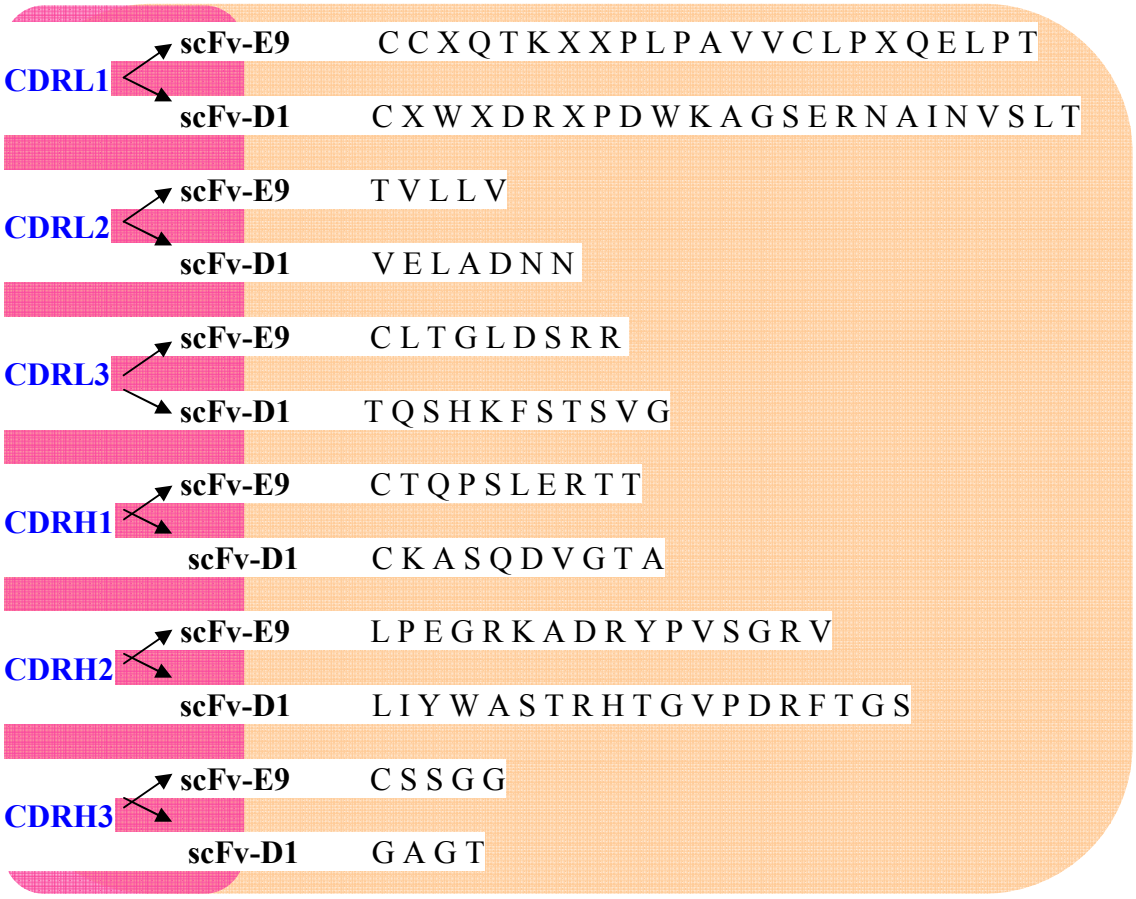


Figure 3.15: Deduced amino acid sequence of scFv-E9 and D1. *The complementary determining regions (CDRs) were determined following Kabat rules.*

3.2.3.9 CM5 chip immobilization with AFB_2

Immobilization of AFB_2 (Figure 3.16) onto the dextran surfaces was performed as described in section 2.2.3.18. The carboxyl groups on the sensor chip surface were activated with mixture of EDC and NHS to produce reactive succinimide esters. Due to the lack of amine groups on AFB_2, amine groups were created on the CM5 chip surface through the addition of Jeffamine. The carbonyl groups at position 1 of AFB_2 were coupled directly to amino group of protein using CMO spacer (Cervino *et al.*, 2007).

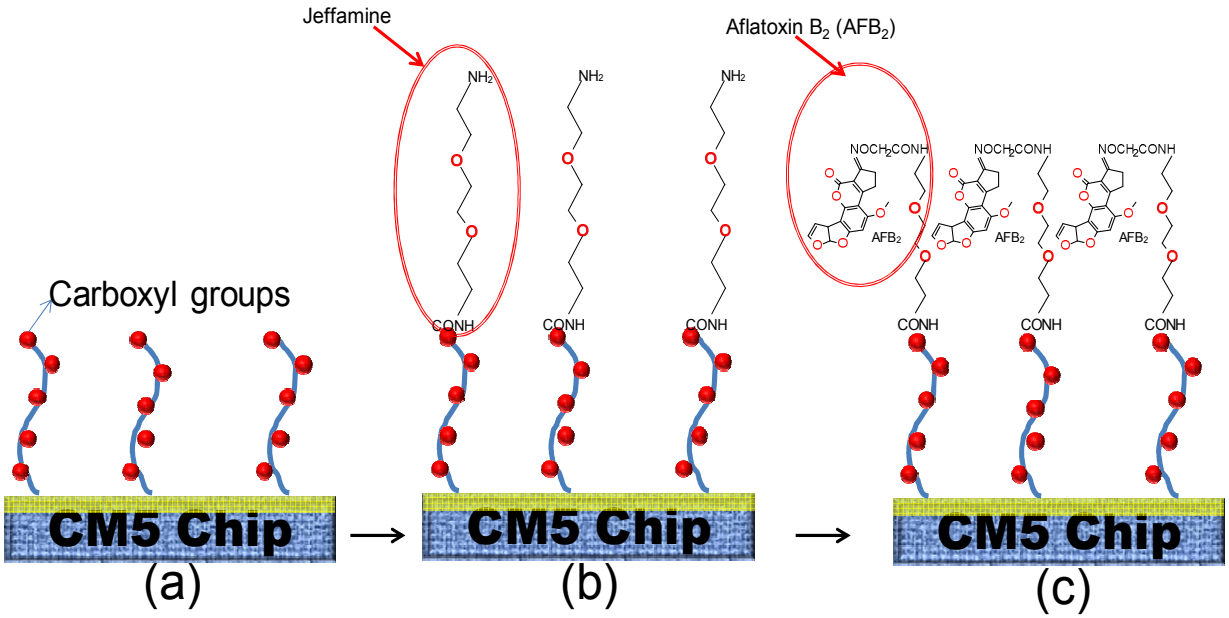


Figure 3.16: Schematic representation of immobilization procedure of AFB_2 onto the CM5 chip*. The first stage (a) involved in the immobilization of AFB_2 onto CM5 chip was surface activation with EDC and NHS. In the second stage amine groups were generated through treating with Jeffamine (b). The third stage involves the covalent attachment of AFB_2 to the amine groups on the CM5 chip (c).*

3.2.3.10 Analysis of scFv-E9 on AFB_2 immobilized sensor chip

After immobilizing the CM5 chip with AFB_2, varying dilutions (1/100, 200, 400 & 800) of IMAC purified scFv-E9 was passed over the sensor chip to record the optimal responses. A 1/100 dilution of scFv-E9 (Figure 3.17) resulted in approximately 359.68 RU. As negative control anti-CRP scFv antibody was passed over the AFB_2 immobilized sensor chip, which doesn't yield any notable responses.

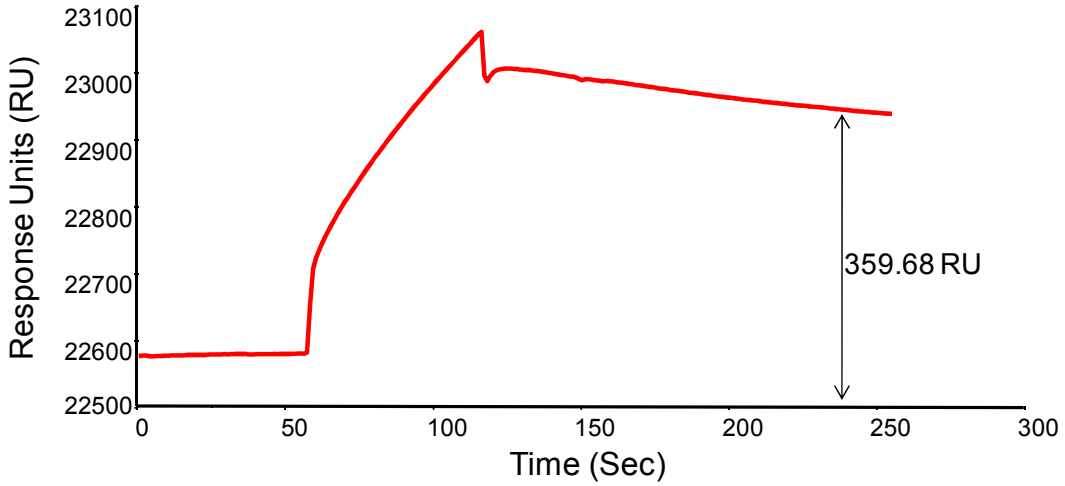


Figure 3.17: Sensogram depicting the binding of E9 to AFB_2 immobilized CM5 chip. *A 1/100 dilution of scFv-E9 in HBS buffer was passed over flow cell 1 of the AFB_2 immobilized sensor chip at a flow rate of 15 µL for 1 min. The net binding of response of E9 was 359.68 RU.*

3.2.3.11 Kinetic analysis of scFv-E9

The association (K_a) and dissociation (K_d) rate constants of the anti-AFB_2-scFv-E9 were determined on SPR using the kinetic analysis command using varying concentrations of scFv-E9 (43.7-350 nM). The data were evaluated using BIAevaluation software (Figure 3.18) K_D value was found to be 2.8 (± 0.13) X 10^{-8} M, which indicates the nanomolar affinity to AFB_2.

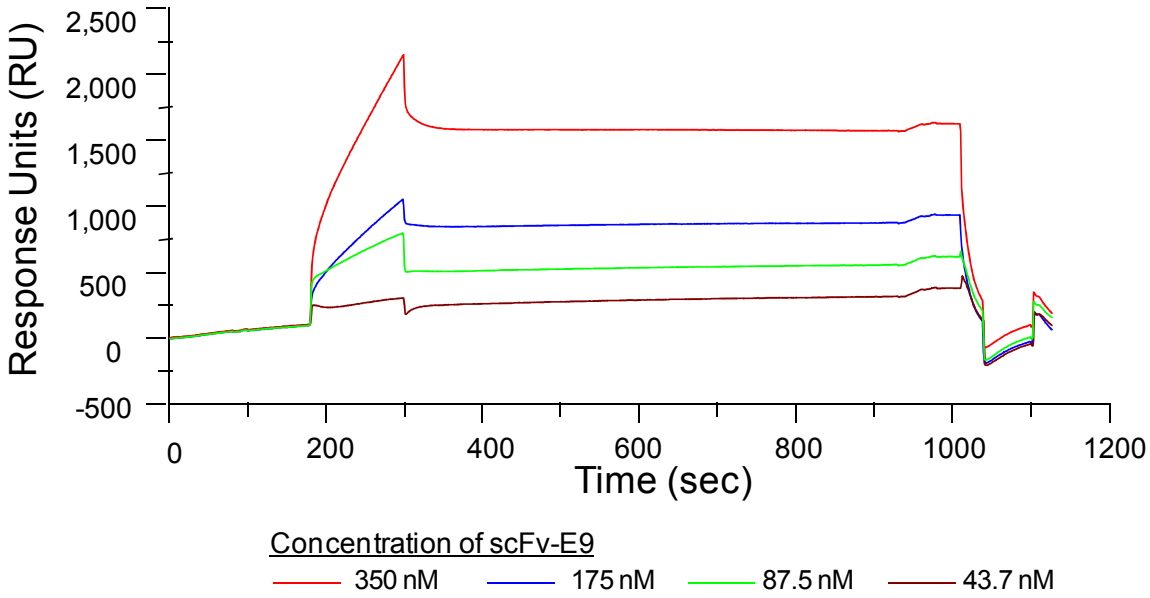


Figure 3.18: SPR-based kinetic analysis of scFv-E9. *Four varying concentrations of scFv-E9 were injected over the AFB_2 immobilized sensor chip. The values were fitted in 1:1 Langmuir binding model to measure the K_D value.*

3.2.3.12 SPR-based competitive inhibition assay using scFv-E9

To assess the binding ability of anti-AFB_2 scFv-E9 in solution phase, an inhibition assay was performed as described in section 2.2.3.21. In this assay, a 1:1 ratio of scFv-E9 was inhibited using varying concentration of AFB_2 from 0.48-125 ng/mL at 37^0C for 30 mins. A calibration curve (Figure 3.19) was plotted with response units on Y-axis against known concentration of AFB_2. The SPR analysis revealed that the scFv-E9 had an IC_{50} concentration of 8 ± 0.08 ng/mL. The limits of detection and quantification were 0.9 ± 0.09 and 2.4 ± 0.1 ng/mL (Mean ± S.D.).

In order to determine the accuracy and precision of the SPR-based inhibition assay, intra- and inter-day variability studies were performed (Figure 3.20). The results obtained from intra- and inter-day were presented in Table 3.4. The percentage coefficient of variance (% CVs) ranged from 1.7-8.9% and 0.9-8.3% as observed from intra- and inter-day studies, respectively, indicating good degree of precision of the SPR-based inhibition assay.

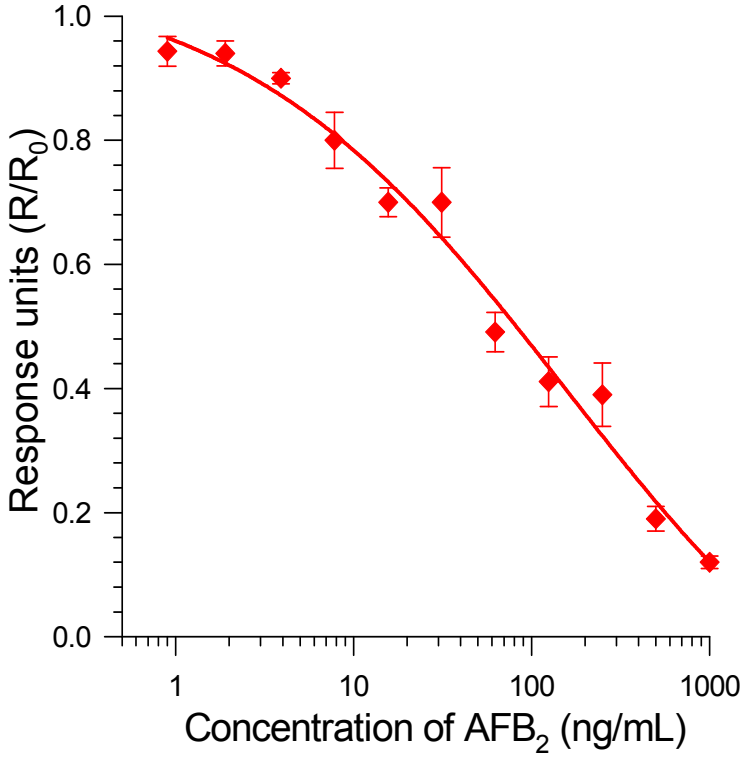


Figure 3.19: Representative SPR-based competitive inhibition curve for scFv-E9. *A 1/100 dilution of scFv-E9 was incubated with 0.48-125 ng/mL of AFB_2 at 37^0C for 30 mins. This antigen antibody mixture was passed over the CM5 sensor chip immobilized with AFB_2 toxin. The response units in the presence of varying concentration of AFB_2 (R) along with the responses in the presence of zero AFB_2 (R_0) concentration was recorded. R/R_0 was calculated and the error bars indicate the standard deviation of three independent determinations.*

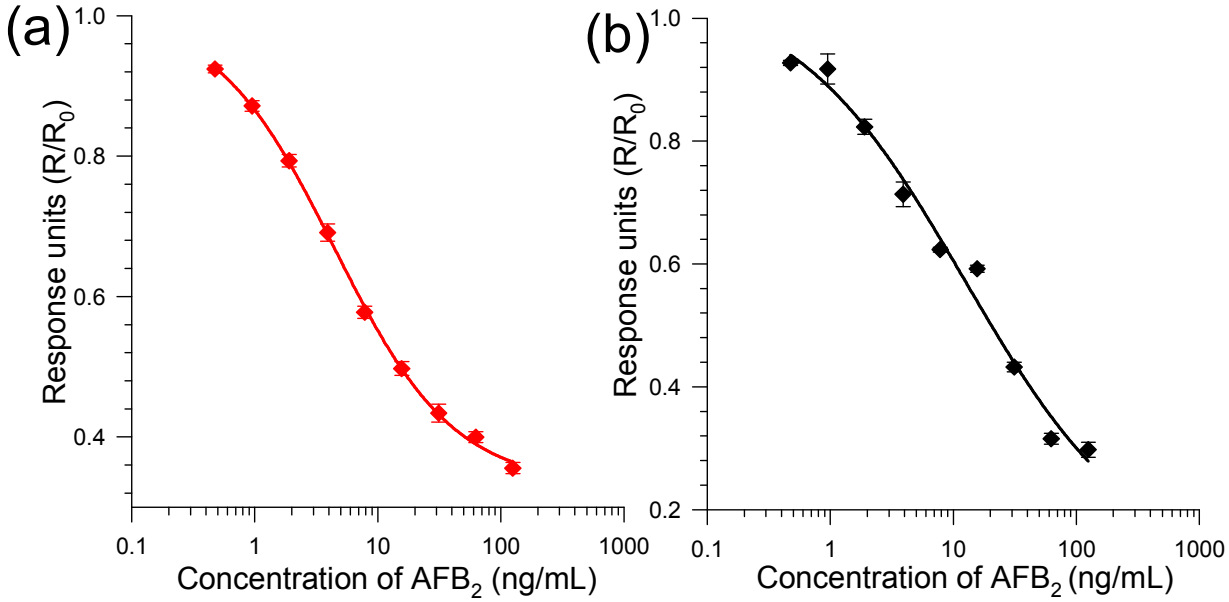


Figure 3.20: Intra- (a) and inter-day (b) variations of SPR-based inhibition assay using scFv-E9 in solution. *A 1/100 dilution of anti-AFB_2 scFv was incubated with 0.48-125 ng/mL of AFB_2 and passed over the AFB_2 immobilized sensor chip. The results were normalized by dividing the response units in presence of AFB_2 (R) by the response units of zero concentration of antigen (R_0) in the assay. For intra-day (a) studies the S.D. was computed from 3 validation run on the same day and inter-day (b) S.D.s were determined over 5 validation runs on 5 separate days. A 4-parameter logistic curve was fitted to the data set.*

Table 3.4: *Intra- and inter-day coefficient of variance (CVs) for anti-AFB_2 scFv-E9 used in SPR-based inhibition assay described in section 2.2.3.21. CV was calculated using the formula % CV= (S.D./Mean) X 100.*

AFB_2 concentration (ng/mL)	Intra-day assay				Inter-day assay			
	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies
125	124.25	0.3557	8.9	99.4	131.75	0.2976	3.1	105.4
62.5	65.56	0.3998	6.4	104.9	57.19	0.3154	2.8	91.5
31.2	29.6	0.4339	3.2	94.9	31.51	0.4322	8.3	101
15.6	15.22	0.4975	7.4	97.6	17.55	0.5923	7.3	112.5
7.8	7.72	0.5775	2.5	99	7.67	0.6234	0.9	98.4
3.9	3.83	0.6910	6.2	98.2	3.75	0.7134	3.2	96.2
2	1.97	0.7934	1.7	98.9	2.01	0.8232	1.9	100.9
0.98	0.98	0.8715	4.3	100	1.05	0.9173	2.6	107.4
0.49	0.5	0.9240	2.9	103.1	0.48	0.9272	4.2	97.5

3.2.3.13 Evaluation of matrix Spiking and recovery studies of almond samples

Almond nut sample was extracted using 80% (v/v) methanol (Whitaker *et al.*, 2006). Five mL of the almond extract was evaporated to dryness in a rotary evaporator and resuspended in 5 mL of HBS buffer. To 1 mL of almond sample in HBS buffer AFB_2 (156.25 ng/mL) was added, whose recovery (measured by fluorescence assay (n=3)) was found to be 84.3%.

3.2.3.14 Intra- and inter-day validaton of SPR-inhibition assay using spiked almond samples

The spiked samples were mixed with antibody (1:1 spiked sample/antibody) and injected (flow rate 15 µL) onto the surface of the sensor chip, with a contact time of 90 sec. The surface was regenerated with 30 mM NaOH for 30 sec twice (flow rate 30 µL/min). The precision of the assay was measured through performing intra-day (within runs) and inter-day (between runs) assay (Table 3.5 & Figure 3.21).

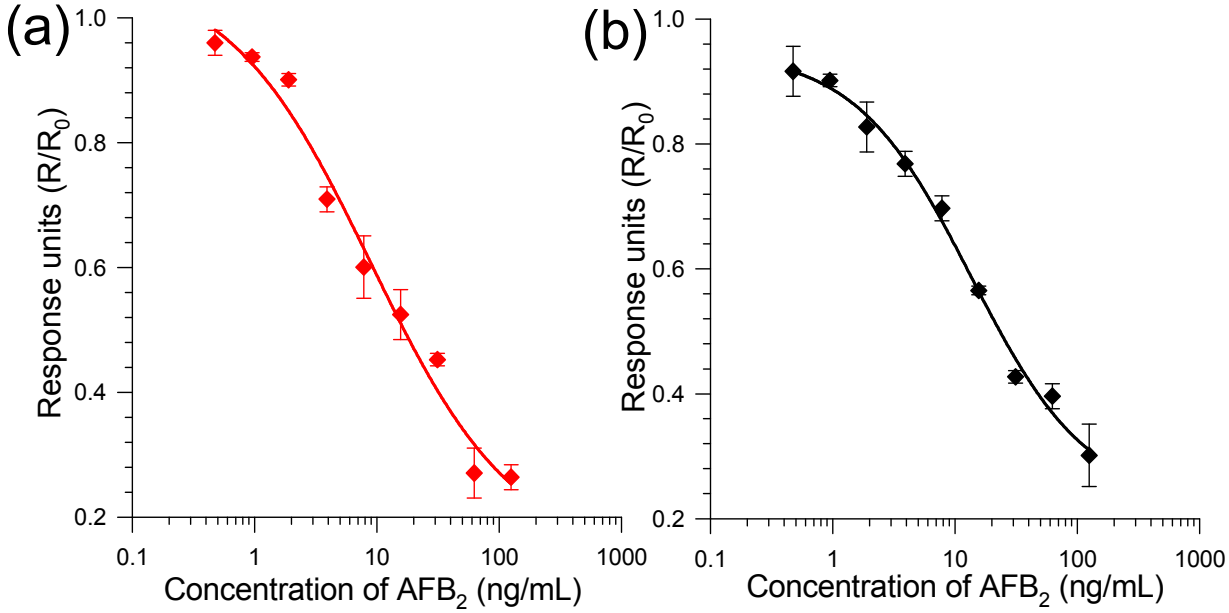


Figure 3.21: Intra- (a) and inter- (b) day validation of SPR-based competitive inhibition assay performed using almond spiked samples. *1/100 dilution of anti-AFB_2 scFv was incubated with almond sample containing 0.48-125 ng/mL of AFB_2 and passed over the AFB_2 immobilized sensor chip. The results were normalized by dividing the response units in presence of AFB_2 (R) by the response units of zero concentration of antigen (R_0) in the assay. For intra-day (a) studies the S.D. was computed from 3 validation runs on the same day and inter-day (b) S.D.s were determined over 5 validation runs on 5 separate days. A 4-parameter logistic curve was fitted to the data set.*

Table 3.5: *Intra- and inter-day coefficient of variance for the determination of AFB_2 in almond sample using SPR-based competitive inhibition assay. Three sets of nine standards were assayed on five different days and CVs were calculated.*

AFB_2 concentration (ng/mL)	Intra-day assay				Inter-day assay			
	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies
125	130.5	0.2642	3.2	104.4	128.12	0.3016	6.9	102.5
62.5	57.56	0.2708	5.6	92.1	67.56	0.3963	7.3	108.1
31.2	37	0.4525	8.2	118.6	29.85	0.4273	10.6	95.7
15.6	15.77	0.5248	5.1	101.1	15.49	0.5655	6.2	99.3
7.8	7.67	0.6007	6.7	98.3	8.12	0.6972	9.5	104.1
3.9	3.67	0.7094	5.7	94.2	3.84	0.7684	3.7	98.6
2	2.33	0.9007	3.2	116.5	1.89	0.8273	5.1	94.5
0.98	0.98	0.9372	6.6	100	1.03	0.9018	4.0	105.3
0.49	0.46	0.9598	8.4	95.1	0.49	0.9165	8.1	100

3.3 Discussion

This chapter deals with the generation of anti-AFB_2 scFv recombinant antibodies. AFB_2 posses a carbonyl functional group that can be conjugated to carrier proteins such as BSA. The AFB_2-BSA yielded a lower serum titration values in mice (Hastings *et al.,* 1988). In order to attain higher serum titer values against AFB_2, an attempt was made to conjugate AFB_2 to the KLH carrier protein using a two steps NHS ester method, which, ultimately was proven to be an excellent approach for obtaining specific AFB_2 antibodies with no cross reactivity to other aflatoxins.

AFB_2-KLH conjugate led to the generation of a very high immune response with a titre of 1/512,000 in a Balb/c mouse. This serum titre value was deemed sufficient to utilize the immunized mouse for the isolation spleenomic RNA, followed by cDNA synthesis. A single chain Fv gene fragment was assembled together by Splice by Overlap Extension of the variable heavy and light chains with a flexible linker. The SOE product was successfully ligated into pComb 3XSS vector followed by electroporation into XL1-Blue electrocompetent cells. This resulted in a library size of 2.19 X 10^7 transformants, which was within the range of the expected 10^6 and 10^8 transformants.

Anticipating the presence of diverse anti-AFB_2 scFvs, the antibody library was subjected to panning on the solid surface of an immunotube that was coated with AFB_2-BSA, as stated by (Willats, 2002). Since the possibility of obtaining highly specific clones will be widened through decreasing coating concentration (Charlton *et al.*, 2001), lower concentrations of AFB_2-BSA were used in successive rounds of panning. Trypsinization (Lin *et al.*, 2000) was used for the elution of bound phage from immobilized AFB_2-BSA. During the panning procedure, the titre of the eluted phage dropped after the third and fourth rounds to 10^4, which might be due to the low amplification titres after the second and third rounds of panning, respectively. A slight increase in the titre of eluted phage (10^5) obtained from the fifth round of panning was noticed. The phage from each of the rounds of panning were subsequently

analysed by polyclonal phage ELISA. An increase in the signal of the phage obtained from round 5, when titrated against AFB_2-BSA, indicated the presence of higher numbers of AFB_2 encoding genes. In this instance the background values obtained against BSA and milk Marvel were 7 times lower than the signal obtained when the phage was titrated against AFB_2-BSA. The phage from fifth round of panning were then infected into Top 10F′ cells for the soluble expression of antibodies following the procedure stated in section 2.2.3.13. Single scFv clones (384) were picked and expressed.

The selection of scFv antibodies from a recombinant library expressed on M13 bacteriophage led to the isolation of set of antibodies that bound to AFB_2 with high specificity. Over 95% of the clones tested were found to be positive-binders for AFB_2-BSA. Low background values were obtained when clones were tested against BSA and KLH. The presence of a high number of positive scFv clones that specifically bind to AFB_2 represents a significant enrichment of phage with the required antibody gene, thus, ultimately resulting in a successful panning procedure.

Ten representative positive clones were selected and analysis of the lysates carried out by indirect and competitive ELISA, as described in section 2.2.3.15. The titre values of the scFv-producing clones were found to be greater than 1/500. The ten scFv-producing clones were then assayed for their recognition of AFB_2 in solution and seven (A8, D1, H8, A1, A2, E9 & H12) recognized AFB_2. Due to the minimal differences between the aflatoxin congeners generation of antibody that is absolutely specific to one type of aflatoxin is highly difficult. Most of the antibodies developed either through immunizing AFB_1 or B_2 conjugates resulted in the generation of antibodies with either wide or narrow range of cross reactive characteristics. Hence cross reactivity studies were performed with four structurally similar aflatoxins (AFB_1, G_1, G_2 & M_1) by competitive ELISA assay described in section 2.2.3.15. These studies revealed the presence of five scFvs (A8, D1, H8, A1 & A2) with varying percentage cross reactivity to aflatoxin congeners and two scFvs (E9 & H12) without any affinity towards other

aflatoxin types that were tested (Table 3.2). The earlier reports suggests, that the monoclonal antibodies developed against AFB_2 exhibit higher cross reactivity with AFB_1 and, in a few cases, these antibodies were unable to recognize AFB_2 itself. In contrast to these results, the scFvs developed in the present work were able to specifically recognize only AFB_2, which is highly desirable for the development of specific assays.

The results of cross reactivity studies, indicating the presence of different scFv types were further confirmed through restriction digestion of scFvs with B*st*N1, which also revealed the presence of three different sets of population in the seven competent scFvs. Further sequencing analysis of the scFv-E9 (no cross reactivity) and D1 (with cross reactivity) were performed to trace the CDR that might play a crucial role in the attributing the cross reactivity characteristic to the scFv. There was variation in the sequences of both scFv-E9 & D1 (Figure 3.15). Based on this it is hard to assess the role played by the CDRs for cross reaction.

