การคัดเลือกโมโนโคลนอลแอนติบอดีต่ออะฟลาท๊อกซิน จากคลังแอนติบอดีบนผิวเฟจ

นางสาวกุณฑลี ร่างน้อย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2552

SELECTION OF MONOCLONAL ANTIBODIES SPECIFIC TO AFLATOXIN FROM PHAGE DISPLAY ANTIBODY LIBRARY

KUNTALEE RANGNOI

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biotechnology

Suranaree University of Technology

Academic Year 2009

SELECTION OF MONOCLONAL ANTIBODIES SPECIFIC TO AFLATOXIN FROM PHAGE DISPLAY ANTIBODY LIBRARY

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

Thesis Examining Committee

_____

(Assoc. Prof. Dr. Neung Teaumroong)

Chairperson

_____

(Assoc. Prof. Dr. Montarop Yamabhai)

Member (Thesis Advisor)

_____

(Dr. Amara Chinaphuti)

Member

_____ _____

(Prof. Dr. Sukit Limpijumnong) (Asst. Prof. Dr. Suwayd Ningsanond)

Vice Rector for Academic Affairs Dean of Institute of Agricultural Technology

กุณฑลี ร่างน้อย : การคัดเลือกโมโนโคลนอลแอนติบอดีต่ออะฟลาท็อกซินจากคลังแอนติบอดีบนผิวเฟจ (SELECTION OF MONOCLONAL ANTIBODIES SPECIFIC TO AFLATOXIN FROM PHAGE DISPLAY ANTIBODY LIBRARY) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.มณฑารพ ยมาภัย, 83 หน้า.

อะฟลาท็อกซินเป็นสารพิษที่เกิดจากเชื้อรา ซึ่งพบปนเปื้อนในอาหารคนและสัตว์ รวมทั้งผลิตผลทางการเกษตรในหลายพื้นที่ วิธีการตรวจหาอะฟลาท็อกซินที่ใช้กันอยู่ในปัจจุบัน ได้แก่ วิธีโครมาโตรกราฟี แบบแผ่นบาง และแบบของเหลวประสิทธิภาพสูง หรือวิธีทางอิมมูน ซึ่งเป็นวิธีการที่มีขั้นตอนที่สะดวก รวดเร็ว และมีความคุ้มทุนมากที่สุด โดยวิธีการนี้ต้องใช้แอนติบอดีที่จำเพาะต่ออะฟลาท็อกซินเป็นสารสำหรับตรวจสอบ เทคโนโลยีการแสดงโปรตีนบนผิวเฟจ นับเป็นทางเลือกหนึ่งที่มีประสิทธิภาพสูงในการผลิตแอนติบอดี งานวิจัยนี้ได้ทำการคัดเลือกโมโนโคนอลแอนติบอดีที่จำเพาะต่ออะฟลาท็อกซินบี 1 จากคลังเฟจซึ่งแสดงแอนติบอดีแบบเส้นเดี่ยว เอสซีเอฟวีบนผิวเฟจ โดยได้ทำการคัดเลือกจำนวน 3 รอบ แล้วนำเฟจจำนวน 96 โคลนที่คัดหามาได้ไปผลิตเป็นโมเลกุลแอนติบอดีเส้นเดี่ยวที่ไม่ติดอยู่บนผิวเฟจ จากแบคทีเรียสายพันธุ์ อีโคไล เอชบี 2151 จากนั้นนำแอนติบอดีเหล่านี้ไปทดสอบความสามารถในการจับกับอะฟลาท็อกซิน โดยใช้วิธีการอีไลซ่าแบบแข่งขัน จากนั้นจึงได้นำโคลนที่แสดงความสามารถในการจับอะฟลาท็อกซินอย่างจำเพาะเจาะจงมาศึกษาลำดับเบสของดีเอนเอ โดยการวิเคราะห์ด้วยเครื่องวิเคราะห์ลำดับเบสอัตโนมัติ และนำแอนติบอดีที่ได้นำมาทำการแยกให้บริสุทธิ์ แล้วทำการศึกษาโครงสร้างโดยใช้วิธีเอสดีเอสเพจ และวิธีเวสเทิร์น บลอต รวมทั้งทำการวิเคราะห์คุณสมบัติในการจับจำเพาะต่ออะฟลาท็อกซินโดยใช้วิธีการอีไลซ่า แบบแข่งขัน นอกจากนั้นแล้ว ได้นำแอนติบอดีที่คัดเลือกแล้วนี้ไปสร้างให้เป็นโมเลกุล เอสซีเอฟวีที่เชื่อมต่อกับ เอนไซม์อาลคาไล ฟอสฟาเตส (scFv-AP) เพื่อให้สะดวกในการตรวจจับด้วยวิธี อีไลซ่า แอนติบอดีเชื่อมต่อที่ได้สร้างขึ้นนี้ มีความสามารถในการจับกับอะฟลาท็อกซินดีกว่าเมื่อเปรียบเทียบกับ โมเลกุลแอนติบอดีเส้นเดี่ยวประมาณ 3-4 เท่า โดยแอนติบอดีนี้สามารถใช้ในการตรวจจับอะฟลาท็อกซินบี 1 ได้ต่ำสุดประมาณ 0.006 ถึง 0.03 ไมโครกรัมต่อมิลลิลิตร (6-10 ส่วนในล้านส่วน) และพบว่าค่าความสามารถในการยับยั้งการจับกึ่งหนึ่งอยู่ในช่วงระหว่าง 0.035-0.02 ไมโครกรัมต่อมิลลิลิตร เมื่อนำแอนติบอดีที่เชื่อมต่อกับเอนไซม์อาลคาไล ฟอสฟาเตส นี้ไปทดสอบการจับกับอะฟลาท็อกซินชนิดอื่นๆ ได้แก่ อะฟลาท็อกซิน บี 2, จี 1, จี 2 และ เอ็ม1 พบว่าร้อยละความสามารถในจับกับจี 1 มีมากที่สุด ผลจากการศึกษานี้แสดงว่าสามารถใช้เทคโนโลยีการแสดงโปรตีนบนผิวเฟจในการคัดเลือกแอนติบอดีชนิดเส้นเดี่ยวที่มีความจำเพาะต่ออะฟลาท็อกซินบี 1 ได้ และยังสามารถทำการตัดต่อยีนเพื่อสร้างเป็นแอนติบอดีที่เชื่อมต่อกับเอนไซม์อาลคาไล ฟอสฟาเตส ซึ่งสามารถนำไปใช้ประโยชน์ในการพัฒนาเป็นชุด

ตรวจสอบ สำหรับตรวจวิเคราะห์สารพิษอะฟลาท็อกซินที่ปนเปื้อนในผลิตผลทางการเกษตรได้ต่อไป

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2552

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

KUNTALEE RANGNOI : SELECTION OF MONOCLONAL ANTIBODIES SPECIFIC TO AFLATOXIN FROM PHAGE DISPLAY ANTIBODY LIBRARY. THESIS ADVISOR : ASSOC. PROF. MONTAROP YAMABHAI, Ph.D., 83 PP.

PHAGE DISPLAY/SCFV/ ANTIBODY/ NAÏVE /BIO-PANNING/MONOCLONAL /RECOMBINANT /AFLATOXIN/ALKALINE PHOSPHATASE FUSION

Aflatoxins are one of the major mycotoxins that contaminate several agricultural products and human food in many areas of the world. Several methods for aflatoxin determination have been developed, including thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), and immunological method. Among these, the most cost-effective method is immunological analysis that uses monoclonal antibodies as detection reagents. Phage display technology is a powerful alternative method for the production of monoclonal antibody. A specific antibody to aflatoxin B_1 (AFB_1), the most toxic and prevalence isoform, could be isolated by bio-panning from Phage-displayed human single-chain-variable-fragment (scFv) antibody libraries. After three rounds of affinity selection, ninety-six positive clones were induced for the production of soluble scFv antibody fragments by using *Escherichia coli* non-suppressor strain (HB2151), and tested for their ability to bind soluble AFB_1 by competitive enzyme-linked immunosorbent assay (ELISA). Amino acid sequence analysis of the selected clones was performed using information obtained from automated DNA sequencing. ScFv antibodies that could be inhibited by soluble AFB_1 were purified to analyze their structure by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis, and confirm their specific binding to AFB_1 by competitive ELISA. In addition, selected scFv clones were engineered to create scFv- alkaline phosphatase (scFv-AP) fusions and used as

reagents for one-step detection in ELISA format. The scFv-AP fusions showed 3-4 fold improved binding property when compared to soluble scFv form. The IC_{50} of scFv-AP by AFB_1 varied between 0.035-0.02 μg/ml and the limit of detection was approximately 0.006-0.03 μg/ml (6-30 ppb). The selected antibodies were specific to AFB_1 and could cross-react with AFG_1, but not to other aflatoxins, namely AFB_2, AFG_1, AFG_2, AFM_1. These results indicated that phage display technology could be used to obtain a specific antibody against aflatoxin, and that the scFv-AP fusion was an efficient detection reagent that could be further developed to generate a cost-effective diagnostic kit or biosensor for the detection of aflatoxin contamination in agricultural commodities and products in the future.

ACKNOWLEDGMENTS

The completion of this thesis was made possible through the support and inspiration of several individuals all of whom have my gratitude.

I would like to express my deepest gratitude to my advisor, Assoc. Prof. Dr. Montarop Yamabhai for her year encouragement, network, patient, and support in completing this thesis.

I am grateful to my co-advisor, Dr. Amara Chinaphuti, for her advice on aflatoxin fields, production of polyclonal antibody from animals and her valuable suggestion.

I would like to sincerely thank Prof. Dr. Richard O'Kennedy at Applied Biochemistry Group, Dublin City University in Ireland, who gave me a good opportunity to had experience in his lab and his valuable suggestions.

I would like to thanks to Potjamas Pansri, Nanthanit Jaruseranee, Suphap Emrat, Onladda Juajun, Sirima Sukasem, Puntarika Pesutcha and Bancha Buranabanyat for their year of sharing, not only their scientific and technical knowledge, but also spirited prodding has kept me go through many years of work.

I would like to thank my friends, Nareerat Ma-on, Ratsamee Wasuwan, Nantida Watanarojanaporn, Apiradee Sripiromruk, Kirana Yoohat, Jenny Fitzgerald, Weili Guo, P'Wan, P' Lai, P'Ton, P'Jub, P'May, P'Zai, Gustavo and Monica for their encouragement and friendship.

Throughout this thesis work, exciting moments and warm working atmosphere have been shared with many people at F3 lab at SUT and Applied Biochemistry Group at Ireland.

I would like to express my special thanks to Panuwat Booncheua for his love and soul support.

Finally, my special thanks are given to my beloved family, my father, my mother and my little sister, without their love and support, I would never have achieve this far.

Kuntalee Rangnoi

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

°C	degree Celsius
µg	microgram
µl	microlitre
bp	base pair
DNA	deoxyribonucleic acid
et al.	Et alia (and other)
g	gram
h	hour
Kda	Kilo daltal
l	litre
mg	milligram
min	minute
ml	milliliter
mM	millimolar
ng	nanogram
pmol	picomol
rpm	revolution per minute
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

CHAPTER I

INTRODUCTION

1.1 Significant of this study

Aflatoxin is a potent mycotoxin produced by certain strains of *Aspergillus flavus* and *A. parasiticus* (Woychik et al., 1984). Among 18 different types of aflatoxins identified, major members are aflatoxin B_1, B_2, G_1, and G_2. Aflatoxin B_1 (AFB_1) is normally predominant in amount in cultures as well as in food products. These toxins especially AFB_1 are highly carcinogenic agents consistently found as contaminants in human food supplies and animal feeds in many areas of the world (Taylor et al., 1997). As a consequence, there is a need for routine screening of products destined for animal and human consumption. The methods of analysis include thin layer chromatography (TLC), gas liquid chromatography (LC), high-pressure and liquid chromatography (HPLC), which are laborious and require expensive instrumentation and clean-up of the samples (Devi et al., 1999). Immunological methods are cost-effective and adaptable to the situation of developing countries. Commercial test kits for detection of aflatoxin have been developed, which used polyclonal or monoclonal antibody as detection reagents. These techniques need the immunization of animals to obtain specific antibodies, which are costly and time-consuming. Thus, an alternative method for large scale production of specific monoclonal antibody at lower cost in the form of a recombinant proteins obtain from phage display technology is attractive. Phage display antibody technology is also beneficial for the production of convenient test kits that can be distributed to a wide range of customers and user at lower cost, resulting in a better health of the populations in the future

(Yuan et al., 1997). Also, the monoclonal antibodies have proven to be useful analytical tools for quantifying the aflatoxins.

Several *in vitro* methods have been developed for the production of antibodies an alternatives to *in vivo* animal-based methods (Bradbury et al., 2003). The most commonly used technology among the various *in vitro* strategies is phage display (Hust and Dubel, 2004) which is, recognized as a powerful tool for selecting recombinant antibody fragments with specific binding properties from a vast number of variants against a wide range of target molecules, such as proteins, glycoproteins, oligosacharides, nucleic acid toxins or low molecular weight compounds – haptens (Willats, 2002; Yau et al., 2003). Phage display, first described by Smith (1985), refers to the display of functional foreign peptides, proteins or antibody fragments on the surface of a bacteriophage (Clackson et al., 1991).

Filamentous bacteriophages have been used to display single-chain variable fragment (scFv) and Fab antibody fragments by fusion to the phage minor coat protein pIII (Hoogenboom et al., 1991; McCafferty et al., 1990). The antibodies phage display library can be used to select antibodies against any antigen, including foreign antigen, non-immunogenic antigens and toxic antigens. Display of large and diverse antibody fragment gene repertoires on phages permits the direct selection of monoclonal antibodies using antigen-affinity chromatography without the need for immunization (Marks et al., 1991).

The procedure for selecting phage is simple and inexpensive. Using recombinant DNA technology and microbiological methods, peptides or proteins are fused to one of the phage capsid proteins by genetic engineering into appropriate vector (plasmid or phagemid). The peptides or proteins that displayed on the phage coat proteins are accessible to various molecular interactions. Affinity selection of antibodies is accomplished by exposing the phage library to immobilized antigen molecules (binding clones are captured and non-binding clones are washed away). The captured phage particles are eluted from antigen, amplified by infecting *Escherichia coli* host cells and used in a

subsequent round of affinity selection. After the final round of affinity selection phage particles are amplified in order to prepare and characterize their displayed antibodies individually (Petrenko and Vodyanoy, 2003). Finally, the monoclonal phage population with the desired binding specificities can be isolated (Willats, 2002).

Advantages of phage-display antibody technology include (i) selection of antibodies from a phage library is unbiased by the immunogenicity and less dependent on quantitative abundance of the target ligands (Schier et al., 1996), (ii) antibodies are produced in DNA-encoded plasmids that are readily cloned and modified, (iii) antibodies can be produced in large quantities from *Escherichia coli* without the use of animals, (iv) the genes of the antibody can be further engineered to obtain improved antibody property such as sensitivity.

In this thesis, scFv monoclonal antibody which is specific to aflatoxin was produced by using phage display technique. The phage displayed antibody fragment was induced to produce soluble scFv fragment against aflatoxin. In addition, a fusion of scFv to bacterial alkaline phosphatase (AP) was generated. This construct was used as convenient one step detection reagents for Enzyme-linked Immunosorbent Assay (ELISA). This scFv-AP can be developed for the detection of aflatoxins in agricultural products and food products in the future.

1.2 Research objectives

1. To select phage displayed scFv antibodies which are specific to aflatoxin by bio-panning.
2. To produce scFv fragments that can interact specifically with aflatoxin.
3. To create scFv-AP fusion for one-step detection of aflatoxin in ELISA format.

1.3 Scope and limitation of the study

In this study, the specific antibodies to aflatoxin B_1 were selected from human scFv antibody libraries by using *in vitro* panning process. Antibody was purified using Ni^{2+}-NTA resin and was analysed by SDS-PAGE and western blot. Amino acid sequences of each scFv antibody were determined by DNA sequence analysis. Selected clones were engineered to create scFv-alkaline phosphatase fusions (scFv-AP) and used as convenient one-step detection probes for competitive ELISA. The sensitivity and cross-reactivity to unconjugated aflatoxin B_1, B_2, G_1, G_2 and M_1 was determined by competitive ELISA.

CHAPTER II

LITERATURE REVIEWS

2.1 Aflatoxins

Aflatoxins molecules were first discovered in the early 1960 when 100,000 turkeys died in England after consuming contaminated brazillian peanut meal (Hartley et al., 1963). These toxins are highly carcinogenic secondary metabolites of *Aspergillus flavus* and *A. parasiticus* which produced during fungal infection of a susceptible crop in the field or after harvest. The productions of aflatoxins occur at high temperatures and humidity, therefore their contamination are most common in tropical and subtropical countries. Contamination can occur in the field, during harvest, transportation and storage under optimal conditions for fungal growth (Bedard and Massey, 2006). Conditions that contribute to fungal growth and the production of aflatoxins are hot and humid climate, kernel moisture, favorable substrate characteristics, and factors that decrease the host plant's immunity (insect damage, poor fertilization, and drought).

There are four principle types of aflatoxin: B_1, B_2, G_1 and G_2 plus two additional metabolic products, M_1 and M_2 which are named for their respective innate fluorescent properties and mobility in chromatogram (Pitt, 2000). Whereas the B designation of aflatoxins B_1 and B_2 resulted from the exhibition of blue fluorescence under UV-light, while the G designation refers to the yellow-green fluorescence of the relevant structures under UV-light. Aflatoxin M_1 and M_2 are major metabolites of aflatoxin B_1 and B_2 respectively. The aflatoxins M_1 and M_2 were first isolated from milk of lactating animals fed with aflatoxin. AFB_1 is the most toxic and most prevalent compound, followed by G_1, B_2 and G_2 with decreasing toxicity (Busby and Wogan, 1984).

Aflatoxins have been found in many foods and animal feeds. These toxins dissolve in various polar organic solvents including methanol, chloroform, dimethyl sulfoxide (DMSO), aqueous acetone and aqueous hexane-acetone-water azeotrope that developed for extraction procedures of natural products. They are heat stable, even at temperatures in excess of 100^{o}C and are difficult to destroy. These toxins have closely similar structures and form a unique group of highly oxygenated, naturally occurring heterocyclic compounds. Their molecular formulas as established from elementary analyses and mass spectrometric determinations are B_1: C_{17} H_{12} O_6, B_2: C_{17} H_{14} $O_{6,}$ G_1: C_{17} H_{12} O_7 and G_2: C_{17} H_{14} $O_{7.}$ Aflatoxins B_2 and G_2 were established as the dihydroxy derivatives of B_1 and G_1, respectively. Whereas, aflatoxin M_1 is 4-hydroxy aflatoxin B_1 and aflatoxin M_2 is 4-dihydroxy aflatoxin B_2. The chemical structures of these aflatoxins are shown in Figure 1. Some physical properties of the compounds are summarized in Table 1.

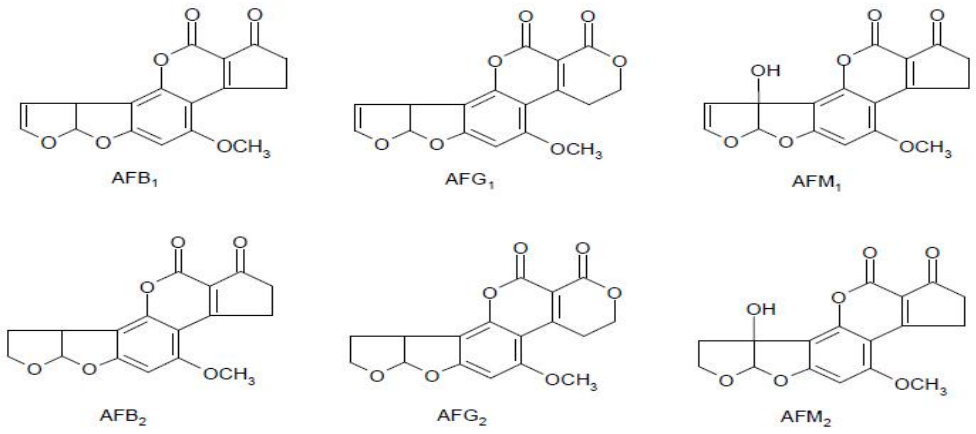


Figure 1. Chemical structures of major aflatoxins namely aflatoxin B_1, G_1 and M_1 with the double bonds in 8-9 positions and aflatoxins B_2, G_2 and M_2 without the double bond. Other methabolites are AFM_1 and AFM_2 have been found in milk and urine of lactating animals. AFM_1 and AFM_2 are produced from AFB_1 and $AFB_{2,}$ respectively, by hydroxylation in lactating

Table 1. Chemical and physical properties of aflatoxins. Adapted from Wogen (1966).

Aflatoxin	Molecular formula	Molecular weight	Melting point (^{o}C)
B1	$C_{17}H_{12}O_6$	312	268-269
B2	$C_{17}H_{14}O_6$	314	286-289
G1	$C_{17}H_{12}O_7$	328	244-246
G2	$C_{17}H_{14}O_6$	330	237-240
M1	$C_{17}H_{12}O_7$	328	299
M2	$C_{17}H_{14}O_6$	330	293

2.1.1 Effects of aflatoxin contamination

Agricultural products are often contaminated with fungi that can produce toxic metabolites referred as mycotoxins. Among these, aflatoxins have assumed economic importance because of their influence on the health of human beings and livestock and on the marketability of agricultural products. The tolerance levels currently set by the regulatory bodies worldwide are typically 0.05 ppb for AFM_1 in milk, 10 ppb for AFB_1 and 20 ppb for total aflatoxins in food intended for human consumption and 20-300 ppb for total aflatoxins in animal feeds (Lee et al., 2004). Food and Drug Administration (FDA) action levels for aflatoxin present in human food is 20 ppb with the exception of milk is 0.5 ppb. The action level for most feed is 20 ppb.