Large-scale expression and IMAC purification of scFv-E9 was followed by affinity (IC_{50}) determination, which was 85 ng/mL. The IC_{50} value is higher than the earlier reported for monoclonal antibodies by Hastings *et al.* (1988) at (50 pg/mL), Rieger *et al.* (2009) at (0.0027 ng/mL) and Cervino *et al.* (2008) at (0.042 ng/mL).

SPR-based system is a versatile technique to determine the biomolecular interactions due to the low sample requirement and less time-consumption than ELISA (De Crescenzo *et al.*, 2008). For the AFB_2 sensor chip generation, AFB_2 toxin was used instead of AFB_2 protein conjugate in order to avoid the regeneration problems encountered by Daly *et al.* (2000). Amine coupling strategy was employed for the immobilization of AFB_2 onto the CM5 chip. Varying dilutions of scFv-E9 were passed over the AFB_2 immobilized sensor chip to determine the optimal response units around 300-400 RU. A 1/100 dilution of scFv-E9 produced binding response of 359 RU. Kinetic analysis of scFv-E9 indicated the low nanomolar affinity levels. The competitive efficiency of scFv-E9 to AFB_2 in solution phage was determined and IC_{50} value where half maximal inhibition of antibody in the presence of antigen was measured. The SPR

inhibition curve in Figure 3.19 revealed the IC_{50} value of 8 ng/mL (buffer) and 11 ng/mL (matrix) for scFv-E9. Intra- and inter-day variations showed that the scFv-E9 has a range of detection of free AFB_2 from 0.48-125 ng/mL with CVs between 1.7-8.9% and 0.9-8.3%, respectively, which fall within the 20% limit as reported by (Findlay *et al.*, 2000). The results indicate the reproducibility of the assay. To assess the feasibility of detecting AFB_2 in food matrix, almond was purchased and spiked with known concentration of AFB_2. The results of inter- and intra-day (Table 3.5) SPR-inhibition assay suggest that scFv-E9 can readily be used for the detection of AFB_2 in almonds. The limit of detection increased from 0.9 ± 0.09 ng/mL (buffer) to 3.6 ± 0.13 ng/mL (matrix).

In conclusion, an scFv with higher specificity towards AFB_2 was isolated from the recombinant antibody library. To our knowledge this is the first successful recombinant anti-AFB_2 scFv antibody without any cross reactivity to aflatoxin congeners. ELISA and SPR- based inhibition assay were developed with the scFv-E9 that had a IC_{50} of 85 & 8 ng/mL respectively, which falls within the maximum permissible limits of 20 ppb. This scFv was further used for the immuno-affinity column development described in chapter 6.

Chapter 4
Generation of anti-Zearalenone murine scFv antibodies

4.1 Introduction

Zearalenone (ZEN) is the most prevalent of the *Fusarium* toxins (Glenn, 2007). Cross reactive monoclonal antibodies against ZEN, developed to-date, were generated following the immunization of Balb/c mice (Dixon *et al.*, 1987; Teshima *et al.*, 1990) and rabbits (Burkin *et al.*, 2002) with ZEN-BSA conjugates. Only one report exists for the generation of a scFv from a monoclonal antibody by Yaun *et al.* (1997). This scFv antibody exhibits higher cross reactivity with other structurally similar toxins to ZEN than does the parent monoclonal antibody. No scFv antibodies were reported for ZEN that were generated using cDNA libraries. Hence, it was decided to generate a highly specific anti-ZEN scFv antibody from a cDNA library (Figure 4.1), with the aim of isolating scFvs with little or no cross reactivity to ZEN variants like alpha and beta zearalenol.

Spleenomic RNA from mice, immunized with a ZEN-KLH conjugate, was used to generate a recombinant scFv library, which was subsequently subjected to panning against ZEN-BSA. A scFv with an IC_{50} of 80 ng/mL was successfully isolated and incorporated into both ELISA and SPR-based inhibition assays. The latter was applied for the detection of ZEN in sorghum.

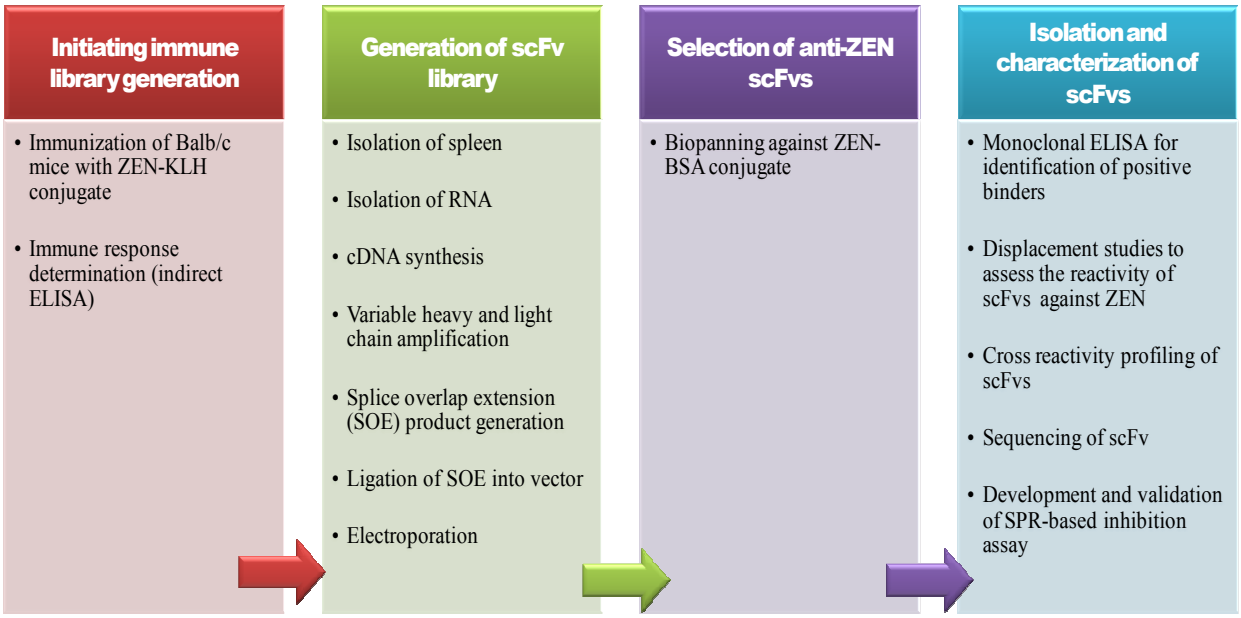


Figure 4.1: Strategy adopted for the generation of anti-ZEN scFvs. *The generation of scFvs started with the immunization of Balb/c mice with ZEN-KLH conjugate, followed by the spleenomic RNA isolation and cDNA synthesis. From the cDNA variable and heavy chains of the antibody were amplified, followed by the ligation of the glycine-serine linked antibody chains (SOE product) into a vector, which was then electroporated into XL1-Blue E. coli cells. The entire ZEN library was biopanned on solid immunotube surfaces, which were pre-coated with ZEN-BSA. The isolated scFvs were incorporated into ELISA and SPR-based assays.*

4.2 Results

4.2.1 Serum titre determination and cDNA synthesis

Following immunization of Balb/c mice the serum titration against ZEN-BSA (after 3rd boost), as determined by indirect ELISA, was found to be in excess of 1/256,000 (Figure 4.2). The immune responses of the serum against BSA and KLH were also determined. However, no response to KLH was detected, which was not expected. The results indicate that the anti-serum was specific to ZEN. Spleenomic RNA was isolated from the immunized mouse after a final intravenous boost (after 4th boost). RNA (4.5 ng) was used for the generation of cDNA (yield; 137.6 ng/µL), from which variable heavy and light chain genes were amplified using specific primers defined for use with mice (Kabat *et al.*, 1991; Kettleborough *et al.*, 1993).

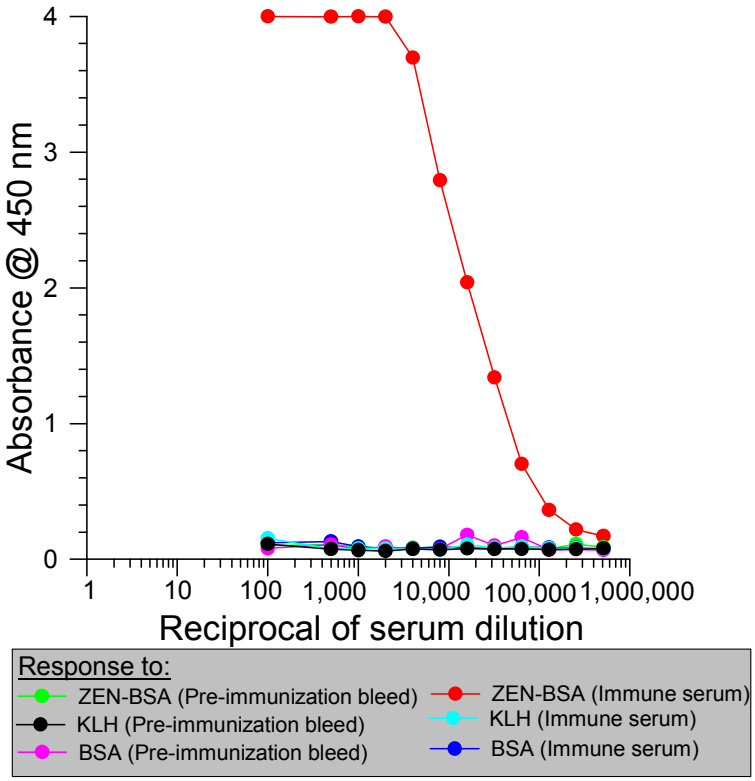


Figure 4.2: Serum titre of ZEN-KLH-immunized mouse, determined by indirect ELISA against ZEN-BSA. *Serum dilutions (1/100 to 1/512,000) were added to the immuno-plate coated with ZEN-BSA, BSA and KLH. A 1/1,000 dilution of AP-labelled goat anti-mouse IgG was added followed by visualization with pNPP. The figure shows that there was a very low signal, when the serum titration was performed against KLH. The higher signal of the serum generated against ZEN-BSA conjugate is indicative of the immune response of the mouse to ZEN.*

4.2.2 Amplification of variable heavy and light chain genes

Optimal conditions for amplification of the heavy and light chain genes were determined using 1, 1.5, 2 & 3 mM of $MgCl_2$. Due to the presence of a single band in Figure 4.3, 1 mM of $MgCl_2$ was selected for the large-scale (20X) reactions to amplify V_k, V_λ and V_H, respectively.

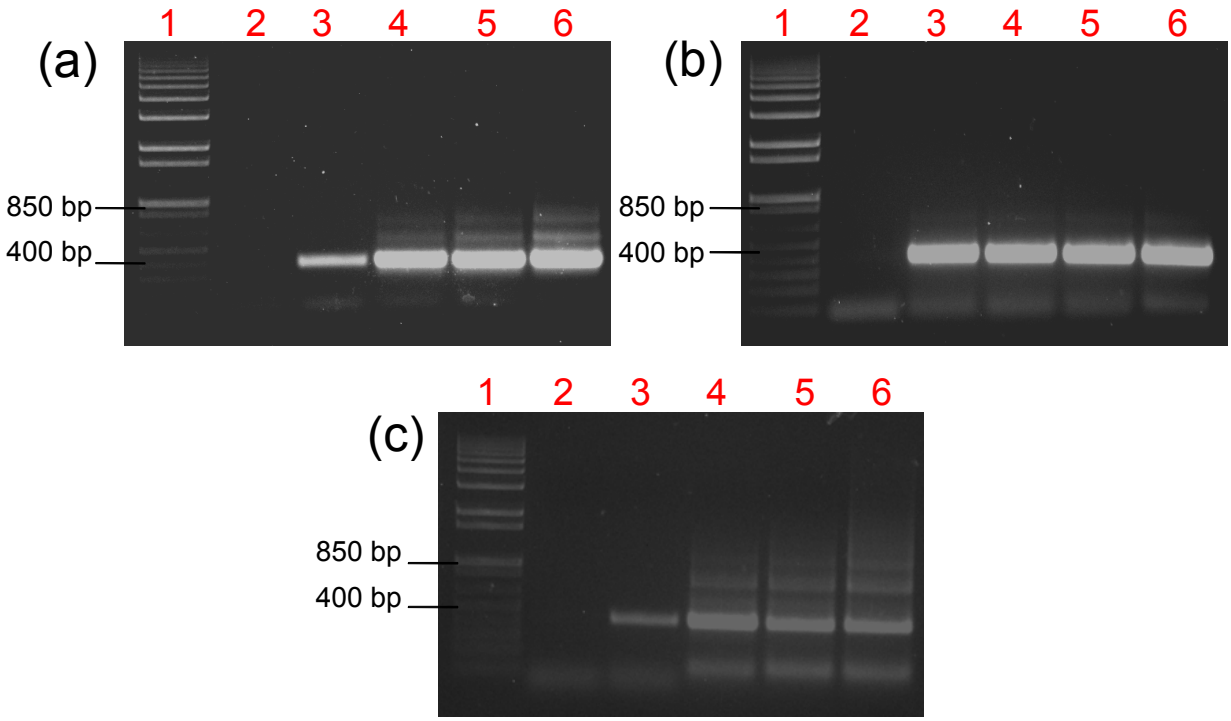


Figure 4.3: Optimization of the magnesium concentration for the amplification of V_k (a), V_λ (b) and V_H (c). *The PCR reaction mixture is composed of 0.5 µL of cDNA, 100 pmol of mouse primers, 5x PCR buffer, 200 µM dNTPs, 0.25 µL of GoTaq® DNA Polymerase and 1-3 mM of $MgCl_2$. The PCR amplification conditions were as follows: initialization for 5 mins at 94^0C; 30 cycles of denaturation; annealing and elongation for 15, 30, 90 sec at 94^0, 56^0 and 72^0C, respectively; followed by the final elongation step for 10 mins at 72^0C. The representation of the various lanes is as follows: lane 1: 1 Kb plus DNA ladder; lane 2: negative PCR reaction; lanes 3-6: PCR products amplified using four different concentrations of $MgCl_2$ (1, 1.5, 2 & 3 mM).*

4.2.3 Splice overlap extension (SOE) of the amplified heavy and light chain genes

The amplified heavy and light chain genes were linked by an SOE-PCR reaction. The optimal amplification condition was set up by running a $MgSO_4$ gradient (1, 1.5, 2 & 4 mM); (Figure 4.4). A $MgSO_4$ concentration of 2 mM, in the case of SOE (ʎ) and 4 mM for SOE (k), were used for the further amplification of the SOE products. The yields were 13.4 & 10.9 µg/240 µL, respectively.

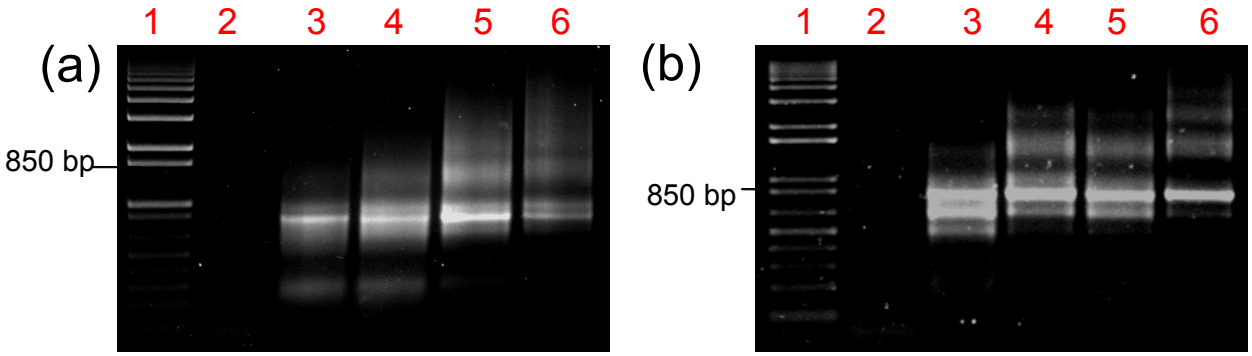


Figure 4.4: Optimization of $MgSO_4$ concentration for SOE product generation. *Figure 4.4(a) indicates SOE (ʎ) and 4.4(b) represents SOE (k). The PCR reaction mixture is composed of 100 ng of V_L and V_H were mixed with 100 pmol of CSC-F and CSC-B, 10x PCR buffer, 200 µM dNTPs, 0.2 µL of Platinum® Taq DNA polymerase and 1-4 mM of $MgSO_4$. The PCR amplification conditions were as follows: initialization for 5 mins at 94^0C; 30 cycles of denaturation; annealing and elongation for 30, 30, 120 sec at 94^0, 56^0 and 72^0C, respectively; followed by the final elongation step for 10 mins at 72^0C. The representation of the various lanes is as follows: lane 1: 1 Kb plus DNA ladder; lane 2: negative PCR reaction; lanes 3-6: SOE obtained using four different concentrations of $MgSO_4$ (1, 1.5, 2 & 4 mM).*

4.2.4 S*fi*I restriction digestion and ligation of SOE into pComb 3XSS

The vector (pComb 3XSS) was restriction digested with S*fi*I, X*ho*I, X*ba*I and Antarctic phosphatase. The SOE products (ʎ and k) were restriction digested with S*fi*I alone. The restriction digested vector and SOE products were ethanol-precipitated overnight followed by purification using a Nucleospin Extract II kit, after running the products (SOE and vector) on 0.5% (w/v) agarose gel. The SOE product was ligated into the vector at a ratio of 3:1.

4.2.5 Electroporation and biopanning

The ligated mixture (4 µL) was electroporated into XL1- Blue electrocompetent cells. A library size of 1.05 X 10^8 was obtained. The input and output titres after each round of the panning were recorded (Table 4.1). To determine the presence of scFv-expressing genes in the cells, a colony pick PCR (Figure 4.5) was performed using randomly picked clones from the titration plates. The PCR product was resolved on a 1% (w/v) agarose gel, which indicated the presence of a scFv insert in all of the clones selected.

Table 4.1: *Input and output titres of phage collected from six rounds of panning of the ZEN library against ZEN-BSA conjugate using M13K07 as the helper phage for the enrichment of scFv recognizing ZEN*

Round	Input titre (cfu/mL)	Output titre (cfu/mL)
Round 1	1×10^{11}	1×10^{7}
Round 2	2×10^{11}	1×10^{7}
Round 3	2×10^{11}	5×10^{5}
Round 4	1×10^{11}	5×10^{5}
Round 5	9×10^{11}	1×10^{4}
Round 6	4×10^{11}	2×10^{6}

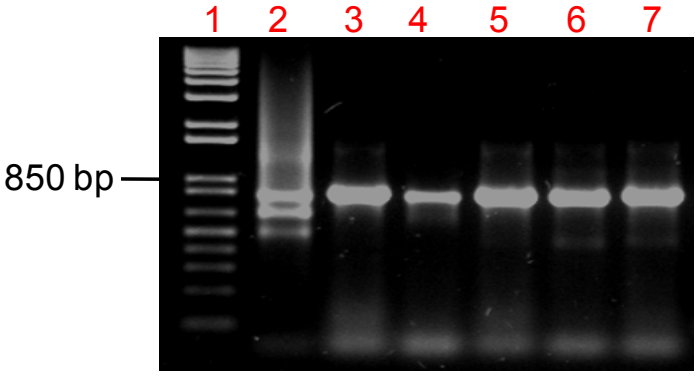


Figure 4.5: Colony pick PCR of randomly selected clones from the ZEN-panned library*. A single bacterial colony carrying the required scFv insert was amplified using the primers designed for SOE generation under the PCR conditions described for SOE-PCR reaction.The amplification of a band at approximately 750 bp indicates the presence of the scFv insert. The representation of the various lanes is as follows: lane 1: 1 Kb plus DNA ladder; lane 2-7: amplification of inserts in randomly picked colonies.*

4.2.6 Polyclonal phage ELISA

Rescued phage, from each round of panning, were screened against ZEN-BSA to determine the binding activity to ZEN. The binding of phage to BSA and milk Marvel was also determined. The bar chart generated from the polyclonal phage ELISA data indicate higher reactivity of the phage, obtained from round 5 & 6 (Figure 4.6) of panning, toward ZEN-BSA. An increase in the reactivity of phage towards BSA and Marvel was also noticed, which was still low, when compared to the signal obtained when phage titration was performed against ZEN-BSA. The phage from rounds 5 & 6 were solubly expressed through infecting the phage into Top 10F′ cells.

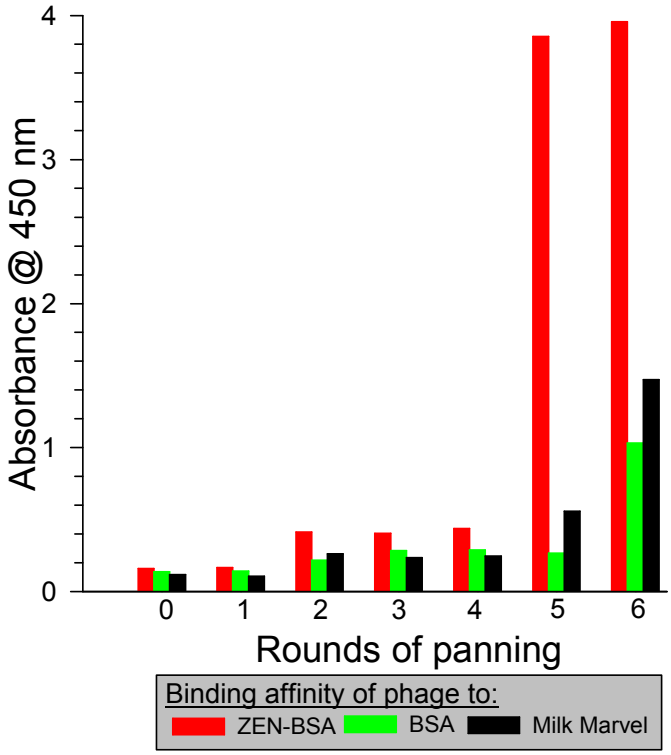


Figure 4.6: Polyclonal phage ELISA of the ZEN-panned library against ZEN-BSA. *Each of the analytes (ZEN-BSA, BSA and milk Marvel) were coated on the microtitre plate (in triplicate). The phage (1/2 dilution) from the six rounds of panning were analyzed against the three analytes. HRP-labelled mouse anti-M13 monoclonal antibody (1/1,000 dilution) was added, followed by the addition of TMB solution. Significant enrichment of the phage was seen in rounds 5 and 6, which indicates the presence of ZEN-positive binders.*

4.2.7 Monoclonal ELISA using solubly expressed anti-ZEN scFv producing clones

Indirect ELISA was performed using individual clones (384) that were grown and induced with IPTG. The plates were freeze-thawed (X3 times) and the culture was spun down and the lysates tested against ZEN-BSA. The results indicate that even though a significant enrichment of the phage was evident from the results of polyclonal phage ELISA, the number of ZEN-positive binders were low (Figure 4.7).

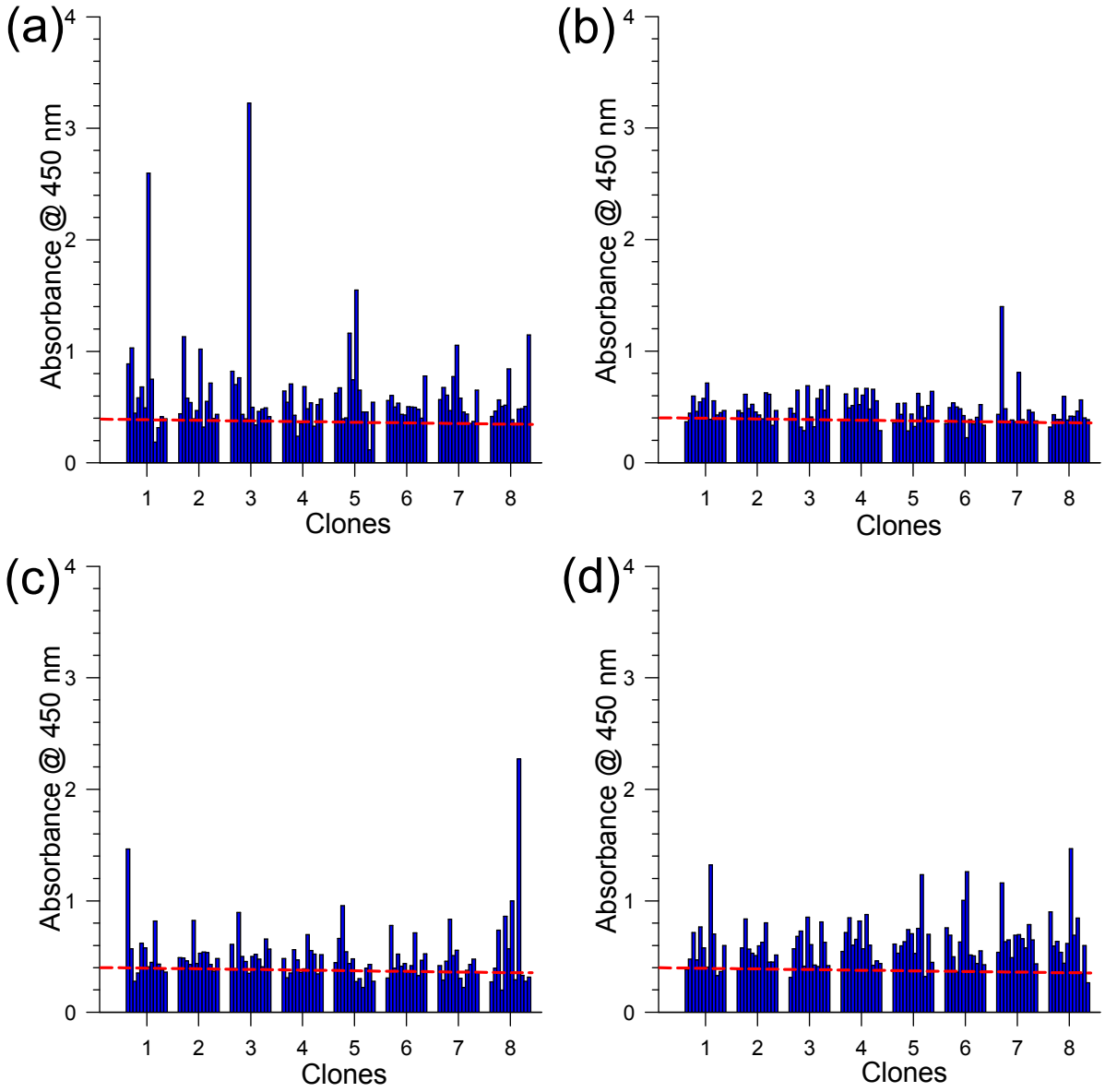


Figure 4.7: Graphical representation of monoclonal ELISA of anti-ZEN solubly expressed clones against ZEN-BSA-coated microtitre plates. *Dilutions (1/2) of supernatants were added to the ZEN-BSA-coated and milk Marvel-blocked immunoplate. A chromogenic reporter (HRP-labelled rat anti-HA monoclonal antibody) and its visualizer (TMB) were added and the absorbance read on a Tecan Saffire II plate reader. Figures a, b, c and d correspond to plates 1, 2, 3 and 4, respectively. The red line is the reference value, above which the clones were selected as being ZEN-positive binders.*

4.2.8 Immuno-reactivity of the anti-ZEN scFv-P2A2

The immunoreactivity of scFv-P2A2 (with highest displacement or best expression profile as determined from preliminary analysis) was evaluated through ELISA (indirect and competitive indirect ELISA). The titre value of scFv-P2A2 as determined by indirect ELISA, was in excess of 1/60,000. An inhibition ELISA was developed using scFv-P2A2, where the optimal dilution (1/800) of the scFv was incubated with 0.004-5 µg/mL of ZEN in the ratio of 1:1. The absorbance of antibody recorded in the presence of analyte (A) was divided by the absorbance of antibody in the absence of analyte (A_0). A semi-log plot was drawn with A/A_0 on the Y-axis against concentration of ZEN on the logarithmic X-axis (Figure 4.8). The mid-inflection point, where half-maximal inhibition (IC_{50} value) of 200 ± 0.05 ng/mL (Mean ± S.D.) was determined. The limit of detection and quantification were 10 ± 0.07 ng/mL and 28 ± 0.09 ng/mL. The blank value was determined by recording absorbance of antibody in the presence of zero analyte (n=20). The formulae used for the calculating the LOD and LOQ were:

LOD = Blank value + 3 S.D. of blank value

LOQ = Blank value + 10 S.D. of blank value

Precision of the competitive inhibition ELISA developed using scFv-P2A2 was determined through intra- and inter-day validation (Figure 4.9) and represented as percentage CVs (Table 4.2). For intra-day variation the assay was repeated three times on the same day and inter-day variations were assessed by performing the assay on five different days. The CVs for both intra- and inter-day assays were below 7.43 and 11.34%, respectively (Table 4.2).