Aflatoxins are both acutely and chronically toxic to animals, including man, causing acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects (Stoloff, 1977). In humans, numerous studies have linked the incidence of primary hepatocellular carcinoma with the intake of aflatoxins, leading to the classification of AFB_1 as class 1 human carcinogen by the IARC (IARC, 1993). Toxic and especially carcinogenic effects of aflatoxins have been reported in several different animals, but susceptibility to these toxins

varies greatly with sex, age, species and strain within a species (Busby and Wogan, 1984; CAST, 2003). Numerous animal studies have shown that the liver is the main target organ and therefore the main symptoms of aflatoxin exposure in domestic and laboratory animals are hepatic injuries. Aflatoxin is hepatotoxic and its acute and chronic effects in swine are largely attributable to liver damage (Armbrecht, 1978). In cattle, the primary symptom is reduced weight gain as well as liver and kidney damage. Milk production is reduced in cow (Keyl, 1978). Lower aflatoxin doses may lead to milder hepatic injuries and reduced growth rate, especially in young animals (Pier, 1992). Domesticated animals are variably susceptible to aflatoxins; some oral LD_{50} values (lethal dose for 50% of animals) are given for aflatoxin B_1 in several animal species in Table 2.

Table 2. Oral LD_{50}'s of Aflatoxin B_1. Adapted from Robens and Richard (1992).

Animal	**sex**	**Age/size**	**LD_{50} (mg/kg)**
Duckling	M	1 d	0.37
Rat	M-F	1 d	1.0
Rat	M	21 d	5.5
Rat	F	21 d	7.4
Dog	M-F	Adult	0.5
Hamster	M	30 d	10.2

As for the mechanism of AFB_1 induced mutagenicity and carcinogenicity, AFB_1-epoxide adducts to DNA, preferably guanine nucleotides, causing point mutations mainly G-C to T-A (94%) or A-T (6%) (Bailey et al., 1996). Depending on the location this mutation, activation of proto-oncogenes or silencing of suppressor genes will cause initiation of the cancer process. Mutations in the *p53* tumor suppressor gene at the hot spot

of codon 249, are discussed in the context of AFB_1, but relations between this mutation, AFB_1 and hepatitis B virus infection are not entirely clear (Smela et al., 2001).

It is clear that exposure to aflatoxins is hazardous to human and animal health. For that reason, most countries have regulations governing the allowable concentrations of aflatoxin in food and feed (Van Egmond, 1989).

2.1.2 Recent methods of analysis for aflatoxins

Aflatoxin contamination is widespread in staple crops like peanut, maize, sorghum, pearl millet, chillies, pistachio, cassava etc., and compromises the safety of food and feed supplies (Waliyar et al., 2009). It is important to be able to detect and quantify aflatoxins in commodities to protect human and animal health. Food and feed industries should monitor their products routinely to ensure that aflatoxins levels are below the regulatory limits. Sensitive, accurate, and precise methods of analysis are needed for any monitoring programme to be effective. Many analytical methods including thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), gas-liquid chromatography (GLC), immunoassay and combined methods have been employed to detect AFB_1. TLC, GC and HPLC methods for determining aflatoxins in food are widely accepted as official methods. However, these methods are laborious and time consuming. Often these techniques require knowledge and experience chromatographic techniques to solve separation and interference problems. Immunoassays are preferred over analytical methods because of their simplicity and cost-effectiveness. However, commercial kits based on immunological methods are expensive, and may be difficult to import them. These methods have a unique ability to routinely handle a large number of samples and do not require time-consuming procedures and sophisticated equipment, many imunoassays for aflatoxin have been developed. The described immune assays have integrated either polyclonal or monoclonal antibodies. Antibody-based techniques, such as ELISA, are widely used for routine screening of possible mycotoxin contamination. Diagnostic assays usually are based

on competitive ELISA formats in which the binding of the anti-hapten antibodies to a labeled tracer molecule is inhibited by free antigen from the sample. The decrease of labeled tracer bound to the antibody is then determined (Lauer et al., 2005).

In the present day, DOA-Afatoxins ELISA test kit was approved for determination of aflatoxins at the level of 0.4 ppb by using polyclonal for detection agents (according to the manufacture leaflet, Department of Agricultural, Thailand). However, these kits require immunization of animals to produce polyclonal antibody. The alternative method to produce antibodies at lower cost by using recombinant proteins obtains from phage display technology. There have been previous reports on the isolation single chain variable fragments (scFv) specific to aflatoxin B_1 from phage display antibody library (Daly et al., 2002; Dunne et al., 2005; Moghaddam et al., 2001; Yang et al., 2009).

2.2 Phage display technique

Phage display was first described by Goerge P. Smith in 1985 (Smith, 1985). This technique refers to the display of functional foreign peptides, proteins or antibody fragments on the surface of a filamentous bacteriophage. A gene of interest is fused to a gene encoding a phage coat protein, resulting in phage particles that display the encoded protein and contain its gene, providing a direct link between phenotype and genotype (Figure 2). This connection between genotype and phenotype enables large libraries of proteins to be screened and amplified in a process called *in vitro* selection (bio-panning).

Two key discoveries were essential for the development of antibody phage display technology. First, the demonstration that foreign DNA inserted into filamentous phage gene III (g3) is expressed as a fusion protein and displayed on the surface of the phage (Smith, 1985). Second, the successful expression of functional antibody fragments in the periplasmic space of *E. coli* (Better et al., 1988; Skerra and Plu¨ckthun, 1988)

The advantages of phage display are easy to manipulate, rapid, inexpensive and appropriate for large scale production. Many recombinant anibodies with specific for various antigens such as human-self antigen, non-immunogenic antigens and small haptens were produced by phage display technology (Yau et al., 2003). This technique can also be used to study protein-ligand interactions (Cesareni, 1992), receptor and antibody-binding sites (Griffiths, 1993; Winter et al., 1994), and to improve or modify the affinity of proteins for their binding partners (Burton, 1995; Neri et al., 1995).

Large repertoires of antibodies (from 10^9- to 10^{12}) were constructed and displayed on the surface of filamentous phages, thus exceeding the natural repertoire by several magnitudes (Hoogenboom et al., 1998). These libraries can then be easily used to select and purify specific phage particles bearing sequences with desired binding specificities from the non-binding variants.

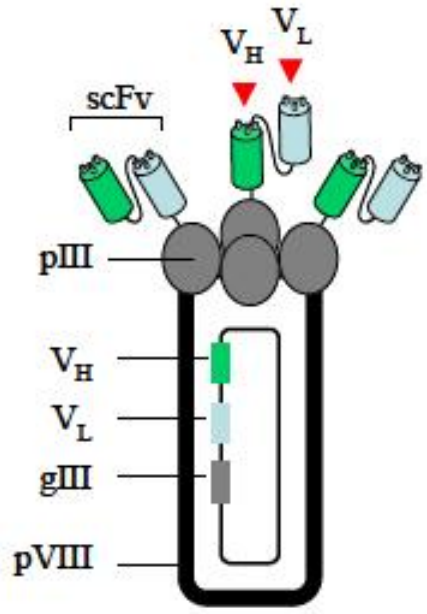


Figure 2. Diagram of filamentous phage displaying scFv antibody fragments (Byrne et al., 2009). The phage consists of circular ssDNA surrounded by a coat protein. g8p (pVIII) is the major coat protein g3p, at the tip of the phage, is one of the minor coat proteins. The genes encoding variable domain of the scFv are fused to gene III in the genome of the filamentous phage. The scFv is displayed as a fusion to pIII protein at the top of the phage. The phage retains its ability to infect bacteria since the scFv is not fused to all pIII protein molecules. Four g3p molecules are illustrated in the figure, three of which display scFv molecules.

2.2.1 Biology of the filamentous bacteriophage

2.2.1.1 Structure

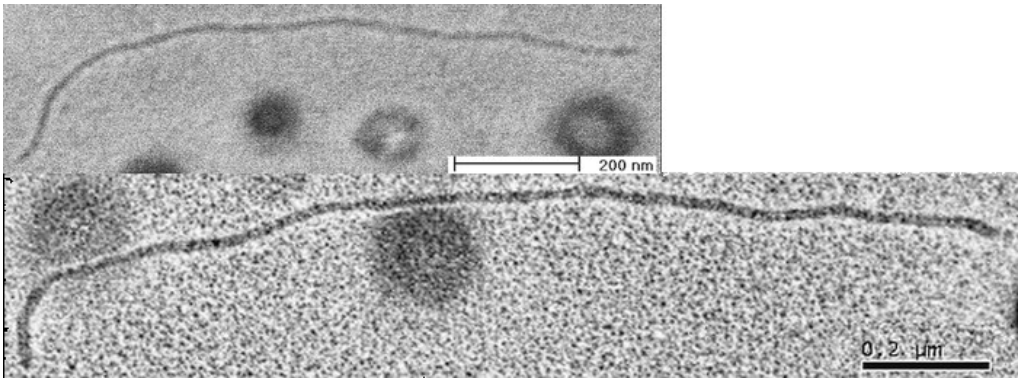
Filamentous phage have been identified which are able to infect a variety of gram negative bacteria. The best characterized of these nonlytic bacteriophage (M13, fl and fd) infect *E. coli* containing the F conjugative plasmid. The genomes of these three bacteriophage have been completely sequenced and are 98% homologous (Beck and Zink, 1981; Hill and Petersen, 1982; Van Wezenbeek et al., 1980). M13, Fl and fd are

collectively refered to as the Ff phage because of their dependence on the f plasmid for infection and their similarity. Ff phage has a single strand, covalently closed DNA genome which is enclosed in a long cylinder of approximately 7 nm wide by 900 to 2000 nm in length. Each genome encodes 11 genes, the products of which are listed in Table 3. The genes are grouped in the genome according to their functions in the life cycle of the phage. There are three groups of genes. One group (gene II, V, and X) encodes proteins required for DNA replication of the phage genome (Rapoza and Webster, 1995). The second group (gene III, VI, VII, VIII and IX) encodes the proteins that make up the capsid proteins for phage particle (Endemann and Model, 1995; Rapoza and Webster, 1993; Simons et al., 1981). Among these, pVIII is the major coat protein (with about 2700 copies per phage), while pIII, pVI, pVII and pIX are minor coat proteins (each with five copies per phage). Protein pIII, which is commonly fused with recombinant proteins (Smith, 1985), is presented as approximately 3-5 molecules. The last group (gene I, IV, and XI) encodes three proteins that are involved in the membrane associated with assembly of the bacteriophage. Gene products pI, pIV, and pXI are required for phage assembly (Horabin and Webster, 1988; Rapoza and Webster, 1995), but they are not part of the phage particle.

Table 3. Genes and gene products of fl bacteriophage (Webster, 1996)**.**

Gene	Function	Amino acid
II	DNA replication	410
X	DNA replication	111
V	Binding of ssDNA	87
VIII	Major capsid protein	50
III	Minor capsid protein	406
VI	Minor capsid protein	112
VII	Minor capsid protein	33
IX	Minor capsid protein	32
I	Assembly	348
IV	Assembly	405
XI	Assembly	108

A



B

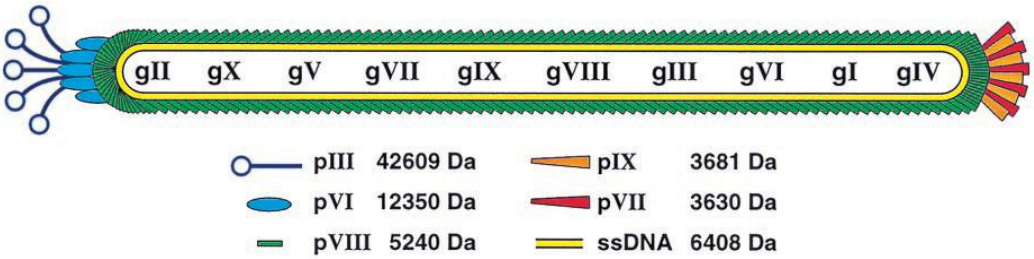


Figure 3. (A) Electron microscopic of filamentous bacteriophage (http://www.mardre.com /homepage/mic/tem/samples/bio/virus/m13a.htm). (B) Filamentous phage fd architecture (Gao et al., 1999). Its single-stranded DNA genome is encased in a cylindrical protein coat. The long site of the cylinder consists of about 2,800 molecules of the major coat protein (pVIII). On one end, the particle is capped by about five copies of the proteins pVII and pIX. During the phage assembly process, this end emerges first from the bacterium. The incorporation of the minor coat proteins pIII and pVI on the other end of the particle terminates the assembly process. Protien III is the first protein to interact with the host cell during infection.

2.2.1.2 Life cycle of phage

The general stages to a phage life cycle are infection, replication of the viral genome, assembly of new viral particles and then release of the progeny particles from the host. Infection is a multistep process requiring interactions with the F conjugative pilus and the bacterial TolQ, R and A cytoplasmic membrane proteins. The infection begins when pIII attaches to the F pilus of *E.coli.* The phage genome is then transferred to the cytoplasm of the bacterial cell where resident proteins convert the single stranded DNA genome to a double stranded replicative form ("RF") (Webster, 1996). This RF undergoes rolling circle replication to make ssDNA, and serves as a template for expression of phage capsid proteins, and other phage proteins involved in the assembly process. Once the phage DNA has been fully coated with pVIII, the secretion terminates by adding the pIII and pVI cap (Marvin, 1998), and the new phage detaches from the bacterial surface. The bacterial host can continue to grow and divide, allowing this process to continue indentifinity. Filamentous bacteriophage does not produce lytic infection in their host.

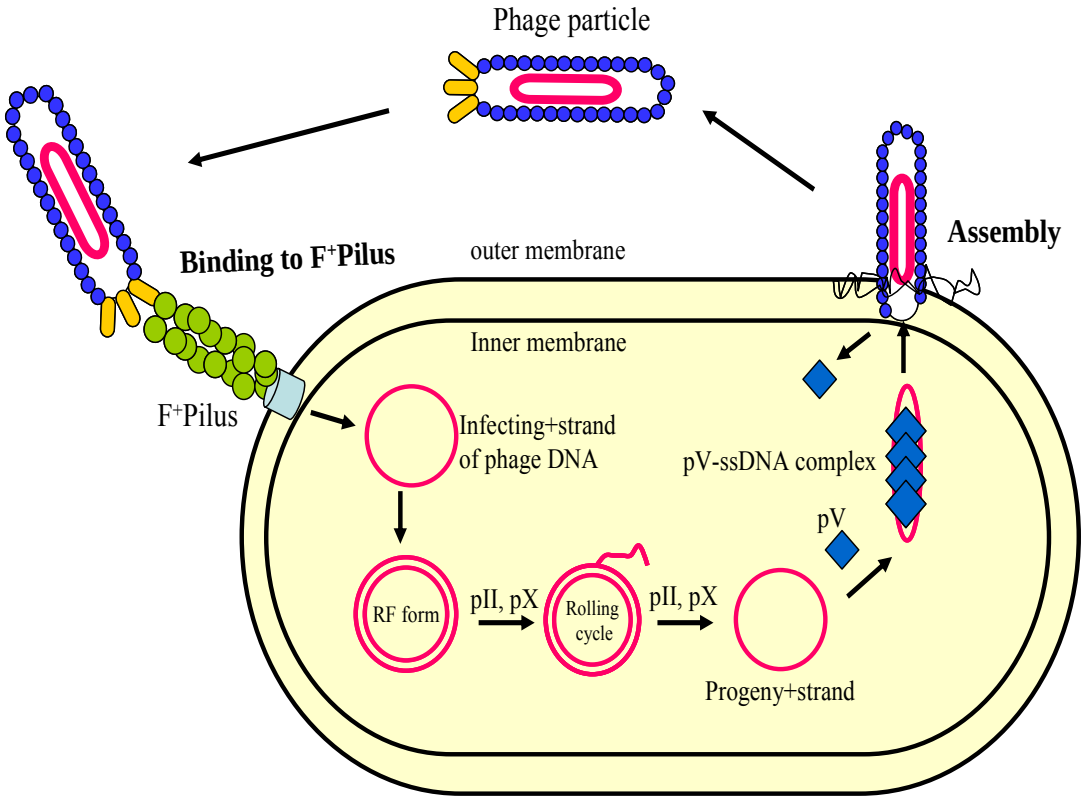


Figure 4. Life cycle of filamentous phage. Phage particle infected to bacterial host cell by binding of pIII to the tip of the F pilus and entry of the single stranded DNA (ssDNA) into the cytoplasm. The ssDNA is converted by host enzymes to a double stranded replicative form (RF). The RF undergoes rolling circle replication to make ssDNA and also serves as a template for expression of the phage proteins gIIIp and gVIIIp. Phage progeny are assembled by packaging of ssDNA into protein coats and extruded through the bacterial membrane into the medium.

2.2.1.3 Phage vector

Phagemids are the most popular vector for display which is hybrids of phage and plasmid vectors. These vectors contain the origins of replications for both M13 phage and *E. coli*, gene coding part of pIII protein with the appropriate cloning site, and an antibiotic-resistance gene (Azzazy and Highsmith, 2002). Moreover, phagemids often contain an amber stop codon, to allow host specific expression of pIII fusion protein or soluble fusion partner, respectively (Hoogenboom et al., 1991), and a gene encoding one coat protein that will be fused to the foreign DNA that is to be expressed (Barbas et al., 1991; Kang et al., 1991).

If a phagemid vector is used, phage particles will not be released from the *E. coli* cells until they are infected with helper phage, which enables packaging of the phage DNA and assembly of the mature virions with the relevant protein fragment as part of their outer coat on either the minor (pIII) or major (pVIII) coat protein. The incorporation of many different DNA fragments into the pIII or pVIII genes generates a library from which members of interest can be isolated.

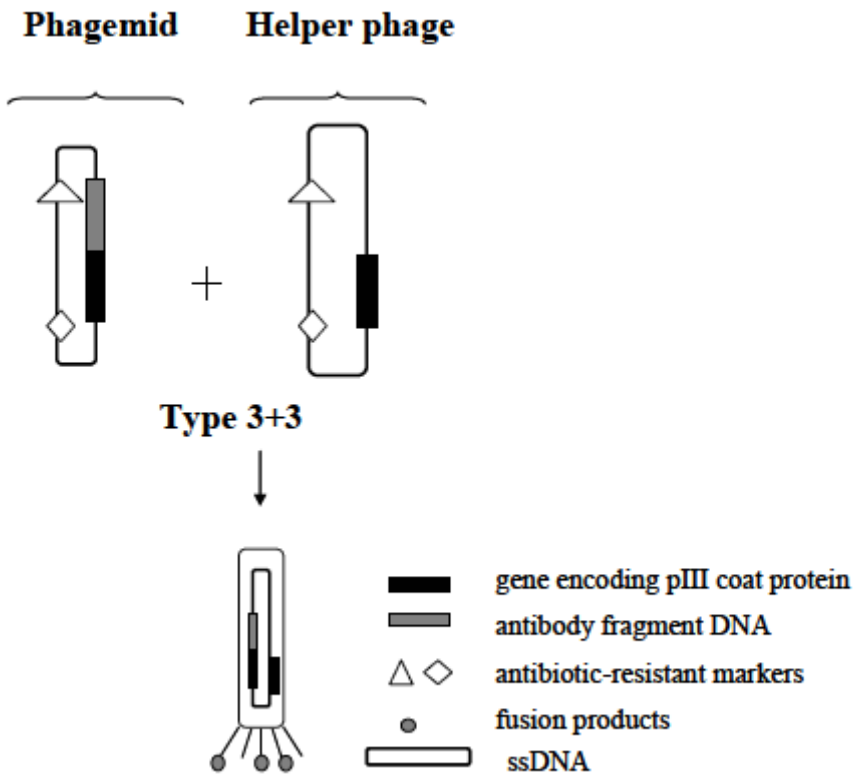


Figure 5. Schematic picture of a phagemid vector used to display antibody fragment on the surface of filamentous phage through pIII coat protein (Yau et al., 2003). Gene pIII is represented as a black box, the antibody fragment DNA insert as a grey box. The phagemid vector replicated in *E. coli*, but co-infection with a helper phage is required for the production of phage particles.

2.3 Phage display antibody library

Phage display has been used for isolation recombinant antibodies with a range of unique specificities and produces many high affinity antibodies without immunization. The surface of filamentous bacteriophage can be used displayed to antibody fragments in different formats, including Fab fragment (Cabilly, 1989), Fv fragment (Skerra and Plu¨ckthun, 1988), scFv fragment (McCafferty et al., 1990), and diabody fragments(Holliger et al., 1993).

In general, two kinds of library can be used: naïve and immune. The naïve libraries are derived from natural unimmunized human rearranged V genes (Sblattero and Bradbury, 2000; Sheets et al., 1998), synthetic human V genes (Knappik et al., 2000) or shuffled V genes (Rader et al., 1998). Immune libraries are created from V genes from immunized humans (Dubrovskaya et al., 2007) or mice (Orum et al., 1993). Antibodies selected from immunized libraries tend to have much higher affinities for the antigen used for immunization when compare to a naïve library of equivalent size.

Key advantages of naïve library or single-pot repertoires include: (i) isolation of human antibodies to self, nonimmunogenic or toxic antigens; (ii) a single library can be used for all antigens; (iii) short time needed for antibody generation (2—4 rounds of selection in two weeks); and (iv) direct isolation of high affinity antibodies when very large repertoires are used (de Haard et al., 1999; Vaughan et al., 1996). Disadvantages of naïve libraries are: (i) low affinity of antibodies isolated from small sized libraries; (ii) the time needed to construct large libraries, and (iii) content and quality of the library are unequal.

Advantages and disadvantages of immune libraries include: (i) the long time requirement for animal immunisation, (ii) easier preparation than naive libraries (only medium sized repertoires, 10^6–10^7 of different phage particles are desired), (iii) the unpredictability of the immune response of the animal to an antigen of interest, (iv) the lack of immune response to some antigens, (v) the restrictions in generating human antibodies,

(vi) construction of a new library for each new antigen which increases the total time of the procedure by 1–3 month.

Thus, Phage display antibody has many advantages for production of antibody over conventional methods. Researcher can use appropriate kind of library, depending on their research objectives.

2.3.1 Phage display antibody expression formats

The antibody molecule has four polypeptide chains, two heavy (H) chains with molecular weights of 50 kDa and two light (L) chains (25 kDa molecular weight) linked by disulphide bonds. The chains have both constant (C) and variable (V) regions. The antigen-binding site of an intact antibody consists of a paired of variable domains VH and VL.

Antibodies have been displayed as functional binding molecules in different formats of antibody fragments. Effective display formats of antibodies are single-chain fragment variable (scFv) (Clackson et al., 1991; Marks et al., 1991), Fab (fragment antibody binding) fragments (Kang et al., 1991), Fv's (fragment variable) with an engineered intermolecular disulphide bound to stabilize the V_{H}- V_{L} pair (dsFv's) (Brinkmann et al., 1995), and diabody fragments (Holliger et al., 1993; McGuinness et al., 1996).

ScFv are relatively small (26-27 kDa), generally quite stable, and are encoded by a single gene, which simplifies genetic manipulations. The most common polypeptide linker to fuse the V_{H} and V_{L} chains together into a scFv antibody fragment is $(Gly_4Ser)_3$. The two variable domains can be connected either as V_{H}-linker-V_{L} or V_{L}-linker-V_{H}.

The other commonly used recombinant antibody fragments are Fab. Fab consist of two polypeptide chains, one containing the light chain variable and constant domains, V_{L}-$C_{\kappa \text{ or } \lambda}$, the other a truncated heavy chain containing the variable domain and one constant domain, V_{H}-C_{H1}. The illustrating picture of antibody fragment is shown in Figure 5, and the characteristics of different antibody fragments are presented in Table 4.

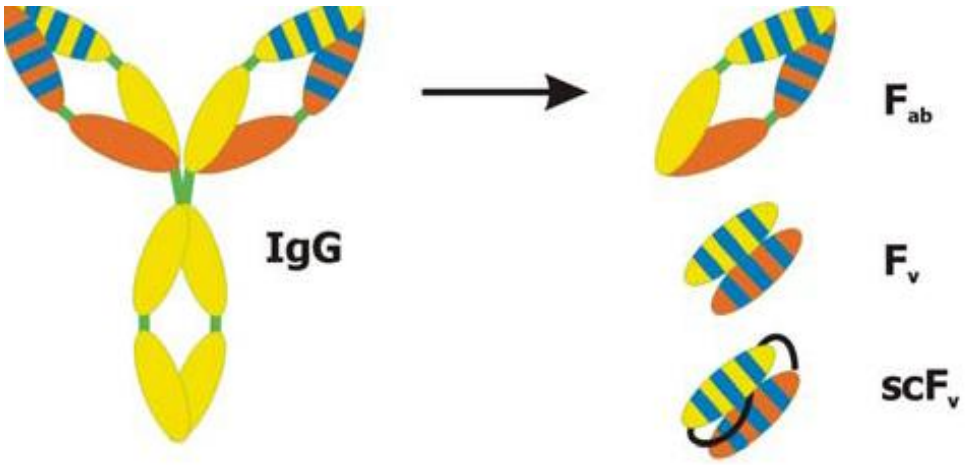


Figure 6. A cartoon representation of Immunoglobulin G (IgG) and antibody fragments. (Fj llman and Hall, 2005) Heavy chains are light yellow, whereas light chains are dark orange. Striped domains represent the variable domains ontaining complement determining regions (CDRs) in blue. The antigen binding fragment (Fab) is a large and stable fragment, whereas the variable fragment (Fv) is an unstable association of the two original variable domains. The single chain variable fragment (scFv) is recombinantly expressed with a linker to make it a small and stable antibody fragment.

Table 4. Characteristics of antibody fragments. Adapted from Azzazy and Highsmith (2002).

Antibody fragment	Size (kDa)	Paratopes (valency)	Structure
scFv	25-30	1	V_H and V_L domains are linked by a linker peptide. Changing the linker length can lead to the formation of diabody (60 kDa), triabody (90kDa), or tetrabody (120 kDa)
Fv	25	1	V_H and V_L with no linker between the V domain
Fab	50	1	Composed two chains of V_H-C_H1 and V_L-C_L
$F(ab)_2$	100	2	Two Fab molecules
IgG	150	2	Parent antibody molecule consists of two heavy chain (V_H-C_H1-C_H2-C_H3) and two light chain (V_L-C_L)

2.3.2 Application of phage display antibody

The most successful applications of phage display antibody include (i) the de novo isolation of high affinity human antibodies from non-immune and synthetics libraries (Knappik et al., 2000); (ii) isolation of genes encoding antigen-specific antibodies from immunized animals and from hybridomas; (iii) the discovery of antibodies with unique properties from non-immune (Huie et al., 2001) and immune libraries from animal or human donors (Kramer et al., 2003). Antibody libraries displayed on phage are frequently used in the pharmaceutical industry for isolation of therapeutic antibody leads, for development into primarily anti-cancer or anti-inflammatory antibody drugs.

Phage display is particularly useful in cases where monoclonal antibodies could not be obtained by classical hybridoma technique such as antibodies against nonimmunogenic or toxic antigens such as Aflatoxin, which is hapten molecule (Daly et al., 2002; Moghaddam et al., 2001). Phage antibody technology can also be used to clone and rescue monoclonal antibodies from genetically unstable hybridomas. Phage antibody genes can be easily sequenced, mutated, and engineered to improve antigen binding (Azzazy and

Highsmith, 2002). Finally, soluble recombinant antibodies can be produced quickly and economically and can be used as *in vitro* diagnostic reagents.

2.3.3 Selection of monoclonal antibody by in vitro bio-panning

The specifically binding clones are selected from phage display antibody libraries by using bio-panning method. This technique is used to enrich phage particles from phage-display libraries. The phage display antibodies are incubated with immobilized target or interested antigen. The unbound phages are removed by washing with washing buffer such as Phosphate Buffer Saline buffer (PBS) or PBS containing Tween. Phage antibodies bound to target can be eluted in different ways namally; acidic solution such as glycine-HCl buffer (Kang et al., 1991; Parmley and Smith, 1988), basic solutions like triethylamine (Marks et al., 1991), by enzymatic cleavage of protease site engineered between the antibody and gene III (Ward et al., 1996) or by competition with excess antigen (Clackson et al., 1991) or antibodies to the antigen (Meulemans et al., 1994). After elution, the specifically bound phages are amplified in *E.coli* cells for further rounds of bio-panning. Ideally, only one round of selection should be required, non-specific binding limits the enrichment that can be achieved per selection round. In most cases, several rounds (approximately 2-4 rounds) of selection and amplification are needed to select the best binders from the phage display library. Figure 7 show illustration diagram of in vitro bio-panning method.

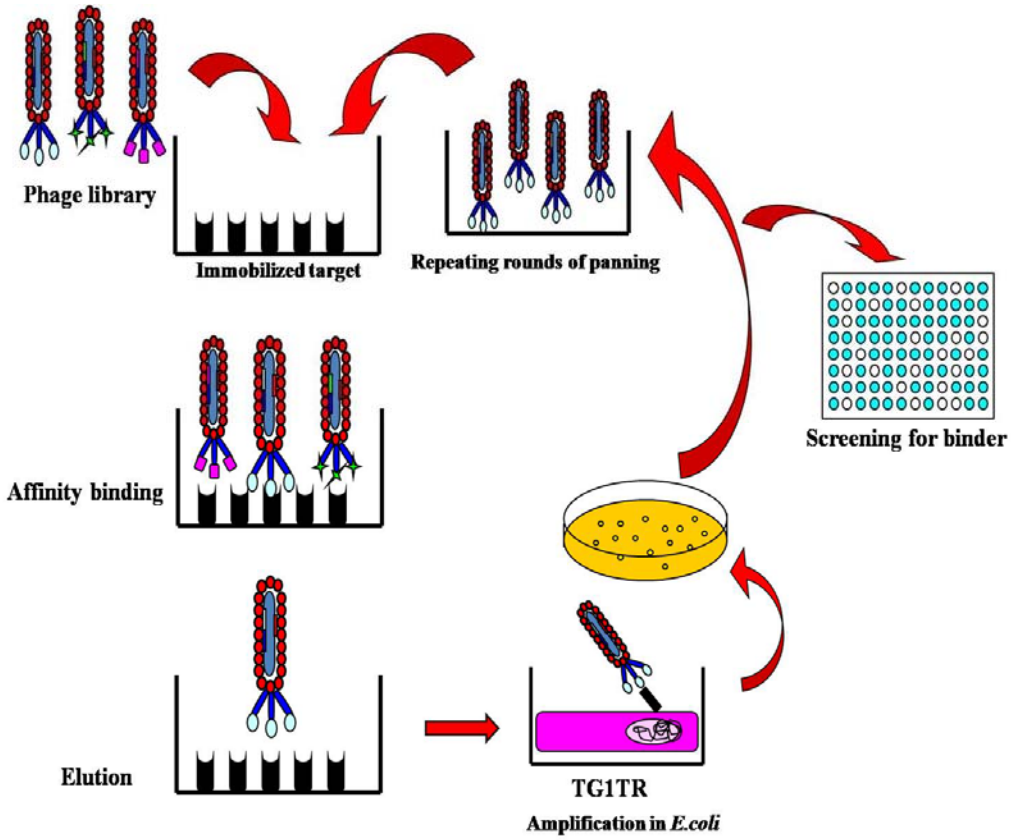


Figure 7. Overview of bio-panning method. The target or antigens of interest are immobilized on to immuno tube or ELISA plate. The phage display antibodies are leaved to bind to target protein. Unbound phages are washed away. After that, the bound phages are eluted appropriate methods as described in the text. The specific phages are amplified in *E.coli.* and are enriched in each round of selection. The binder phages are screened in ELISA technique.

2.3.4 Production of soluble scFv fragments

If the phagemid contain amber stop codon after the scFv gene, the soluble scFv can be produce by infecting *E. coli* strain HB2151 is nonsuppressor cells as they recognize an amber stop codon, engineered between the scFv gene and gIII in the phagemid. Thus only express the scFv will be expressed without the g3p protein. Phagemid that is used in this thesis also designed to introduce polyhistidine tag fused to the expressed scFv, thereby permitting rapid and simple protein purification by immobilized metal affinity chromatography. Depending on the isolated clone, soluble antibodies may be present in the culture supernatant, the bacterial periplasm, and inside the bacterial cells. All three fractions must be isolated and analyzed by western blot, using a commercially available conjugated antihistidine (His) tag antibody, to determine the location of the soluble antibodies. Soluble scFv are relatively simple to isolate, can be economically produced in bacteria in very large quantities (Kipriyanov et al., 1997; Mavrangelos et al., 2001), and do not entail complex refolding procedures (Sánchez et al., 1999).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Phage

Griffin.1, TomlinsonI and TomlinsonJ scFv libraries from the MRC, Cambridge Centre for Protein Engineering, Cambridge England were kindly provided by Prof. Greg Winter and Dr. Ian Tomlinson, respectively.

KM13K07 helper phage NEBa (Vieira and Messing, 1987) and Propagated as described in the MRC phage display protocols.

3.1.2 Antigen

Aflatoxins conjugated with BSA and soluble Aflatoxin B_1, B_2, G_1, G_2 were prepared from *Aspergillus flavus* (Sigma, Germany).

Bovine albumin serum BSA was natural purified protein obtained from Fluka, USA.

3.1.3 Bacteria

E. coli TG1: Suppressor strain was used for cloning and for amplification of phage between rounds of panning (Supplied by MRC, Cambridge, UK).

E. coli HB2151: Non-Suppressor strain was used for inducing scFv fragment.

3.1.4 Antibodies

Anti-M13 Horseradish peroxidase (HRP): (Amersham-Pharmacia Biotech, Sweden)

Anti-Protein L peroxidase HRP (Sigma, Germany)

3.1.5 Instruments

Autoclave:	Hiclave HA-3000MIV, Hirayama, Japan
Balance:	Precisa 205A, Precisa Instruments, Switzerland
	Precisa 3000C, Precisa Instruments, Switzerland
Centrifuge machine:	Sorvall RC5C plus, Kendro laboratory Products, USA
	Eppendrof centrifuge 5810 R, Eppendrof, USA
Deep freezer -70 °C:	Heto, Ultra Freeze, Denmark.
ELISA reader:	Sunrise, TECAN, Austria
Electroporator :	Eppendrof 2510, Eppendrof, USA
Freezer -20 °C:	Heto, HLLF 370, Denmark.
	MyBio LFT420, DAIREI, Denmark
Gel dryer:	Drygel sr. SLAB GEL Dryer model SE1160, Hoefer Scientific Instruments, USA
Gel electrophoresis apparatus:	Mini Protean® 3 cell, BioRad, USA
Heat Box:	HB1, Wealtee Corp., USA
Incubator shaker:	C24 Incubator shaker, New Brunswick Scientific, USA
Incubator:	Memmert, BE 500, WTB Binder BD115, Shel-Lab 2020 Low Temperature Incubator, Sheidon, USA
Laminar hood	Holten LaminAir HBB 2448, Denmark.
	BH2000 Series ClassII Biological Safety Cabinets,
	BHA120 & BHA180, Clyde-Apac,
Membrane transfer	Semi Phor, Hoefer Scientific instruments, USA
Microcentrifuge:	Mini spin plus, Eppendrof, USA
	Eppendorf 54154, Eppendorf, Germany

pH meter:	Ultra Basic pH meter, Denver Instruments, Germany
Rotator:	Certomat TCC, B. Braun Biotech International, Germany
Sequencing machine:	ABI prism model 310 Genetic Analyzer, Applied Biosystems, USA
Shaker:	Innova 2300 platform shaker, New Brunswick Scientific, UK Certomat TC2, B. Braun Biotech International, Germany
Sonicator:	Waken GE100 Ultrasonic processor, Japan
Spectrophotometer:	Ultrospec 2000, Pharmacia biotech, UK
Stirrer:	Variomag Electronicrührer Poly 15, Germany Magnetic stirrer MSH300, USA
Thermomixer:	Thermomixer compact, Eppendrof, USA
Vortex:	Vortex-Genie 2 G506, Scientific Industries, USA

3.2 Methods

3.2.1 Selection of scFv fragment from naive semi-synthetic scFv antibody library

3.2.1.1 Amplification of phage libraries for panning

The Tomlinson I and J library are contributed by MRC (Cambridge, UK). These are naive semi-synthetic scFv antibody library that modify at CDR3 region. The 500 µl libraries stock (Tominson I and J) were added into 50 ml pre-warmed 2XYT (16g/L tryptone, 10g/L yeast extract and 86mM NaCl) containing 100 µg/ml ampicillin and 1% (w/v) glucose and grown shaking at 37^{o}C OD_{600} 0.4 (approximately 2 hours). The 1×10^{11} KM13 helper phage was added into 50 ml cultures and incubated at 37^{o}C for 30 minutes. Then, the cultures were centrifuged at 4,000 rpm for 10 minutes and resuspended in 100 ml of 2XYT containing 100 µg/ml ampicillin, 50 µl/ml kanamycin and 0.1% (w/v) glucose. The cultures were incubated shaking at 30^{o}C for overnight. The next day, the overnight

cultures were spun at 4,000 rpm for 30 minutes. The 20 ml PEG/Nacl (20% Polyethylene glycol 6000, 2.5 M NaCl) was added into 80 ml supernatant and left for 1 hour on ice and spun again at 4,000 rpm for 30 minutes. The pellet was resuspended with 4 ml Phosphate Buffer Saline (PBS) with 15% glycerol and stored at -70^{o}C. These libraries were used for the selection of the specific scFv clones to aflatoxin.

3.2.1.2 Selection of phage antibodies (Bio-panning)

The selection was done on Nunc Maxisorp immuno tube (Nunc, Denmark). Aflatoxin B_1 conjugated with Bovine serum albumin (BSA) (Sigma, Germany) in 100 µl phosphate buffered saline (PBS) was immobilized on immune tube and incubated for overnight (16-18 hours) at 4^{o}C. This experiment was done 3 round of selection. Amount of targets were used for selection in each round 50 µg, 15 µg and 5 µg respectively. The immune tube were washed three times with PBS and blocked with PBS supplemented with 4% (w/v) skimmed milk (4% MPBS) for 2 hours at room temperature. After, removal of the blocking solution, the tube was washed three times with PBS. The libraries were pre-incubated in the 4% (w/v) MPBS and 1% (w/v) BSA for 30 minute at room temperature. After incubation for 30 minute at room temperature, the libraries were added to immune tube and incubated at room temperature for 2 hours. Unspecific phages were washed away three times with PBS supplemented with 0.05% (v/v) Tween 20 (PBST) and three times with PBS. Bound phages were eluted with 500 µl of 1µg/ml trypsin buffer (50 µl trypsin in 450 µl PBS) incubating the immune tube at room temperature for 20 minutes with rotating.

The eluted phages were recovered by infection of 350 µl of exponentially growing *E. coli* TG1 at 37^{o}C for 30 minute. Then, phages infected were performed 10-fold-serial dilution (10^2-10^6) and spread each dilution on TYE agar plates (10g/L tryptone, 5g/L yeast extract, 137mM NaCl and 15g/L Bacto-agar) supplemented with 100 µg/ml ampicillin and 1% (w/v) glucose. The agar plates were incubated at 37^{o}C for overnight.

3.2.1.3 Further rounds of selection

1 ml 2XYT media were added to the agar plates that have a lawn of infected bacteria and loosen the cells with glass spreader. The scraped cells were kept as 15% (v/v) glycerol stock at -70^{o}C. The 10 µl of scraped bacteria were added into 10 ml 2XYT supplemented with 100 µg/ml ampicillin and 1% w/v) glucose and incubated at 37^{o}C with shaking until OD_{600} was 0.4 (approximately 2 hours). After this procedure, the phages were rescued by 5×10^{10} helper phage KM13 (0.65 µl) and incubating 37^{o}C without shaking for 30 minutes. After centrifugation at 4,000 rpm, 4^{o}c for 15 minutes, supernatant was removed and resuspend the pelleted bacteria with 5 ml of 2XYT containing 100 µl/ml ampicillin, 50 µl/ml kanamycin and 0.1% (w/v) glucose prior to incubated at 30^{o}c with shaking for overnight. Next day, the overnight culture was centrifuged at 4,000 rpm, 4^{o}C for 15 minutes and kept the supernatant. After, 1ml PEG/NaCl was added with 4 ml of the supernatant and kept on ice for 1 hour and then spun at 4,000 rpm, 4^{o}c for 30 minutes. The supernatant was removed and the pellet was resuspened in 100 µl PBS for the next round of selection. Three rounds of selections were performed.

3.2.1.4 Polyclonal phage ELISA

The Immuno 96 MicroWell^{TM} plate (Nunc, Denmark) was immobilized with 5 µg of AflatoxinB1-BSA and 1% (w/v) Bovine serum albumin BSA in 100 µl PBS buffer as control. After incubating plate at 4^{o}C for overnight, the plate was washed 3 times with PBS. The wells were blocked with 4% (w/v) skimmed milk for 2 hours at room temperature. Then, wells were washed 3 times with PBS and added 50 µl precipitated phage from the end round of selection in 50 µl 4% (w/v) MPBS. The plate was incubated at room temperature for 2 hours after that, phage solution was discarded. Unspecific phages were washed away three times with PBST, followed by three times with PBS and incubated for 1 hour with a 1:5,000 dilution of a mouse anti-M13 phage-horseradish peroxidase (HRP) conjugate (Amersham-Pharmacia Biotech, Sweden) in 2% (w/v) MPBS. The plate was

washed again as described earlier. The 100 µl 3,3', 5,5'- Tetramethybenzidine (TMB; Sigma, Germany) substrate was added. The reaction was developed to be a blue color at 37°C for 30 minutes and was then stopped using 50 µl/well of 10% (v/v) HCl. The absorbance was read at 450 nm, using a Sunrise absorbance reader (TECAN, Austria).

3.2.1.5 Preparation of monoclonal phage

In the 1st and 3rd selection round, individual clone was randomly picked out from the TYE plate to different well (100 µl of 2XYT with 100 µg/ml ampicillin and 1% (w/v) glucose was added) of immuno plate (Nunc, Denmark). After culturing, small inocula (5 µl) from each well was transferred to a second ELISA plate containing 200 µl of 2XYT with 100 µg/ml ampicillin and 1% (w/v) glucose per cell. The original ELISA plate or master plate was added with glycerol to a final concentration of 20 % and kept at -20°C. The transferred plate was shaken at 37°C for 2 hours, and phages were rescued in *E.coli* TG1 cells by adding 10^{10} helper phage to each well, the plate was shaken at 37°C for 1 hour before spinning the plate at 4,000 rpm for 10 minutes. The supernatant was thrown away and the pellet was resuspended in 200 µl of 2XYT containing 100 µg/ml ampicillin and 50 µg/ml kanamycin, and cultured at 30°C 250 rpm for overnight. The overnight culture was spun at 4,000 rpm for 10 minutes and 50 µl of the supernatant containing phage was used in monoclonal phage ELISA.