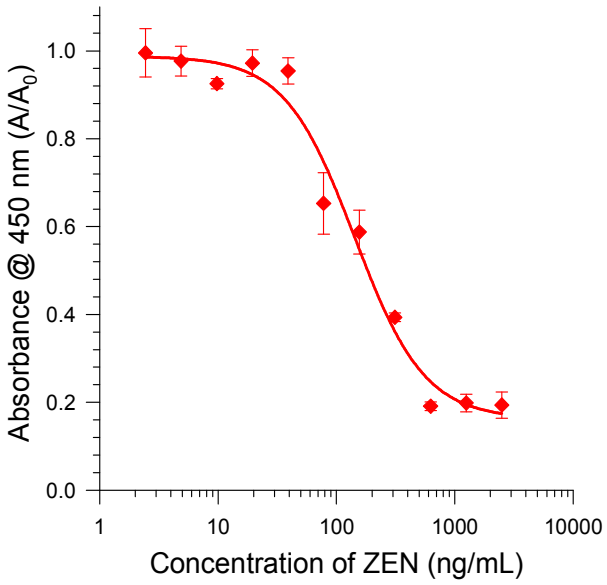


Figure 4.8: Inhibition ELISA for the detection of Zearalenone using scFv-P2A2. *The ELISA plate was coated with 5 µg/mL of ZEN-BSA and blocked with 4% (w/v) milk Marvel. Decreasing concentrations of ZEN were incubated with 1/800 dilution of anti-ZEN scFv-P2A2 in a ratio of 1:1 for 30 mins at 37^0C prior to the addition of the mixture to the previously blocked microtitre plate. This was then probed with anti-HA HRP-labelled secondary antibody, followed by visualization with TMB. The inhibition curve generated indicates a decrease in antibody binding with increasing concentration of ZEN. The IC_{50} value of scFv-P2A2 was 200 ng/mL.*

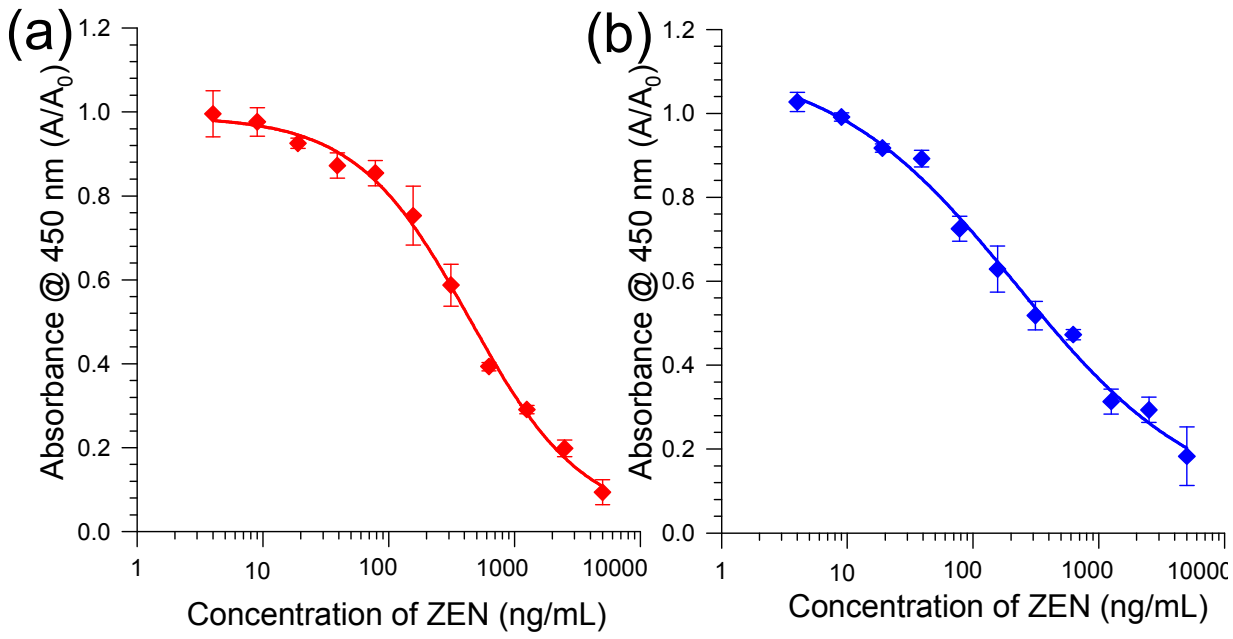


Figure 4.9: Intra- and inter-day competitive inhibition ELISA using scFv-P2A2. *A 5 µg/mL ZEN-BSA coating concentration was used with a 1/800 dilution of anti-ZEN scFv that was incubated with ZEN (0.004-5 µg/mL). For intra-day (a) studies the S.D. was computed from 3 validation runs on the same day and inter-day (b) S.D. was determined over 5 validation runs on 5 separate days. A 4-parameter logistic curve was fitted to the data set.*

Table 4.2: *Intra- and inter-day assays coefficient of variance (CVs) for anti-ZEN scFv in buffer. CVs were computed from the 3 replicate analyses carried out on the same (intra-day) and on five different days (inter-day).*

ZEN concentration (ng/mL)	Intra-day assay				Inter-day assay			
	Back-calculated concentration (ng/mL)	A/A_0	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	A/A_0	% CVs	% Accuracies
5000	4965	0.0938	4.45	99.3	4950	0.1832	7.54	99
2500	2610	0.1984	2.46	104.6	2580	0.2936	5.77	103.2
1250	1260	0.2910	6.33	101	1210	0.3133	1.34	97.4
625	607	0.3933	3.56	97.2	656	0.4729	8.43	105
312.5	312	0.5874	7.43	100	305	0.5183	4.56	98
156.25	158	0.7527	1.34	101.6	152	0.6292	9.43	97.8
78.125	79	0.8542	3.56	102	74	0.7253	11.3	95.2

4.2.9 Cross reactivity of anti-ZEN scFv-P2A2

Cross reactivity studies were carried out using the scFv-P2A2 and two structurally related ZEN (α-Zearalenol and β-Zearalenol). Inhibition curves were generated for all the three analytes tested (ZEN, α-Zearalenol and β-Zearalenol) in the same way as for ZEN, as described in section 4.2.7. The percentage cross reactivity was calculated by taking the IC_{50} value of clone when tested against ZEN as 100% and through substituting in the formula

$$\text{Percentage cross reactivity} = \frac{IC_{50} \text{ against ZEN derivative X } 100}{IC_{50} \text{ against ZEN}}$$

The results (Table 4.3) indicates that scFv-P2A2 was highly specific to ZEN with least cross reactivity to other ZEN types i.e., α-Zearalenol and β-Zearalenol.

Table 4.3: *Cross reactivity studies of scFv-P2A2 by competitive inhibition ELISA and its cross reactivity comparison with other reported anti-ZEN antibodies.*

Type of antibody	Cross reactivity percentage		Reference
	α-Zearalenol	β-Zearalenol	
Reported antibodies			
Monoclonal antibody	26	11	(Yuan *et al.*, 1997)
scFv (generated from monoclonal antibody)	82	26	(Yuan *et al.,* 1997)
Monoclonal antibody	42	< 1	(Burmistrova *et al.*, 2009)
Polyclonal antibody	0.15	<0.02	(Burkin *et al.,* 2002)
Monoclonal antibody	0.9	<0.1	(Teshima *et al.*, 1990)
Monoclonal antibody	3.6	14.4	(Dixon *et al.,* 1987)
Polyclonal antibody	3.14	1.96	(Gao *et al.*, 2011)
Monoclonal antibody	0.65	0.94	(Gao *et al.,* 2011)
Monoclonal antibody	108.1	119.3	(Ferreira Junior *et al.*, 2012)
Developed antibody			
scFv-P2A2	<0.01	0.1	

4.2.10 SDS-PAGE and western blotting of scFv-P2A2

Commassie staining of the SDS-PAGE gel was performed as described in section 2.2.4.10.3. Western blot analysis was carried out using the scFv-P2A2 to demonstrate that the secondary anti-HA could specifically detect the purified scFv. The purified scFv was electrophoresed using SDS-PAGE and then transferred onto nitrocellulose, which was blocked with 4% (w/v) milk Marvel and probed with anti-HA-HRP-labelled rat monoclonal antibody. Figure 4.10 indicates that the secondary antibody (anti-HA) enabled specific detection of scFv. A single band was visible at approximately 24 *k*Da.

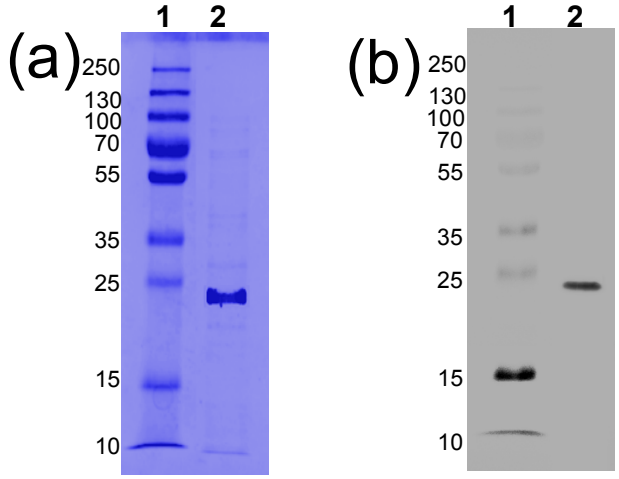


Figure 4.10: SDS-PAGE (a) and western blotting (b) of scFv-P2A2 after subjecting to IMAC purification. *A 12.5% SDS gel was run for 1.5 hrs at 100 V and the gel was stained with Commassie dye solution (Commassie blue R-250 (2 g), methanol (450 mL), acetic acid (100 mL) and water (450 mL) and destained overnight at room temperature with mixture containing acetic acid, methanol and water in the ratio of 2:5:13. For western blotting the SDS-PAGE gel was transferred onto the nitrocellulose membrane for 15 mins at 15 V and the membrane was blocked with 4% (w/v) milk Marvel for 1 hr. This was probed with 1/5,000 dilution of anti-HA HRP-labelled rat monoclonal antibody, followed by visualization of band using TMB substrate. The representation of the various lanes is as follows: lane 1: Fermentas Pageruler plus marker and lane 2: IMAC purified P2A2 clone. The purified P2A2 clone revealed the presence of scFv band at approximately 24 kDa.*

4.2.11 Sequencing of scFv-P2A2

Plasmid DNA of scFv-P2A2 was sent to Source Biosciences for sequencing. The linear nucleotide sequencing results were translated into amino acid sequences using the Expasy translate tool. The CDRs are represented in Figure 4.11.

Figure 4.11: Deduced amino acid sequence of scFv-P2A2. *The heavy and light chain CDRs present in the scFv-P2A2 are shown.*

4.2.12 Zearalenone immobilization on CM5 sensor chip

Standard amine coupling chemistry leads to stable bonding between toxins and activated sensor chip surfaces (Tudos *et al.*, 2003). This approach was adopted for the immobilization of ZEN-CMO-NHS synthesized using the procedure described in section 2.2.4.11. The ZEN-CMO-NHS was attached to the amine groups generated on a CM5 chip surface through treatment with Jeffamine (Figure 4.12).

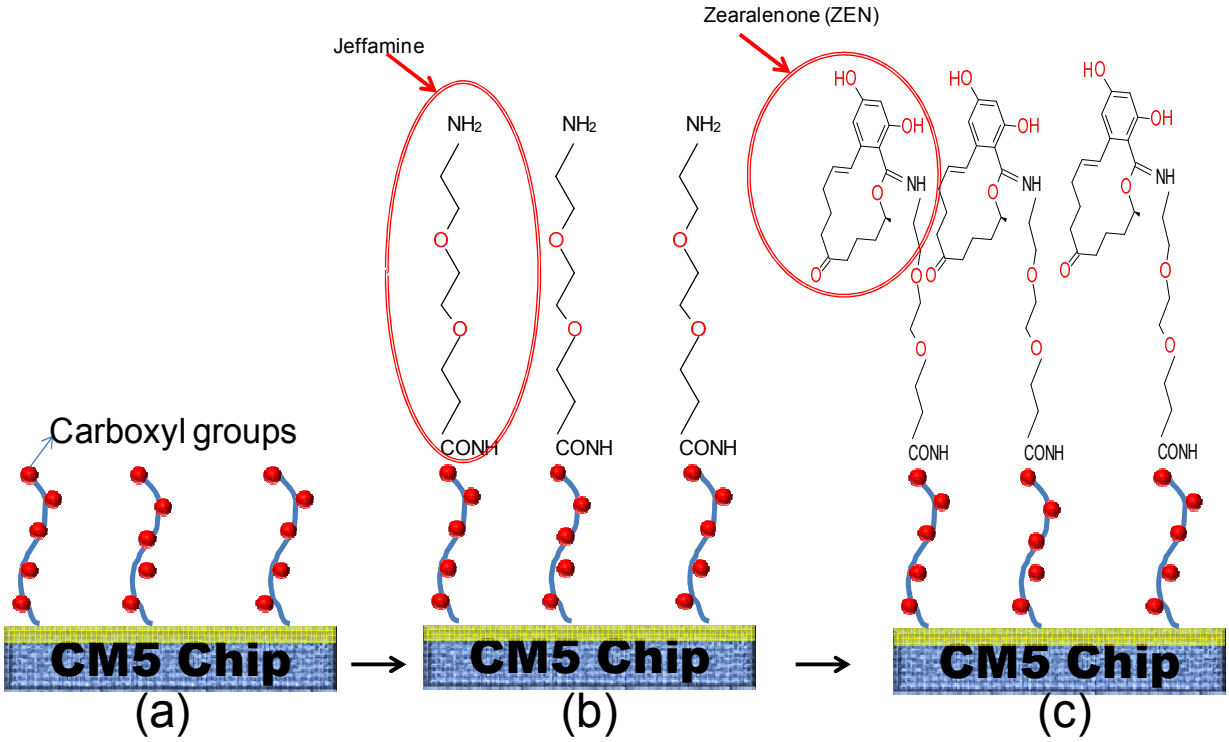


Figure 4.12: Schematic representation of immobilization of ZEN onto CM5 sensor chip. *The first stage (a) involved in the immobilization of ZEN onto CM5 chip was surface activation with EDC and NHS. In the second stage amine groups were generated through treating with Jeffamine (b). The third stage involves the covalent attachment of ZEN to the amine groups on the CM5 chip (c).*

4.2.13 Binding analysis of P2A2 to ZEN-immobilized CM5 sensor chip

The optimal antibody dilution was determined by passing 1/50, 1/100, 1/200/ 1/400 and 1/800 dilutions of IMAC-purified anti-ZEN scFv-P2A2 over the ZEN-immobilized sensor chip. A 1/100 dilution P2A2 scFv yielded a binding response of 110 RU (Figure 4.13). The binding specificity of the ZEN-immobilized chip was tested using an anti-AFB_2 scFv (negative control) (1/200 dilution, flow rate of 15 µL/min for 3 mins). Negligible binding (< 10 RU) of anti-AFB_2 scFv indicates that there was no binding between the anti-AFB_2 scFv and ZEN, which was covalently attached to the CM5 chip. Surface regeneration conditions were optimized using NaOH concentrations ranging from 10-50 mM and it was found that 20 mM is required for efficient chip regeneration (flow rate 30 µL/min for 30 sec).

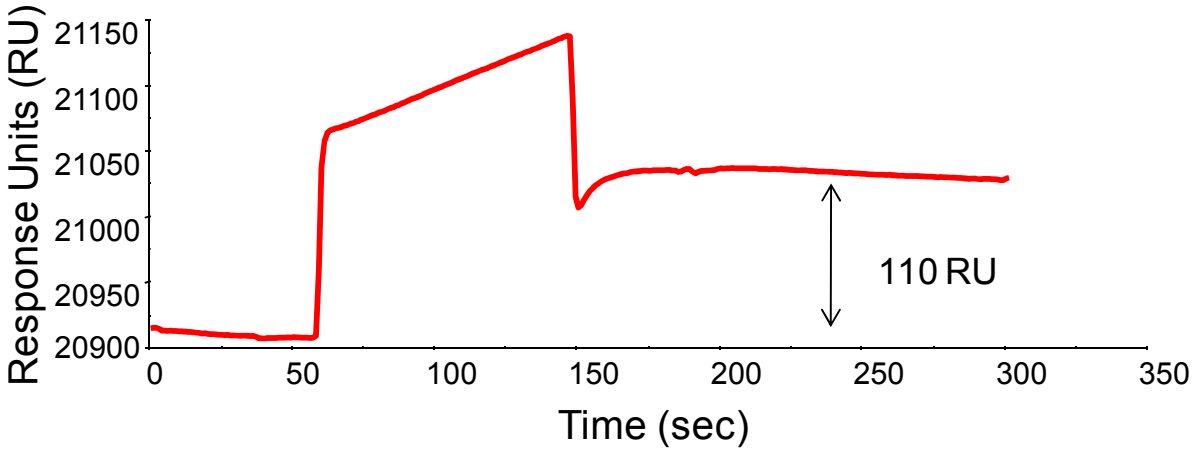


Figure 4.13: Sensogram depicting the binding of P2A2 scFv to ZEN-immobilized CM5 sensor chip. *The scFv-P2A2 was passed over flowcell 2 of ZEN-immobilized CM5 chip at a flow rate of 15 µL/min for 3 mins. The net response units achieved by P2A2 were 110 RU.*

4.2.14 SPR-based kinetic analysis of scFv-P2A2

The K_a and K_d values were 5.72 X 10^4 $M^{-1}s^{-1}$and 2.28 X 10^{-5} s^{-1}, respectively for scFv-P2A2. Thus the calculated K_D value for P2A2 scFv/ZEN interaction was 3.9 X 10^{-10} M (Figure 4.14) ($K_D = K_d/K_a$).

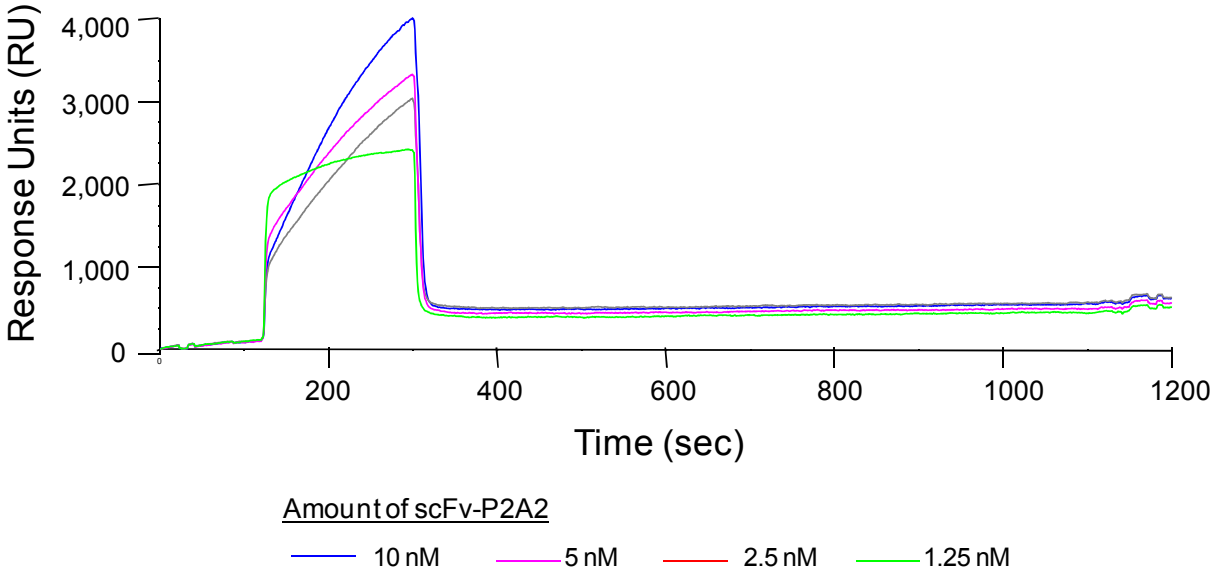


Figure 4.14: Kinetic analysis of scFv-P2A2. *The dissociation of varying concentrations (1.25-10 nM) of P2A2 clone was used to perform kinetic analysis. The data obtained was fitted into Langmuir 1:1 fitting model and the K_D value was found to be 3.9 X 10^{-10} M after normalization.*

4.2.16 SPR-based solution-phase ZEN binding assay using scFv-P2A2

An inhibition assay for the detection of ZEN was developed using a ZEN-immobilized CM5 chip and anti-ZEN scFv-P2A2. To assess the binding ability of P2A2 scFv to ZEN in solution, free ZEN standards, ranging from 0.004-1 µg/mL were mixed with 1/100 dilution of scFv-P2A2 in the ratio of 1:1 and incubated for 30 mins at 37^0C. The samples were then passed randomly over the sensor chip and the response units of the scFv in the presence of each individual concentration of ZEN was divided by the response units of the scFv alone in the absence of free ZEN (Figure 4.15).

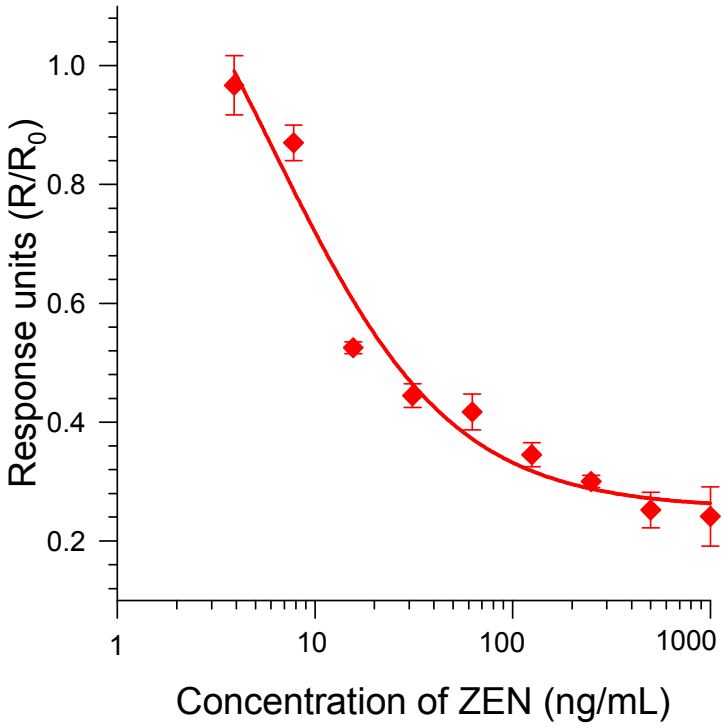


Figure 4.15: SPR-based competitive inhibition curve of scFv-P2A2 in solution. *The R/R_0 was obtained by dividing response units obtained at 9 different ZEN concentrations (0.004-1 µg/mL) by the RU response of scFv-P2A2 without ZEN. A 4-parameter logistic curve was fitted to the data set and each point is the mean of three replicate measurements.*

Intra and inter-day variability studies (Figure 4.16) were carried out to assess the reproducibility of the SPR-based inhibition assay. Intra-day variations were measured by repeating the assay three times on one day and mean response units of antibody bound to each varying concentration of ZEN was plotted against log concentration of ZEN. The percentage variations of intra-day studies were represented as percentage coefficient of variance (% CVs), which were below 6%. Like-wise, inter-day variation was assessed by repeating the inhibition assay on five different days with variability determined to be less than 9%. The CVs for intra- and inter- day variability studies (Table 4.4) indicates the reproducibility of the assay.

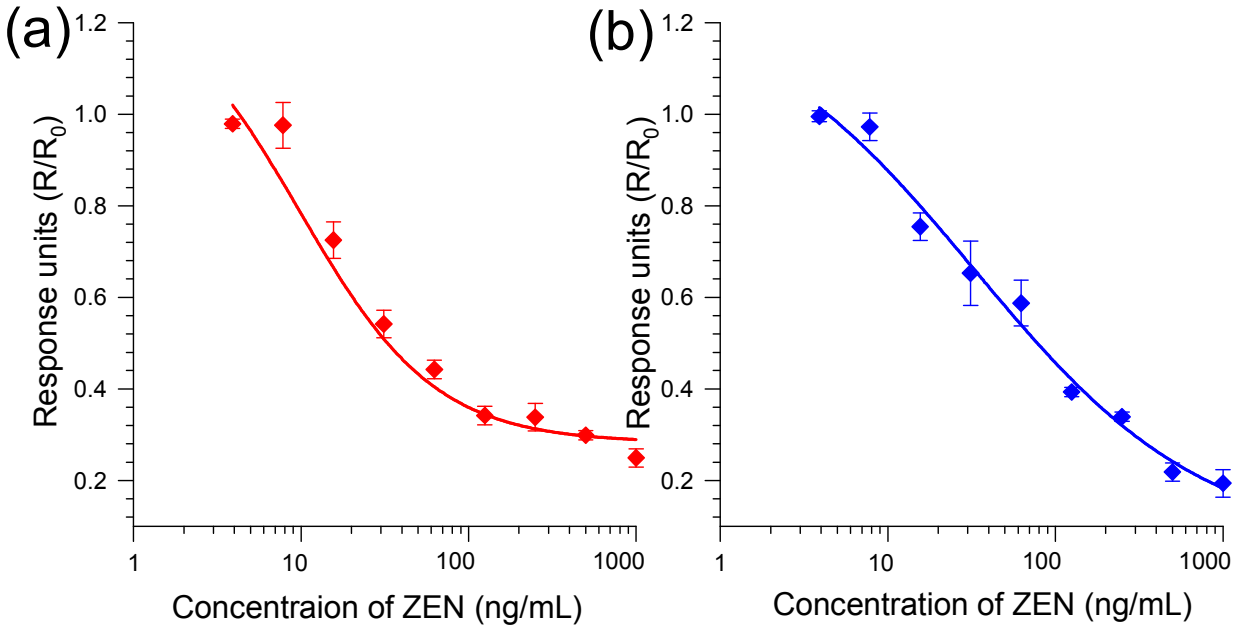


Figure 4.16: Intra- (a) and inter-day (b) variations of SPR-based inhibition assay using scFv-P2A2 in solution. *A 1/100 dilution of anti-ZEN scFv was incubated with 0.004-1 µg/mL of ZEN and passed over the ZEN-immobilized sensor chip. The results were normalized by dividing the response units in presence of ZEN (R) by the response units of zero concentration of antigen (R_0) in the assay. For intra-day (a) studies the S.D. was computed from 3 validation runs on one day and inter-day (b) S.D.s were determined over 5 validation runs on 5 separate days. A 4-parameter logistic curve was fitted to the data set.*

Table 4.4: *Intra- and inter-day assays coefficient of variance (CVs) for anti-ZEN scFv in buffer. Percentage CVs were computed from the 3 replicate analyses carried out on the same (intra-day) and on five different days (inter-day).*

ZEN concentration (ng/mL)	Intra-day assay				Inter-day assay			
	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies
125	122	0.3419	5.35	97.6	123	0.3933	1.35	98.4
62.5	67	0.4425	3.12	108	67	0.5874	8.75	107.7
31.25	32	0.5419	1.35	101.3	29	0.6527	6.33	93.3
15.625	17	0.7251	3.42	110.2	14	0.7542	3.56	88.5
7.812	9	0.9760	2.46	116.5	9	0.9723	7.53	111
3.906	4	0.9792	5.24	93.7	4	0.9953	6.68	99

4.2.16 Extraction of ZEN from sorghum and its application in SPR-based inhibition assay

A sorghum reference sample with 48.6 ± 0.03 ng/mL ZEN obtained from Triology laboratories was extracted using the procedure described in section 2.2.4.13. The mean recovery of ZEN from the 10 extractions was 42.5 ± 0.2 ng/mL (Mean ± S.D.). The working concentration of the SPR-based inhibition assay using sorghum extract was set to 3.9-125 ng/mL. For intra-day validation the assay was run three times on the same day and inter-day assays were performed on five different days (Figure 4.17). The mean response units were recorded and the responses of antibody in the presence of varying concentrations of ZEN were divided by the responses of antibody in the absence of ZEN. The IC_{50} value was found to be 22 ± 0.02 ng/mL. The CVs for intra- and inter-day assays fall below 7% and 12%, respectively (Table 4.5). The limit of detection of 7.8 ± 0.07 ng/mL was measured through determining responses of antibody in the presence of ZEN-free sorghum sample, where n=20. The limit of quantification was 13 ± 0.03 ng/mL (Mean ± S.D.).