3.2.1.6 Confirmation of binding activity of monoclonal phage clones by ELISA

Immuno 96 Wells™ Plate was coated and blocked as in polyclonal phage ELISA. After incubation for 2 hours at room temperature, the wells were washed 3 times with PBS. Then 50 µl of phage supernatant and 50 µl 4% (w/v) MPBS were added into each well of the plate. Unspecific phages were washed away 3 times with PBST and 3 times with PBS. After stand on the bench 2 hours, the plate was washed 3 times with PBST and followed 3 times with PBS. Subsequently, 100 µl HRP-anti-M13 (1:5000 dilution in 2% (w/v) MPBS)

was added to each well. After incubation for an additional 1 hour at room temperature, the wells were washed again and 100 µl substrate solutions (TMB, SIGMA) was added to each well and incubated at 37°C for 30 minutes. The reaction was stopped with 10% (v/v) HCl. The yellow color was developed after adding HCl. Absorbance was measured at 450 nm.

3.2.2 Expression and purification of scFv antibody

3.2.2.1 Induction of soluble antibody fragment

In total, 35 positive clones from Tomlinson J and 61 clones from Tomlinson I library that shown high activities were infected into a non-suppressor strain HB2151 which reads the amber codon as a stop signal, thereby solubly expressing the antibody fragment's. The 5 µl of phage particles from master glycerol stock were infected to 150 µl exponentially growing *E.coli* HB2151 bacterial at an OD_{600} of 0.4, and incubated at 37°C for 30 minutes. After that, the infected bacteria were streak on TYE agar plates containing 100 µg/ ml ampicillin and 1% (w/v) glucose. The plate**s** were incubated at 37°C for overnight. The next day, single colonies were picked with pipette tips and used to inoculate 100 µl of 2xTY, containing 100 µg/ ml ampicillin and 1% (w/v) glucose in 96 immuno plate. The culture was incubated with shaking at 37°C for overnight. The following day, the 8 µl cultured were transferred to 96 Deep Well immune plate containing 800 µl 2XYT, 100 µg/ ml ampicillin and 0.1% (w/v) glucose and incubated shaking at 37°C until OD_{600} reached 0.9. The master plate was added glycerol to a final concentration of 15% and stored at -70°C. The 100 µl 2XYT, 100 µg/ ml ampicillin and 9 mM isopropyl-β-d-thiogalactopyranoside (IPTG), achieving a final concentration of 1mM were added into 96 deep well plate. The incubation was continued at 30ºC for overnight. The secreted antibody could be found in the supernatant.

3.2.2.2 Small scale production of soluble scFv antibody

A single colony of HB2151 *E.coli* cells containing the pIT2 bearing the 3I-F6 encoding gene was inoculated into 5 ml 2XYT broth containing 100µg/ml Ampicillin, 1% (v/v) glucose and was grown overnight at 37^{o}C, with shaking at 250 rpm. The next day, overnight culture was inoculated into 15 ml 2XYT medium containing 100µg/ml Ampicillin, 0.1% (v/v) glucose at 37^{o}C for 3 hours with shaking at 220 rpm. After 3 hr, the culture was induced with 1mM IPTG and incubated overnight at 30^{o}C with shaking at 220 rpm. The culture was centrifuged at 4,000 rpm for 20 min at 4^{o}C and the supernatant was discarded. The pellet was resuspended in 1ml of lysis buffer (20mM Tris; pH 8.0, 150mM NaCl and 1mM PMSF). The cells were sonicated twice on ice for 30sec at 50% output. They were then centrifuged in microcentrifuged tube at 14,000 rpm at 4^{o}C for 10 minutes. The supernatant is cell lysate fraction and was used to purify scFv fragment.

3.2.2.3 Small scale purification by using Ni-NTA resin

ScFv antibody containing a hexahistidine tag was purified using Ni^{++} ions immobilized on resin by covalent linkage to nitrilotriacetic acid (NTA) (Novagen). The high affinity interaction between the histidine residues and the Ni^{++} ions allow the specific purification of histidine tagged recombinant proteins. The antibody fragments were eluted with imidazole, which competes for binding to the Ni^{++} ions. Before use of the Ni^{++} ions, the resin was equilibrated with lysis buffer (20mM Tris; pH 8.0, 150mM NaCl, 1mM PMSF). Thus, the same pH and buffer composition with that of the Ni^{++} ions resin ensuring that the sample would bind properly. The cell lysate fraction was incubated in a microcentrifuge tube with 200 µl Ni-NTA resin for 2 hour to allow the binding of hexahistidine tag to Ni-NTA resin. The solution was centrifuged at 2000 rpm for 5 min. The flow-through from the wash was collected for further analysis. The washing buffer 1 (20mM Tris; pH 8.0, 150mM NaCl, 5mM imidazole) was added into the microcentrifuge tube and spun again. The Ni-NTA matrix was washed with washing buffer 2 (20mM Tris;

pH 8.0, 150mM NaCl, 20mM imidazole). After that, the elution buffer (20mM Tris; pH 8.0, 150mM NaCl, 250mM imidazole) was added into the tube and they were centrifuged again. The scFv fragment should be presented in the eluted fraction. To monitor antibody purity, SDS-PAGE electrophoresis was used. To determine the efficiency of purification, the flow through fraction, wash fraction and elution fraction were compared by SDS polyacrylamide gel and western blot analysis. ELISA was used to detect the antibody binding.

3.2.2.4 Large scale production of soluble scFv antibody

A single colony of HB2151 *E.coli* cells containing pIT2 bearing the 3I-F6 encoding gene was inoculated into 5 ml 2XYT broth, containing 100µg/ml Ampicillin, 1% (w/v) glucose and was grown overnight at 37^{o}C, with shaking at 250 rpm. The next day, an overnight culture was inoculated into 500 ml Terrific broth, containing 100µg/ml ampicillin and 0.1% (w/v) glucose at 37^{o}C for 4 hours (OD_{600} is 0.9) with shaking at 220 rpm. And then, the culture was induced with 1mM IPTG and incubated overnight at 30^{o}C at 220 rpm. The culture was poured into 250 ml centrifuge bottles and centrifuged at 10,000 rpm for 20 min at 4^{o}C and the supernatant was discarded. The pellet was resuspended in 30 ml of sonication buffer (1xPBS, 0.5M NaCl and 20mM Imidazole). The resuspended solution was aliquoted in 1 ml fractions into 2 ml centriguation tubes. The cells were sonicated on ice for 45second min at 40% output for three times. The cells were centrifuged in microcentrifuged tube at 14,000 rpm at 4^{o}C for 10 minutes. The cell lysate fractions were passed through 0.2µm filter for sterisation.

3.2.2.5 Purification scFv fragment by immobilized metal affinity chromatography (IMAC) using Ni-NTA

The column was prepared by loading 4 ml of Ni-NTA resin (Novagen, USA) and left to form a 2 ml packed resin. The column was equilibrated with running buffer (1xPBS, 0.5M NaCl, 20mM Imidazole and 1% (v/v) Tween). The filtered supernatant was transferred to prepared IMAC columns and passed through the column twice and the flow

through was collected. The column was washed once with 30 ml running buffer to remove any non-specifically proteins. The scFv was eluted with 20 ml of 100mM sodium acetate, pH 4.4 and collected in 1.5 ml microcentrifuge tubes (final volume 500µl in each tube) containing 50µl 10x PBS (filtered through a 0.2 µm filter) and 50 µl 100 mM NaOH. The eluted antibody was buffer exchange into PBS and concentrated by ultrafiltration (Vivaspin, Sartorius) and then the scFv solution was dispensed in 20 µl volumes and stored at -20^{o}C until required. SDS-PAGE electrophoresis was used to monitor antibody purify. The efficiency of purification, the flow through fraction, wash fraction and elution fraction were compared by SDS polyacrylamide gel and western blot analysis. ELISA was used to detect the antibody binding.

3.2.2.6 ScFv antibody ELISA

Immuno 96 Wells^{TM} Plate was performed by coating microtitre plates with 5 µg of AflatoxinB_1-BSA in a volume of 100 µl and with 1% (w/v) BSA as control. The ELISA plate were washed 3 times with PBS and blocked with PBS supplemented with 3% (w/v) BSA. After incubation for 2 hours at room temperature, the wells were washed three 3 times with PBS. Then 50 µl of scFv antibody and 50 µl 3% (w/v) BSA were added into each well of the plate. After stand on the bench 2 hours, the plate was washed 3 times with PBST and followed 3 times with PBS. Subsequently, 100 µl Protein L peroxidase (1:5000 dilutions in 3% (w/v) BSA) was added into each well. After incubation for an additional 1 hour at room temperature, the wells were washed again and 100 µl substrate solutions (TMB, SIGMA) was added to each well and incubated at 37^{o}C for 30 minutes. The reaction was stopped with 10% (v/v) HCl. The yellow color was developed after adding HCl. Absorbance was measured at 450 nm.

3.2.2.7 Sequence analysis

Plasmid DNA from three different clones were extracted and purified from overnight culture by using a commercially plasmid preparation kit (Mini Preps: Qiagen, Germany) and were completely sequenced by Macrogen (Seoul, Korea) using primers: For VH use link seq new CGA CCC GCC ACC GCC GCT G and For Vκ use pHEN seq CTA TGC GGC CCC ATT CA. Immunogenic analysis of variable regions was done using IgBLAST and V-Base programme.

3.2.2.8 SDS polyacrylamide gel

SDS polyacrylamide gel was used to determine the efficiency of purification, the flow through fraction, wash fraction and elution fraction were compared. A SDS polyacrylamide gel, consisting of 12 % running gel and 5 % stacking gel in SDS-PAGE electrophoresis buffer. The samples were boiled for 5 min before they were loaded onto the gel. The proteins were detected with Coomassie Brilliant Blue (CBB) staining solution for 15 minutes, followed by destaining with destaining solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid). The gel was then dried using the Gel Drying System (Drygel sr. SLAB GEL Dryer model SE1160, Hoefer Scientific instruments, USA).

3.2.2.9 Western Blot analysis

For western blot analysis, samples will be run on SDS-PAGE gel and the scFv were blotted on to nitrocellulose membrane or PVDF membrane. After run SDS-PAGE, The membrane and filter papers were cut to the same size of gel. All of them were incubated in transfer buffer (25 mM Tris, 192 mM glycine, 10% (v/v) methanol) for 30 minutes at room temperature. The proteins on the SDS polyacrylamide gel were transferred to membrane using a membrane transfer machine (Semi Phor, Hoefer Scientific Instruments, USA). And, membrane was blocked with 3% (w/v) BSA in PBS for 1 hour at room temperature. Then, the membrane was washed 3 times with PBST following 3 times with PBS by rotating for 10 minutes. After that, the membrane was incubated with Protein L peroxidase (a dilution

of 1:5000 in 3% (w/v) BSA) for 1 hour. Membrane was washed the same as previous step. The 3,3',5-5'-Tetramethylbenzidine (TMB) substrate (Sigma, Germany) was used according to the manufacturers protocol for developing the blue color on the membrane. Stop reaction was done by adding 1M EDTA.

3.2.2.10 Competition ELISA for anti Aflatoxin B_1

For the inhibition assay, 5µg of AFB_1-BSA was coated on an ELISA plate. The antibodies, at dilutions previously determined by an antibody titre, were pre-incubated with varying concentrations of soluble aflatoxin-B1 from 5 µg to 0.019 µg. After incubation at 37 ºC for 30 minutes, the mixture was then transferred to the previously coated and blocked microtitre plate and incubated for 1 hour. The unbound antibodies were washed away 3 times with PBS containing 0.05% (v/v) Tween 20 and 3 times with PBS. The secondary antibody (Protein L peroxidase) was added to wells and incubated for 1 hour at room temperature. Following this, the substrate solution was added and incubated at 37 ºC for 30 minutes, 10% (v/v) HCl was used to stop the reaction. The reaction was then measured by reading the absorbance at 450 nm using the Tecan plate reader.

3.2.3 Cloning of scFv fragment into vector containing alkaline phosphatase gene

The anti-AFB_1 selected from Tomlinson I library (TomI-F6) and YAMO 1 library (YM1-C3) were engineered to create scFv-AP fusion.

3.2.3.1 Cloning of scFv fragment into pKP300ΔIII

pKP300ΔIII was generated from Brian, Kay Lab (UIC, USA). It is expressions vector that containing alkaline phosphatase gene. The scFv fragments DNA were inserted into pKP300ΔIII between *NcoI* and *Not*I sites. The DNA of scFv fragments and pKP300ΔIII vector was double digested with *NcoI* (10U/µl, NEB, USA) and *Not*I (10U/µl, NEB, USA) enzymes, to generate compatible sticky ends. The digestion reactions of scFv fragments and pKP300ΔIII vector were performed separately, each in a total volume of 30

µl. The reaction mixtures consisted of 1 µg of insert DNA, 0.5 µg of vector DNA, 1x NEB buffer 3, 1 µg/ml BSA, 5U of *NcoI* (10U/µl, NEB, USA) and 5U of *Not*I (10U/µl, NEB, USA). The reactions were incubated at 37°C for 3 hours. The reactions were heat inactivated at 65ºC for 20 minutes. After heat inactivate enzyme, the *Nco*I/*Not*I digested vector was dephosphorylated by adding 0.5 µl of CIP enzyme (10U/µl, NEB, USA) and incubated at 37ºC for 1 hour. The inserts and vectors were separated from stuffer fragments by gel electrophoresis followed by Wizard clean up kit (Promega, USA). The scFv DNA was ligated into pKP300ΔIII vectors at a 3:1 ratio. The total volume of ligation reaction was 10 µl, which consisted of 24.6 µg of inserted DNA, 12.1 µg of pKP300ΔIII vector, 1x T4 DNA ligase buffer and 1 µl of T4 DNA ligase (400U/µl, NEB, USA). The ligation reaction was incubated at 25ºC for 2 hours and heat inactivate enzyme at 65ºC for 10 minutes. Then, the ligation reaction was transformed into 100 µl of *E.coli* TG1 cells by electroporation. The electroporation was performed at 1.8 kV, 18 µF, 200 Ω and τ approximately 4 msec using an electroporation machine (Eppendrof 2510, Eppendrof, USA). The cuvette was added immediately with 900 µl of 2XYT medium room temperature. The 1 ml of combined transformed cells was incubated at 37ºC for 1 hour. After that the transformed cells were spread on LB plates, containing 100 µg/ml ampicillin and incubated for overnight at 30ºC. Five of individual clones were picked and analyzed by double digestion with *NcoI/NotI* and ELISA format.

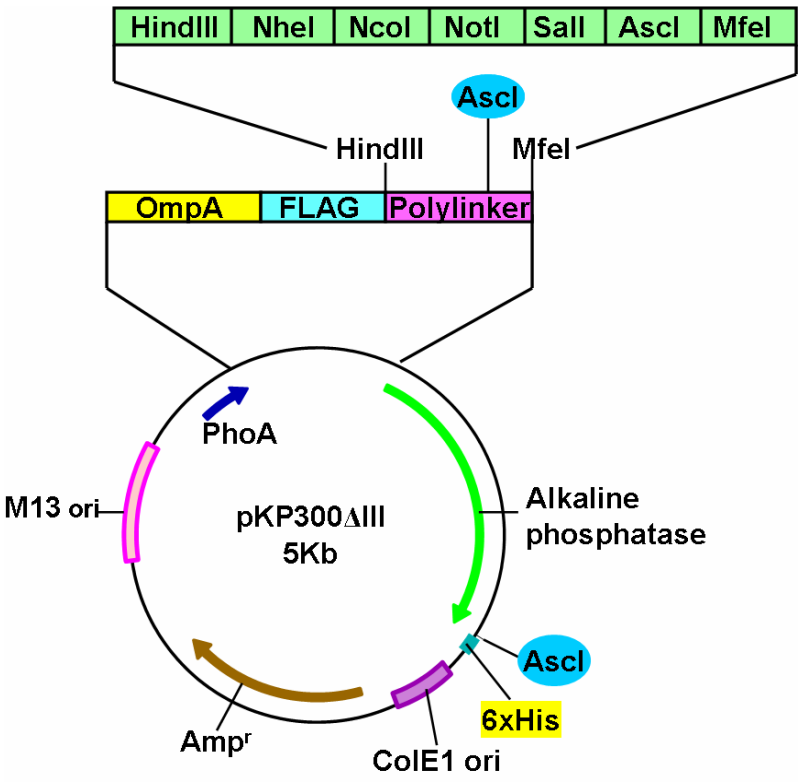


Figure 8. Map of pKP300ΔIII (Krittika, personal contact). The arrow shows the position of PhoA promoter, alkaline phosphatase and ampicillin resistance genes.

3.2.3.2 Digestion to check positive clones

Five individual colonies were picked with sterilized toothpicks into 5 ml of low phos media (30 mM $(NH_4)_2SO_4$, 2.4 mM $(CH_2COONa)_2.2H_2O$, 14 mM KCl, 5.36g/L yeast extract, 5.36g/L Hy-case SF casein hydrolysate, 7mM $MgSO_4$, 14mM glucose, pH 7.3 adjust by KOH). After incubation for 18 hour, the samples were centrifuged at 4000 rpm for 10 minutes. The supernatants were tested the activity specifically to aflatoxin on ELISA format. The DNA of each clones were extracted DNA by using Qiagen mini prep (Qiagen, Germany). The digestion reactions of DNA were performed, in a total volume of 10 µl. The reaction mixtures consisted of 1 µl of DNA, 0.5 µg of vector DNA, 1x NEB buffer 3, 1 µg/ml BSA, 1U of *NcoI* (10U/µl, NEB, USA) and 1U of *Not*I (10U/µl, NEB, USA). The reactions were incubated at 37°C for 1 hour and were run on 2% (w/v) agarose gel in TAE

buffer at 100 volts for 40 minutes. The positive clones were shown 2 bands in approximately size 5kb and 800 bp.

3.2.3.3 ScFv-AP ELISA

Immuno 96 Wells^{TM} Plate was performed by coating microtitre plates with 5 µg of AflatoxinB$_1$-BSA in a volume of 100 µl and with 1% (w/v) BSA as control. The ELISA plates were washed three times with Tris-buffered saline (TBS; 25mM Tris-HCl, pH 7.5, 140 mM Nacl, 3mM KCl) supplemented with 0.05% (v/v) Tween 20 (TBST) and blocked with TBS supplemented with 3% (w/v) BSA. After incubation for 1 hour at room temperature, the wells were washed 5 times with TBST. Then 50 µl of scFv-AP supernatant and 50 µl 3% (w/v) BSA were added into each well of the plate. After standing on the bench for 2 hours, the plate was washed 5 times with TBST. Subsequently, 100 µl p-Nitrophenyl phosphate disodium salt hexahydrate (PNPP; SIGMA, USA) was added into each well. After incubation for 20 minute at room temperature, an absorbance was measured at 405 nm.

3.2.3.4 Expression and purification scFv-AP by using Ni-NTA column

Individual colony was picked with sterilized toothpicks into 10 ml of low phos media. After incubation for 20 hour, the samples were centrifuged at 4000 rpm for 10 minutes. The pellet was resuspended in 1 ml of 1X BugBuster lysis buffer (Novagen, USA) and incubated on ice for 20 minutes. The solution was centrifuged at 14,000 rpm for 10 min. The supernatant was kept to run SDS-PAGE. The Ni-NTA column was equilibrated with 600 µl of lyses buffer and spun at 2,000 rpm for 2 minutes. The cell lysate fraction was added into column and spun at the same speed again. The flow through was collected for SDS-PAGE. The washing buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole; pH 8.0) was added to column and spun. The column was washed anther two times. After that, 200 µl the elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole) was added into the column and they were centrifuged again. The scFv fragment should be presented in

the eluted fraction. To monitor antibody purity, SDS-PAGE electrophoresis was used. To determine the efficiency of purification, the flow through fraction, wash fraction and elution fraction were compared by SDS polyacrylamide gel.

3.2.3.5 Extraction soluble Aflatoxin from peanut

The 20 grams of peanut were blended with mortar. After that, 100 ml of 70% (v/v) methanol were added into sample. The sample was shaking at 180 rpm for 30 min. Then, the sample was passed though filter papers. Sample was diluted with 5% (v/v) methanol in TBST before using in competitive ELISA analysis.

3.2.3.6 Cross reactivity scFv-AP fusion in competitive ELISA

ScFv-AP antibody as assayed against a range of soluble aflatoxins B_1, B_2, G_1 and G_2. Stock solutions of aflatoxin were prepared in 100% methanol and diluted using TBST containing 5% (v/v) methanol. The assays were immobilized and blocked as described in 3.2.3.3. After washing 3 times with TBST, the dilution antibodies were pre-incubated with varying concentrations of soluble aflatoxin from 5 µg to 0.019 µg and 1:2 dilution of peanut's extraction. After incubation at 37 ºC for 30 minutes, the mixture was then transferred to the previously coated and blocked microtitre plate and incubated for 1 hour. The unbound antibodies were washed away 5 times with TBST. Following this, the PNPP substrate solution was added and incubated at room temperature for 30 minutes. The reaction was then measured by reading the absorbance at 405 nm using the Tecan plate reader.

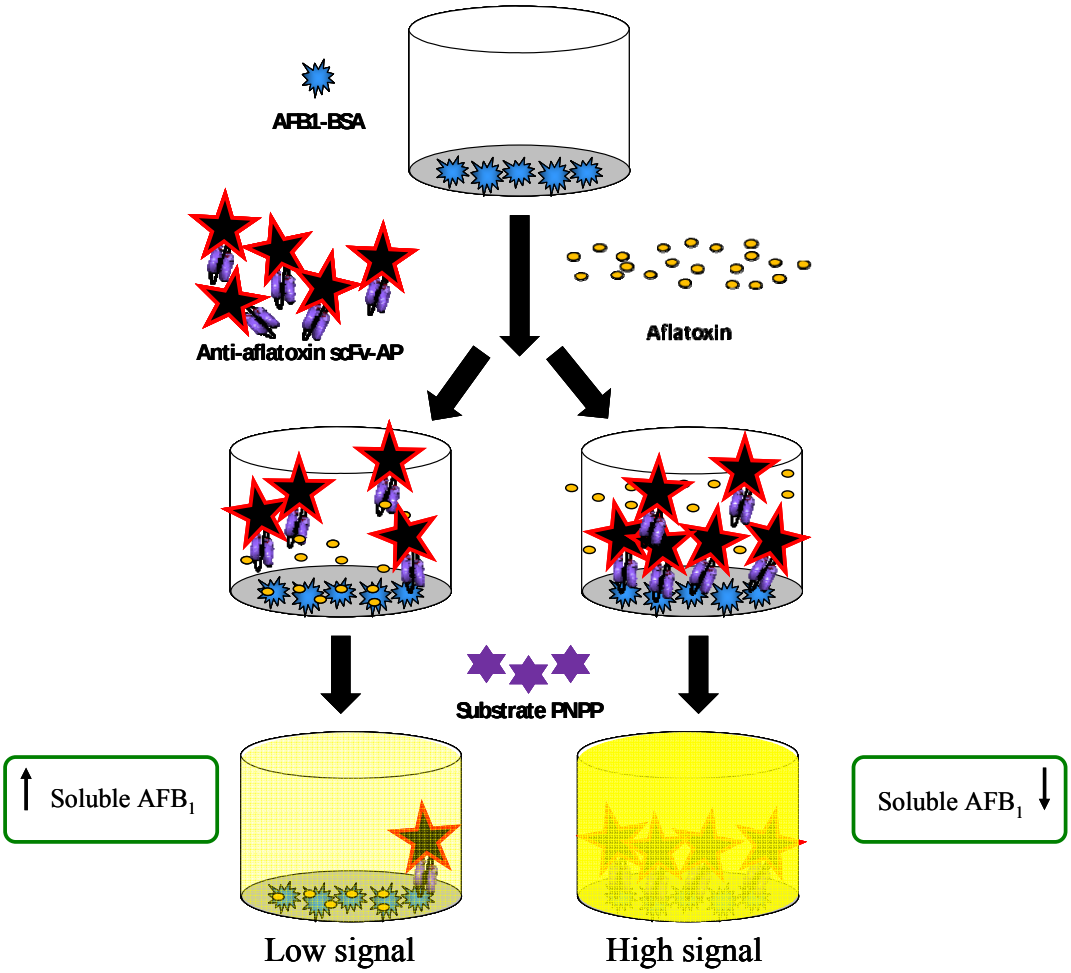


Figure 9. Schematic diagrams of competitive ELISA to test the binding of scFv-AP to soluble AFB_1. The AFB_1-BSA was immobilized on ELISA plate. The mixtures of scFv-AP and soluble aflatoxin were added to plate. The binding of soluble scFv-AP to AFB_1-BSA, hence the ELISA, depend on the amount of soluble AFB_1 which is a competitor. In the presence of high amount of soluble AFB_1, low scFv-AP can bind to AFB_1-BSA (low signal). On the contrary, if the amount of soluble AFB_1 is low, ELISA signal will be high.

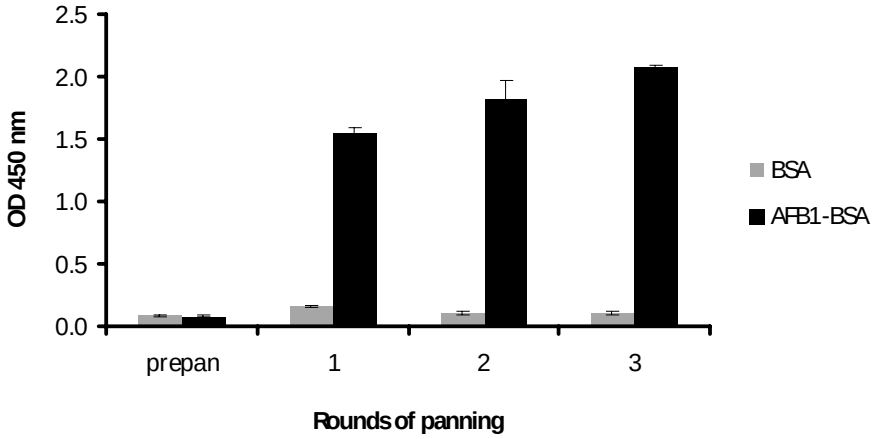
CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Polyclonal phage ELISA

The human naïve scFv Tomlinson I and J libraries were used for selection of scFv specific to aflatoxin. Three rounds of panning were performed. After scraped cell in each rounds of selection, the polyclonal phages that bind to aflatoxin B_1 were analyzed by polyclonal phage ELISA to monitor the enrichment of the phage population of the selection process using mouse anti-M13 antibodies conjugated to HRP for detection. Polyclonal phages selected from Tomlinson I library showed positive signal in every rounds of selection (Figure 10A). The results indicated that the phage display antibodies specific to aflatoxin B_1 conjugated BSA were enriched in each round of selection (Figure 10) whereas, screening of polyclonal phage antibodies from Tomlinson J library did not show any enrichment after second round of selection (Figure 10B). Yang et al. reported the signals of polyclonal phage antibodies against AFB_1 increased after each round of selection which similar as this result.

A



B

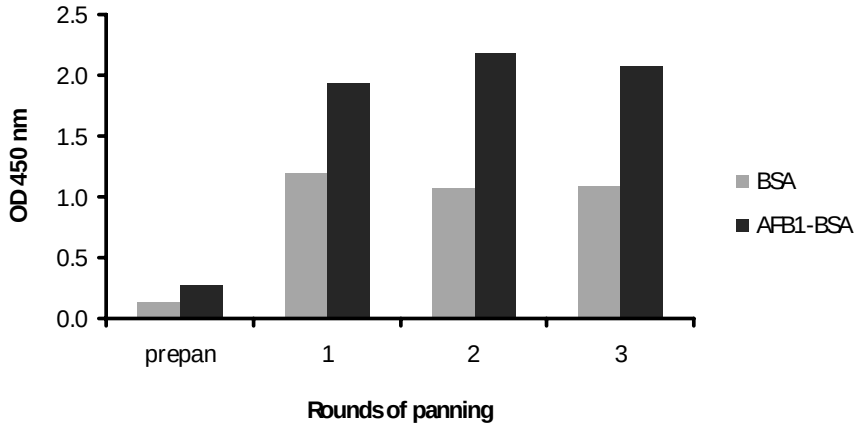


Figure 10. Polyclonal phages ELISA of (A) Tomlinson I library, (B) Tomlinson J library specific to AflatoxinB_1-BSA (AFB_1-BSA) were detected with HRP conjugated mouse anti-M13 antibodies. After each round of selection, polyclonal phages were prepared and analyzed for binding to AFB_1-BSA. 1% BSA was used as negative control in this experiment. All analyses were performed in duplicate.

4.2 Selection of scFv fragments specific to aflatoxin by using biopanning

The Tomlinson I and J libraries were grown to use for selecting scFv specific to Aflatoxin. Three rounds of selection were performed. Titration of eluted phage from Tomlinson I and J library showed that there was significant enrichment in their number between the first and second rounds of panning, but not in the third round (Table 5).

The results in Table 5 showed that the phages were amplified the same antibody formats after 3rd round of selection and antibody can bind to AFB_1-BSA more than soluble AFB_1. This problem can be solved by;

(i) decreasing the coating concentration of antigen in each round (Strachan et al., 2002) or increasing more times of washing.

(ii) The free aflatoxin may be used to elute the aflatoxin specific antibodies

(iii) The phage antibodies are pre-incubated with the carrier protein prior to incubation with the aflatoxin–protein conjugate. Subtractive panning should remove any antibody binding to the carrier protein, but not those antibodies binding to the bridging group linking the protein and the afaltoxin (Sheedy et al., 2007).

Nonetheless, such panning optimization strategies have led to the isolation of antibodies with higher specificity and lower levels of cross-reactivity (Chames et al., 1998).

However, with naïve libraries, the isolation of antibodies binding strictly to the free hapten such as aflatoxin is difficult, and only few publications have addressed the problems associated with the selection of antibodies that only recognize the hapten when the latter is conjugated with its carrier protein (Moghaddam et al., 2001). This study can use standard bio-panning method to isolate antibody that bind to soluble aflatoxin B_1, when using BSA-conjugated toxin as a target, as demonstrated by competitive ELISA.

Table 5. Overview of selection of antibodies against Aflatoxin B_1-BSA.

scFv library source	**Tomlinson I**	**Tomlinson J**
Number of clone after 1st panning	3.2×10^3	4×10^2
Number of clone after 2nd panning	1×10^5	1.4×10^5
Number of clone after 3rd panning	2.2×10^5	2×10^6
Number of positive phage clones after 1st round	6/96	8/96
Number of positive phage clones after 3rd round	71/96	32/96
Number of soluble scFv expessed	19/62	2/34
Number of scFv fragments that bound soluble Aflatoxin B_1	3/8	NA
Number of scFv producing different clones	1/3	NA

* The number of positive clones / the number of screened clones. NA is an abbreviation for not applicable.

4.2.1 Monoclonal phage ELISA in 1st round of selection.

The phage clones specific to aflatoxin B_1- BSA from 1st round were screened for the positives clones by ELISA method using mouse anti-M13 antibodies conjugated to HRP for detection. The phage clones were scored as positive by ELISA when the absorbance value on AFB_1-BSA coated wells was at least two times higher than binding of phage to 1% (w/v) BSA coated-wells. Six positives individual clones from Tomlinson I (Figure 11A) and eight clones from Tomlinson J (Figure 11B) from 1st round of panning showed positive signal from 96 picked clones from each library.

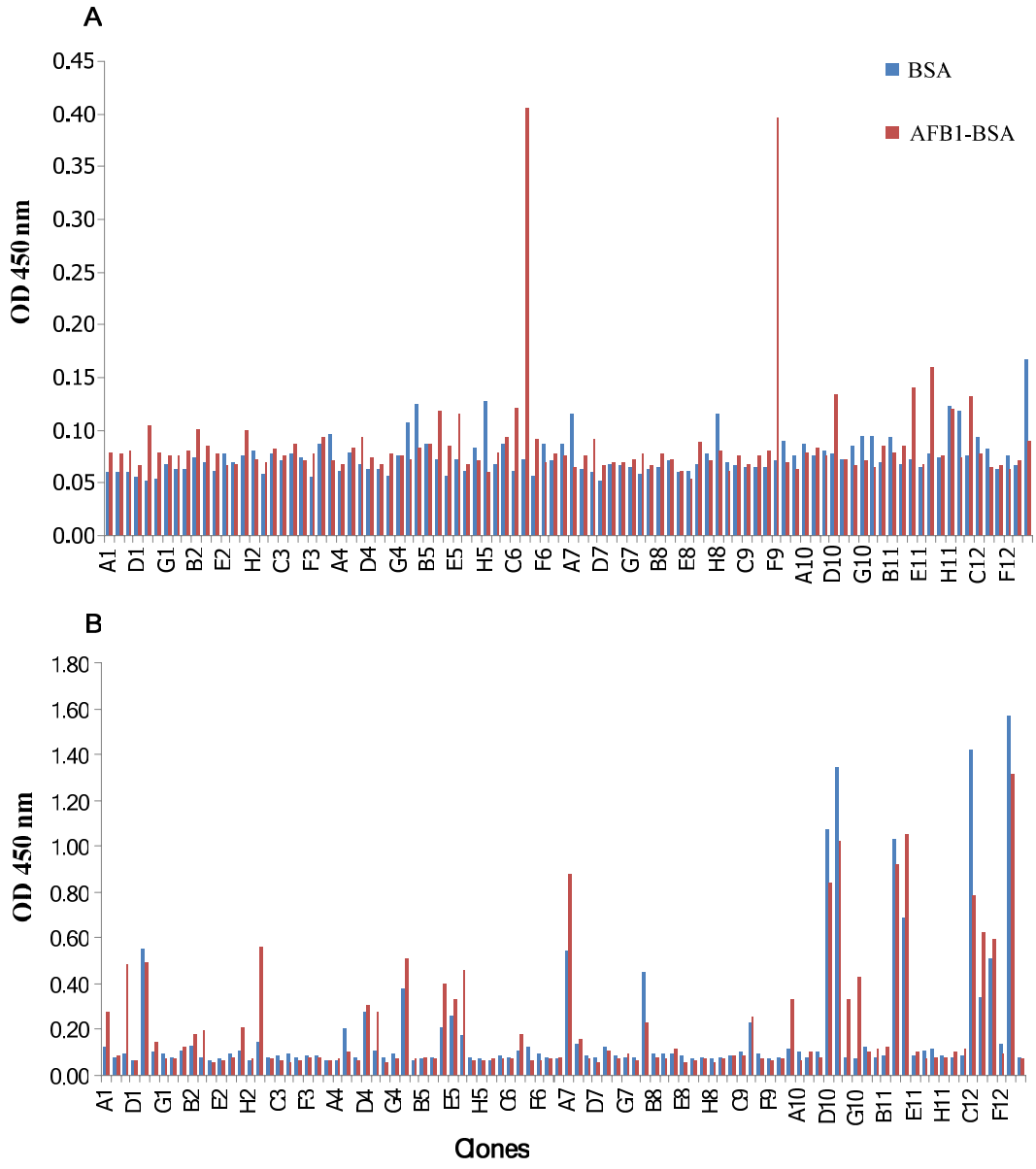


Figure 11. Binding of specific phage to AFB_1-BSA (Aflatoxin B_1 conjugated with BSA) from 1st round of panning. Clones were selected from (A) Tomlinson I antibody library and (B) Tomlinson J antibody library. Signal developed using TMB substrate and monitored by optical density at 450 nm. Six positive clones (B12, D6, D10, D11, F9 and F11) from Tomlinson I and eight clones (C1, C2, A3, E4, F5, H9, F10 and G10) from Tomlinson J library showed specific binding to AFB1-BSA and not to 1% (v/v) BSA (negative control).

4.2.2 Monoclonal phage ELISA 3rd round of selection.

After 3rd round of selection, individual 96 clones were randomly picked and their binding were tested by phage ELISA using mouse anti-M13 antibodies conjugated to HRP for detection. Seventy-one positive clones from Tomlinson I (Figure 12A) and thirty-two clones from Tomlinson J (Figure 12B) library showed specific binding to AFB1-BSA. The positive phage clones shown absorbance two times higher than the phage binding to 1% (w/v) BSA. These positive phage clones were enriched from 1st round of selection.

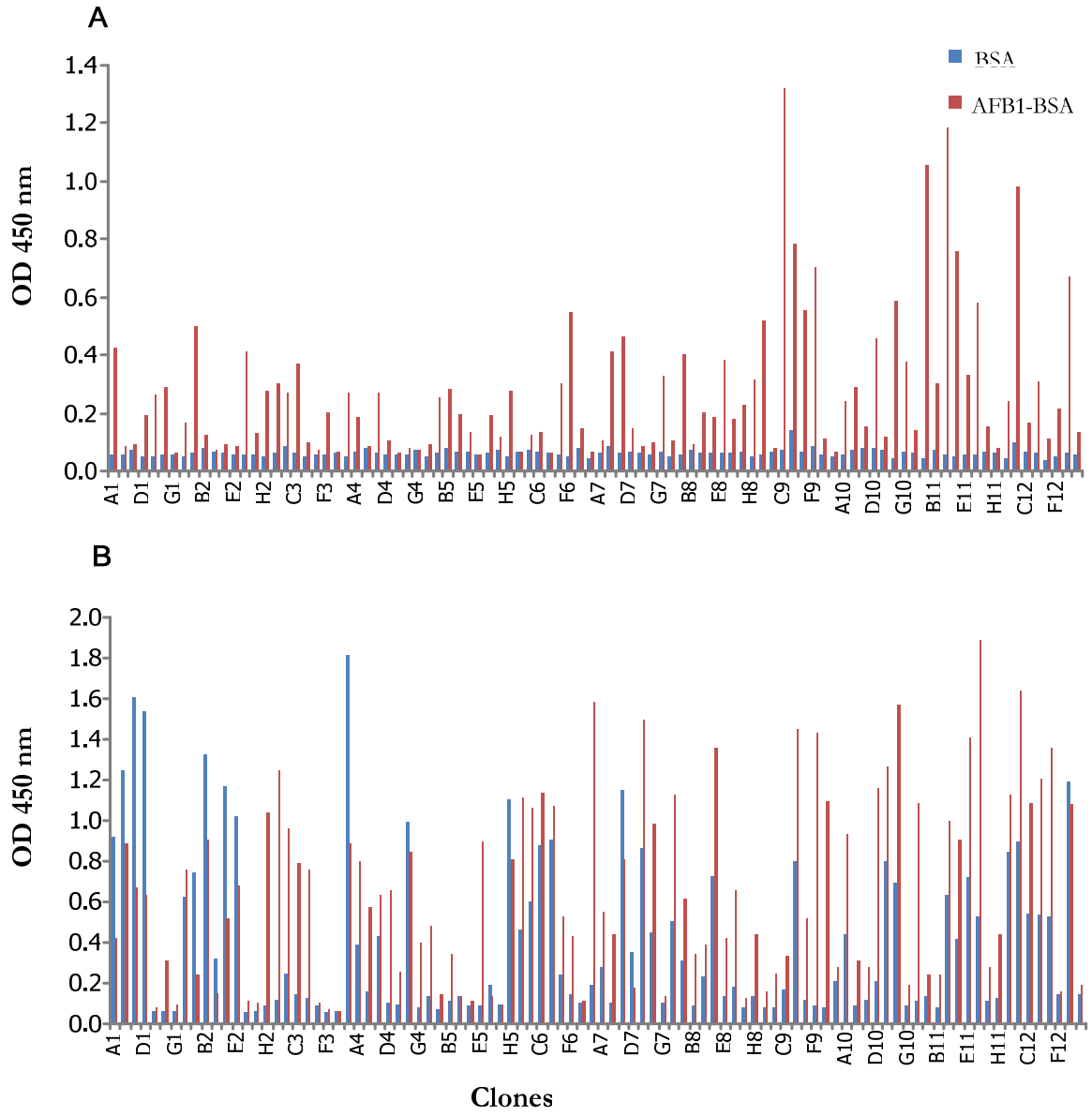


Figure 12. Binding of specific phage to AFB_1-BSA (Aflatoxin B_1 conjugated with BSA) from 3rd round of panning. Clones were selected from (A) Tomlinson I antibody library and (B) Tomlinson J antibody library. Data is expressed as absorbance at 450 nm. Seventy-one positive clones from Tomlinson I and thirty-two clones from Tomlinson J library showed specific binding to AFB1-BSA and not to 1% (v/v) BSA (negative control).

4.3 Production of soluble antibody fragments

From total, 62 positive clones from Tomlinson I and 34 clones from Tomlinson J library were brought forward to be assessed in induction of soluble antibody formats. The 96 selected clones were infected into a non-suppressor *E.coli* strain HB2151 (non-supressor strain) which reads the amber codon (TAG) as a stop signal, thereby solubly expressing the antibody fragment's. The scFv-gene III fusion is under the control of the LacZ promoter. Expression was induced by the addition of IPTG. The scFv fragments are then secreted in the supernatant or cell lysate.

ScFv fragments specific to aflatoxin B_1 were determined by ELISA. Bound scFv were detected using an anti-protein L-HRP labeled antibody. The substrate used to detect bound secondary antibody was TMB (Fluka) and the reaction was stopped with 10% (v/v) HCl. The reaction was measured by reading the absorbance at 450 nm with Tecan plate reader.

Nineteen clones were shown to secrete scFv antibody specifically binding to AFB_1-BSA detected in cell culture supernatants by ELISA (Figure 13). From these, eight clones which showed the highest signal were brought forward for competitive studies.