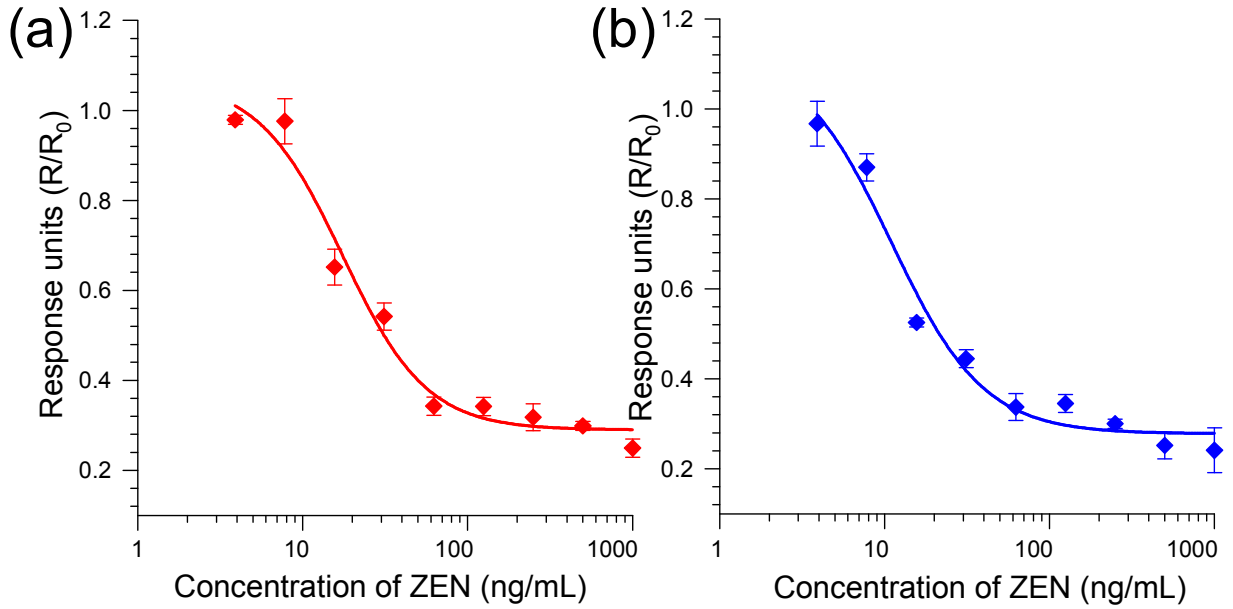


Figure 4.17: Intra- and inter-day SPR-inhibition assay using sorghum samples along with scFv-P2A2. *A 1/100 dilution of anti-ZEN scFv was incubated with sorghum sample containing 0.004-1 µg/mL of ZEN and passed over the ZEN immobilized sensor chip. The results were normalized by dividing the response units in presence of ZEN (R) by the respone units of zero concentration of antigen (R_0) in the assay. For intra-day (a) studies the S.D. was computed from 3 validation runs on one day and inter-day (b) S.D. was determined over 5 validation runs on 5 separate days. A 4-parameter logistic curve was fitted to the data set.*

Table 4.5: *Intra- and inter-day assays coefficient of variance (CVs) for anti-ZEN scFv in matrix. CVs were computed from the 3 replicate analyses carried out on the same (intra-day) and on three different days (inter-day).*

ZEN concentration (ng/mL)	Intra-day assay				Inter-day assay			
	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies
62.5	59	0.3425	1.45	95.2	61	0.3372	8.97	98.4
31.25	32	0.5419	3.12	103.2	31	0.4446	9.24	100
15.625	14	0.6518	2.24	87.5	13	0.5251	11.2	81.2
7.812	9	0.9760	1.45	112.5	8	0.8700	3.56	108.3

4.3 Discussion

The work presented in this chapter describes the generation of a murine scFv phage display library to produce soluble anti-ZEN scFv antibodies and characterization of the scFv by ELISA, SDS-PAGE, western blotting and SPR-based analysis. Earlier Balb/c mice were immunized with ZEN-BSA. After the serum titre value reached 1/30,000 (data not shown), the spleenomic RNA was used for the synthesis of cDNA, followed by the amplification of heavy and light chains for linking them through glycine-serine for SOE formation. The generated antibody-phage library was subjected to panning on an ELISA plate coated with varying concentrations of ZEN-KLH conjugates. Unfortunately, scFvs that could recognize ZEN were not isolated from this library. The failure to isolate anti-ZEN scFvs may be mainly attributed to the low immune response of the mice (<1/30,000) and also the lower surface area on microtitre plate, which was used for performing biopanning.

To attain a higher titration value, ZEN-KLH conjugate was used as an immunogen instead of ZEN-BSA. Following the standard immunization protocol with ZEN-KLH conjugate, an immune response of 1/256,000 was achieved. Comparing the immune response attained upon administering BSA and KLH conjugates of ZEN, synthesized employing the two-step NHS ester method, resulted in a 8.5 fold increase in the titration values of the sera from Balb/c mice, which was mainly due to presence of a larger number of ZEN molecules on the surface of the KLH molecule, when compared to BSA, as is evident from the results of MALDI-TOF and TNBS assays (Refer Appendix III). The immune response was considered to be of sufficient level for the isolation of spleenomic RNA from which the scFv library was subsequently generated, using a set of primers (designed for chimeric antibody generation) and optimized PCR reactions.

The other parameter that was taken into consideration to successfully isolate anti-ZEN scFvs was the usage of immunotubes for biopanning (Kehoe *et al.*, 2006), which have a larger surface area for efficient binding of the phage to the surface that has adsorbed ZEN-BSA conjugate.

During the panning procedure a significant drop in the phage output titre to 10^4 from 10^7, after the fifth round of panning, was noticed. The phage output after the 6^{th} round of panning increased to 10^6, which represents the higher amplification titres. The selected phage antibodies were expressed using *E. coli* host expression system (Top 10F′), which is considered an effective expression system when compared to others like *B. subtilis* and *B. brevis*. Less than 50% of the scFv-producing clones were found to yield ZEN-positive binders.

Five representative scFvs (P1C6, P1G2, P2A2, P2H9 and P2G2) were analysed through indirect and competitive ELISA adopting the procedure described in section 2.2.4.9. The titre values of all the clones were found to be higher than 1/50. These scFvs were assayed to determine the affinity towards ZEN in solution, and, all of the five scFvs were found to recognize ZEN. The IC_{50} of scFv-P2A2 (80 ng/mL) was lower when compared to the other scFvs and, hence, further analysis of P2A2 was carried out. The cross reactivity profile of scFv-P2A2 with alpha and beta zearalenol was analyzed (competitive indirect ELISA), indicating minimal cross reactivity.

In contrarst to the SPR developed by Urraca *et al.* (2005) and Choi *et al.* (2009) for the detection of ZEN, ZEN was immobilized directly onto the surface of the amine-activated CM5 chip using a slightly modified immobilization procedure from that developed by Van der Gaag *et al.* (2003). In the initial phase of the SPR assay development the scFv-P2A2 was passed over the surface of a ZEN-immobilized sensor chip to determine the optimal response units. The affinity of scFv-P2A2 was evaluated through kinetic analysis using varying concentrations of scFv, which resulted in K_a and K_d values of 5.72 X 10^4 $M^{-1}s^{-1}$and 2.28 X 10^{-5} s^{-1}, respectively. Thus the calculated K_D value for scFv-P2A2/ZEN interaction was 3.9 nM (Figure 4.14).

For the determination of optimal range of ZEN for SPR-based inhibition assay a range of ZEN concentrations were prepared and pre-mixed with scFv-P2A2. The samples were then passed over the surface of sensor chip followed by regeneration steps with 20 mM NaOH in between the runs. A standard calibration curve was generated from the ratio of mean binding slope of

standard ZEN to the standard scFv-P2A2 (without ZEN) against log concentration of ZEN. The range of detection for the scFv was found to be 0.0039-1 μg/mL.

Direct coupling of ZEN was performed by Van der Gaag *et al.* (2003), but their assay was not validated using 'Real' sample to determine the feasibility of the assay for food analysis. In the present studies the assay was proven to be applicable for the detection of ZEN in sorghum and the assay may be applied for other types of foods. The detection limits of the SPR assays developed to-date were 0.01 ng/mL (Van der Gaag *et al.,* 2003), 0.3 ng/mL (Choi *et al.,* 2009) and 0.007 ng/mL (Urraca *et al.,* 2005). The SPR-based inhibition assay developed using scFv-P2A2 has a limit of detection of 7.8 ng/mL (matrix).

In conclusion, an anti-ZEN scFv that is highly specific to ZEN was developed and applied in an ELISA and SPR-based inhibition assays with IC_{50} values of 200 & 10 ng/mL, respectively. which falls within the 200 ng/mL maximum permissible limits for ZEN. The SPR-based assay was proven to be feasible for the detection of ZEN in sorghum samples. To our knowledge this is the first reported SPR-based inhibition assay developed using scFv for the specific detection of ZEN. The limit of detection is far lower than the permissible limits of 200 ng/mL, which would make this antibody useful for ZEN analytical purposes. Furthermore, this scFv was used to generate an immuno-affinity column described in chapter 6.

Chapter 5
Generation and characterization of anti-T-2 toxin IgY

5.1 Introduction

T-2 toxin is not inactivated by food processing techniques (Sokolovic *et al.,* 2008). Due to its high incidence, the regulatory authorities in the EU are trying to set maximum permissible levels for T-2 in food and feed (http://services.leatherheadfood.com/eman/FactSheet .aspx?ID=79).

To-date, avian species were rarely used for the development of antibodies against mycotoxins and only one report exists, for the successful antibody generation in chickens against T-2 toxin (Jaszczuk *et al.,* 1995). Egg yolk-derived antibodies offer potential applications in various fields including therapeutics (Lee, 2002), immuno-diagnostics (Sunwoo *et al.,* 2006) and food toxin detection (Allen and Smith, 1987). The various advantages offered by the utilization of egg yolk antibodies, including the reduction in the need for sacrifice of animals, the presence of large concentrations of antibodies in eggs (Hau and Hendriksen, 2005) and resistance of IgY to acid and heat (Losch *et al.,* 1986), make the utilization of IgY a viable alternative source for generating polyclonal antibodies, with sensitivities comparable to anti-T-2 monoclonal antibodies developed to-date. Monoclonal antibody-based assays generated for the detection of T-2 toxin have detection limits between 0.05-100 ng/mL (Gendloff *et al.,* 1984; Barna-Vetro *et al.,* 1994; Kononenko *et al.,* 1999; Takumi *et al.,* 2004). These antibodies exhibited cross reactivity with other mycotoxins. Hence, there is a need to develop an affinity purification technique capable of isolating anti-T-2 specific (mono-specific) IgY.

It was decided to generate and affinity purify an anti-T-2 toxin IgY (from egg yolk) with subsequent incorporation into a Biacore-based competitive inhibition assay format (Figure 5.1) based on reported immunoassays using IgY for detection of other mycotoxins such as ZEN (Pichler *et al.,* 1998) and DON (Schneider *et al.,* 2000).

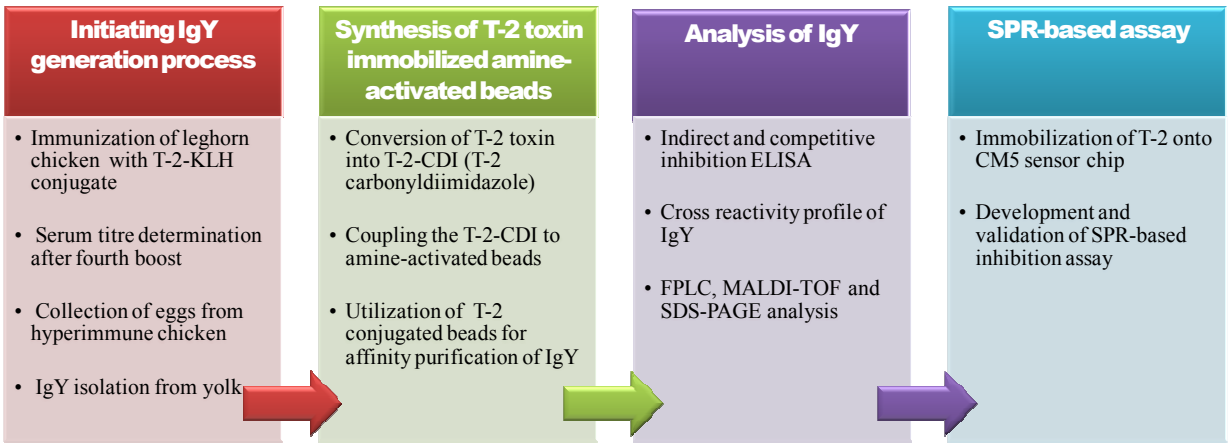


Figure 5.1: Multi-stage approach adopted for isolation of mono-specific anti-T-2 IgY and its subsequent incorporation into SPR-based inhibition assay. *In the first stage IgY was isolated from the eggs of T-2 KLH immunized chicken. The second stage comprises of synthesis of T-2 toxin immobilized amine-activated beads for subjecting the IgY to affinity purification to isolate T-2 specific IgY with minimal cross reactivity to other mycotoxins. After affinity purification the purity, identity and molecular mass of IgY was analyzed by FPLC, SDS-PAGE and MALDI-TOF, respectively. The specificity and sensitivity of IgY was determined by ELISA's. Later SPR-based inhibition assay was developed and validated.*

5.2 Avian immune response to T-2 toxin

Following immunization of a leghorn chicken with T-2-KLH-adjuvant emulsion mixture four times, the antibody response was evaluated through determining the antigen-specific titre of the serum. Positive and negative controls were incorporated to confirm the specific immune response towards T-2 toxin. The pre- and post-immunization serum titre of the chicken was determined against T-2-BSA, KLH and BSA. As expected, there was a response of the serum against KLH. A semi-log plot was generated with the reciprocal of serum dilutions (X-axis) against absorbance values @ 450 nm (Y-axis). From the graph (Figure 5.2) an antibody titre of 1/120,000 was found after the fourth boost.

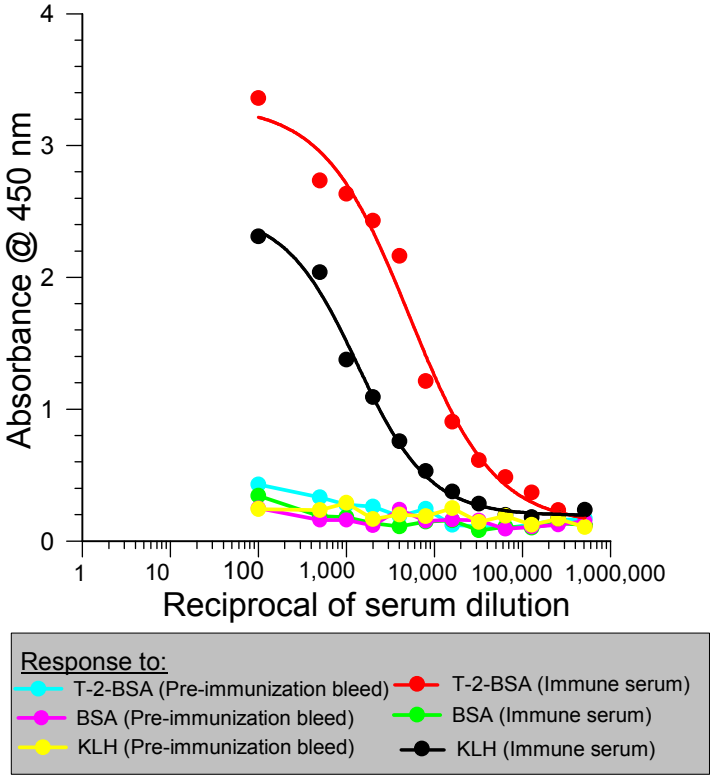


Figure 5.2: Immune response of T-2 KLH conjugate immunized chicken. *Doubling dilution of immune and pre-immunization sera (1/100 to 1/512,000) were tested against T-2 BSA, BSA and KLH in duplicate. A 1/1,000 dilution of HRP-labelled rabbit anti-chicken Ig secondary antibody was added, followed by the visualization with TMB solution. The immune response was recorded to be in excess of 1/120,000.*

5.3 Isolation, affinity purification and analysis of anti-T-2 IgY

5.3.1 Isolation of anti-T-2 IgY from egg yolks

Chicken eggs collected after the fourth boost were used for the isolation of anti-T-2 toxin IgY. A commercial kit was used for IgY isolation, following the protocol outlined in section 2.2.5.3 of chapter 2. The re-suspended IgY precipitate was quantified on the Nanodrop™. The concentration of IgY obtained was 10 mg/mL. The overall yield of IgY from one egg was approximately 120 mg. Subsequently, mono-specific IgY was attained after subjecting the IgY to affinity purification on T-2 toxin-immobilized amine-activated sepharose beads.

5.3.2 Immobilization of T-2 toxin on amine-activated beads

The immobilization procedure, described in section 2.2.5.4 of chapter 2, was adopted for covalent linkage of T-2 toxin to the beads. A T-2 toxin carbonyldiimidazole derivative (T-2 carbamate), which has greater capacity for coupling to the amine-activated beads (Hermanson, 1996) was prepared through activating hydroxyl group at the C3 position of T-2 toxin with carbonyldiimidazole (CDI) and used for immobilization. The sequential steps involved in T-2 toxin immobilization onto the amine-activated beads are depicted in Figure 5.3. Free amine groups were estimated using the Ninhydrin test. A decrease in the number of free amine groups after T-2 immobilization was established. A colorimetric determination of the amine-activated beads was carried out through reading the absorbance @ 280 nm on Tecan Saffire plate reader. The difference in the absorbance value of T-2 immobilized beads and un-immobilized beads indicates the utilization of amine groups for the coupling of T-2 toxin.

5.3.3 Affinity purification of IgY using T-2 toxin-immobilized amine-activated beads

The T-2 toxin-immobilized beads were equilibrated with PBS. After equilibrating the column, 10 mg of IgY were added and incubated for 2 hrs at 4^0C, followed by washing the column with PBS. The bound IgY was eluted using 8 mL of 100 mM glycine-HCl in water (pH 3.0). One mL eluted fractions of IgY were collected and neutralized with 50 µL of 1 M Tris (pH 9.0). The percentage recovery of IgY was 10-20% (calculation based on equation = (amount of IgY added to the beads – amount of IgY recovered) X 100 / amount of IgY added to the beads). This loss can be attributed to the elimination of IgY that is not specific to the T-2 toxin.

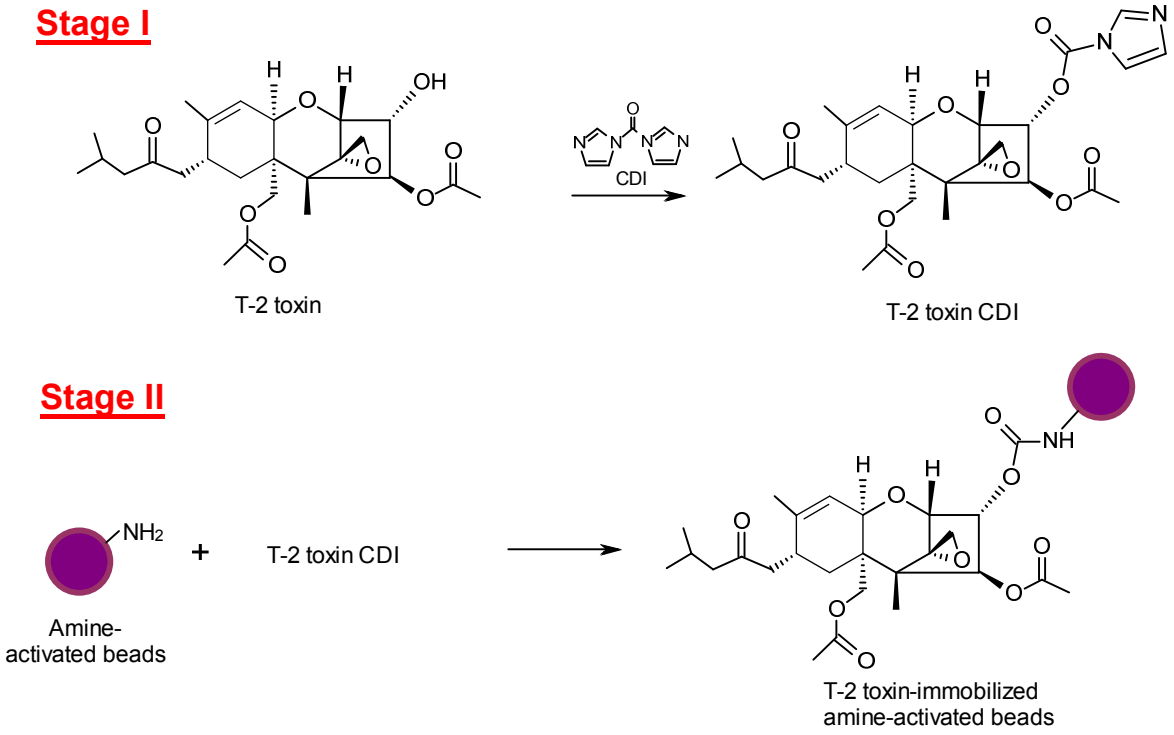


Figure 5.3: T-2 toxin immobilization on amine-activated beads. *The T-2 toxin was first reacted with carbonyldiimidazole and the reaction mixture was added to the amine-activated beads in the presence of coupling buffer.*

5.3.4 ELISA assay using anti-T-2 toxin IgY

5.3.4.1 Indirect ELISA

An indirect ELISA assay (Figure 5.4) was performed on a microtitre plate coated overnight at 4^0C with T-2-BSA or BSA. Equal starting concentrations (1 mg/mL) of IgY (before and after affinity purification) were used to perform the assay. Varying dilutions of IgY from 1/100 to 1/512,000 were used. From the semi-log plot drawn with the reciprocal IgY dilution values on X-axis against absorbance @ 450 nm (Y-axis), a 50% increase in the titre value of pure IgY after affinity purification was recorded. Overall, the titre values before and after affinity purification were 1/80,000 and 1/120,000, respectively.

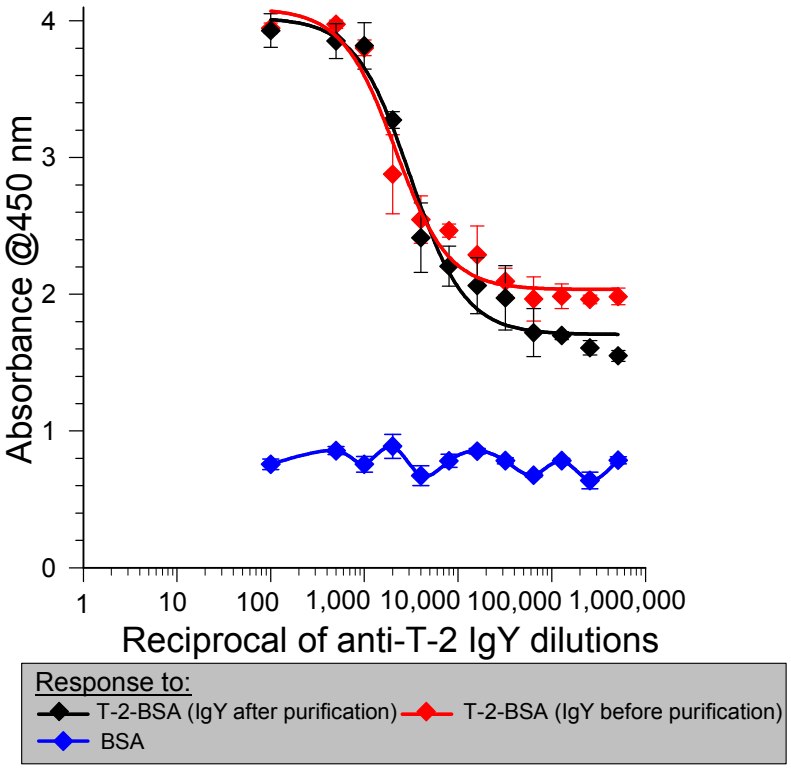


Figure 5.4: Titration of anti-T-2 IgY. *Varying dilutions of IgY before and after affinity purification were tested against T-2-BSA and BSA. HRP-labelled rabbit anti-chicken Ig (1/1,000 dilution) was added followed by the addition of TMB solution. The error bars indicates S.D. of the results obtained from the experiments where n=3 independent determinations. The absorbance of BSA-coated wells was taken as background values.*

5.3.4.2 Competitive indirect ELISA (CI-ELISA)

CI-ELISA (Figure 5.5) was performed using a 1/1,000 dilution of IgY that was incubated for 30 mins with varying concentrations (0.004-2.5 µg/mL) of T-2 toxin. In this experiment, the toxin conjugate (T-2-BSA) was immobilized on the immunoplate. Competition for antibody binding occurs between the immobilized toxin and the free toxin added (Section 2.2.5.5). A semi-log plot was drawn with A/A_0 (A= absorbance of antibody inhibited with varying concentration of T-2 toxin & A_0= absorbance of antibody with no T-2 toxin) on Y-axis against concentration of T-2 toxin on a logarithmic X-axis. The mid inflection point (IC_{50} value) was 40 and 80 ng/mL when IgY before and after affinity purification, respectively were used.

For intra- and inter-day assay variations (Figure 5.6), each concentration was assayed three times on the same day and five times on different days, respectively. The mean absorbance of

bound antibody for each antigen concentration was plotted against log concentration of antigen. The percentage CVs (coefficient of variance) for inra- and inter-day assays were below 8 and 11%, respectively (Table 5.1).

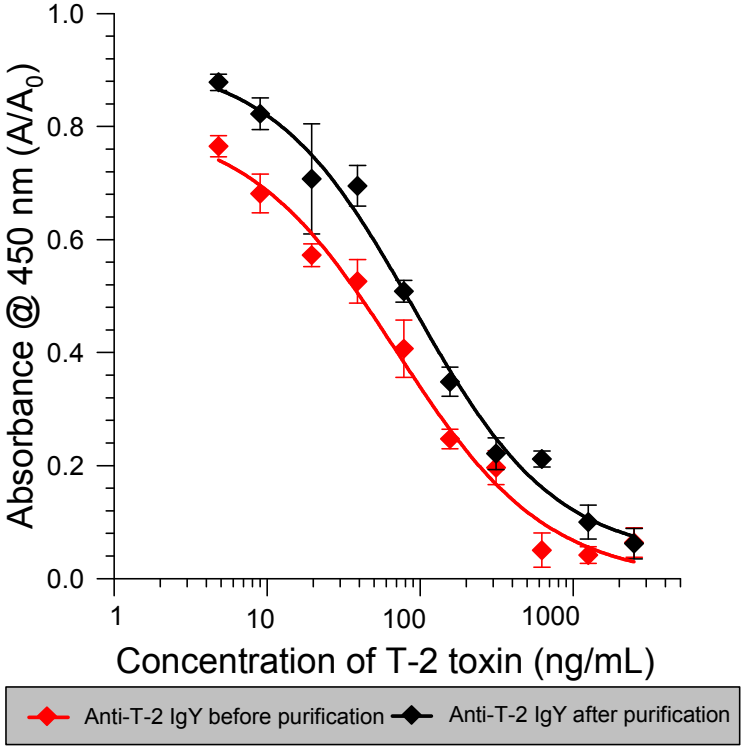


Figure 5.5: Competitive inhibition curve of anti-T-2 IgY. *A 1/1,000 dilution of both mono-specific and crude IgY were incubated for 30 mins at 37^0C with varying concentration of T-2 toxin (0.004-2.5 µg/mL) in the ratio of 1:1. The error bars indicates standard deviation (S.D.) calculated where n=3 independent determinations.*

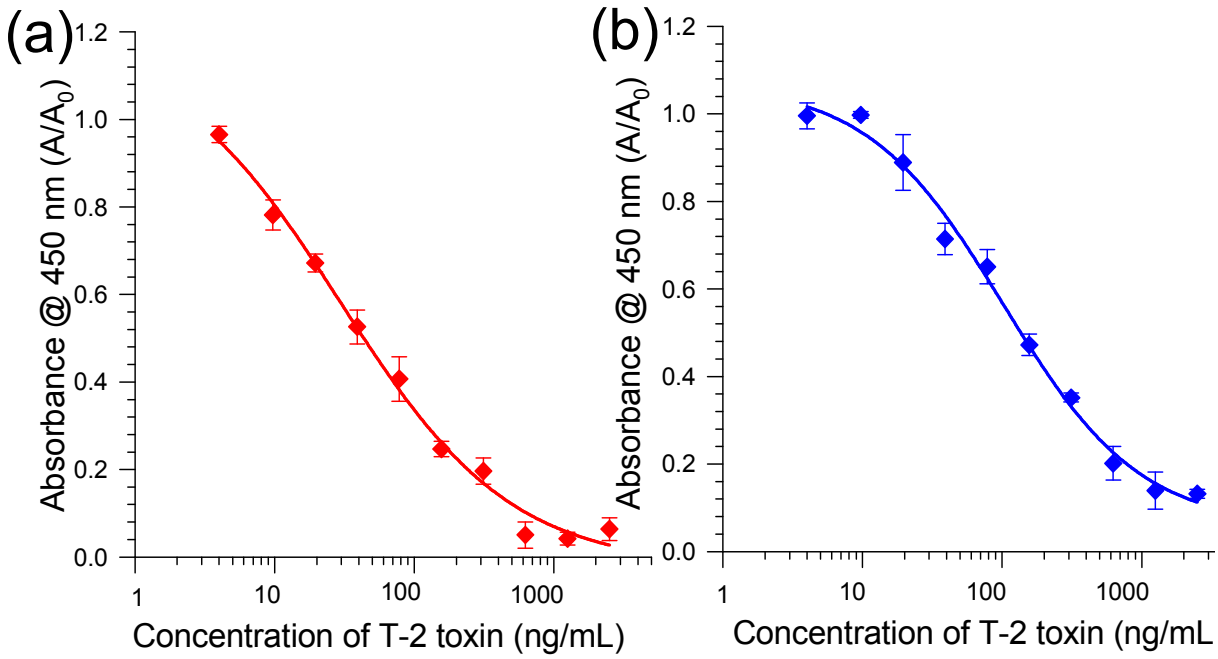


Figure 5.6: Intra- and inter-day variation of competitive ELISA for T-2 toxin using affinity purified mono-specific IgY. *A 5 µg/mL T-2-BSA coating concentration was used with a 1/1,000 dilution of anti-T-2 IgY that was incubated with a T-2 toxin (0.004-2.5 µg/mL). For intra-day (a) studies the S.D. was computed from 3 validation runs on the same day and inter-day (b) S.D. was determined over 5 validation runs on 5 separate days. A 4-parameter logistic curve was fitted to the data set.*

Table 5.1: *Intra- and inter-day coefficient of variance for the determination of free T-2 toxin using anti-T-2 IgY. Three sets of ten standards were assayed on five different days and CVs were calculated.*

T-2 toxin concentration (ng/mL)	Intra-day assay				Inter-day assay			
	Back-calculated concentration (ng/mL)	A/A_0	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	A/A_0	% CVs	% Accuracies
312.5	347	0.1965	7.59	111.2	318	0.2210	9.56	101.9
156.25	149	0.2471	2.89	95.5	156	0.3483	5.22	100
78.125	84	0.4069	1.61	107.7	81	0.5084	1.45	103.8
39.062	39	0.5260	2.59	100	35	0.6950	5.65	89.7
19.531	19	0.6722	6.15	100	18	0.7074	6.67	94.7
9.765	8	0.7815	2.35	88.9	10	0.8226	4.77	111.1
4.882	4	0.9654	1.39	100	4	0.8783	5.67	100

5.3.4.3 Cross reactivity studies

The cross reactivity of anti-T-2 toxin IgY for HT-2, deoxynivalenol, zearalenone, fumonisin B_1 were determined employing the procedure described in section 2.2.5.5.2. The cross reactivity is defined as the point where the 50% reduction in the signal (in the presence of analyte) is noticed in comparison to the signal obtained in the presence of no analyte. The percentage cross reactivity was calculated by taking the IC_{50} value against T-2 toxin as 100%. The calculation was done using the formula

$$\% \text{ cross reactivity} = \frac{IC_{50} \text{ value of mycotoxins}}{IC_{50} \text{ value of T-2 toxin}} \times 100$$

From the % cross reactivity data (Table 5.2) it was found that affinity purification of IgY led to a decrease in the cross reactivity percentage of IgY to HT-2 toxin and also to the other mycotoxins.

Table 5.2: *Cross reactivity profile of anti-T-2 IgY before and after affinity purification*

Toxin	% Cross reactivity	
	Before affinity purification	After affinity purification
T-2	100%	100%
HT-2	46%	5%
Deoxynivalenol	0.2%	0.11%
Zearalenone	0.15%	0.12 %
Fumonisin B_1	0.32%	0.21%

5.3.5 IgY confirmatory tests

5.3.5.1 FPLC and SDS-PAGE analyis of IgY

IgY (M.wt.~180 *k*Da) was analysed on FPLC using size exclusion chromatography. A set of molecular weight markers (Ovalbumin (43,000 Da), Conalbumin (75,000 Da), Aldolase (158,000 Da), Ferritin (440,000 Da)) were injected into the FPLC. The chromatogram (Figure 5.7(a)) was generated, which indicates that the peak of IgY corresponds to approximately 180 *k*Da.

To confirm IgY purity and identity, SDS-PAGE analysis (Figure 5.7(c)) under reducing and non-reducing conditions were performed. Under reducing conditions two bands corresponds to the light chain (~24 *k*Da) and heavy chain (~65 *k*Da) were seen. One band at approximately 180 *k*Da was identified when SDS-PAGE gel was run under non-reducing conditions.

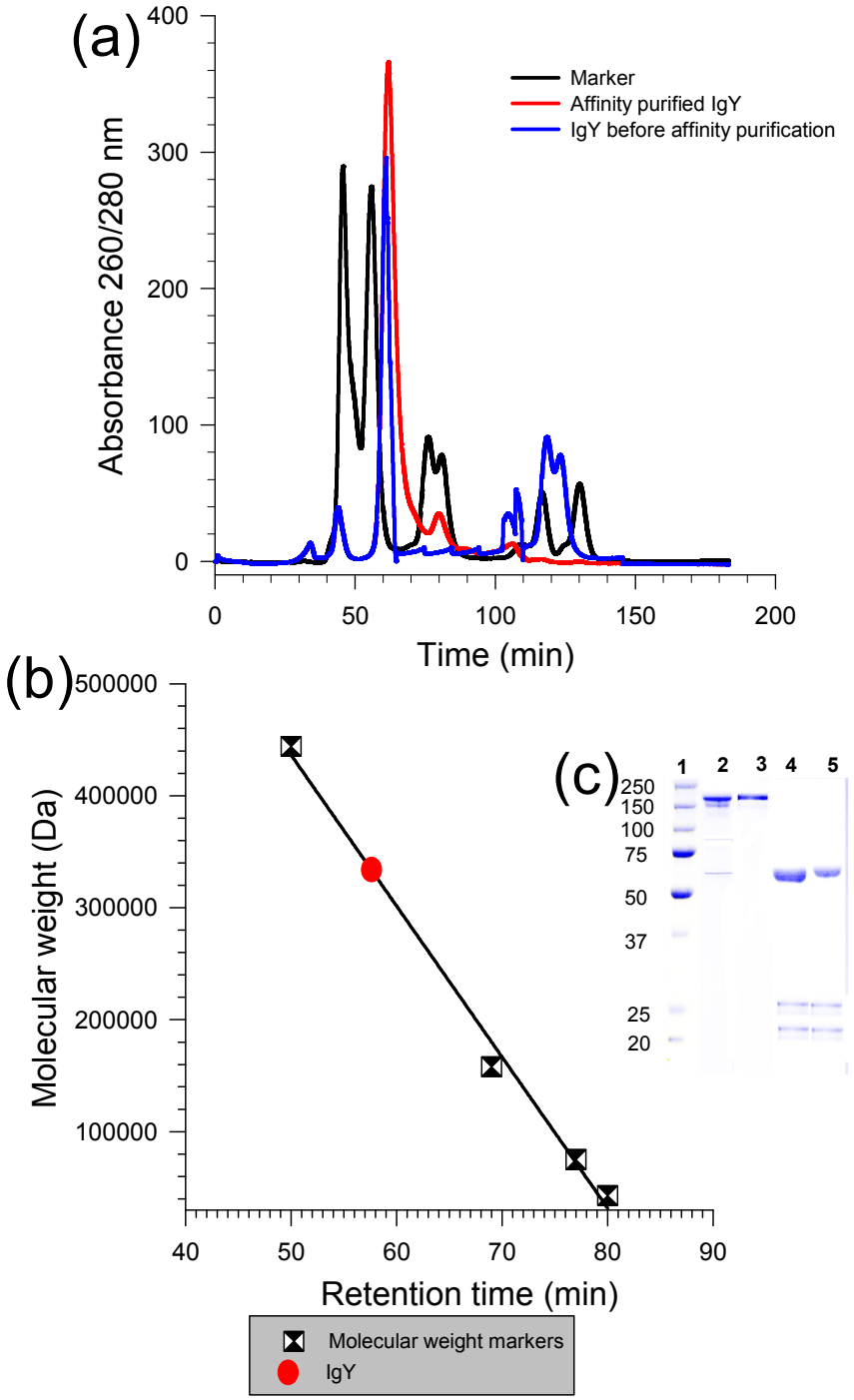


Figure 5.7: FPLC and SDS-PAGE analysis of IgY. *Equal concentrations of IgY, before and after affinity purification, were used. A range of molecular weight markers were also injected, in parallel, to confirm the IgY peak through comparing the peak positions obtained from the chromatogram (Figure (a)). Figure (b) indicates retention time of calibration standards versus the retention of IgY .The estimated molecular weight of IgY was ~180 kDa. Figure (c) represents the SDS-PAGE gel of the IgY under reducing and non-reducing conditions. The representation of the various lanes is as follows: lane 1: Sigma wide range molecular weight markers; lanes 2 & 3: non-reduced IgY before and after purification and lanes 4 & 5: reduced IgY before and after purification, respectively.*

5.3.5.2 MALDI-TOF analysis

The molecular mass of purified IgY was analyzed through MALDI-TOF analysis (Figure 5.8), which indicates the presence of four different fragments in the whole IgY molecule. The molecular mass of the light chain of the IgY is approximately 31,000-37,000 Da. The heavy chain's molecular mass is approximately 69,000-85,000 Da. The mass of the intact IgY molecule is approximately 165,000-172,000 Da.

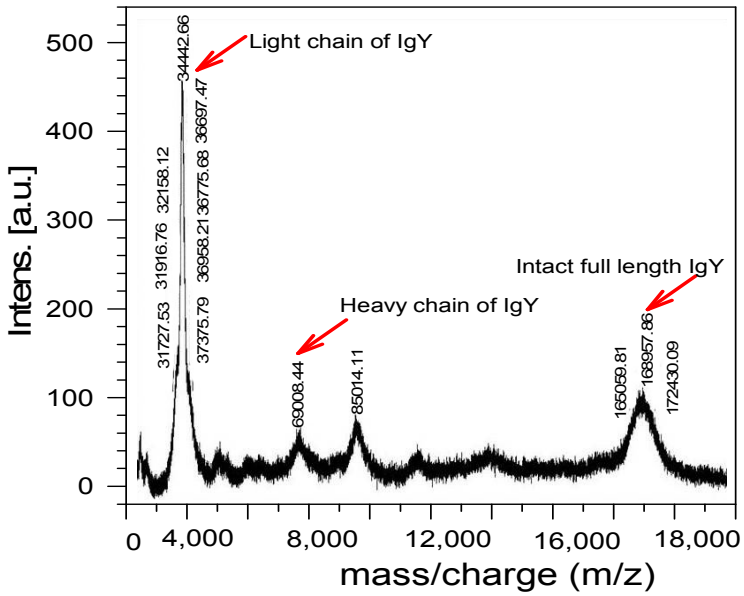


Figure 5.8: MALDI-TOF analysis of the anti-T-2 toxin IgY. *The IgY (1 mg/mL) and sinnapic acid were mixed in the ratio of 1:1 and added to the chip surface in the presence of trifluroacetic acid. From the figure it can be seen that besides the intact IgY, there are also fragmented portions of IgY. The arrows indicates the major peaks of the IgY.*

5.4 Immobilization of T-2 toxin on CM5 sensor chip

Analysis of the binding of anti-T-2 toxin IgY was performed using the Biacore™ 3000 biosensor. Prior to anti-T-2 toxin IgY assay, the CM5 chip was immobilized with T-2 toxin (Figure 5.9). The various steps involved in this process were, priming of CM5 chip with HBS buffer, followed by surface activation with 1:1 ratio of EDC-NHS. The EDC-NHS surface activated CM5 chip was then incorporated with amine groups through the addition of Jeffamine, followed by the immediate blockage of un-reacted amine groups with ethanolamine. Then a T-2-CDI mixture was added leading to the generation of T-2 toxin immobilized CM5 chip.

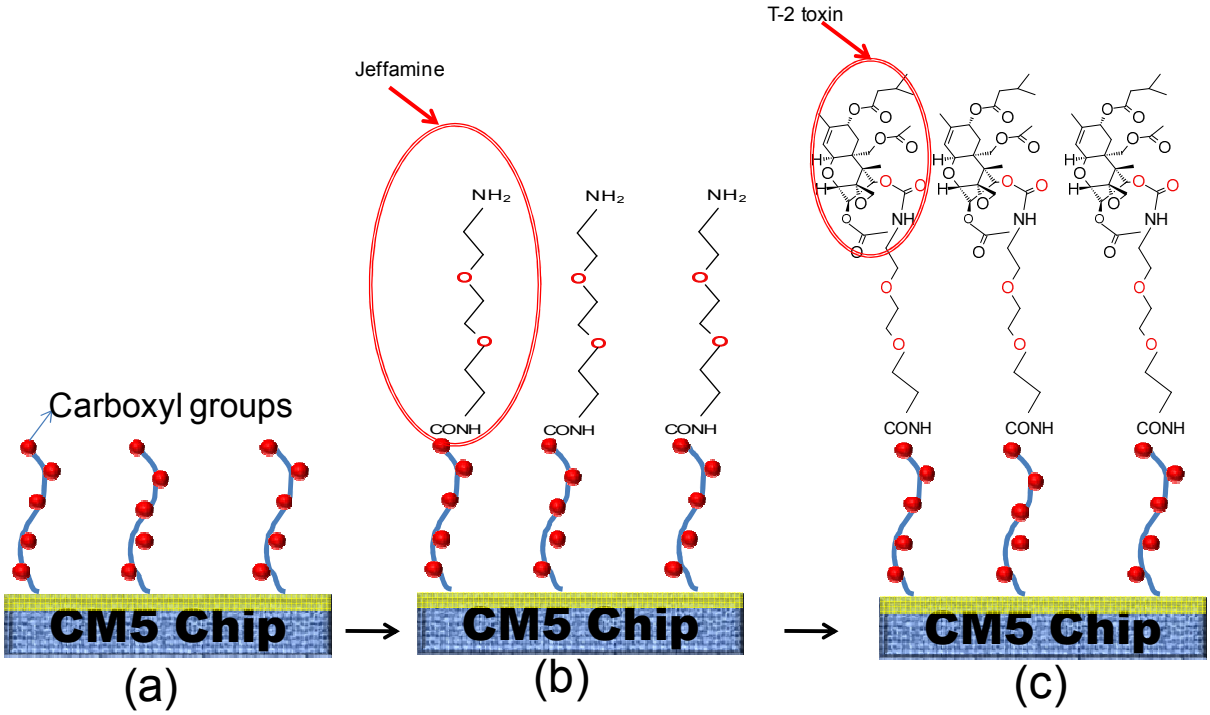


Figure 5.9: Schematic representation of T-2 toxin immobilization on a CM5 sensor chip. *The first stage (a) involved in the immobilization of T-2 toxin onto CM5 chip was surface activation with EDC and NHS. In the second stage amine groups were generated through treating with Jeffamine (b). The third stage involves the covalent attachment of T-2 toxin to the amine groups on the CM5 chip (c).*

5.5 Analysis of anti-T-2 toxin IgY on T-2 toxin immobilized sensor chip

After immobilization of T-2 toxin on sensor chip, the affinity purified anti-T-2 IgY was passed over the sensor chip. Figure 5.10 shows that anti-T-2 IgY recognizes T-2 toxin in its unconjugated form. A negative control antibody (anti-CRP scFv antibody) was also used in order to prove the specific binding the anti-T-2 toxin IgY on the CM5 chip.

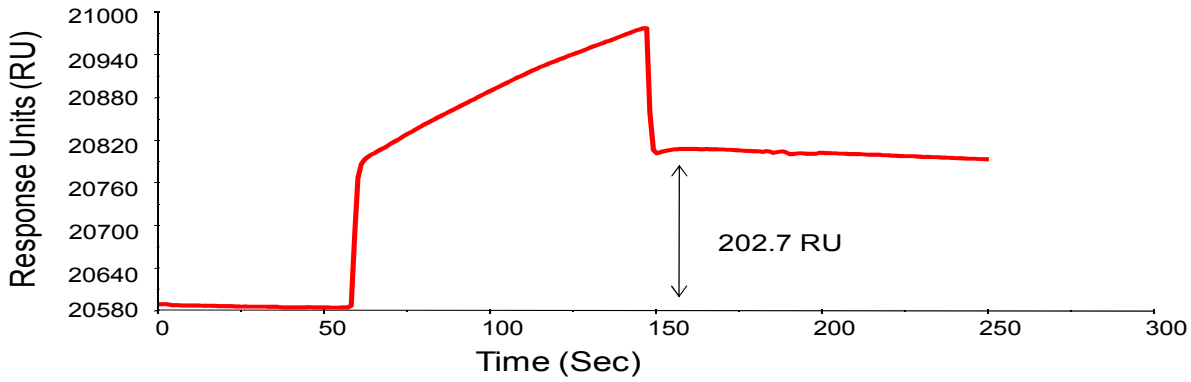


Figure 5.10: Sensogram depicting the binding of anti-T-2 toxin IgY to T-2 toxin. *A 1/2,500 dilution of anti-T-2 toxin IgY was passed over the flow cell 1 of T-2 toxin immobilized sensor chip, which resulted in a net binding response of 202 RU. The exact response units were measured through subtracting the RU obtained when non-specific antibody was passed over the surface.*

5.6 Solution phage T-2 toxin binding assay

In this assay anti-T-2 IgY was prevented (inhibited) from binding to the immobilized T-2 toxin through the addition of varying concentrations of T-2 toxin (0.007-1 µg/mL) in solution (Figure 5.11). The SPR analysis revealed that the anti-T-2 toxin IgY had an IC_{50} value of 42 ng/mL (Figure 5.11). The LOD and LOQ of anti-T-2 toxin IgY was 8 ± 0.15 ng/mL and 12 ± 0.21 ng/mL. Intra- (n=3) and inter-day (n=5) assays were performed and graph was plotted using the mean A/A_0 (A= absorbance of antibody in presence of T-2 toxin and A_0= absorbance of antibody alone) against concentration of T-2 toxin (Figure 5.12). The percentage coefficient of variance (% CVs) for both intra- and inter-day assays are presented in Table 5.3.

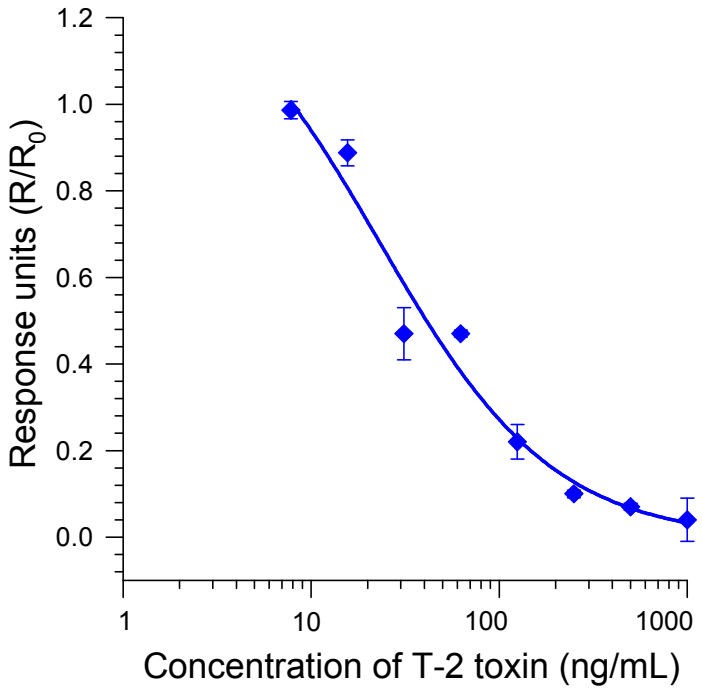


Figure 5.11: SPR-inhibition curve of the anti-T-2 toxin IgY. *The R/R_0 was calculated by dividing the response units obtained at 8 different T-2 toxin concentrations (0.007-1 µg/mL) by the response units obtained upon passing anti-T-2 toxin IgY alone, without T-2 toxin. A 4- parameter logistic curve was fitted to the data set using SigmaPlot software. The error bars indicates the S.D. of three replicate measurements.*

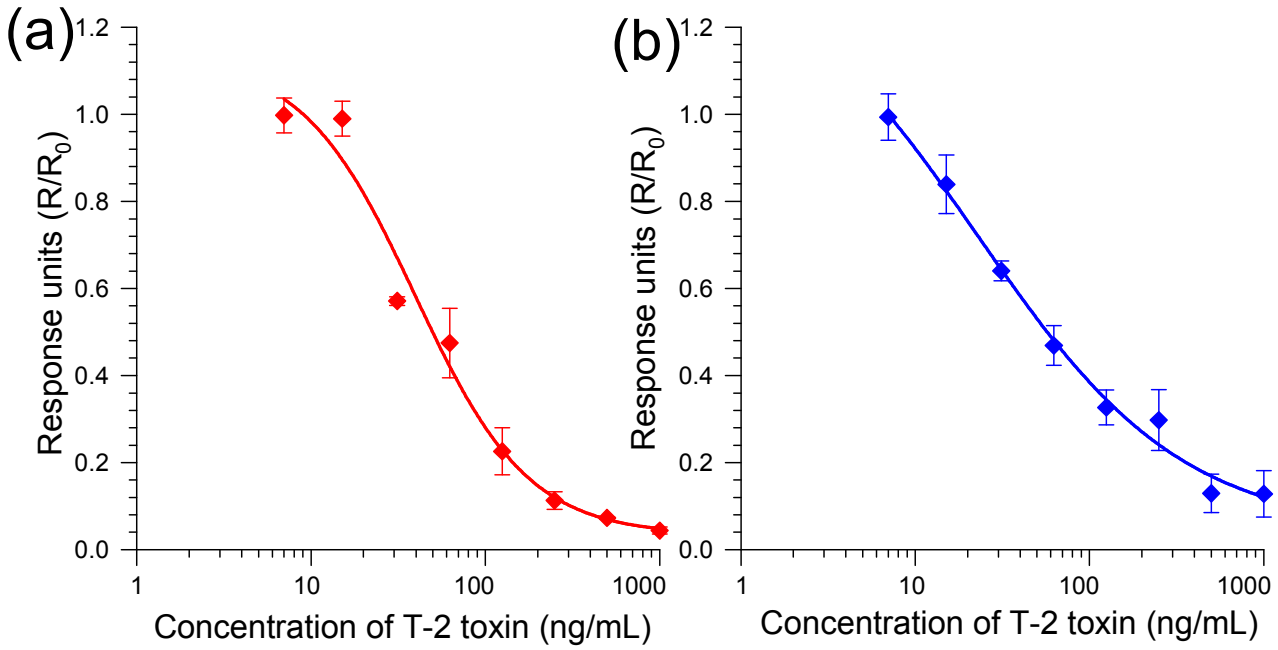


Figure 5.12: Intra- (a) and inter- (b) day SPR-inhibition calibration curves in solution. *A 1/2,500 dilution of anti-T-2 IgY was incubated with 0.007-1 µg/mL of T-2 toxin and passed over the T-2 toxin immobilized sensor chip. The results were normalized by dividing the response units in presence of T-2 toxin (R) by the response units of zero concentration of antigen (R_0) in the assay. For intra-day (a) studies the S.D. was computed from 3 validation runs on the same day and inter-day (b) S.D. was determined over 5 validation runs on 5 separate days. A 4-parameter logistic curve was fitted to the data set.*

Table 5.3: *Intra- and inter-day coefficient of variance for the determination of free T-2 toxin using anti-T-2 IgY. Three sets of eight standards were assayed on five different days and CVs were calculated.*

T-2 toxin concentration (ng/mL)	Intra-day assay				Inter-day assay			
	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies
1000	1113	0.0440	2.72	111.3	1046	0.1283	3.21	104.6
500	510	0.0730	6.33	102	446	0.1296	11.4	89.2
250	249	0.1130	8.93	99.6	293	0.2977	2.47	117.2
125	123	0.2260	3.02	98.4	117	0.3268	3.63	93.6
62.5	73	0.4746	8.08	117.7	61	0.4690	6.12	98.4
31.25	27	0.5711	1.31	87.1	31	0.6402	1.45	100
15.625	19	0.9900	5.45	126.7	16	0.8390	8.35	106.7
7.8125	6	0.9976	2.45	85.7	7	0.9936	7.67	100

5.7 Spiking of wheat samples with T-2 toxin

Wheat samples (10 grams) were ground and extracted with 30 mL ethanol/water 85:5 (v/v) by constant stirring on a magnetic stirrer for 30 mins. The extract was filtered using Whatman Grade 1 filter paper. The filtrate was evaporated to dryness in a rotary evaporator and dissolved in 10 mL HBS buffer (pH 7.4). T-2 toxin was then added. The percentage recovery (n=3) attained from SPR inhibition analysis when the wheat sample extract was spiked with 1 µg/mL of T-2 toxin was 100%.

5.8 Intra- and inter-day validation of SPR inhibition assay using wheat sample extract

Affinity purified anti-T-2 IgY was used to develop an inhibition assay for the detection of T-2 toxin, as described in section 2.2.5.7. The assay was performed using wheat sample extract spiked with T-2 toxin ranging from 0.007-1 µg/mL, assayed in triplicate and repeated over five days. Figure 5.13 represents the intra-day inhibition standard cruve (four parametric fit) using SigmaPlot software. The percentage coefficient of variance, which are indicative of the precision of the both inter- and intra-day assays (Figure 5.13) were tabulated (Table 5.4). The inter-day coefficient of variance between 1.7 and 5.4% (Table 5.4). The limit of detection and limit of quantification (LOQ = LOB + 10 X S.D. of LOB) were 11 ± 0.23 and 23 ± 0.31 ng/mL (Mean ± S.D.).

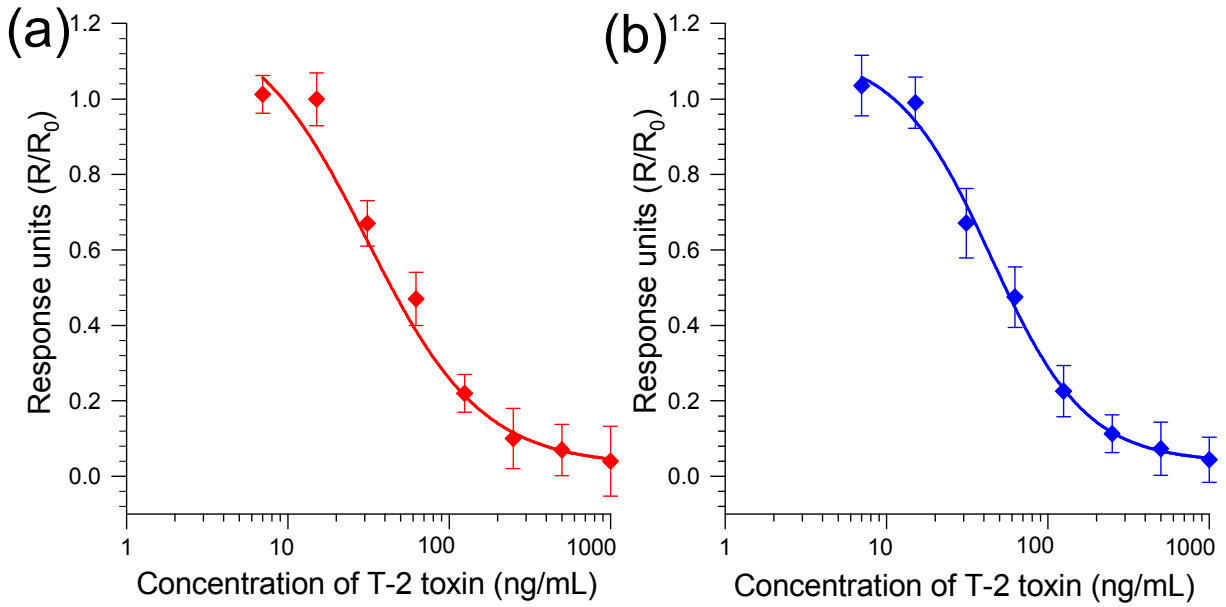


Figure 5.13: Intra-day and inter-day calibration curves of SPR-based inhibition assay using mono-specific IgY. *A 1/2,500 dilution of anti-T-2 IgY was incubated with spiked wheat sample extract containing 0.007-1 µg/mL of T-2 toxin and passed over the T-2 toxin immobilized sensor chip. The results were normalized by dividing the response units in presence of T-2 toxin (R) by the response units of zero concentration of antigen (R_0) in the assay. For intra-day (a) studies the S.D. was computed from 3 validation runs on the same day and inter-day (b) S.D. was determined over 5 validation runs on 5 separate days. A 4-parameter logistic curve was fitted to the data set.*

Table 5.4: *Intra- and inter-day coefficient of variance for the determination of T-2 toxin in spiked wheat sample extract using anti-T-2 IgY. Three sets of eleven standards were assayed on five different days and CVs were calculated.*

T-2 toxin concentration (ng/mL)	Intra-day assay				Inter-day assay			
	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	R/R_0	% CVs	% Accuracies
125	126	0.2200	3.57	100.8	124	0.2260	3.67	99.2
62.5	66	0.4700	4.28	106.4	62	0.4746	5.12	100
31.25	31	0.6700	1.31	100	31	0.6711	2.45	100
15.625	16	0.9990	2.45	106.7	15	0.9900	3.65	100
7.8125	6	1.0122	1.45	85.7	6	1.0356	2.67	85.7

5.9 Discussion

T-2 immunogens induce slower immune response in both rabbits and mice due to the instability of the hemisuccinate (HS) of T-2 (a T-2 derivative that was commonly synthesized to perform conjugation) and also due to immunosuppressive effects (Hunter *et al.,* 1985). To circumvent this problem, the conversion of T-2 to T-2-HS was avoided. Instead, an alternative strategy of conjugating T-2 to the carrier protein using the CDI method, employing a slightly modified procedure developed by (Xiao *et al.,* 1995) was adopted. An immune response of 1/120,000 was obtained, which was higher than the earlier reported response obtained through immunizing a rabbit with a T-2-KLH conjugate synthesized following EDC-NHS ester chemistry (Wang *et al.,* 2010). The five factors that might influence the immune response, including the dose and molecular weight of the antigen, type of adjuvant used, route of immunization, frequency of immunization and intervals between immunizations (Schade and Hinak, 1996), were taken into consideration when developing avian anti-T-2 toxin antibodies. Based on the finding of (Schwarzkopf *et al.,* 2000), that an antigen concentration ranging from 10 µg/mL to 1 mg/mL has to be used to generate a good immune response, 200 µg/mL of the T-2 KLH conjugate (1 mL volume of T-2 conjugate Fruends adjuvant emulsion) was administered to the chicken. Fruend's adjuvant is cost-effective for eliciting immune responses in laboratory animals. This is the most commonly used adjuvant for the production of antibodies in chickens. A combination of Fruends's complete adjuvant (FCA) and Fruend's incomplete adjuvant (FIA) was used for the successful generation of an antibody response (Li *et al.,* 2006).