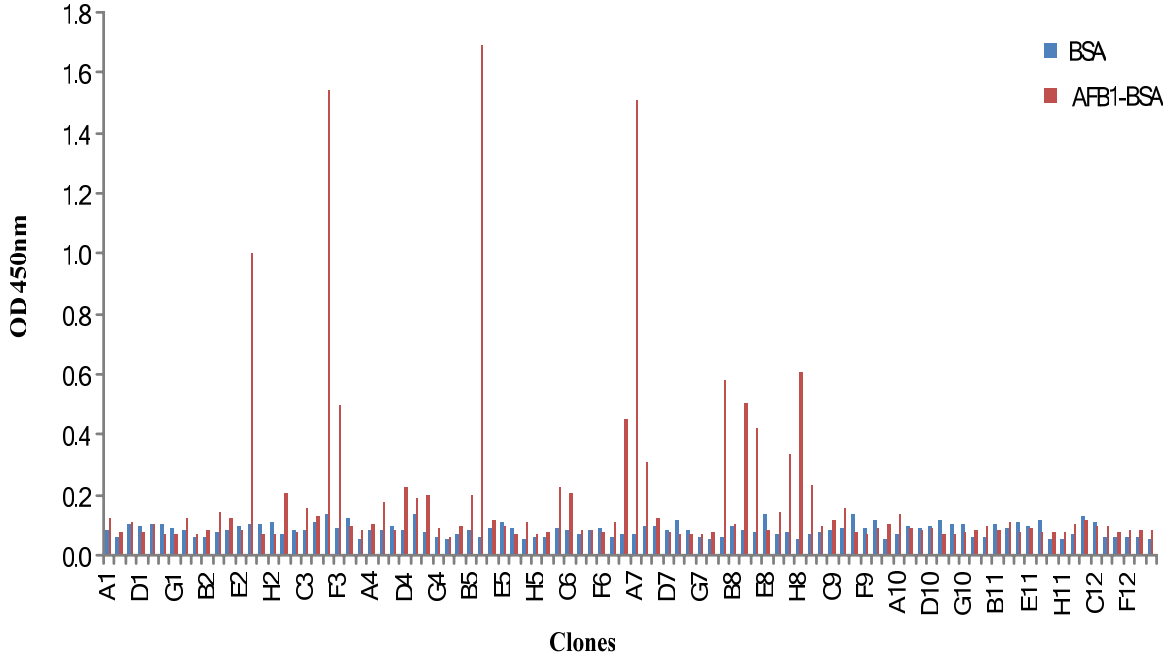


Figure 13. Binding of scFv antibody fragments to AFB_1-BSA. Sixty-one positive clones from Tomlinson I and thirty-five clones from Tomlinson J library were induced and scFv fragments binding to AFB_1-BSA in ELISA was analysed. The data is expressed as absorbance at 450 nm. Nineteen clones were shown to secrete scFv antibody specifically binding to AFB1-BSA and not to 1% (v/v) BSA (negative control).

4.4 Competitive ELISA of scFv fragments

The eight positive clones that shown highest absorbance from the earlier experiment were tested the binding to soluble AFB_1 using competitive ELISA. The positive result was determined if the absorbance decreased when the concentrations of soluble AFB_1 increased. TomI-F6, TomI-H2 and TomI-E9 were inhibited from binding immobilized aflatoxin by free toxin, indicating specific binding to soluble AFB_1 (Figure 14).

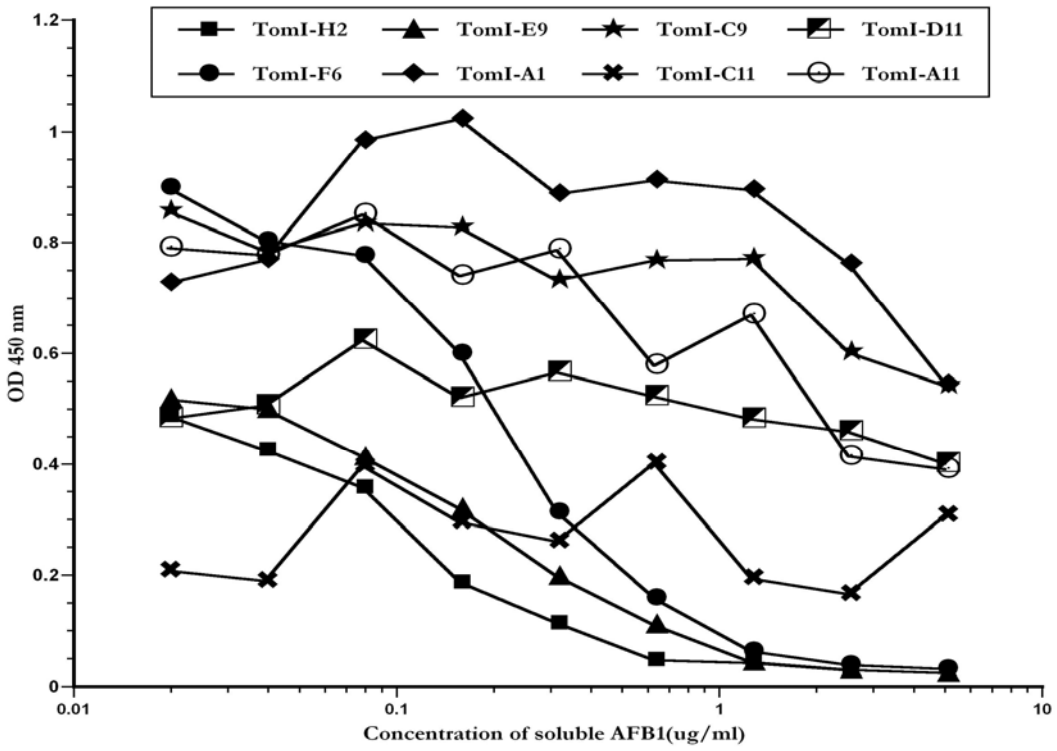


Figure 14. Binding of scFv fragments to AFB_1-BSA in an inhibition assay. The three clones exhibiting specific binding for free aflatoxin are TomI-H2, TomI-F6 and TomI-E9. This can be seen from the decreasing absorbance shown when higher concentrations of aflatoxin B_1 are present.

4.5 Sequence analysis of selected clones

Three clones (TomI-F6, TomI-H2 and TomI-E9) that can be inhibited by soluble aflatoxin B_1 were selected for sequence analysis. After sequencing, the origin of the V gene and complimentarity determining region 3 (CDR3) lengths were determined using V BASE immunoglobulin V gene database (Tomlinson et al., 1996) and IgBLAST program. The analysis showed that the three clones of phages displaying anti-aflatoxin B_1, TomI-F6, TomI-H2 and TomI-E9 were identical. The closest germline sequence for anti-aflatoxin scFv antibody could be identified by comparison to database. The deduced amino acid sequences of V_H and V_L are illustrated in Figure 15. According to the amino acid sequences, the V_H was classified as human heavy chain family V_H3 derived germline V3-

23*05 while V_L fell into human kappa light chain family Vκ1 derived germline V1D-39*01. The numbers of different amino acids from germ line of V_H and V_L of phage clone were 7 and 2 amino acids, respectively. The CDR3 of the binders had lengths 9 amino acids for the VH genes, and for the VL genes as shown in Table 6. TomI-A8, TomI-A11, YM1-C5 and YM-D2 that the clones not bind to soluble aflatoxin were identified sequences (Table 6). The CDRH3 is critical importance in the interaction of an antibody with its respective antigen. This region, along with the CDRL3, is considered to make the most significant contributions to affinity and specificity.

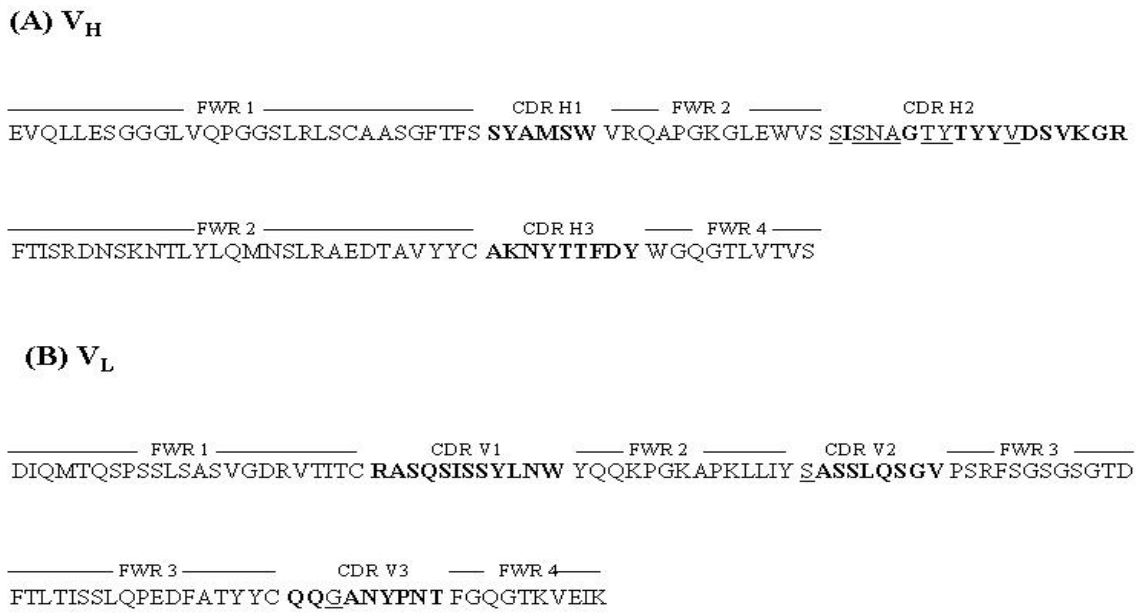


Figure 15. Amino acids sequence alignment of scFv antibody fragments (TomI-F6) selected against AFB_1.The heavy (A) and light chain (B) complementarity determining regions (CDRs) were defined according to IgBLAST program and are shown in bold letters. The underline letters has shown amino acids difference from germ line.

Table 6. Amino acids sequence of selected clones.

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
VH									
TomI-F6	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYAMSW	VRQAPGKGLEWVS	SISNAGTYTYYVDSVKGR	FTISRDNSKNTLYLQMNSLRAEDTAVYYC	AKNYTTFDY	IGHV3-23*05	7	VH3
TomI-A8	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYAMSW	VRQAPGKGLEWVS	SIAGSGNYTSYADSVKGR	FTISRDNSKNTLYLQMNSLRAEDTAVYYC	AEASGAFDY	IGHV3-23*04	6	VH3
TomI-A11	EVQLVESGGGLVQPGS*TRLSCAASGFTFS	SYAMRW	VH*APGKGLEWVS	AISGYGSYTTYADSVKGR	FTISRDNSKNTLYLQMNSLRAEDTAVYYC	AKGGYSFDY	IGHV3-23*04	11	VH3
YM1-C3	QVQLVQSGAEVKKPASVKVSCKAS	GYTFTSYA	MHWVRQAXGQRLEWMGW	INAGNGNT	KYSQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYCAR	ADDYGSGSYGFDY	IGHV1-3*01	1	VH1
YM1-C5	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	SYAMRW	VRQAPGKGLEWVS	AISGSGGSTYYADSVKGR	FTISRDDSKNTAYLQMNSLKTEDTAVYYC	SRVGLWGPRYYYYGMDVW	IGHV3-23*04	4	VH3
YM1-D2	QMQLVQSGAEVKKTGSSMKVSCRVSGYTFT	NRYLHW	VRQAPGQALEWMG	WITPFNGNTNYAQKFQDR	VTITRDRSMSTAYLELSSLRSEDTAMYYCAS	GGPLDY	IGHV1-45*02	5	VH1
VL									
TomI-F6	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLNW	YQQKPGKAPKLLIY	ASSLQSGV	PSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQGANYPNT	IGKV1D-39*01	2	VK1
TomI-A8	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLNW	YQQKPGKAPKLLIY	NASALQSGV	PSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQNAYSPDT	IGKV1D-39*01	2	VK1
TomI-A11	DIQMTQSPSSLSASVGDRVTITC	RASQSISSYLNW	YQQKPGKAPKLLIY	ASNLQSGV	PSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQNADSPAT	IGKV1D-39*01	2	VK1
YM1-C3	DTVMTQSPSSLSASVGDRVTITC	RASQSISSYLNW	YQQKPGKAPRLLIYA	ASSLQSGV	PSRFSGNGSGTDFTLTISSLQPEDFATYYC	QQSYSTPYA	IGKV1D-39*01	4	VK1
YM1-C5	SYVLTQPPSVSLTLGQTARITCE	GNSIGRKHVHW	YQQKPGQAPVLVYR	DIQRPSGI	PERFSGSNSGNTATLTITGVQVGDDSDYYC	QVWDRDSRTIV	IGLV3-9*01	16	VL3
YM1-D2	QSALTQPASASGSPGQSVTISCT	GTGSDVGGYNYVSW	YQQHPGKAPKLMIYE	VSKRHSGV	PDRFSGSKSGNTASLTVSGLQAEDEADYYC	SSYAGSNNLV	IGLV2-8*01	3	VL2

4.6 Purification of soluble scFv antibodies

The pIT2 vector of the Tomlinson's library encodes a carboy-terminal hexahistidine tag, which allowed for rapid purification of the recombinant antibodies using IMAC. Clone TomI-F6 that showed to be inhibited by soluble aflatoxin was selected to purify. For small scale production, induction was performed at 30^{o}C for 20 hours, using 1mM IPTG in a total culture volumn of 15 ml. The antibody fragment was purified from cell lysate by using Ni-NTA resin, and analyzed by SDS-PAGE and Western Blot (Figure 16). All factors affecting purification, including the quantity of resin and concentration of imidazole in both the wash and elution buffers. Imidazole were incorporated in the wash buffer, reduced the non-specific binding of contaminant proteins to the resin. scFv fragments were eluted with 250mM imidazole. The results indicated that the purity of antibodies greatly increased following elution with imidazole in comparison to the crude cell lysate.

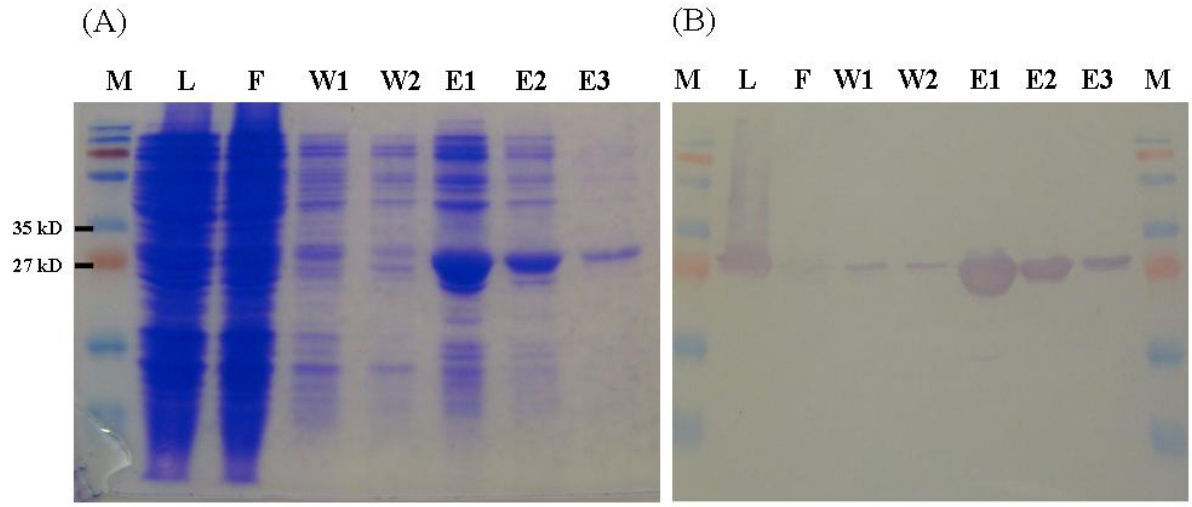


Figure 16. SDS-PAGE (A) and western blot analysis (B) of purified scFv antibody in small scale production (anti-Aflatoxin clone TomI-F6) by IMAC. SDS-PAGE stained with CBB. Western blot probed with anti-protein L peroxidase labeled antibody. Color was developed by using TMB substrate. Lane (M): Protein Molecular weight Markers (Sigma Colour burst prestain); Lane (L): cell lysate fraction; Lane (F): Flow through fraction; Lane (W1-3): Wash fraction; Lane (E1-3): Elution fraction containing purified scFv antibody.

For the large scale production of soluble scFv antibody, the culture volumn were increased to 500 ml. For high levels of expression, antibody was grown in Terrific broth, which contains high concentrations of tryptone and yeast extract also includes glycerol as source of carbon and potassium phosphate salts as a buffering agent to prevent cell death at decreased pH. The results of SDS-PAGE and Western Blot analysis (Figure 17) showed that the scFv-hexahistidine fusion antibodies with a size of about 30 kDa were successfully purified from lysed cells using Ni-NTA resin. However, the contaminated protein appeared to co-purifying along with desired scFv antibodies. The contaminants removal approach will be necessary in further purification step. The western blot result showed that only monomer scFv was obtained and that it was functionally active. The quality of product

seems to be lower than small scale production because sometimes inclusion body format can occur during production scFv, which results in poor expressivity (Brichta et al., 2005). This problem can be solved by cloning this scFv to pKP300ΔIII (expression vector) encode an carboxy-terminal $(His)_6$ tag, which allowed for the rapid purification of recombinant antibodies using immobilized metal ion affinity chromatography (IMAC).

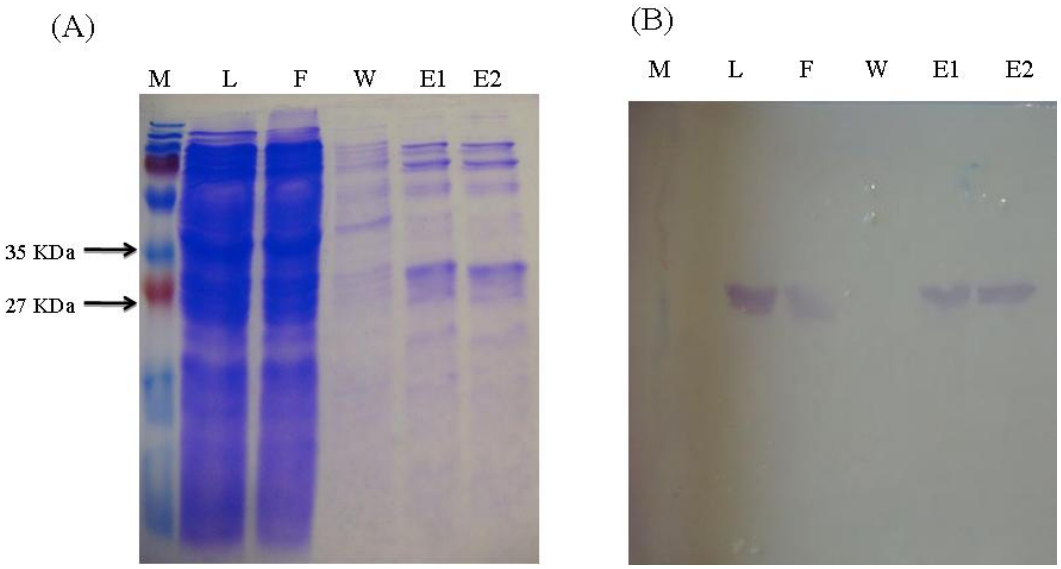


Figure 17. SDS-PAGE (A) and Western blot analysis (B) of purified scFv antibody in largescale production (anti-Aflatoxin clone TomI-F6) by IMAC. SDS-PAGE stained with CBB. Western blot probed with anti-protein L peroxidase labeled antibody. Color was developed by using TMB substrate. Lane (M): Protein Molecular weight Markers (Sigma Colour burst prestain); Lane (L): cell lysate fraction; Lane (F): Flow through fraction; Lane (W): Wash fraction; Lane (E1-2): Elution fraction containing purified scFv antibody.

4.7 Cloning of scFv fragments into pKP300ΔIII vectors

The scFv fragments against aflatoxin B_1 that selected from compact human naïve library (Pansri et al., 2009) and a semi-synthetic library (Tomlinson; TomI-F6) as engineered to create scFv-alkaline phosphatase fusions (scFv-AP) and used as convenient one-step detection probes for competitive ELISA.

The resulting scFv fragment (TomI-F6) and pKP300ΔIII vector were digested with restriction enzymes *NCoI* and *Not I*. The digested products were assessed on agarose gel and the resulting gel images are shown in Figure 18 with bands visible at approximately 5,000 bp representing vector and 750-800 bp representing insert.

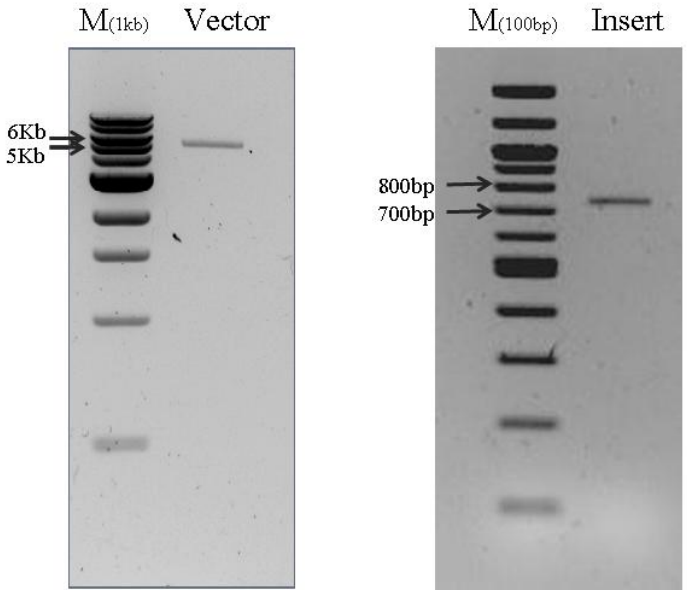


Figure 18. Agarose gel analysis of *NCo*I and *Not*I digestion products. M, 1 kb DNA ladder and 100 bp ladder. A vector (pKP300ΔIII) and insert (scFv-F6) band can be seen at approximately 5,000 bp and 800 bp, respectivetly. The concentration of DNA of vector and insert were 12.1 µg/ml and 12.3 µg/ml, respectively.

Full length scFvs had been prepared; they were ligated into pKP300ΔIII vector at *NCoI* and *NotI* sites. The ligation reactions were transformed to *E.coli* TG1 by electroporation. After incubation for 16 hour, the 5 individually colonies were randomly picked and grown in Low phos media at 30^{o}C for 20 hour. The scFv-AP was expressed and secrete into culture media. Cell pellets were used to extracted plasmid by using Plasmis mini Prep (Qiagen, Germany). Plasmids were digested with *NCoI* and *Not I* for checking the scFv were clone into expression vector. The result showed that all of 5 clones were successfully cloned into vector (Figure 19).

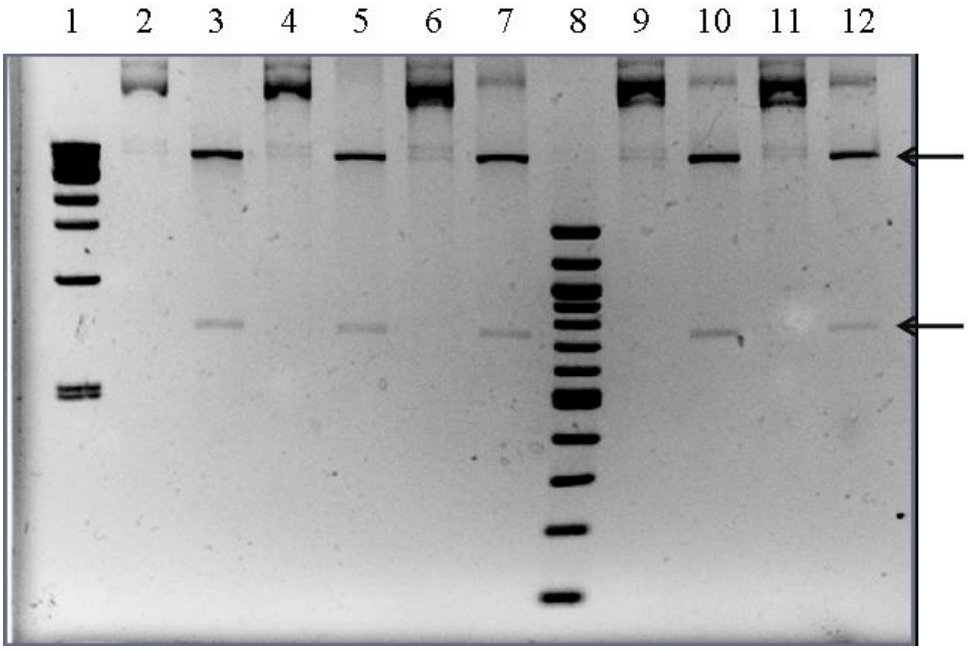


Figure 19. Agarose gel analysis of *NCo*I and *Not*I digestion products to check positive clones. Lane 1, 1 kb DNA ladder; lane 2, 4, 6, 9, 11, undigested of clone 1-5; lane 3, 5, 7, 10, 12, clone 1-5 were digested with enzymes; lane 8, 100 bp DNA ladder. The digestion products were seen in two sites indicated by arrow.

4.8 ScFv-AP ELISA

The five scFv-AP clones were tested the specific to bind AFB_1-BSA by ELISA using PNPP as substrate. The yellow color was developed and measure absorbance at 405 nm. Clone 1 that showed the highest signal bound to AFB_1-BSA (Figure 20) was assessed to determine the optimal antibody dilution to the further step in competitive ELISA. The result showed that an antibody dilution of 1/10 (TomI-F6) and 1/20 (YM1-C3) were appropriated for applies in a competitive ELISA to obtain the half maximal inhibitory concentration (IC_{50}) of soluble AFB_1 and % cross-reactivity (Figure 21).

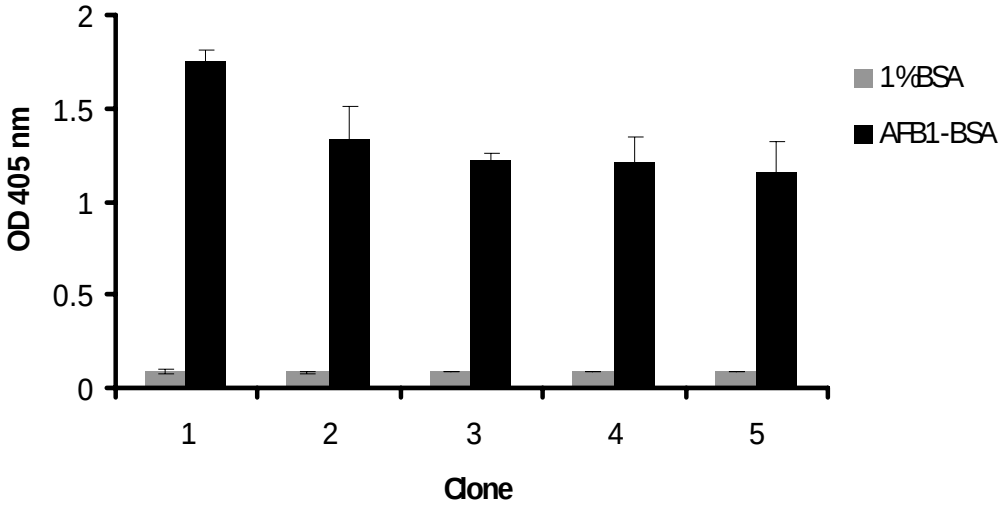


Figure 20. The binding scFv-F6-AP specific to AFB_1-BSA. PNPP was used to be substrate for AP. Absorbance signals were presented with standard deviation of triplicates.

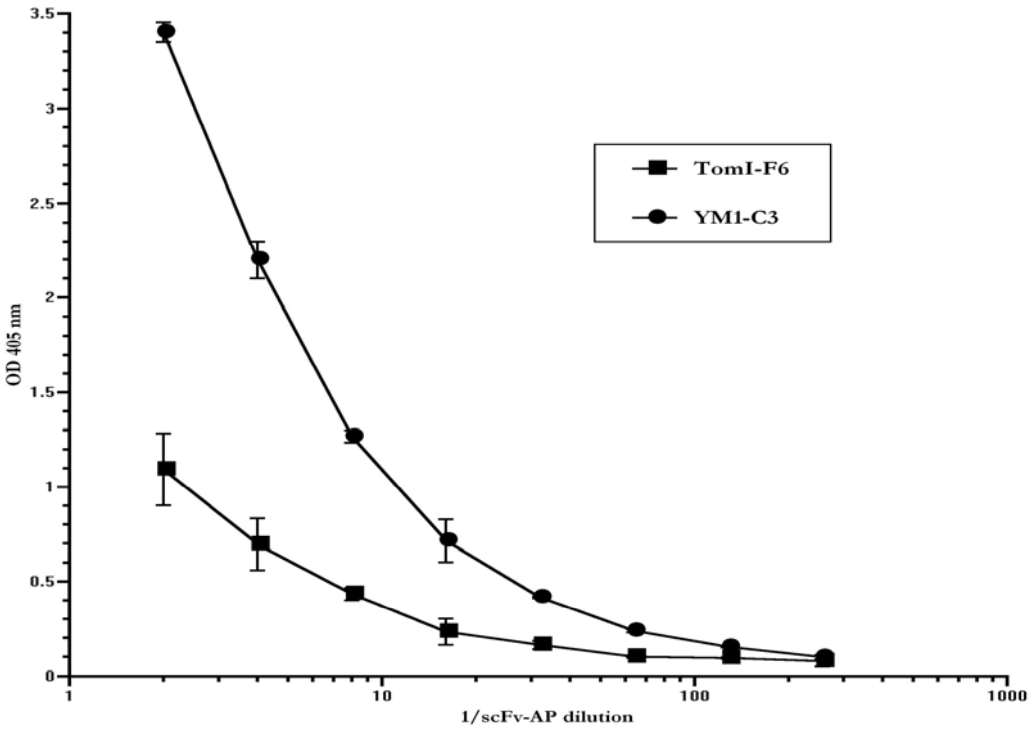


Figure 21. ELISA for determination of scFv-AP antibody dilution for use in a competitive ELISA. Antibody dilutions from 1:2 to 1:256 were assayed. Absorbance signals were presented with standard deviation of triplicates.

4.9 scFv-AP competitive ELISA

Various 1:3 (B: YM1-C3) and 1:2 dilutions (A: TomI-F6) of soluble AFB_1 from 5.0 µg/ml-0.762 ng/ml in 5% methanol in TBST were incubated with two scFv-AP (TomI-F6 and YM1-C3) at 37^{o}C for 30 minutes before adding into wells of Immuno 96 MicroWell^{TM} plates, which coated with 5µg AFB_1-BSA. The plated were washed five times with TBST after 1 hour of incubation. PNPP were used as substrate for AP. The IC_{50} values for the TomI-F6-AP and YM1-C3-AP were found to be 0.2 µg/ml and 0.035 µg/ml, respectively. This result showed that the YM1-C3 demonstrated approximately 10 fold increase in assay sensitivity when compared to TomI-F6. ELISA inhibition curves for both antibodies are shown in Figure 22. The limit of detection (LOD) is 0.006-0.03 µg/ml. This is the smallest concentration of the analyses that produces a signal. This value was determined by selecting

the mean normalized absorbance minus three standard deviations for the negative standard (no AFB_1). Moghaddam et al., and Yang et al. reported that the IC_{50} of the selected clones were about 400, 0.4 ng/ml, respectively. The previous study showed more sensitivity than this study. In the future study, the sensitivity of my antibodies can be further improved by various methods of affinity maturation.

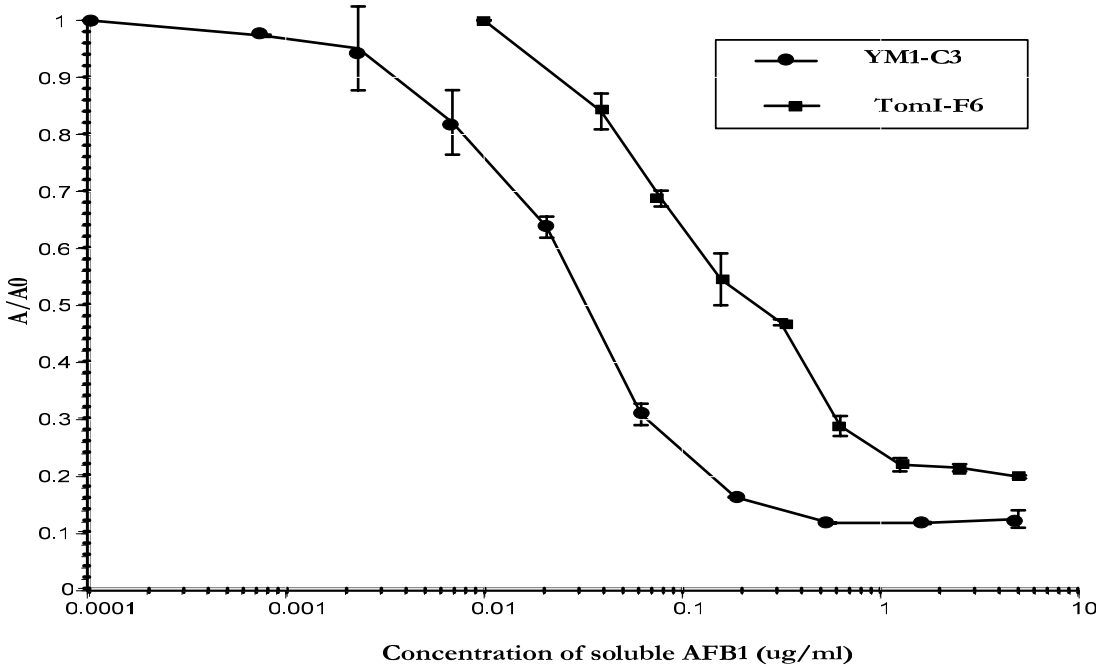


Figure 22. Competitive ELISA of scFv-AP antibody for detection of different concentration of AFB_1. The average absorbance at a wavelength of 405 nm and S.D are shown. Data was normalized by dividing the mean absorbance obtained at each AFB_1 concentration (A) by the absorbance value determined in the presence of zero toxins (A_0). Normalized absorbance values (A/A_0) were plotted against the logarithm of AFB_1 concentration. All analyses were performed in triplicate. The limit of detection of TomI-F6 and YM1-C3 were found to be 0.003 and 0.02 μg/ml, respectively.

4.10 Comparison sensitivity of scFv fragment and scFv-AP

The results of competitive ELISA employing the scFv YM1-C3 antibody fragments were compared to scFv-AP YM1-C3 in terms of assay sensitivity. Standard of each potential, ranging from 2.3 ng/ml to 5,000 ng/ml were prepared in TBST containing 5% (v/v) methanol and mixed with an equal volume of antibody at the optimal concentration. The IC_{50} values for the scFv YM1-C3 and YM1-C3-AP were found to be 0.13 μg/ml and 0.035 μg/ml, respectively. The scFv-AP fusions showed improved binding affinity of approximately 3-20 folds better than the soluble form and phage-displayed scFv formats, respectively (Pansri et al., 2009).

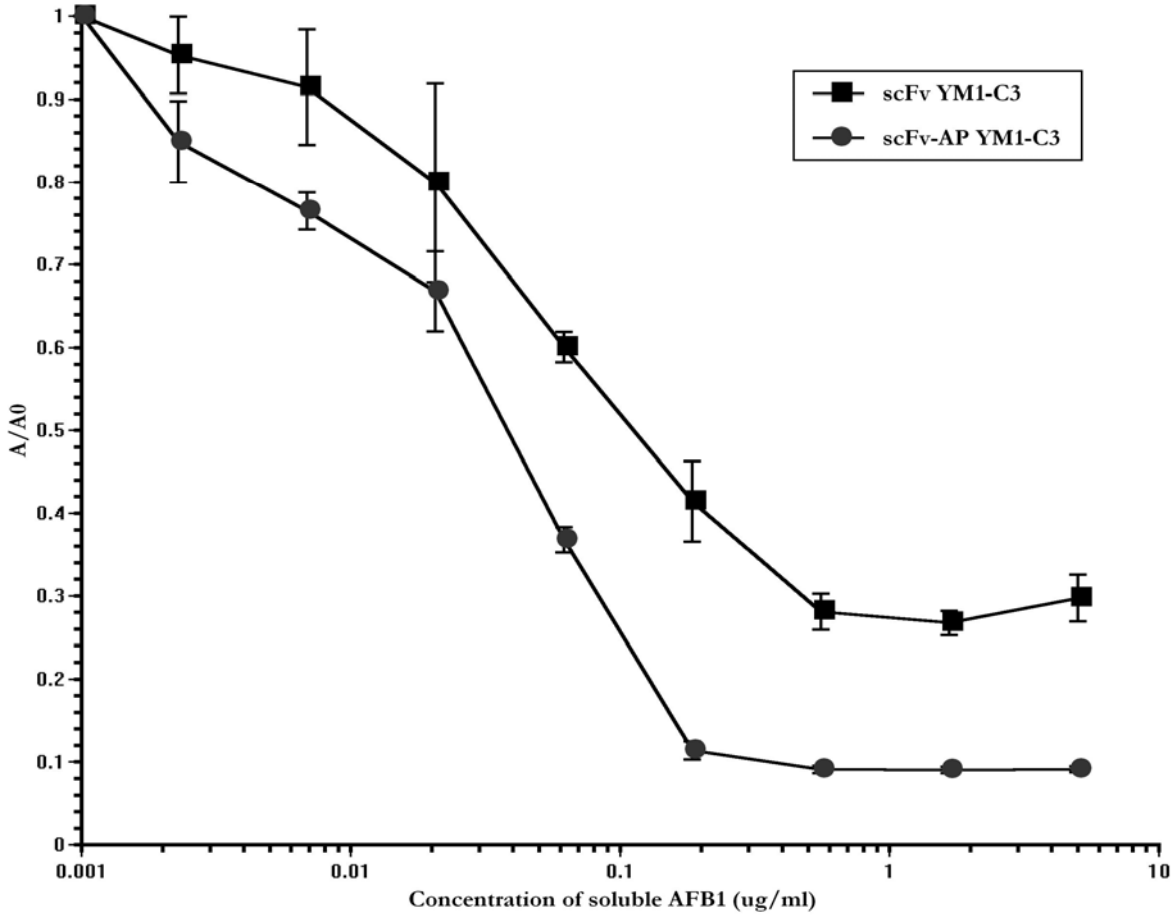


Figure 23. Competitive ELISA for the detection of AFB_1 using the scFv antibody fragment and scFv-AP fusion antibody. The average absorbance at a wavelength of 405 nm and S.D are shown. Data was normalized by dividing the mean absorbance obtained at each AFB_1 concentration (A) by the absorbance value determined in the presence of zero toxins (A_0). Normalized absorbance values (A/A_0) were plotted against the logarithm of AFB_1 concentration. All analyses were performed in triplicate. The IC_{50} values for the scFv YM1-C3 and YM1-C3-AP were found to be 0.13 µg/ml and 0.035 µg/ml.

4.11 Cross-reactivity

The anti-aflatoxin antibodies were demonstrated for cross-reactivity with three structurally related aflatoxins; aflatoxin B_2, G_1, G_2 and M_1 using competitive ELISA. Standard of each potential cross-reactant, ranging from 2.3 ng/ml to 5,000 ng/ml were prepared in TBST containing 5% (v/v) methanol and mixed with an equal volume of antibody at the optimal concentration. The cross-reactivity potential was approximated at the IC_{50}. IC_{50} value is the analyze concentration that results in 50% inhibition. The percent inhibition was determined according to the equation: % inhibition = [(OD sample/ OD control)] × 100 (Figure 24). The percentage cross-reactivity determined at IC_{50} (%CR_{50}) was expressed as 50% inhibitory concentration of aflatoxin B_1 divided by the 50% inhibitory concentration of other aflatoxin and multiplied by 100.

A

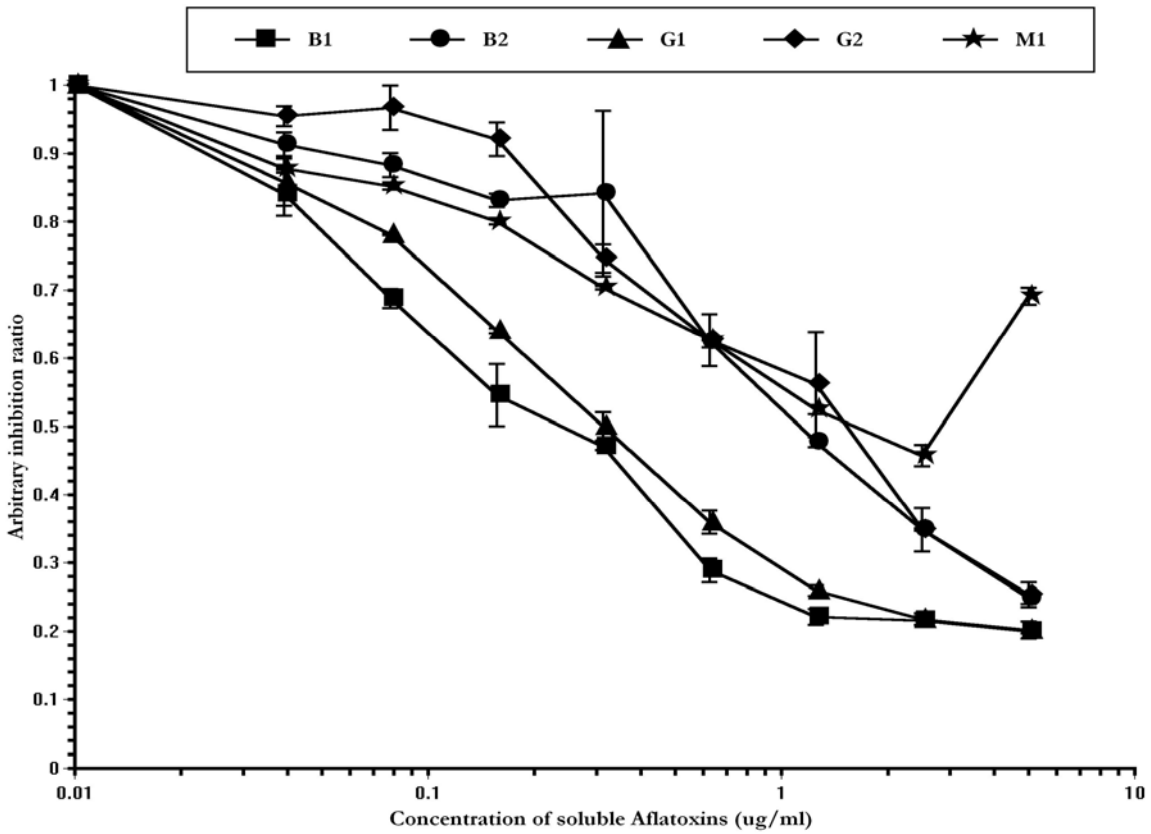


Figure 24. Percent inhibition of aflatoxin antibody (A) TomI-F6 and (B) YM1-C3 was calculated from ELISA value of competitive ELISA. Results were plotted as the mean percent inhibition against the logarithm of soluble aflatoxins concentrations. Data were presented with standard deviation of triplicates.