The egg yolks are the main reservoir of IgY (Kovacs-Nolan and Mine, 2005). Eggs from a host with hyper-immune serum to T-2 toxin were used for the isolation of polyclonal IgY. The affinity purification technique can be used for the isolation of mono-specific antibodies from a polyclonal antibody pool (Josic *et al.,* 1994). To-date, most of the IgY purification techniques are mainly FPLC-based methods. A T-2 toxin affinity column with 1.3 mg/mL of T-2 and

loading value of 22 μmol amine per millilitre of beads was developed and used for affinity purification with an average IgY recovery of 10-20%, which may be associated with the presence of 2-10% antigen-specific antibodies in egg yolk (Ferreira Junior *et al.,* 2012). Since pH plays a role in antibody elution (Kang and Bae, 2004), the effect of pH on the elution of the anti-T-2 IgY antibody was determined over a pH range from 2.0-11.0 and the optimized yield was obtained at pH 3.0 (data not shown). Under the above specified conditions the column was used for five successive purifications with minimal loss in the column binding efficiency. The affinity-purified IgY was evaluated through indirect, CI-ELISA and SPR-based inhibition assays. An increased titre after affinity purification with reduction in the cross reactivity of IgY towards HT-2 toxin from 46 to 5% was recorded, which apparently indicates the elimination of immunoglobulins that are not specific to T-2 toxin from the IgY pool. The decreased cross reactivity of IgY after subjecting to affinity purification on T-2 immobilized ligand indicates that this purification technique can readily be applied for the isolation of mono-specific antibodies in order to increase the specificity of immunoassay without changing the intrinsic properties of the antibody itself.

The identity and molecular mass of the IgY were confirmed by FPLC, SDS-PAGE analysis and MALDI-TOF. The results of all these three assays indicate the presence of IgY of approximately 180 *k*Da molecular weight. The MALDI-TOF data reveals the presence of a heavy (68 *k*Da) and light chain (31-37 *k*Da) fragment, which can be seen when SDS-PAGE was run under reducing conditions. The MALDI-TOF data obtained in the present work are similar to the results obtained by (Sun *et al.,* 2001).

The purified IgY was used for the development of two types of assays: an CI-ELISA and SPR-based inhibition assay. The IC_{50} of anti-T-2 toxin IgY before and after affinity purification as determined by both the assays was found to be 40 ± 0.1 and 80 ± 0.4 ng/mL, respectively which falls with in the range of detection of assays developed for T-2 detection using monoclonal antibodies. The SPR assay was validated using wheat sample extract that had been

spiked (post extraction) with known amounts of T-2 toxin (0.007-1 µg/ml). The IC_{50} of IgY in buffer and matrix were 30 ± 0.1 ng/mL and 32 ± 0.1 ng/mL (Mean ± S.D., n=3), respectively. Limit of blank (LOB) was determined by analysis of 20 blank wheat sample extract without any T-2 toxin. The limit of detection and quantification were 8 ± 0.15 ng/mL (buffer) and 11 ± 0.23 ng/mL (matrix) and 12 ± 0.21 ng/mL (buffer) and 23 ± 0.31 ng/mL (matrix) (Mean ± S.D., n=3), respectively. The precision of the assay was determined by intra- (n=3) and inter-day (n=5) assays, which showed a CV less than 6%, and is well within the range of the acceptable CVs of 20%. The results indicate that the IgY can be successfully used for the detection of T-2 toxin in wheat sample extract with detection levels within the permissible limits of 100 ng/mL (Riazipour *et al.,* 2009).

To summarize, a purification method utilizing antigen-immobilized sepharose in conjunction with affinity chromatographic techniques was developed and applied successfully for the isolation of mono-specific antibodies from polyclonal pool of anti-T-2 IgY. Further the application of mono-specific anti-T-2 IgY for detection of T-2 toxin in wheat was demonstrated.

Chapter 6
Immunoassays for the detection of aflatoxin B_1, B_2, ZEN and T-2 toxins

6.1 Introduction

This chapter focuses on the development of immunoassays for the detection of mycotoxins AFB_1, B_2, ZEN and T-2 toxin. A mycotoxin quantification techniques i.e., ELISA assay on AFB_1 covalently-immobilized microtitre plates was developed. An isolation technique (immuno-affinity column) for the specific isolation of AFB_1, B_2 and ZEN, using their respective recombinant antibodies immobilized on silica particles, was also established. The approaches adopted for the generation of mycotoxin immunoassays are depicted in Figure 6.1.

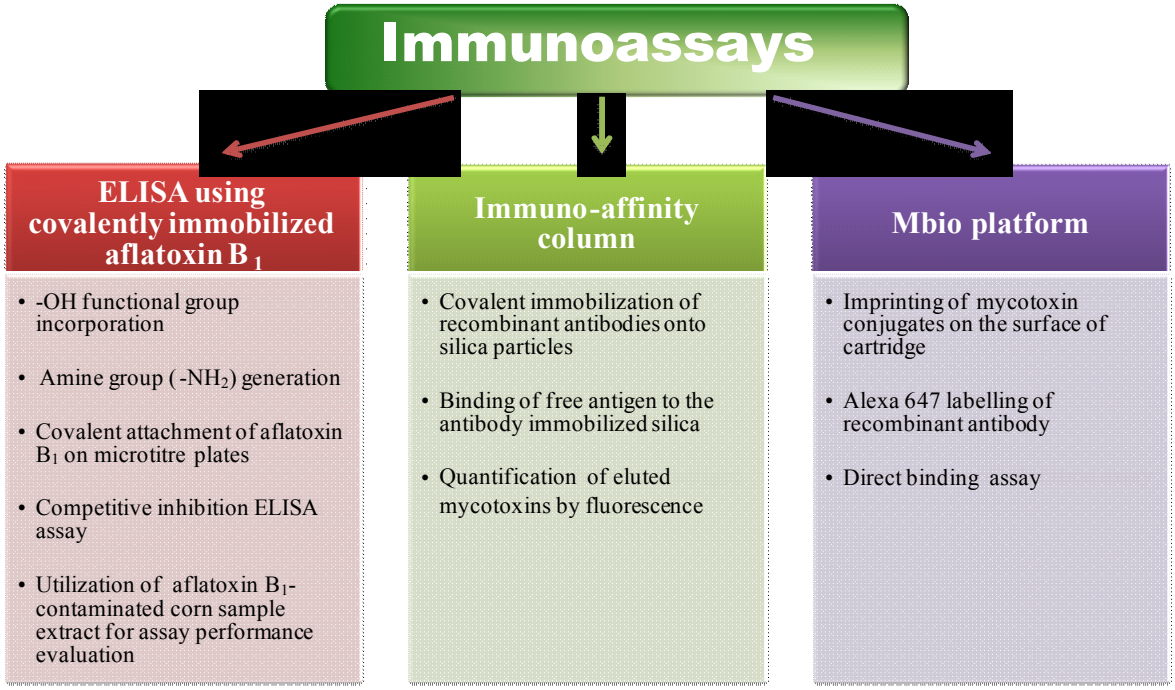


Figure 6.1: Approaches adopted for the development of immunoassays. *Two quantitative approaches (ELISA on covalently immobilized AFB_1 on microtitre plates and the MBio system) and one isolation technique (immuno-affinity column) were developed. Prior to ELISA assay performance the AFB_1 was covalently linked to amine-functionalized microtitre plates. The ELISA assay was performed in both buffer and matrix to evaluate the feasibility of the developed assay for food analysis. Immuno-affinity columns for the specific isolation of AFB_1, B_2 and ZEN were developed individually by immobilizing the anti-AFB_1, B_2 and ZEN antibodies onto silica particles. The stability, reusability and recovery rates of mycotoxins from the column was evaluated. The MBio platform is an array-based assay, measuring fluorescence intensity using planar wave guide technology. BSA conjugates of AFB_1, B_2, ZEN and T-2 were imprinted onto the MBio cartridge. Alexa 647 labelled recombinant (AFB_1, B_2 and ZEN) and polyclonal (T-2 toxin) antibodies were used.*

6.1.1 Covalent immobilization of AFB_1

Amide linkage is one of the most stable methods for immobilizing haptens onto the surface of microtitre plates (Orbulescu *et al.*, 2006). Therefore, amide linkage was selected for the covalent immobilization of carboxylated AFB_1 on amine-activated plates. Amine activation can be achieved using hydrophilic spacer arms such as 3-aminopropyl diisopropyl ethoxysilane, 3-glycidoxypropyl diisopropylethoxysilane or 3-aminopropyl triethoxysilane. The proof of concept of the covalent immobilization approach was studied by indirect and competitive ELISAs using a recombinant anti-AFB_1 Fab antibody fragment with a HA tag (Stapleton *et al.,* 2007) for the detection of AFB_1.

6.1.1.1 Results

6.1.1.1.1 Optimization and implementation of AFB_1 immobilization on microtitre plates

The covalent attachment of AFB_1 (Figure 6.2) on the microtitre plate was optimized through varying the AFB_1-CMO-NHS ester concentration and temperature. A checker-board competitive inhibition ELISA was performed to determine the optimal amount of AFB_1-CMO-NHS ester for covalent immobilization. It was found that 50 μg/mL of ester was more effective in producing an optimal absorbance response, with minimal undesirable background and better 'signal-to-noise' ratios (S/N ratio). The selection of the optimal covalent linkage temperature was performed by overnight incubation at 4^0C, 37^0C and room temperature (RT) after addition of the AFB_1 ester in 1 M carbonate buffer (pH 9.5). It was found that usage of 50 μg/mL of ester at 4^0C incubation was optimal for the generation of an ideal AFB_1-covalently immobilized plate. Incubation at 37^0C and room temperature reduced the efficiency of the covalent linkage of AFB_1 which may be due to increased hydrolysis of NHS ester at higher temperatures leading to reduced amine reactivity.

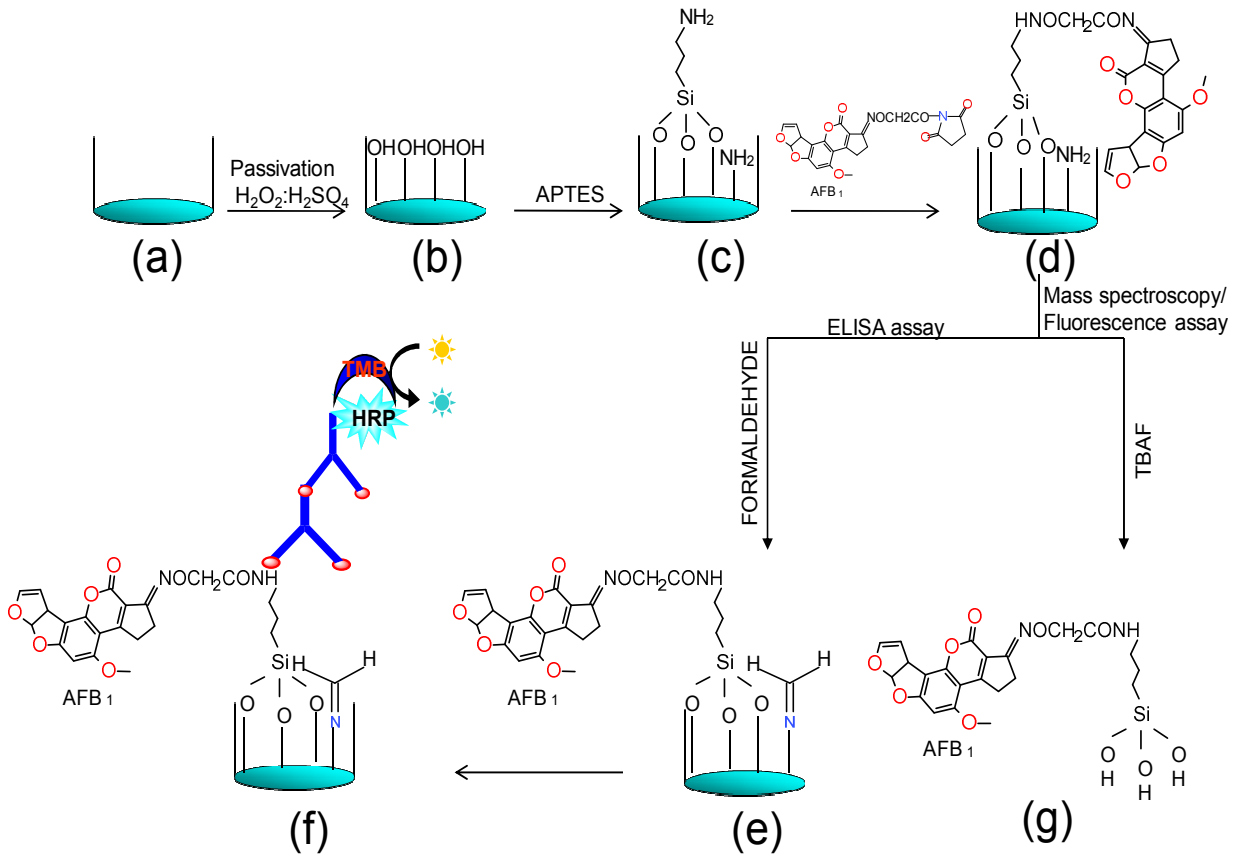


Figure 6.2: Schematic representation of AFB_1 immobilization and assay format development. *The wells of the microtitre plate were activated for the generation of hydroxyl functional groups. (b) Addition of 3-aminopropyltriethoxy silane (APTES) led to the formation of amine groups (c). The modified AFB_1-CMO-ester was attached to the surface through amide bonding (d). Two alternative procedures were performed on these plates. One set of plates was first reacted with 0.2 M formaldehyde to block the unreacted amine groups (e). Anti-AFB_1 antibody, its chromogenic reporter anti-HA-HRP and substrate 3,3′, 5,5′ – tetramethylbenzidine dihydrochloride (TMB) solution were added (f). Another set of plates was used for estimation of the level of AFB_1 immobilized on the surface, through cleaving Si-O- bond from the surface of the modified microtitre plate using 1 M tetrabutyl ammonium fluoride (TBAF). The cleaved product (g) was used for quantification of the covalently-immobilized AFB_1 by fluorescence and the molecular weight of the cleaved compound was determined using ESI mass spectroscopy.*

6.1.1.1.2 Confirmation of AFB_1 immobilization and estimating the amount of covalently immobilized AFB_1

The degree of covalent linkage was determined by quantifying the amount of covalently-immobilized AFB_1 released following cleavage, using a fluorescence-based assay. The cleavage of the AFB_1 from the surface of the microtitre plate was performed through the addition of 1 M TBAF, which cleaves the polystyrene–O–Si bond between the AFB_1 and polystyrene surfaces under neutral conditions. A standard calibration curve was plotted from the fluorescence values of standard AFB_1 concentrations at 360/440 nm (Excitation/Emission) (Figure 6.3(a)). Simultaneously, the fluorescence of the cleaved product was also determined. An initial loading value of AFB_1 of 0.73 nmol/cm^2 was calculated. The molecular weight of the cleaved product was determined by ESI mass spectroscopy. The chromatogram of the cleaved product from the mass spectroscopy data showed a major peak at 503.6 (Figure 6.3(b)), which corresponds to AFB_1-CMO-NH-$(CH_2)_3$-$Si(OH)_3$ (Calculated: 504 Da, Observed: 503.6 Da). From this it can be concluded that neither free AFB_1 (M.Wt. 312.27) nor the ester of AFB_1-CMO-COOH (M.Wt. 385) was present on the surface of the covalently-immobilized plate.

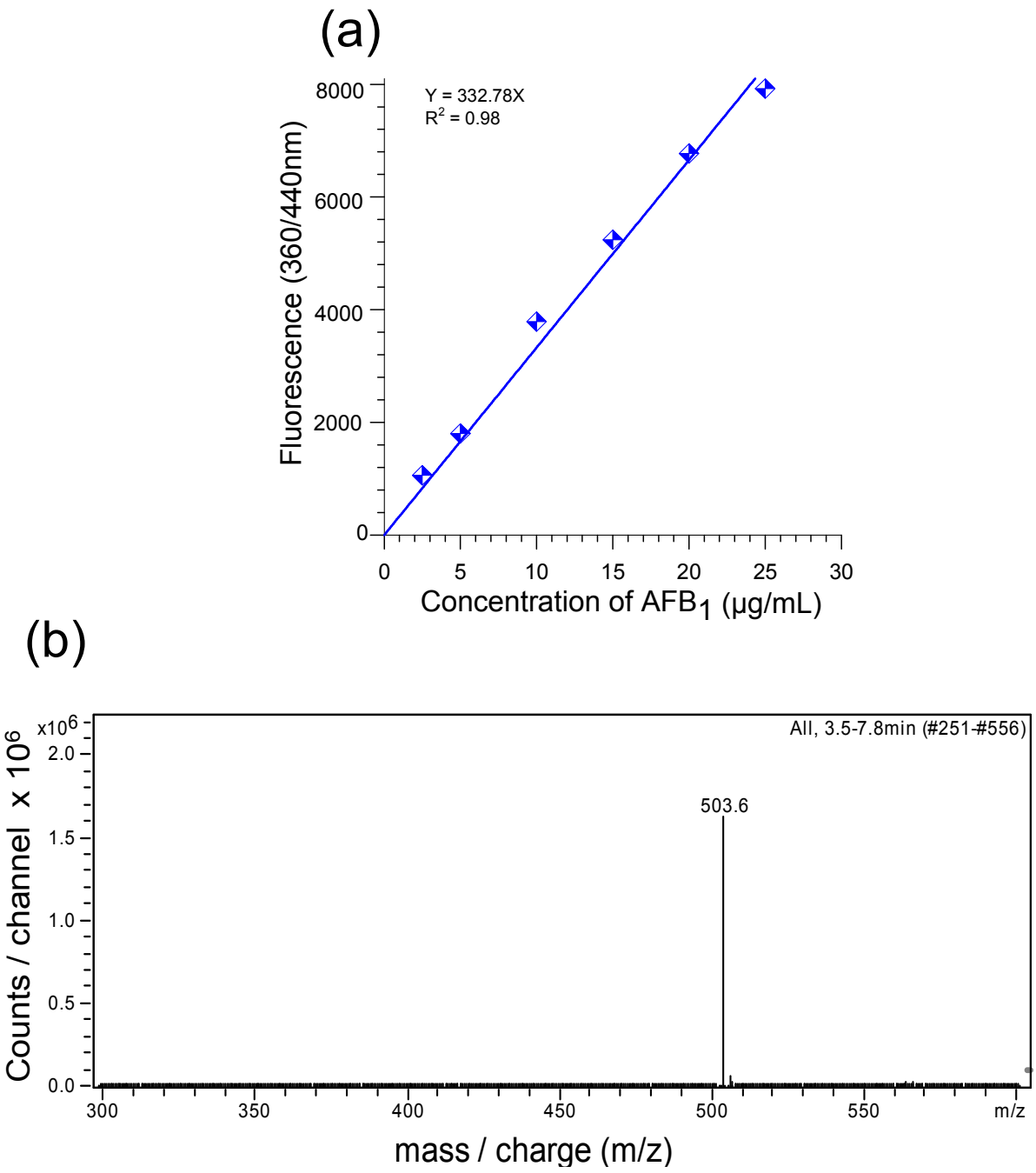


Figure 6.3: Fluorescence (a) and ESI mass (b) analysis of the cleaved AFB_1 that was covalently immobilized on the surface of the microtitre plate. *The covalently linked AFB_1 was cleaved from the surface using 1 M tetrabutyl ammonium fluoride (TBAF). The amount of covalently-immobilized AFB_1 was determined through substituting the fluorescence value of cleaved product into the plot equation drawn using varying concentrations of AFB_1 (2.5-25 µg/mL). An ESI mass spectrum in positive mode was performed to determine the molecular mass of the cleaved product. The molecular weight of the cleaved product corresponds to 503.6 Da, which represents AFB_1-CMO-NH-$(CH_2)_3$-Si-O-polystyrene.*

6.1.1.1.3 Functionality of AFB_1-covalently immobilized microtitre plates

After successful covalent attachment of the AFB_1 on the microtitre plate surface, the next step was immunoassay format development. In the process of assay development, the initial step is blockage of non-specific binding sites. The un-reacted NH_2 groups were blocked using 0.2 M formaldehyde. The blockage of the plates was followed by the determination of functionality of the microtitre plate using indirect and competitive inhibition ELISAs with the anti-AFB_1 Fab antibody that was generated in our lab. An anti-CRP recombinant antibody, which should not bind to the hapten or its conjugate was used as a control. The absence of signal without anti-AFB_1 antibody in the assay, indicated its specificity. The negative control experiments were performed by coating the microtitre plate with milk Marvel and BSA, in the case of the AFB_1 conjugate passively-adsorbed plate, and formaldehyde and PBS, in the case of the AFB_1 covalently-immobilized plates. Three types of AFB_1 conjugates i.e, AFB_1-BSA, AFB_1-OVA and AFB_1-KLH conjugates were used. Due to the lack of variation in the antibody recognition characteristic when assays were performed using either KLH, BSA and OVA conjugates of AFB_1, further competitive inhibition ELISAs were performed on microtitre plates with the passively-adsorbed AFB_1-BSA conjugate. Negative control studies showed no non-specific binding. The IC_{50} value was estimated from the mid-point inflection of the maximum value, on each curve. The IC_{50} results were 27 ± 2.7 ng/mL (Mean ± S.D.; n=3) and 77 ± 2.6 ng/mL (Mean ± S.D.; n=3) in the case of covalently-immobilized AFB_1 and passively-adsorbed AFB_1-BSA conjugate, respectively (Figure 6.4).

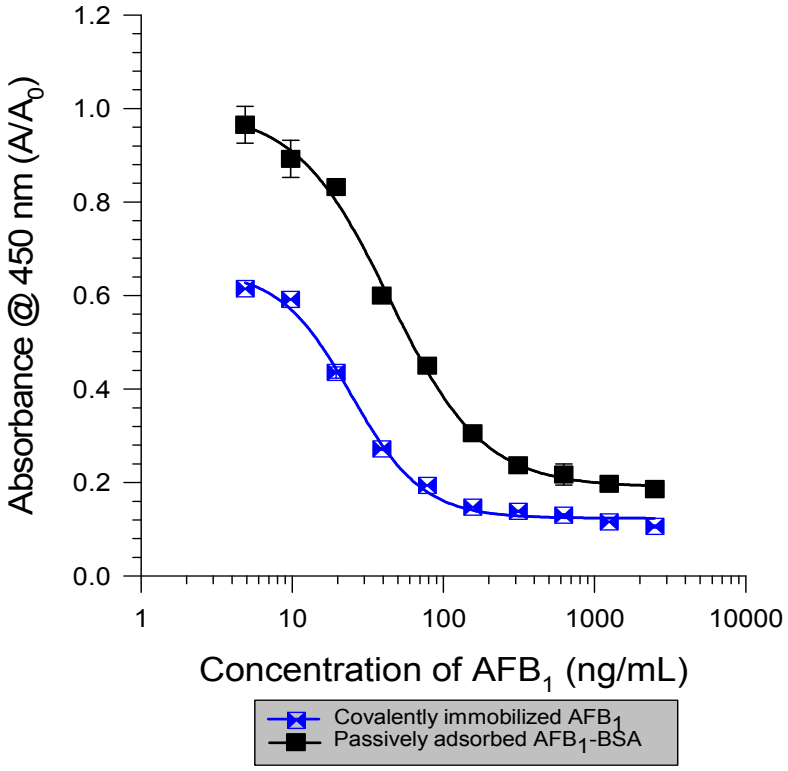


Figure 6.4: Representative standard curve for the competitive ELISA. *The assay was performed in triplicate on the microtitre plates with covalently- (◆) immobilized AFB_1 and passively- (■) adsorbed AFB_1-BSA conjugate. A 1:10,000 dilution of the anti-AFB_1 Fab antibody was used for the inhibition using a free AFB_1 concentration ranging from 0.0048-2.5 µg/mL. The error bars represents the standard deviation (n = 3).*

6.1.1.1.4 Cross reactivity profile of antibody tested on AFB_1 covalently and passively adsorbed microtitre plates

Cross reactivity studies were performed on both covalently-immobilized and passively-adsorbed plates by CI-ELISA (section 2.2.6.1.6) using 0.0048-2.5 µg/mL of AFB_2, G_1 and G_2 and 0.009-10 µg/mL Zearalenone, Fumonisin B_1 and T-2 toxins. It is evident from the results that the antibody is specific to AFB_1 in comparison to other aflatoxin variants, as indicated by the cross reactivity data presented in Table 6.1. The lack of cross reactivity to other mycotoxins such as Zearalenone, Fumonisin B_1 and T-2 toxin (data not shown) indicates the specificity of the antibody to aflatoxins.

Table 6.1: *Cross reactivity profile of the antibody when assayed on AFB_1-covalently immobilized and AFB_1-BSA passively adsorbed microtitre plates*

Mycotoxin	AFB_1-covalently immobilized plate		AFB_1-BSA passively adsorbed plate	
	IC_{50} value (ng/mL ± SD) [a]	% cross reactivity [b]	IC_{50} value (ng/mL ± SD) [a]	% cross reactivity [b]
AFB_1	20 ± 0.6	100%	72 ± 0.2	100%
AFB_2	1,000 ± 0.9	2%	1,600 ± 0.4	4%
AFG_1	110 ± 0.7	18%	240 ± 0.8	30%
AFG_2	1,500 ± 0.6	1%	1,700 ± 0.7	4%

[a] *IC_{50} value is a measure of the amount of AFB_1 required to inhibit the anit-AFB_1 Fab antibody by half the maximum inhibition value.*

[b] *% cross reactivity is calculated using the formula IC_{50} value of AFB_1/ IC_{50} value of test mycotoxin X 100*

6.1.1.1.5 Effect of varying conditions on ELISA assay performance

The effect of incubation times of the primary and secondary antibodies on the assay sensitivity was investigated (Figure 6.5). The sensitivity of the assay performed on microtitre plate with covalently-immobilized AFB_1 remains unaltered, with the increase in incubation time from 20 - 120 mins. From the analysis it was found that 60-80 mins of incubation time is required for attaining the desired sensitivity levels when passively-adsorbed AFB_1-BSA conjugate microtitre plates were used.

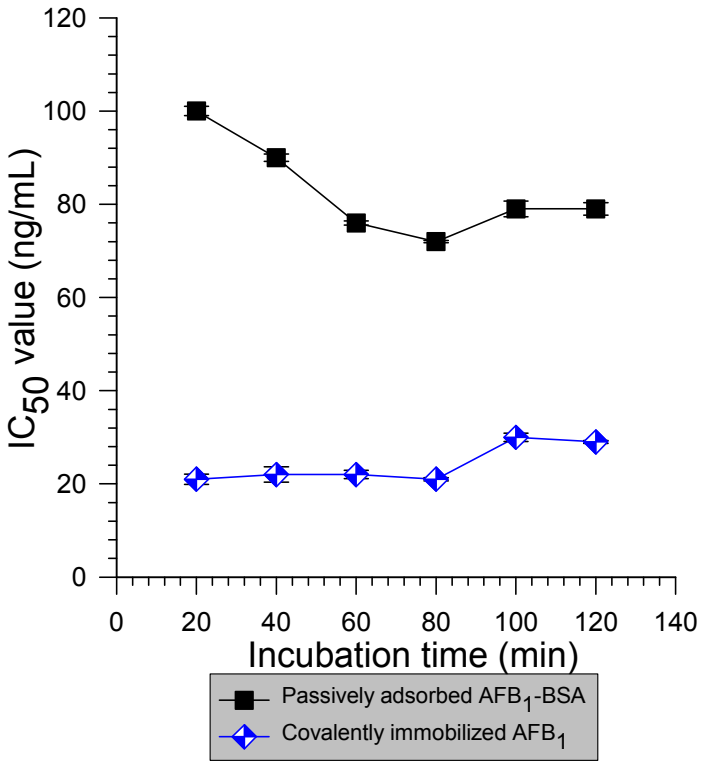


Figure 6.5: Studies on the effect of incubation time on the performance of the competitive inhibition assay. *The primary and secondary antibodies were incubated individually for 20, 40, 60, 80, 100 & 120 mins on different sets of microtitre plates with covalently- (◆) immobilized AFB_1 and passively-(■) adsorbed AFB_1-BSA conjugate. The IC_{50} value of the assay at various incubation times was the same in the case of covalently- immobilized AFB_1. With regard to passively adsorbed AFB_1-BSA the IC_{50} value at 20 and 40 mins of incubation was higher than the normal IC_{50} value which is attained after 60-80 mins of incubation. This shows that the optimal incubation time was 20 mins and 60-80 mins for covalently- immobilized AFB_1 and passively-adsorbed AFB_1-BSA, respectively. Individual points on the graph represent the mean ± S.D. of three determinations.*

The effect of the number of washes on the assay performance was analyzed through increasing the washes from 3 to 12 with PBST and PBS (to remove unbound reagents). From the values obtained it can be deduced that stringent washing does not affect the sensitivity of the assay performed (Table 6.2). The results obtained (Table 6.2), indicate a increase in the IC_{50} value of assay with increasing methanol concentration from 0% to 40% (v/v) i.e., from 75 ± 2.5 ng/mL (Mean ± S.D.; n=3) to 125 ± 2 ng/mL (Mean ± S.D.; n=3) in the case of passively-adsorbed AFB_1-BSA conjugate. The sensitivity of the assay performed on the microtitre plate with covalently-immobilized AFB_1 remains unaltered up to 40% (v/v) methanol, after which the

assay sensitivity was reduced drastically. Furthermore, the effect of pH (Table 6.2) was studied, which shows that increase of pH from 6 to 9 has no significant impact on the assay performance using either type of microtitre plates (covalenly-immobilized AFB_1 or passively-adsorbed AFB_1-BSA conjugate microtitre plates)

Table 6.2: *Evaluation of the factors altering the assay performance (n=3)*

Factor	IC_{50} value[a] (ng/mL ± S.D.)	
	Covalently immobilized AFB_1	Passively adsorbed AFB_1-BSA
Number of washes with both PBST & PBS		
3	17 ± 1	72 ± 1
6	25 ± 1	80 ± 1
9	25 ± 1	75 ± 1
12	22 ± 1	77 ± 1
Methanol (v/v)		
0%	22 ± 2	75 ± 3
10%	32 ± 1	110 ± 1
20%	35 ± 1	112 ± 2
30%	25 ± 2	115 ± 2
40%	33 ± 2	125 ± 2
pH		
6	22 ± 1	79 ± 1
7.4	28 ± 1	80 ± 1
9	26 ± 1	74 ± 2

[a] *IC_{50} value is a measure of the amount of AFB_1 required to inhibit the anti-AFB_1 Fab antibody by half the maximum inhibition value. The units are ng/mL ± S.D. of the value obtained from experiments performed in triplicate. The IC_{50} values of both the covalently-immobilized AFB_1 and passively-adsorbed AFB_1-BSA conjugate were compared under varying experimental conditions.*

6.1.1.1.6 Analysis of the storage stability and re-usability of the covalently-immobilized AFB_1 microtitre plates

To determine the storage stability of the microtitre plates that were covalently-immobilized with AFB_1 and passively-adsorbed with AFB_1-BSA conjugate, competitive inhibition assays were performed every alternative day on both these plates stored in a vacuum dessicator. The results show that the covalently-immobilized AFB_1 and passively-adsorbed AFB_1-BSA conjugate microplates were stable up to 14 and 10 days, respectively, (Figure 6.6) with a significance of $p = 0.0002$ (Student's t-test).

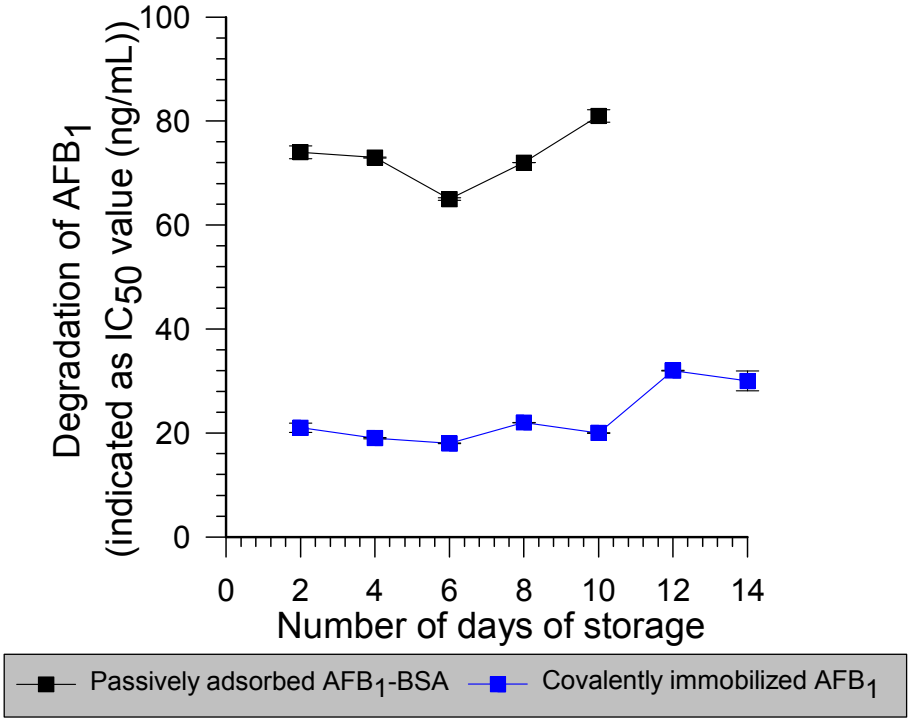


Figure 6.6: Storage stability of the AFB_1-immobilized microtitre plates. *Both the covalently- (■) immobilized AFB_1 and passively- (■) adsorbed AFB_1-BSA conjugate microtitre plates were placed in a vacuum dessicator over a period of 15 days and competitive assays were performed every alternative day. The covalently-immobilized AFB_1 was stable upon storage up to 14 days. The assays performed on the microtitre plates, with passively-adsorbed AFB_1-BSA, have given inconsistent results showing the degradation of the conjugate upon storage after 10 days. The points on the graph represent mean where n = 3 and the error bars represent the standard deviation.*

The re-usability of the microtitre plates was tested by treating the plates with a chaotropic mixture of 8 M urea, sodium dodecyl sulfate and mercaptoethanol. The IC_{50} value of the assay performed on the non-chaotropic-treated microtitre plate was considered as 100%. The percentage variation in the sensitivity of the assay performed on both microtitre plates with covalently-immobilized AFB_1 and passively-adsorbed AFB_1-BSA conjugate, was analyzed using the formula (obtained value/initial value) X 100. Upon successive usages, after treatment with the dissociation buffer, the percentage binding retention when compared to the initial 100% are 96%, 96%, 84%, 80% and 60% in the case of covalently-immobilized microtitre plate, whereas it was 75%, 73.3%, 50% and inconsistent values from the fourth usage onwards, in the case of passively-adsorbed plates (Figure 6.7).

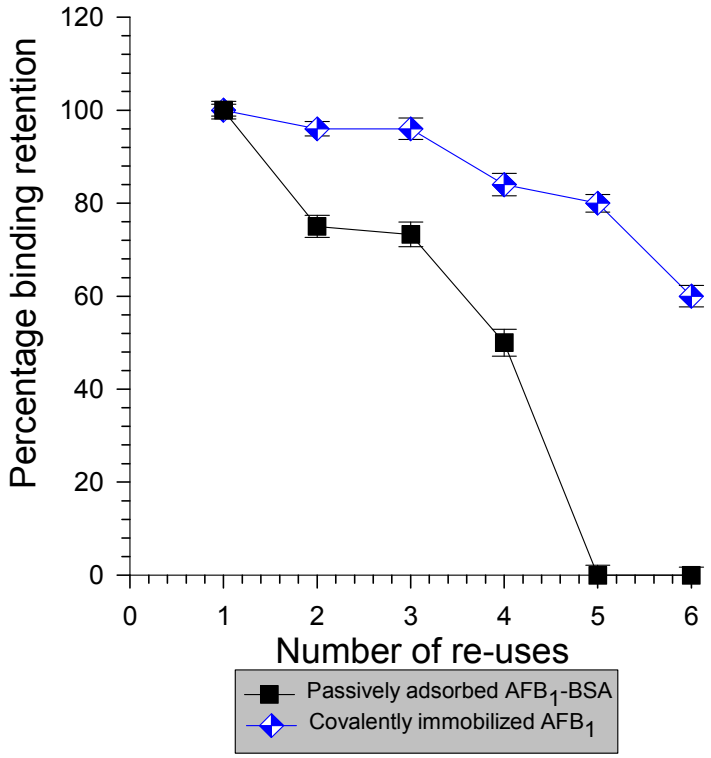


Figure 6.7: Comparative analysis of percentage binding retention for AFB_1 covalently or non-covalently attached to plates. *The AFB_1-immobilized microtitre plates were regenerated with chaotropic agents after the performance of a competitive inhibition enzyme-linked immunoassay. Binding retention value of 60-100% was observed in the assay performed on the covalently-immobilized AFB_1 (◆). With regard to the assay performed on the microtitre plate with passive (■) adsorption of AFB_1-BSA there was 0% binding retention after the fifth chaotropic treatment. The results are the mean ± S.D. for three determinations.*

6.1.1.1.7 Extraction of AFB_1 from corn and its utilization for validating immunoassay developed on AFB_1 covalently-immobilized plates

The amount of recovered aflatoxin from the reference corn material (TR-A100) was quantified by fluorescence estimation at 360/440 nm (Excitation/Emission) using a standard calibration curve (slope equation y = 361.7x (R^2 = 0.99)) with known amounts of AFB_1 (10-320 ng/mL) diluted in 40% (v/v) methanol in water. The certified level of aflatoxins in the corn sample (TR-A100) was 3.7 µg/Kg and the obtained values (fluorescence estimation) were 3.5 ± 0.9 µg/Kg (Mean ± S.D.; n=10). The corn extract was used to perform CI-ELISA on AFB_1 covalently-immobilized microtitre plates (section 2.2.6.1.6). The limit of blank (LOB) was

determined through recording the absorbance value of twenty blank samples. The limit of detection (LOD) was calculated using the formula LOB + 3 X S.D. of LOB. The IC_{50} value of the CI-ELISA performed on AFB_1- covalently immobilized plate using corn matrix was 35 ± 1.1 ng/mL with LOD 12 ± 2.3 ng/mL (Mean ± S.D.; n=3). The trueness of the assay was evaluated through performing intra- (n=3) and inter-day (n=5) assay using the corn extract and represented as percentage coefficient of variance (% CVs) (Table 6.3).

Table 6.3: *Intra- (n=3) and inter-day (n=5) validation of CI-ELISA assay performed on AFB_1 covalently immobilized microtitre plates using corn reference material*

AFB_1 concentration (ng/mL)	Intra-day assay			Inter-day assays		
	Back-calculated concentration (ng/mL)	% CVs	% Accuracies	Back-calculated concentration (ng/mL)	% CVs	% Accuracies
2500	2100	2.4	84	2300	5.1	92
1250	1320	1.4	105.6	1290	3.7	103.2
625	611	5.2	97.8	620	2.8	99.2
312.5	297	1.9	95	289	5.2	92.5
156.25	163.2	0.7	104.5	159.2	4.9	101.9
78.125	75.2	1.8	96.3	76.9	2.0	98.5
39.062	39	4.7	100	41	1.7	105.1
19.531	18	2.6	94.7	19	5.3	100
9.765	10	1.9	111.1	9	1.9	100
4.882	5	4.6	104.2	4.3	1.6	89.6

6.1.2 Immuno-affinity column for the isolation of AFB_1, B_2 and ZEN

The aim of this phase of the research was to use specific recombinant antibodies, immobilized on silica in a column format, for the simultaneous capture, subsequent elution and analysis of mycotoxins. Mycotoxins were added to antibodies bound to silica in the column, incubated for 2 hrs, eluted and quantified. Stability and re-usability studies were undertaken and the feasibility of using column evaluated.

6.1.2.1 Immobilization of anti-AFB_1, AFB_2 and ZEN recombinant antibodies onto the silica

The aminated silica was generated through the treatment of allylated silica (formed as a result of treating silica with allylmethoxysilane in the presence of toluene) with a cysteamine linker under UV light. The initial amine loading value, as determined by the ninhydrin assay, was 1092 μmole of amines/g of silica beads. The NHS groups were incorporated onto the silica surface through treating the aminated silica with sebacic acid. The recombinant antibodies can then be attached to the NHS groups on the silica, leading to the formation of silica beads immobilized with antibodies.

6.1.2.2 Evaluation of functionality of individual immuno-affinity columns (IACs)

6.1.2.2.1 Determination of antigen binding to the affinity columns

The antigen binding capacity of AFB_1, B_2 and ZEN immuno-affinity columns (each containing 200 mg of either anti-AFB_1 or AFB_2 or ZEN antibody-immobilized silicas) were assessed individually by applying 1 mL of 0.3-8 μg/mL of AFB_1, B_2 and ZEN antigens. From Table 6.4, it can be seen that the maximum antigen binding capacities of AFB_1 (with 176.28 μg of anti-AFB_1 Fab antibody/200 mg of silica), B_2 (with 127.6 μg of anti-AFB_2 scFv/200 mg of silica) and ZEN (with 152.8 μg of anti-ZEN scFv/200 mg of silica) columns were 4, 5.4 and 6.7 μg, respectively.

Table 6.4: *Antigen binding capacity of anti-AFB_1, B_2 and ZEN immuno-affinity columns (n=3)*

Amount of mycotoxins added (µg/mL)	Amount of mycotoxins eluted (µg/mL)		
	AFB_1	AFB_2	Zearalenone
0.3	0.2 ± 0.11	0.2 ± 0.21	0.2 ± 0.06
0.6	0.5 ± 0.09	0.5 ± 0.17	0.5 ± 0.31
1.2	1.1 ± 0.25	1.1 ± 0.08	1.2 ± 0.27
2	1.9 ± 0.1	1.9 ± 0.14	1.9 ± 0.18
4	3.9 ± 0.28	3.9 ± 0.09	3.9 ± 0.28
6	3.9 ± 0.14	5.1 ± 0.27	5.9 ± 0.18
8	4 ± 0.08	5.4 ± 0.04	6.7 ± 0.21

6.1.2.2.2 Effect of percentage methanol on elution potential and re-usability of the immuno-affinity column

The role played by the methanol percentage used in elution of mycotoxin (Table 6.5) was evaluated by measuring the % recovery. For this 20, 40, 60, 80 and 100% (v/v) methanol were used for eluting AFB_1, B_2 and ZEN from their respective immuno-affinity columns. The amount of mycotoxins eluted using 100% (v/v) methanol was taken as 100% and percentage recoveries attained with other methanol concentration were calculated using the formula

$$\% \text{ Recovery} = \frac{\text{Amount of mycotoxins eluted using varying methanol concentration}}{\text{Amount of mycotoxins eluted using 100\% (v/v) methanol}} \times 100$$

Table 6.5: *Mycotoxins percentage recovery using varying methanol concentration (n=3)*

% methanol used for elution	% Recovery		
	AFB_1	AFB_2	Zearalenone
20	48 ± 0.2	49 ± 0.8	41 ± 0.4
40	79 ± 0.7	83 ± 0.7	89 ± 0.8
60	83 ± 1	85 ± 1.5	92 ± 0.7
80	92 ± 0.6	93 ± 1.7	94 ± 1.9
100	100 ± 1.8	100 ± 0.4	100 ± 0.3

To facilitate multiple usages of a single immuno-affinity columns for subsequent extraction, the role of methanol elution on column efficiency, as determined by number of re-uses, was evaluated. Table 6.5 indicates that the % recoveries of the three myctoxins using 20% (v/v) methanol falls below 50%. Hence further studies on the re-use were performed using methanol concentrations above 20% for elution. The percentage recovery of mycotoxins after each successive re-usage with 40, 60, 80 and 100% (v/v) methanol for up to 15 re-uses was evaluated. This indicated that using 40% (v/v) methanol the columns could be used more times (13 times for AFB_1 & B_2 column and 15 times for ZEN column) with percentage recoveries above 75%.

6.1.2.2.3 Protein estimation after successive usages of columns

The silica beads (5 mg) obtained after 15 usages were dried in a vacuum desiccator. The amount of antibody bound to the silica surface was estimated by the BCA assay against BSA standard, as described in section 2.2.6.2.5. These results demonstrated that after 15 reuses (using 40% methanol for eluting), there is a decrease in the amount of protein bound to the silica surface (Table 6.6).

Table 6.6: *Amount of protein bound to silica before and after fifteen uses using 40% methanol for elution (n=3)*

Type of antibody immobilized	Amount of antibody bound to 5 mg of silica	
	Prior to first use (µg/5 mg of silica)	After reusing 15 times (µg/5 mg of silica)
Anti-AFB_1 Fab	4.40 ± 1.1	2.57 ± 0.9
Anti-AFB_2 scFv	3.19 ± 0.8	1.74 ± 1.3
Anti-ZEN scFv	3.82 ± 1.5	1.94 ± 1.1

6.1.2.2.4 Storage stability studies of the immuno-affinity columns

To check the stability of silica at varying temperatures, 5 mg of dried silica beads were stored at room temperature, 4^0C and 37^0C for 24 hrs. The amount of antibody bound on the silica was estimated by BCA assay against BSA as a standard. Unmodified silica was used as a control for the assay. The silica was stable at most of the conditions, although a slight decrease in the absorbance was noted after incubation at 37^0C for 24 hrs as shown in Figure 6.8. This study reveals that the covalent conjugation of antibody through amide bonds on silica beads is very stable.

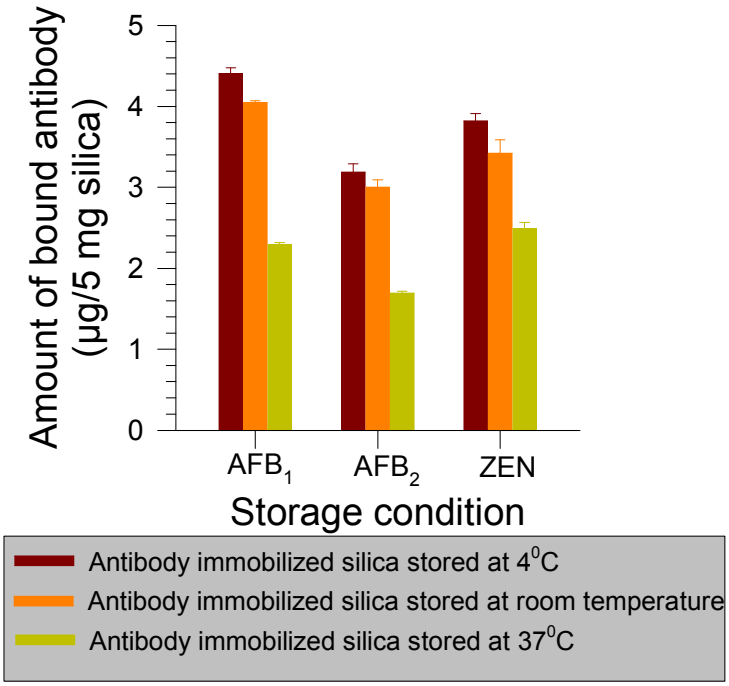


Figure 6.8: Stability of silica-bound protein stored for 24 hrs at different temperatures by BCA assay (n=3).

6.1.2.3 Multi-analyte immuno-affinity column

6.1.2.3.1 IACs washing

After assessing the maximum antigen binding and methanol tolerance of the individual IACs, a multi-analyte column was designed with equal amounts (3 X 200 mg = 600 mg) of silicas immobilized with three different recombinant antibodies. To the multi-analyte column 8 µg of AFB_1, B_2 and ZEN antigens dissolved in 1 mL were added individually, followed by incubation for an hour and washing with 1X PBS (1 mL) for 10 times to ensure complete removal of unbound or loosely bound mycotoxins (AFB_1, B_2 and ZEN) where steady fluorescence reading (360/440 nm for AFB_1 or B_2 and 236/440 nm for ZEN) was recorded (Figure 6.9).

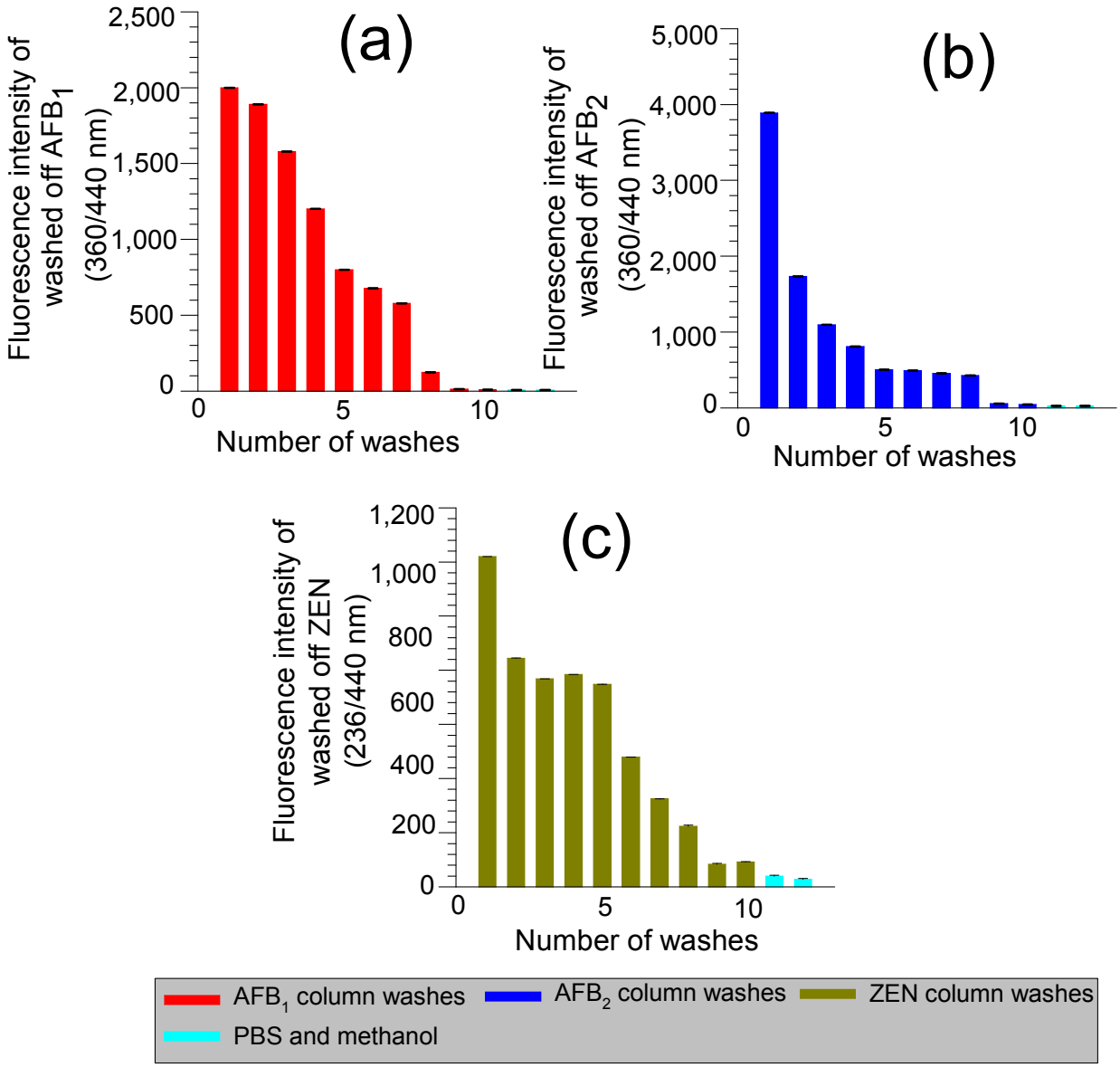


Figure 6.9: Graphical representation of the washing AFB_1, B_2 and ZEN immuno-affinity columns. *To each of the immuno-affinity column 8 µg of mycotoxins were added. After 10 subsequent washes with 1X PBS, the fluorescent reading was stabilized. The stabilized fluorescent reading indicates the removal of loosely bound or excess of mycotoxin from the immuno-affinity column. The negative controls in this experiment include fluorescence readings of methanol and 1X PBS.*

6.1.2.3.2 Quantification of eluted AFB_1, B_2 and ZEN from multi-analyte IAC

After subjecting the multi-analyte column to washing (section 6.1.2.3.1) the antigens bound to the antibodies on the silica surface were eluted using 40% (v/v) methanol. Quantification of the AFB_1, B_2 and ZEN eluted from the column was determined by fluorescence assay. The fluorescence intensity of varying concentrations (0.125-16 µg/mL) of AFB_1, AFB_2 and ZEN standards in 40% (v/v) methanol was determined followed by the generation of calibration curves for each individual mycotoxin. The fluorescence intensity of eluted myctoxins were substituted in the slope equations of calibration curve generated using AFB_1, B_2 and ZEN standard (Figure 6.10) to determine the amount of eluted mycotoxins. The amount of eluted AFB_1, B_2 and ZEN from IAC were recorded as 3.9 ± 1.9, 5.3 ± 0.9 and 6.2 ± 1.5 µg/mL, respectively.

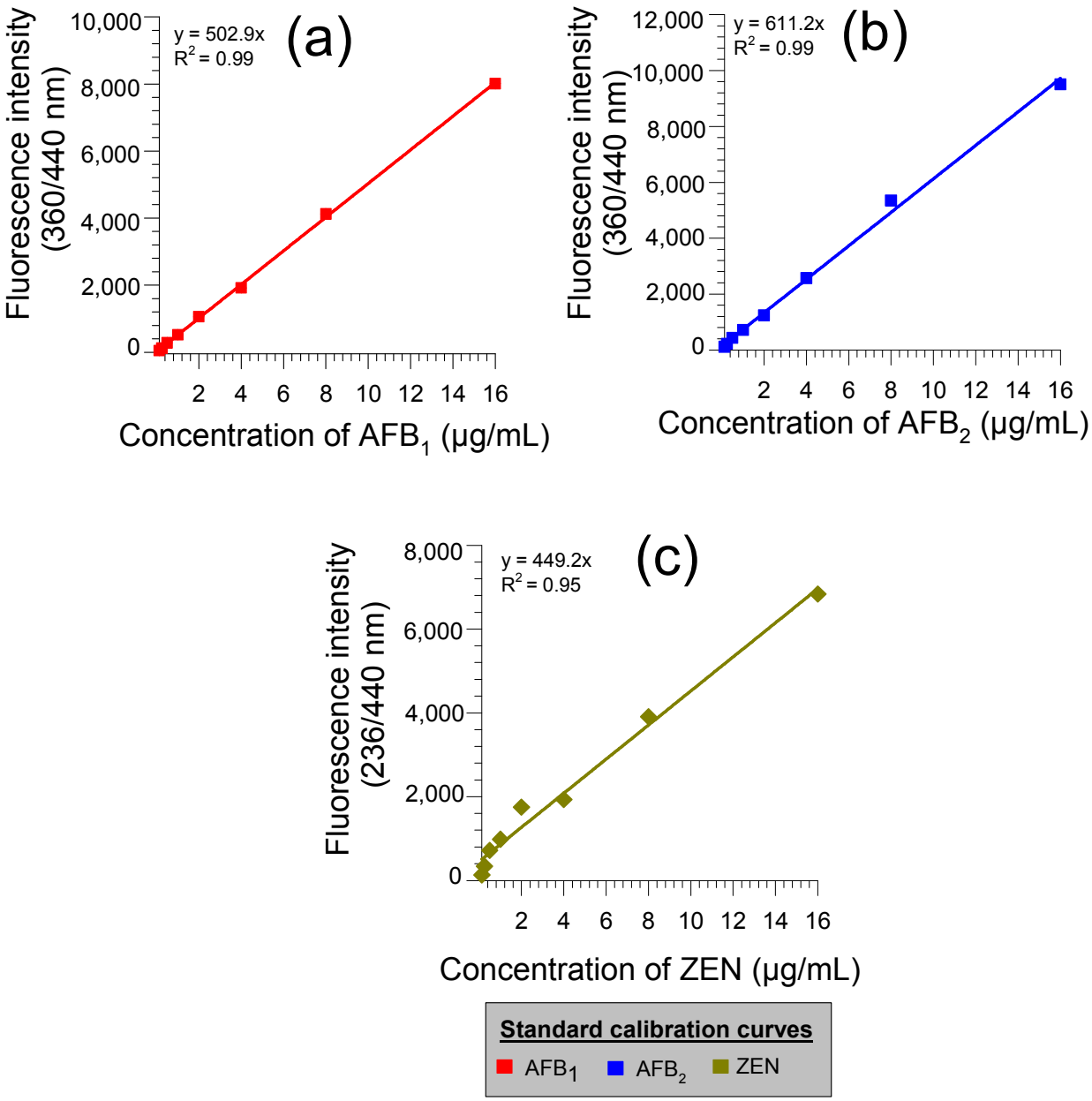


Figure 6.10: Calibration curve for AFB_1, B_2 and ZEN (0.125-16 µg/mL). *The amount of eluted AFB_1, B_2 and ZEN were calculated by substituting the fluorescence reading of the eluants in their respective slope equations.*

6.1.2.3.3 Re-usability of multi-analyte immuno-affinity column

The re-usability of the multi-analyte column (Figure 6.11) was tested by quantifying the eluted amounts of AFB_1, B_2 and ZEN from the immuno-affinity columns (200 mg each of silicas immobilized with three different antibodies) up to 15 times. The elutions of mycotoxins from unmodified silica were also tested (negative control). The re-usability was assessed by measuring the change in the percentage recovery upon successive uses using the formula % recovery = amount of mycotoxin recovered after first use/amount of mycotoxin recovered during the first usage X 100. The results indicate that the generated multi-analyte immuno-affinity column could be used for extraction of AFB_1, B_2 and ZEN up to 6 times with minor loss in the binding capability. The repetitive washing steps might have resulted in the cleavage of the covalently bound antibodies from the silica surfaces.

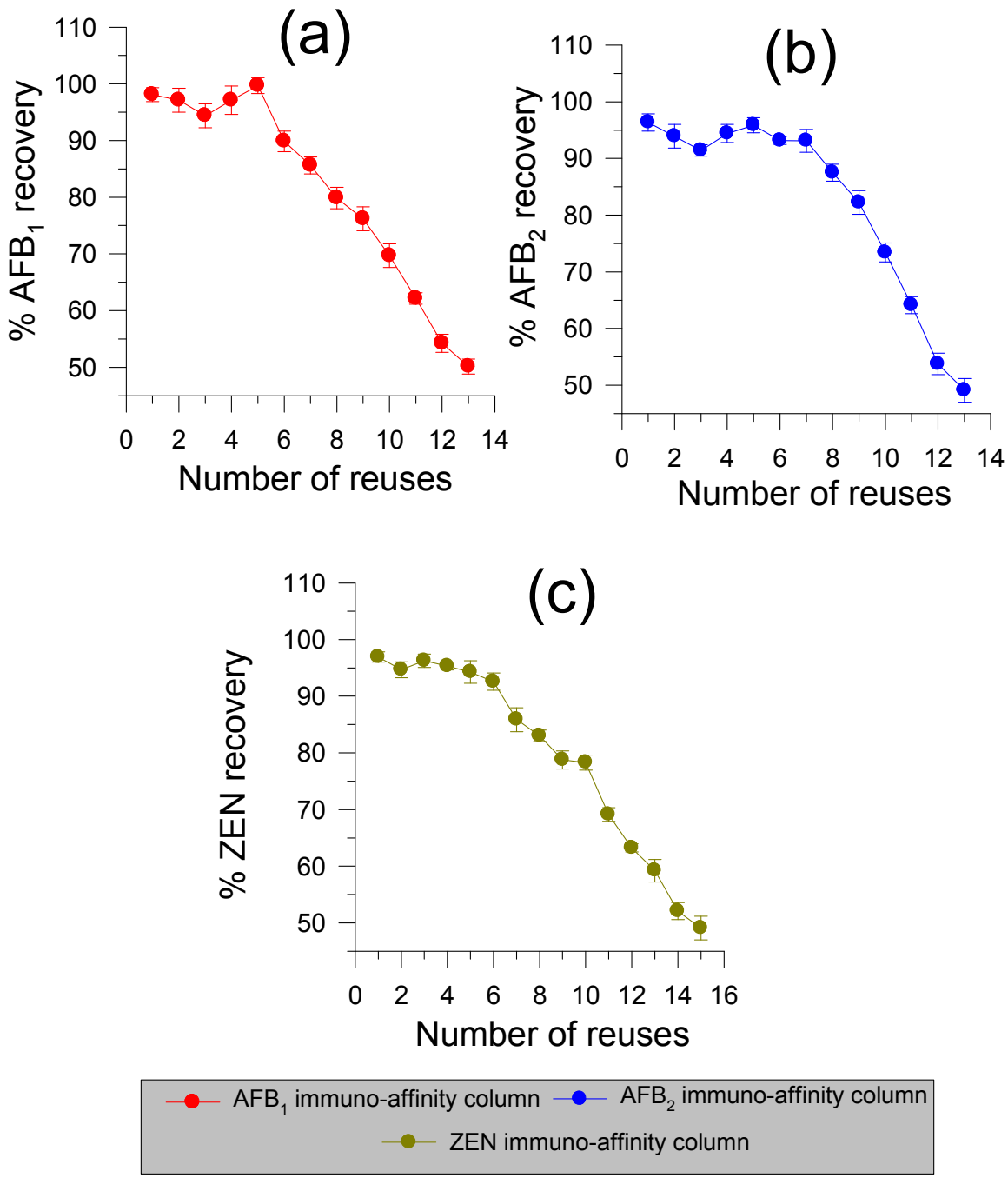


Figure 6.11: Performance of immuno-affinity columns after fifteen successive uses. *The three immuno-affinity columns were used for 15 times and the recoveries of mycotoxins were evaluated. A 50% loss in the activity of the immuno-affinity column was noticed after subjecting the AFB_1, B_2 and ZEN columns to 13, 13 and 15 times of re-uses, respectively.*

6.1.2.3.4 Protein estimation of the multi-analyte column

The silica beads containing three antibodies (5 mg) obtained after 15 usages from the above step were dried in vacuum desiccator. The amount of antibody bound to the silica surface was estimated by BCA assay against BSA standard, as described in section 2.2.6.2.5. The amount of antibodies bound to silica prior to first use was 11.41 ± 1.2 µg/ 5 mg of silica, but whereas after using the silica in an mutli-analyte column for 15 times the amount of bound antibodies decreased to 6.25 ± 0.9 µg/ 5 mg of silica. These results demonstrate that after 15 reuses, there is a almost 50% loss in the amount of bound protein, which will decrease the efficiency of the multi-analyte immuno-affinity column.

6.1.3 Preliminary results of MBio assay

6.1.3.1 Direct binding assay

The utility of Alexa-647 labelled anti-AFB_1, B_2, ZEN and T-2 toxin antibodies was determined by performing direct binding assays using the MBio cartridges that were imprinted with 50 and 100 µg/mL of AFB_1, B_2, ZEN and T-2 toxin BSA conjugates along with controls such as BSA-647 (positive control), buffer and microcystin conjugates (negative control) (Figure 6.12).

Contents	BSA-647	Buffer	Microcystin LR –BSA	Microcystin LR –BSA	Microcystin LR -OVA	Microcystin LR –OVA	Buffer	**AFB_1-BSA**	**AFB_1-BSA**	Buffer	BSA-647	Buffer	**AFB_2-BSA**	**AFB_2-BSA**	Buffer	**ZEN-BSA**	**ZEN-BSA**	Buffer	**T-2-BSA**	**T-2-BSA**	Buffeer	BSA-647
Print Conc.(µg/mL)	**2**	**0**	**50**	**5**	**10**	**5**	**0**	**50**	**5**	**0**	**2**	**0**	**100**	**50**	**0**	**100**	**50**	**0**	**100**	**50**	**0**	**2**

Figure 6.12: Layout of the MBio cartridge imprinted with varying concentrations of conjugates.

The preliminary results of the direct binding assay, which was determined by the addition of 1/100 dilution of Alexa-647 labelled anti-AFB_1, B_2, ZEN and T-2 toxin antibodies to the cartridge, recorded a fluorescence intensity much higher than the background fluroscence values (Table 6.7), thus indicating that preliminary studies refer the assay works.

Table 6.7: *Normalized values obtained upon addition of anti-AFB1, B2, ZEN and T-2 antibodies labeled with ALEXA-647*

Imprinted content	Signal obtained (fluorescence intensity)
BSA-647 (positive control)	83.74
Buffer (negative control)	4.68
Microcystin LR- BSA (negative control)	10.73
Microcystin LR- OVA (negative control)	8.45
AFB_1-BSA 50 µg/mL	674.86
5 µg/mL	95.47
AFB_2-BSA 100 µg/mL	624.46
50 µg/mL	434.88
ZEN-BSA 100 µg/mL	520.31
50 µg/mL	329.12
T-2-BSA 100 µg/mL	77.59
50 µg/mL	43.43

6.1.4 Discussion

The covalent immobilization strategy of haptens involves, the generation of hydroxyl functional groups. Piranha solution (Wang *et al.*, 2008) is generally useful for the generation of hydroxyl groups and this was used for these microtitre plates. Amine (NH_2) groups were incorporated by surface modification with APTES (3-aminopropyltriethoxy silane). To avoid multi-layers of APTES on the surface, the plates were washed thoroughly with 30% (v/v) ethanol and PBS. As baking allows the formation of stable cross-linked attachment of APTES

to the surface (Srinivasan *et al.*, 2011), the plates were then incubated at 80^0C for 2 hrs. After 80^0C incubation, AFB_1-CMO ester was added to the individual wells of the microtitre plate. The amide bond formation, which governs the covalent linkage of AFB_1 to the modified surfaces of the microtitre plate, was achieved through overnight incubation of the plates with AFB_1-CMO ester at 4^0C.

The utilization of the covalently-immobilized plates was initiated through blocking the plates with formaldehyde. Usage of formaldehyde leads to the formation of Schiff's bases (Adhuwalia and Agarwal, 2001), which will block the unreacted amine groups. The blockage of such groups leads to reduced background in the ELISA assay. The lower antibody binding (60%) was noticed when covalently immobilized plates were used (Figure 6.4). This might be due to the reversible nature of the Schiff's base reaction. Under reversible conditions not all of the Schiff's bases gets bound to the amine group on the antibody surface, leading to the reduction in the signal. The results of indirect ELISA testing indicate a 2.7-fold greater sensitivity when compared to the passively-adsorbed AFB_1-BSA conjugate. The specificity of the assays developed on AFB_1 covalently immobilized and AFB_1-BSA passively adsorbed microtitre plates as determined by cross reactivity studies indicate that the percentage cross reactivity exhibited by the antibody was lower when tested on AFB_1-covalently immobilized surfaces.

The key benefits with these covalently-immobilized microtitre plates is that they eliminate longer incubation steps, thus reducing the assay time. In contrast, decreasing the incubation time to 20 mins, led to a decreased assay sensitivity (IC_{50} value) from 79 ± 1.34 ng/mL (Mean ± S.D.; n=3) to 100 ± 0.98 ng/mL (Mean ± S.D.; n=3) for the assays performed on passively-adsorbed AFB_1-BSA conjugate microtitre plates. In comparison, the overall assay time with the covalently-immobilized AFB_1 was 75 mins, which is three times less than that required for the passively-adsorbed AFB_1-BSA conjugate.

Further studies were performed to check the robustness of the assay through investigation of the various physiochemical factors that may affect the sensitivity of the assay through stringent

washes with PBST & PBS, organic solvent concentration and pH. This suggests that the level of hapten availability on surfaces for antibody binding is not affected through stringent washes performed on the plates that were covalently-immobilized with AFB_1 or passively-adsorbed with AFB_1-BSA conjugate. In many analytical sample preparation regimes, AFB_1 will be extracted from food matrices by methanol, so the effect of methanol concentration on the sensitivity of assay was deemed an important parameter to evaluate. In conclusion, the usage of more methanol (above 40%) decreased the sensitivity of the assay by two-fold when the passively-adsorbed AFB_1-BSA conjugate was used, whereas no significant effect on the sensitivity of the assay performed on the covalently-immobilized AFB_1 was detected. Alterations in pH of the diluents used during the performance of an inhibition assay, demonstrated no significant impact on the assay sensitivity performed on both microtitre plate formats. It can be deduced that both covalently-immobilized AFB_1 and passively-adsorbed AFB_1-BSA conjugate are stable with regard to assay sensitivity over the range of pH 6-9.

Storage studies indicate that the covalently-immobilized plates were more stable than the passively-adsorbed microtitre plates. The re-usability of the microtitre plates was tested by treating the plates with a chaotropic mixture of 8 M urea, sodium dodecyl sulfate and mercaptoethanol. This mixture was chosen as ideal in comparison to other dissociation buffers used e.g. Tris-HCl and 2 M NaOH, due to its efficient regeneration effects and its facilitating the re-usage capabilities of the assay. Lower absorbance values with higher background signal were noted with an increase in the number of re-usages. The microtitre plates that were covalently-immobilized with AFB_1 could be re-used for four times with an average agreement of 89% for four successive re-uses.

The % CVs obtained in both intra- and inter-day assays performed using corn extract instead of buffer were less than 5. It is apparent from the results that the corn matrix has not significantly affected the robustness and sensitivity of the CI-ELISA performed on covalently-immobilized AFB_1 microtitre plates. Despite of the matrix effect, the sensitivity of assay developed on

AFB_1-covalently immobilized microtitre plate are within the FDA action levels of 20 ppb (ng/g) and this assay can readily be used for the detection of aflatoxin B_1 in corn samples and might be used for the detection of AFB_1 in other food matrices.

The utilization of immuno-affinity columns for the isolation of mycotoxins from food is gaining importance. Most of the reported immuno-affinity columns developed so far use either polyclonal or monoclonal antibodies. The larger size of both polyclonal and monoclonal antibodies when compared to recombinant antibodies, might affect antigen binding when the antibodies are immobilized on silica particles. Hence usage of recombinant antibodies of smaller size provides a powerful alternative to overcome the limitations of the usage of the larger antibody formats (Inui *et al.*, 2009). In the present study the silica was immobilized with highly sensitive recombinant anti-AFB_1, B_2 and ZEN recombinant antibody fragment.

The nature of solid support plays a crucial role in antibody binding. While selecting the support material considerable attention has to be paid to strategies for minimizing steric hindrances of the attached antibody fragment, which will reduce the active sites of the antibodies (Rejeb *et al.*, 2001). Various solid supports can be used, but in the present work the wakosil silica was selected due to its rigid nature and also it permits higher flow rates and higher pressures than other solid supports. Smaller pore sized silica (100 A^0) was chosen due to the smaller size of the recombinant antibody fragment (Clarke *et al.*, 2000).

The choice of eluting solvent is important as many solvents will play a significant role in the reduction of antibody binding capacity upon successive elution steps. As mild acidic and basic conditions are inefficient in eluting small molecular weight compounds (Sanvicens *et al.*, 2006), methanol elution is a useful quantitative analyte recovery method from the column (Delaunay *et al.*, 2000). After significant washing the column, the bound AFB_1, B_2 and ZEN were eluted using 40% (v/v) methanol. The amount of AFB_1, B_2 and ZEN eluted from the multi-analyte column were 3.9 ± 1.9, 5.3 ± 0.9 and 6.2 ± 1.5 µg/mL, respectively. The antigen binding capacity of the column developed in the present work is higher than the earlier reported

immuno-affinity columns for the isolation of ochratoxin (Uchigashima *et al.*, 2012) and aflatoxins (Uchigashima *et al.*, 2009).

Chapter 7
Overall conclusions and future work

7.1 Conclusions

7.1.1 Anti-mycotoxin antibodies generation

Initially the antigens (AFB_2, ZEN and T-2 toxins) were conjugated to carrier proteins (BSA/KLH), characterized (hapten density per carrier protein molecule) by MALDI-TOF (mycotoxin-BSA conjugates) and TNBS analysis by Dr. Om Prakash. Following conjugate characterization their immuno-potential was assessed by injecting them into Balb/c mice. The results (Appendix IV) of the serum titre of all these mice post-immunization showed that mycotoxin-KLH conjugates are efficient in generating good immune responses, which is necessary for the isolation of specific antibodies.

In chapter 3, generation of anti-AFB_2 scFvs and their subsequent incorporation into assay formats was described. A murine AFB_2 scFv library was generated from the spleen of a AFB_2-KLH-immunized mouse. His-tagged anti-AFB_2 scFvs were purified using immobilized metal affinity chromatography. Cross reactivity studies were performed to find the scFv that was highly specific to AFB_2 with minimal or no cross reaction to aflatoxin variants, which revealed that out of 7 scFvs tested only 2 scFvs were able to specifically recognize AFB_2. A surface plasmon resonance (SPR) kinetic study revealed that anti-AFB_2 scFv-E9 has nanomolar affiniy for AFB_2. The analytical sensitivity of SPR-inhibition assay (indicated as IC_{50}) was lower than that of a competitive inhibition ELISA developed using the scFv. The limit of detection of the SPR-based inhibition assay using spiked almond extract was noted as 3.6 ng/mL, which was within the range of the expected maximum permissible limits of 20 ng/mL.

Chapter 4 outlines, the generation and utilization of anti-ZEN antibodies for the development of ELISA and SPR-based inhibition assays. Recombinant libraries were constructed from the spleens of mice immunized with both ZEN-BSA and KLH conjugates. No anti-ZEN scFvs were isolated from the recombinant library generated from spleen of ZEN-BSA immunized mice. Subsequent attempts were made to build the ZEN scFv library utilizing the genomic material obtained from ZEN-KLH immunized mouse. A positive indication in the polyclonal

phage ELISA indicates the presence of ZEN recognizing scFvs. A few clones from the biopanning process were analysed for the presence of scFv genome, which indicated presence of 100% carry over of the scFv genome in the expressing clones. Monoclonal ELISA results indicated that 25% of the clones were ZEN-positive. The anti-ZEN scFv-P2A2 exhibited minimal cross reactivity when compared to the earlier reported anti-ZEN antibodies. A competitive inhibition ELISA demonstrated that the selected scFv-P2A2 had an IC_{50} value of 200 ng/mL. SPR kinetic study revealed a K_D of 3.9 X 10^{-10} M for the anti-ZEN scFv-P2A2. The IC_{50}s of SPR-based inhibition assay in solution and sorghum matrix were 10 and 22 ng/mL, respectively. The sensitivity of the SPR-inhibition assay is lower than that of the competitive inhibition ELISA in both solution and sorghum matrix. The detection limits of both SPR-inhibition and competitive indirect ELISA assay were within the required limits of 200 ng/mL (ppb also) for ZEN.

In chapter 5, the production, affinity purification and characterization of anti-T-2 IgY for use in the development of a SPR-based inhibition assay was described. A Leghorn chicken was used as the host for the generation of antibodies against T-2 toxin. To our knowledge this is the second successful attempt using chicken (rarely used species for the generation of anti-mycotoxin antibodies) as an immune host for the development of anti-T-2 toxin antibodies. T-2 toxin-specific IgY was isolated through subjecting the IgY to affinity purification on T-2 toxin-immobilized amine-activated beads. A 50% increase in the titration value with a significant decrease in the percentage cross reactivity of the IgY to other tested mycotoxins was noticed after affinity purification of IgY on T-2 toxin-immobilized beads. SPR-based inhibition assay with IC_{50} of 42 ng/mL (sensitivity of SPR was the same as that of ELISA) was developed and applied for the detection of T-2 toxin in spiked wheat sample extracts. Optimized ELISA and SPR assays were highly reproducible. The results of the SPR assay indicate that this assay can be used for the T-2 toxin detection in wheat samples with detection limits below that required (100 ng/mL).

7.1.2 Immunoassay development

The first section (section 6.1.1) of chapter 6, focuses on the development of a competitive inhibition ELISA for the detection of AFB_1 using AFB_1 covalently-immobilized microtitre plates and an anti-AFB_1 recombinant Fab antibody fragments. This covalent immobilization strategy can potentially be applied for various haptens with carboxyl groups. The competitive assay developed on the microtitre plate with covalently immobilized AFB_1 was 2.7-fold more sensitive that the conventional approaches and also the assay time was reduced to 75 mins. The modified plates were able to sustain activity up to 40% (v/v) with methanol. The covalently-immobilized AFB_1 are stable up to 14 days when placed in vaccum dessicator at room temperature, which makes the usage of these modified plates more attractive. The re-usability, storage stability and reduced assay time, make the usage of these AFB_1 covalently-immobilized microtitre plates attractive for use in detection of AFB_1 in corn samples.

7.1.3 Development of immuno-affinity columns

The second section (section 6.1.2) of chapter 6, deals with the development of an extraction technique (immuno-affinity column) using recombinant antibodies immobilized on silica beads. Initial studies were conducted with an anti-AFB_1 Fab antibody using Wakosil and CC grade silica. The results demonstrated better performance with Wakosil silica. Further recombinant antibodies developed against AFB_2 and ZEN were immobilized onto the Wakosil silica. The Wakosil silica immobilized with three antibodies, was used in the construction of a multi-analyte immuno-affinity column. The results show that this column posses binding affinity at µg levels, which is higher compared to the ng affinity of the immuno-affinity columns currently employed for mycotoxin extraction. The developed column can be re-used up to 6 times for AFB_1, B_2 and ZEN with minimal loss in the extraction efficiency. Such a column could have major application in the extraction of mycotoxins from food samples followed by specific detection of individual mycotoxins (i.e., AFB_1, B_2 and ZEN). Existing polyspecific antibodies

used in such an approach, with less affinity for individual toxins such as those highlighted, could lead to under extraction and hence underestimation of actual levels in food samples.

In summary, this work highlights the potential use of immunoassays for the detection of food contaminants. The research also demonstrates the ease of using scFvs derived from immune phage display libraries in a variety of sensitive and specific assay formats for the detection and extraction of AFB_1, B_2 and ZEN. Furthermore, the feasibility of using affinity-purified polyclonal IgY for the specific detection T-2 toxin was also demonstrated. The usage of immuno-affinity column can be used as efficient sample clean up process, which can further be analyzed using analytical techniques.

7.2 Future work

The developed multi-analyte column will be used for the extraction of AFB_1, B_2 and ZEN from contaminated food samples, followed by the HPLC analysis of the recovered toxins from the column. The MBio array assay, where preliminary analysis was carried out for the detection of AFB_1 (data not shown), will be further evaluated using a multiplex assay for the simultaneous detection of AFB_1, B_2, ZEN and T-2. The feasibility of using the MBio array assay for the detection of mycotoxins in food material will be studied.

Chapter 8
Bibliography

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APPENDIX

Appendix I

Validation of immunoassay

The various parameters that need to be considered in order to fully assess the performance of an immunoassay are: accuracy, precision, sensitivity, specificity, suitability and robustness (DeSilva *et al.*, 2003).

Accuracy: Accuracy is measure of percentage bias and is calculated using the formula

Pecentage (%) Bias = (measured value – true value/ true value) X 100

The accuracy value can be obtained from a comparison of the measured value with a known reference standard value is made.

Precision: Precision is a statistical measure of variations between repeated measurements of the same sample. It is a measure of random errors which are determined based on the % CV value (coefficient of variance) or RSD (Relative standard deviation) of replicate measurements. The statistical values can be determined through the performance of either inter- or intra-day assays.

Sensitivity: Sensitivity defines the smallest amount of analyte that can be detected in an assay. To assure the suitability of the assay for intended purpose the sensivity of the assay is considered. At this point the affinity and purity of antibodies plays a major role. During assay optimization the various factors that might effect the affinity of the antibody like the washings, buffer constituents, incubation time etc., need to be considered.

Specificity: The specificity of any immunoassay is the measure of cross reactivity. The specificity of ligands e.g. antibodies plays a crucial role in ligand binding assays (LBA's) in the determination of sensitivity and robustness of the assays.

Robustness: The robustness of an assay is the efficiency of the assay to withstand minor variation in the experimental conditions. This is generally a follow-up method after assay optimization. The validation process also involves the application of various statistical analysis methods.

Limit of Blank (LOB) or Blank value: It is a measure of replicates of blank samples, without the analyte.

Limit of detection (LOD): Limit of detection is the lowest value of an analyte that could be distinguished from the blank value or limit of blank (LOB).

Limit of qantification (LOQ): It is the lowest analyte concentration that can be detected quantitatively with a certain precision. In general the LOQ is higher than LOD.

Appendix II

MALDI-TOF analysis of mycotoxin-BSA conjugates

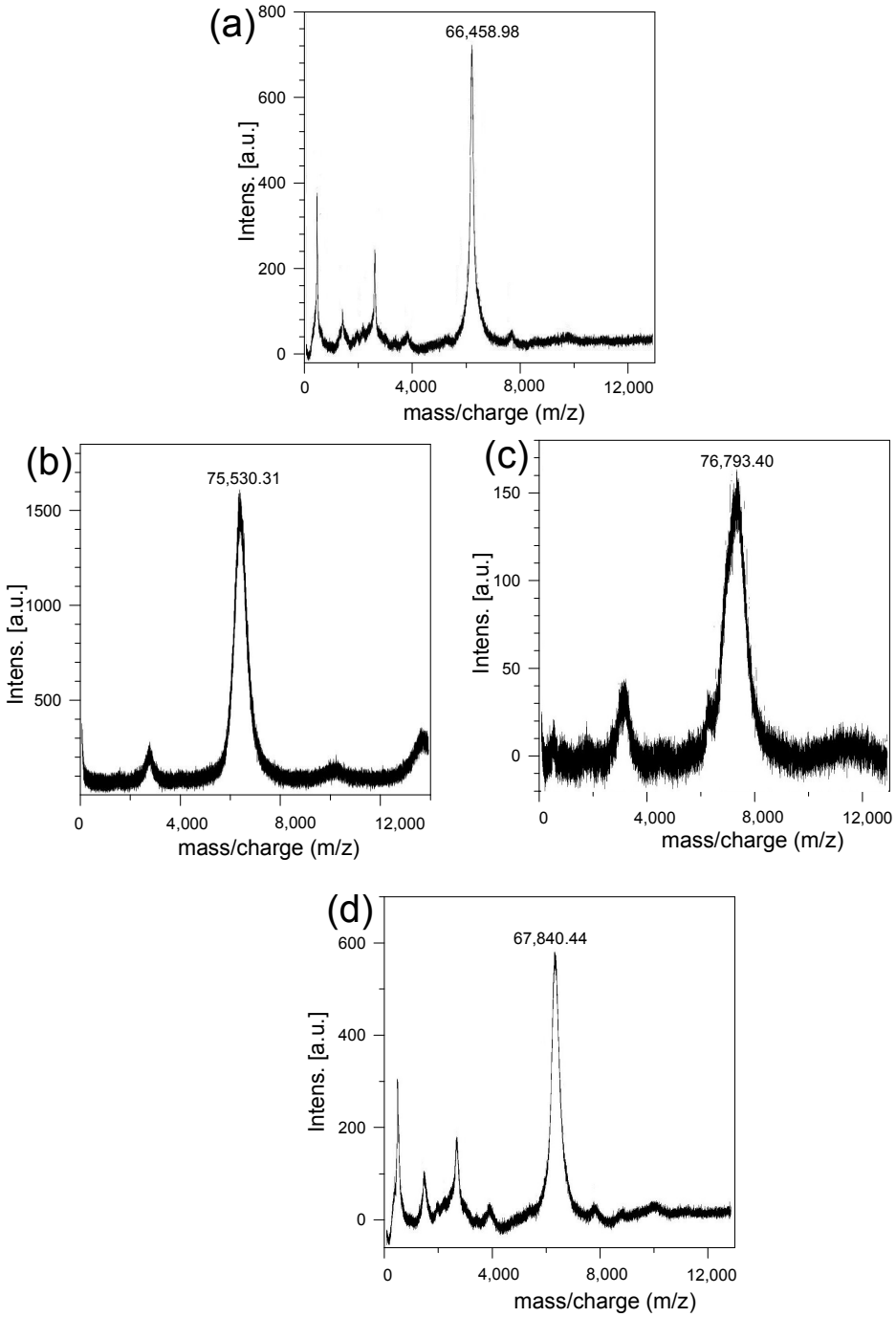

Figure 1: MALDI-TOF analysis of mycotoxin-BSA conjugates. *Molecular mass of (a) BSA (66,458.98 Da), (b) AFB_2-BSA (75,530.31 Da), (c) ZEN-BSA (76,793.40 Da) and (d) T-2-BSA (67,840.44 Da) were shown.*

Appendix III

Mycotoxin conjugate characterization using fluorescence assay and hapten density determination by MALDI-TOF and TNBS assays

Table 1: *Mycotoxin conjugate confirmation (fluorescence assay), hapten density determination (MALDI-TOF & TNBS assays)*

Mycotoxin conjuagte	Fluorescence Estimation	Hapten density	
		MALDI-TOF	TNBS
AFB_2-BSA	863 (Conj) 48 (BSA) 45 (PBS) Ex/Em:360/420nm 906 (Conj) 53 (BSA) 46 (PBS) Ex/Em:360/440nm	29	26
AFB_2-KLH	135 (Conj) 46 (KLH) 45 (PBS) Ex/Em:360/420nm 163 (Conj) 49 (KLH) 46 (PBS) Ex/Em:360/440nm	N/A	120
ZEN-BSA	525 (Conj) 11 (BSA) 8 (PBS) Ex/Em:236/418nm	32	35
ZEN-KLH	923 (Conj) 8 (KLH) 6 (PBS) Ex/Em:236/440nm	N/A	94
T-2-BSA	N/A	20	23
T-2-KLH	N/A	N/A	75

Appendix IV

Murine immune responses to mycotoxin BSA and KLH conjugates

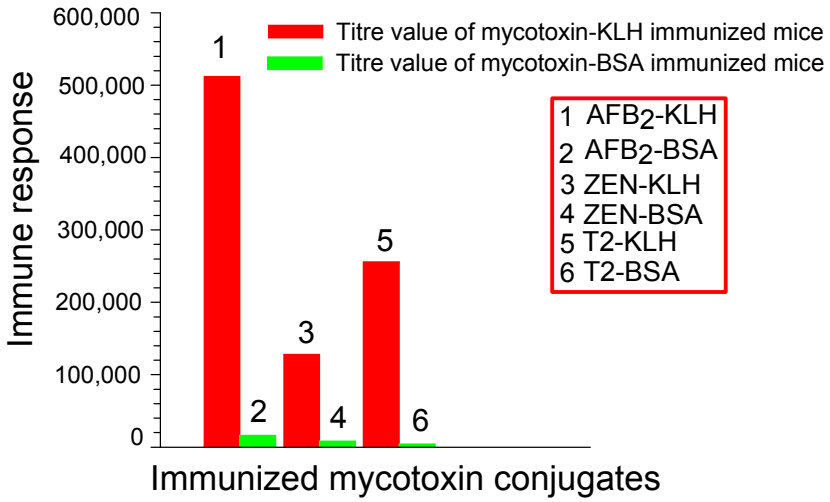

Figure 2: Murine immune response to six different mycotoxin conjugates. *Each of the mycotoxin BSA and KLH conjugates were used for immunizing three Balb/c mice. The antibody response was measured after the third boost by indirect ELISA. The results indicate that the mycotoxin-KLH conjugates are efficient in producing a better immune response than the mycotoxin-BSA conjugates.*