B

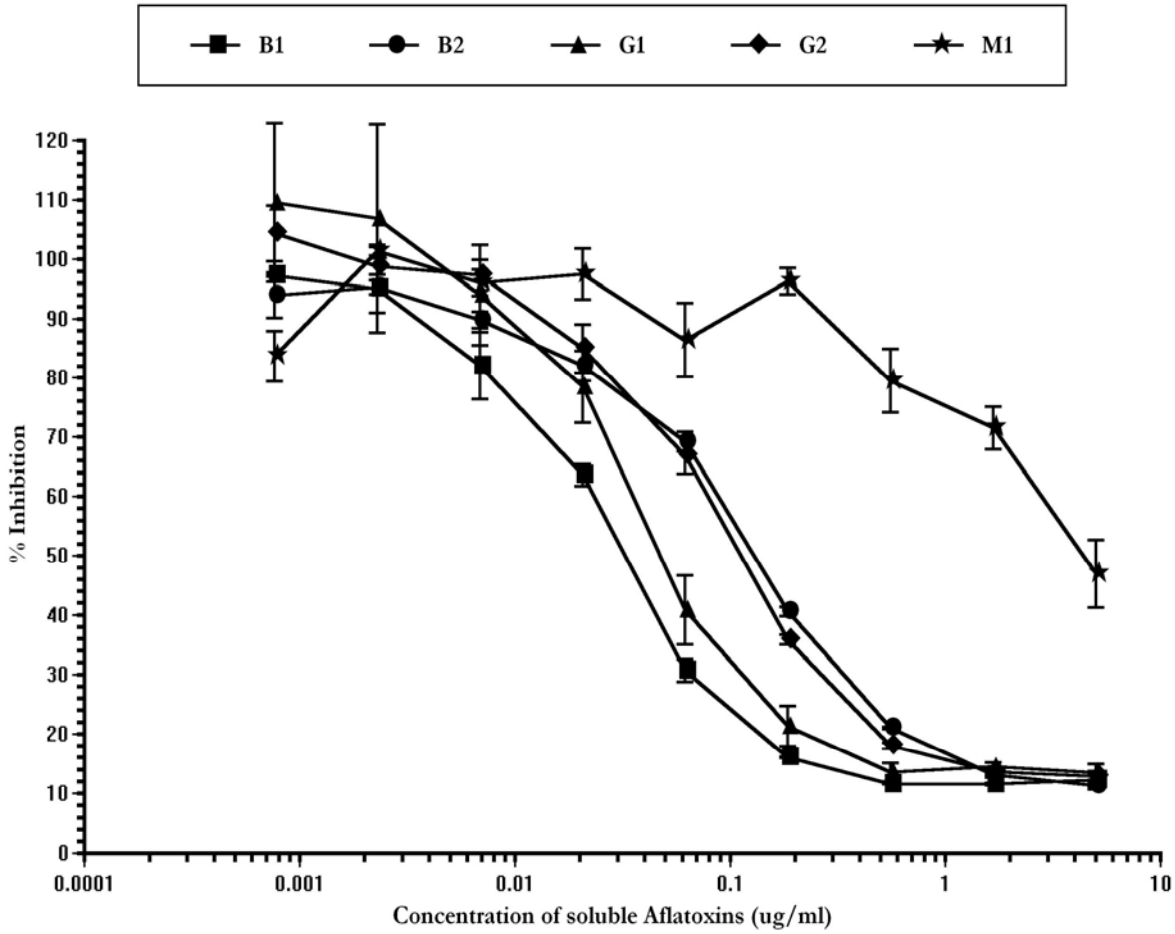


Figure 24. (Continued)

Table 7 shows the results of the studies, which the scFv-AP antibody showed high degree of cross-reactivity, especially to $AFG_1 > AFB_2 > AFG_2 > M_1$ (in decreasing order). Anti-aflatoxin scFv-AP TomI-F6 and YM1-C3 showed the highest level cross-reactivity with aflatoxin G_1 were 68.75% and 70%, respectively. Moghaddam (2001) found that their antibody was able to bind as effectively to AFG_1 as AFB_1. Yang (2009) reported their antibody showed cross-reactivity of 12% with AFB_2, 42% with AFG_1 and 9% with AFG_2. This is similarity in this study. The YM1-C3 showed low percentage cross-reactivity with aflatoxin M_1 (0.88%). This can be shown by the fact that hydroxylation C9 residue in aflatoxin M_1 results in a reduction in binding affinity.

Table 7. Percentage cross-reactivity value of the TomI-F6 and YM1-C3 scFv-AP antibody to various potential cross-reactants. The cross-reactivity potential was proximated at the IC_{50} value, which was estimated at 50% A/A_0.

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

4.12 Purification of scFv-AP

The pKP300ΔIII vector expresses scFv with FLAG tag at N terminal and enzyme alkaline phosphatase and hexahistidine tag fusion at C terminal, which allowed for rapid purification of the recombinant antibodies using IMAC. Clone scFv-AP TomI-F6 was cultured in a total culture volumn of 10 ml. Induction was performed at 30^{o}C for 20 hours. The scFv-AP fusion was purified from cell lysate by using Ni-NTA resin, and analyzed by SDS-PAGE (Figure 25). The result showed that the scFv-AP fusion antibodies with a size of about approximately 70 kDa were successfully purified from lysed cells using Ni-NTA resin.

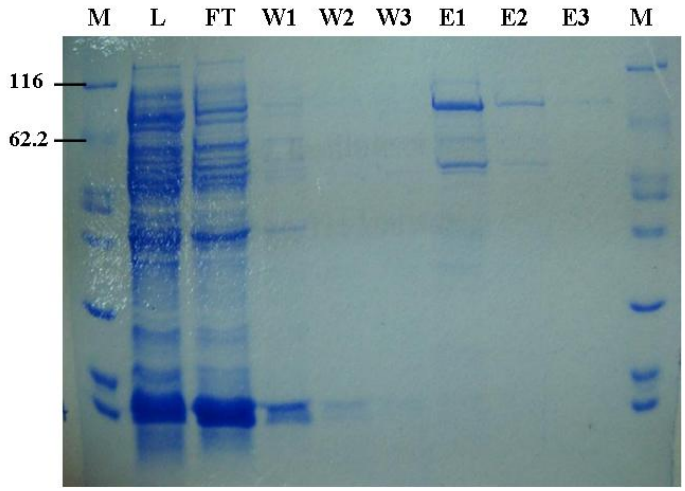


Figure 25. SDS-PAGE purified scFv-AP (TomI-F6) antibody. SDS-PAGE stained with CBB. Lane (M): Protein Molecular weight Markers (Fermentous unstain); Lane (L): cell lysate fraction; Lane (FT): Flow through fraction; Lane (W1-3): Wash fraction; Lane (E1-3): Elution fraction containing purified scFv-AP antibody.

4.13 ScFv-AP binds to AFB_1 in peanut samples

To assess the assay method in practice, peanuts were taken from farmers' fields that were presumed to be at high risk of aflatoxin contamination. These were extracted and assayed by using competitive ELISA. The peanut extract was diluted at 0, 1:16 and 1:256 and was incubated with scFv-AP TomI-F6 before adding to ELISA coated well with AFB_1-BSA. The scFv-AP can bind to AFB_1-BSA (high signal), when the dilution of peanut extract was increased (the concentration of AFB_1 contaminated decreased). The result (Figure 26) showed that this scFv-AP can be used to detected aflatoxin in agricultural product.

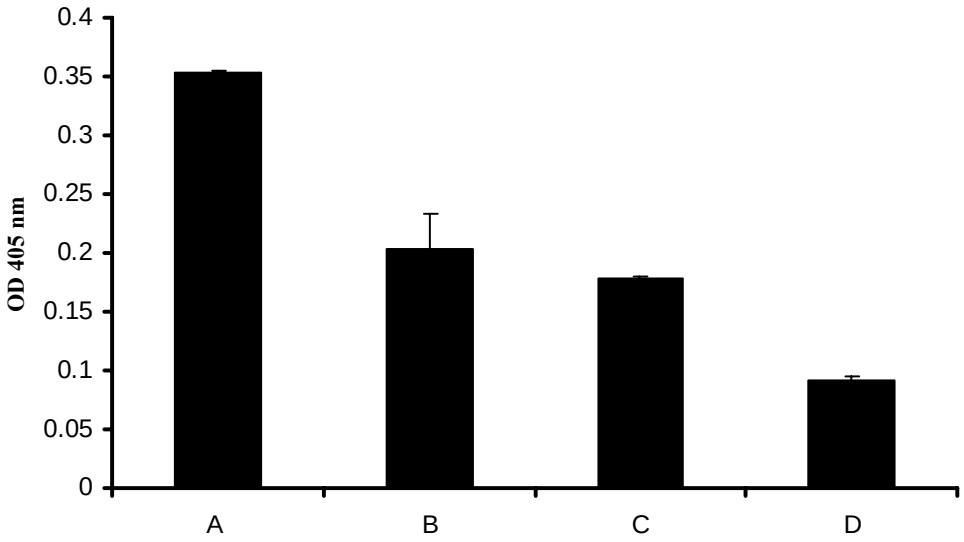


Figure 26. Inhibition of peanuts extract by competitive ELISA. Soluble scFv-AP was incubated with (A) 5% methanol in TBST, (B) 1:256 dilutions of peanut extract, (C) 1:16 dilutions of peanut extract and (D) undiluted peanut extract before transferring to AFB_1-BSA that coated microtitre wells. Absorbance signals were presented with standard deviation of duplicates.

CHAPTER V

CONCLUSION

In summary, scFv antibody specific to aflatoxin B_1 can be selected from naïve semi-synthetic scFv phage display antibody libraries by standard bio-panning. Polyclonal phages showed positive signal in each round of panning. The positive clones from 1^{st} and 3^{rd} round of selection were determined for specific binding to aflatoxin B_1 by competitive ELISA. Three clones from eight clones, that produced high affinity antibody specific to aflatoxin B_1, were selected. All selected clones showed the same nucleotide sequence after automated DNA sequence analysis. DNA sequence of variable regions using IgBLAST and VBASE program analysis revealed that the antibody contained IGHV3 of human V_H region and IGKV1 of human VL region. One of soluble scFv antibody clone that shown high specific to aflatoxin B_1 was expressed and purified by using Ni^{2+}-NTA affinity column chromatography. This scFv antibody and a compact human naïve phage display library (YAMO1) were engineered to create scFv-alkaline phosphatase fusion (scFv-AP) and can be used as convenient one-step detection probe for competitive ELISA. The IC_{50} of scFv-AP by AFB_1 varied between 0.035-0.02 µg/ml and the limit of detection was approximately 0.006-0.03 µg/ml (6-30 ppb). The selected antibodies were specific to AFB_1 and could cross-react with AFG_1, but not to other aflatoxins. These antibodies can be used in therapeutic study in the future.

REFERENCES

Armbrecht, B. H. (1978): Aflatoxicosis in swine. **Mycotoxic Fungi, Mycotoxins, Mycotoxicoses** 2, 227–235.

Azzazy, H. M. E., and Highsmith, W. E. (2002): Phage display technology: clinical applications and recent innovations. **Clinical Biochemistry** 35, 425-445.

Bailey, E. A., Iyer R. S., Stone M. P., Harris T. M., and M., E. J. (1996): Mutational properties of the primary aflatoxin B1 DNA adduct. **Proceeding of the National Academy Sciences of the United States of America** 93, 1535-1539.

Barbas, C. F., Kang, A. S., Lerner, R. A., and Benkovic, S. J. (1991): Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. **Proceeding of the National Academy Sciences of the United States of America** 88 7978–7982.

Beck, E., and Zink, B. (1981): Nucleotide sequence and genome organisation of filamentous bacteriophages fl and fd. **Gene** 16, 35.

Bedard, L. L., and Massey, T. E. (2006): Aflatoxin B1-induced DNA damage and its repair. **Cancer Letters** 241, 174-183.

Better, M., Chang, C. P., Robinson, R. R., and Horwitz, A. H. (1988): Escherichia coli secretion of an active chimeric antibody fragment. **Science** 240, 1041-1043.

Bradbury, A., Velappan, N., Verzillo, V., Ovecka, M., Chasteen, L., Sblattero, D., Marzari, R., Lou, J., Siegel, R., and Pavlik, P. (2003): Antibodies in proteomics I: generating antibodies. **Trends in Biotechnology** 21, 275-281.

Brichta, J., Hnilova, M., and Viskovic, T. (2005): Generation of hapten-specificrecombinantantibodies:antibody phage display technology: a review. **Vet. Med** 50, 231–252.

Brinkmann, U., Chowdhury, P. S., Roscoe, D. M., and Pastan, I. (1995): Phage display of disulfide-stabilized Fv fragments. **Journal of immunological methods** 182, 41-50.

Burton, D. R. (1995): Phage display. **Immunotechnology** 1, 87-94.

Busby, W. F., and Wogan, G. N. (1984): Aflatoxins. **Chemical carcinogens** 2, 945–1136.

Byrne, B., Stack, E., Gilmartin, N., and O'Kennedy, R. (2009): Antibody-Based Sensors: Principles, Problems and Potential for Detection of Pathogens and Associated Toxins. **Sensors** 9, 4407-4445.

Cabilly, S. (1989): Growth at sub-optimal temperatures allows the production of functional, antigen-binding Fab fragments in Escherichia coli. **Gene(Amsterdam)** 85, 553-557.

CAST (2003): Mycotoxins: Risks in Plant, Animal and Human Systems: *Task force report*, Ames, Iowa, USA.

Cesareni, G. (1992): Peptide display on filamentous phage capsids. A new powerful tool to study protein-ligand interaction. **FEBS Letter** 307, 66-70.

Chames, P., Coulon, S., and Baty, D. (1998): Improving the affinity and the fine specificity of an anti-cortisol antibody by parsimonious mutagenesis and phage-display. **Journal of Immunology** 161, 5421-5429.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D., and Winter, G. (1991): Making antibody fragments using phage display libraries. **Nature** 352, 624-628.

Daly, S., Dillon, P., Manning, B., Dunne, L., Killard, A., and Okennedy, R. (2002): Production and characterization of murine single chain Fv antibodies to aflatoxin B1 derived from a pre-immunized antibody phage display library system. **Food and Agricultural Immunology** 14, 255-274.

de Haard, H. J., van Neer, N., Reurs, A., Hufton, S. E., Roovers, R. C., Henderikx, P., de Bruine, A. P., Arends, J. W., and Hoogenboom, H. R. (1999): A large non-immunized human Fab fragment phage library that permits rapid isolation and

kinetic analysis of high affinity antibodies. **Journal of Biological Chemistry** 274, 18218–18230.

Devi, K. T., Mayo, M. A., Reddy, K. L. N., Delfosse, P., Reddy, G., Reddy, S. V., and Reddy, D. V. R. (1999): Production and characterization of monoclonal antibodies for aflatoxin B 1. **Letters in applied microbiology** 29, 284-288.

Dubrovskaya, V., Ulitin, A., Laman, A., Gileva, I., Bormotov, N., Ilyichev, A., Brovko, F., Shchelkunov, S., Belanov, E., and Tikunova, N. (2007): Construction of a combinatorial immune library of human single-chain antibodies to orthopoxviruses and selection of antibodies to recombinant prA30L of the variola virus. **Molecular Biology** 41, 157-167.

Dunne, L., Daly, S., Baxter, A., Haughey, S., and O'Kennedy, R. (2005): Surface plasmon resonance-based immunoassay for the detection of aflatoxin B 1 using single-chain antibody fragments. **Spectroscopy letters** 38, 229-245.

Endemann, H., and Model, P. (1995): Lcoation of Filamentous Phage Minor Coat Proteins in Phage and in Infected Cells. **Journal of Molecular Biology** 250, 496-506.

Fjällman, T., and Hall, J. C. (2005): Antibody engineering—a valuable asset in preventing closed environment epidemics. **Acta Astronautica** 57, 81-88.

Gao, C., Mao, S., Lo, C.-H. L., Wirsching, P., Lerner, R. A., and Janda, K. D. (1999): Making artificial antibodies: A format for phage display of combinatorial heterodimeric arrays. **Proceedings of the National Academy of Sciences of the United States of America** 96, 6025-6030.

Griffiths, A. D. (1993): Production of human antibodies using bacteriophage. **Current Opinion in Immunology** 5, 263-267.

Hartley, R. D., Nesbitt, B. F., and O'Kelly, J. (1963): Toxic metabolites of Aspergillus flavus. **Nature** 198.

Hill, D. F., and Petersen, G. B. (1982): Nucleotide sequence of bacteriophage f1 DNA. **Journal of Virology** 44, 32.

Holliger, P., Prospero, T., and Winter, G. (1993): " Diabodies": small bivalent and bispecific antibody fragments. **Proceedings of the National Academy of Sciences** 90, 6444.

Hoogenboom, H. R., de Bruine, A. P., Hufton, S. E., Hoet, R. M., Arends, J.-W., and Roovers, R. C. (1998): Antibody phage display technology and its applications. **Immunotechnology** 4, 1-20.

Hoogenboom, H. R., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P., and Winter, G. (1991): Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. **Nucleic Acids Research** 19, 4133.

Horabin, J. I., and Webster, R. E. (1988): An amino acid sequence which directs membrane insertion causes loss of membrane potential. **Journal of Biological Chemistry** 263, 11575.

Huie, M. A., Cheung, M. C., Muench, M. O., Becerril, B., Kan, Y. W., and Marks, J. D. (2001): Antibodies to human fetal erythroid cells from a nonimmune phage antibody library. **Proceedings of the National Academy of Sciences of the United States of America** 98, 2682.

Hust, M., and Dubel, S. (2004): Mating antibody phage display with proteomics. **Trends in Biotechnology** 22, 8-14.

IARC (1993): IARC monographs on the evaluation of carcinogenic risks to humans: Some naturally occurring substances food items and constituents, heterocyclic aromatic amines and mycotoxins. **IARC monographs** 56, 245.

Kang, A. S., Barbas, C. F., Janda, K. D., Benkovic, S. J., and Lerner, R. A. (1991): Linkage of recognition and replication functions by assembling combinatorial antibody Fab libraries along phage surfaces. **Proceedings of the National Academy of Sciences of the United States of America** 88, 4363-4366.

Keyl, A. C. (1978): Aflatoxicosis in cattle. **Mycotoxic Fungi, Mycotoxins, Mycotoxicoses** 2, 9–27.

Kipriyanov, S. M., Moldenhauer, G., and Little, M. (1997): High level production of soluble single chain antibodies in small-scale Escherichia coli cultures. **Journal of Immunological Methods** 200, 69-77.

Knappik, A., Ge, L., Honegger, A., Pack, P., Fischer, M., Wellnhofer, G., Hoess, A., Wo lle, J., Plu ckthun, A., and Virneka s, B. (2000): Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. **Journal of Molecular Biology** 296, 57-86.

Kramer, R. A., Cox, F., van der Horst, M., van der Oudenrijn, S., Res, P. C., Bia, J., Logtenberg, T., and , d. K., J., (2003): A novel helper phage that improves phage display selection efficiency by preventing the amplification of phages without recombinant protein. **Nucleic acids research** 31, e59.

Lauer, B., Ottleben, I., Jacobsen, H. J., and Reinard, T. (2005): Production of a single-chain variable fragment antibody against fumonisin B1. **Journal of agricultural and food chemistry** 53, 899-904.

Lee, N. A., Wang, S., Allan, R. D., and Kennedy, I. R. (2004): A rapid aflatoxin B1 ELISA: development and validation with reduced matrix effects for peanuts, corn, pistachio, and soybeans. **Journal of agricultural and food chemistry** 52, 2746-2755.

Marks, J. D., Hoogenboom, H. R., and Bonnert, T. P. (1991): By—passing immunization Human antibodies from V-gene libraries displayed on phage **Journal of Molecular Biology** 222, 581-597.

Marvin, D. A. (1998): Filamentous phage structure, infection and assembly. **Current opinion in structural biology** 8, 150-158.

Mavrangelos, C., Thiel, M., Adamson, P. J., Millard, D. J., Nobbs, S., Zola, H., and Nicholson, I. C. (2001): Increased Yield and Activity of Soluble Single-Chain Antibody Fragments by Combining High-Level Expression and the Skp Periplasmic Chaperonin. **Protein Expression and Purification** 23, 289-295.

McCafferty, J., Griffiths, A. D., Winter, G., and Chiswell, D. J. (1990): Phage antibodies: filamentous phage displaying antibody variable domains. **Nature** 348, 552-554.

McGuinness, B. T., Walter, G., FitzGerald, K., Schuler, P., Mahoney, W., Duncan, A. R., and Hoogenboom, H. R. (1996): Phage diabody repertoires for selection of large numbers of bispecific antibody fragments. **Nature biotechnology** 14, 1149-1154.

Meulemans, E. V., Slobbe, R., Wasterval, P., Ramaekers, F. C., and van Eys, G. J. (1994): Selection of phage-displayed antibodies specific for a cytoskeletal antigen by competitive elution with a monoclonal antibody. **Journal of Molecular Biology** 244, 353-360.

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

Neri, D., Petrul, H., and Roncucci, G. (1995): Engineering recombinant antibodies for immunotherapy. **Cell Biophysics** 27, 47-61.

Orum, H., Andersen, P. S., Oster, A., Johansen, L. K., Riise, E., Bj rnvad, M., Svendsen, I., and Engberg, J. (1993): Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. **Nucleic acids research** 21, 4491.

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

Parmley, S. F., and Smith, G. P. (1988): Antibody-selectable filamentous fd phage vectors-afinity purification of target genes. **Gene** 73, 305-318.

Petrenko, V. A., and Vodyanoy, V. J. (2003): Phage display for detection of biological threat agents. **Journal of microbiological methods** 53, 253-262.

Pier, A. C. (1992): Major biological consequences of aflatoxicosis in animal production. **Journal of Animal Science** 70, 3964-3967.

Pitt, J. I. (2000): Toxigenic fungi and mycotoxins. **British Medical Bullet** 56, 184-192.

Rader, C., Cheresh, D. A., and Barbas, C. F. (1998): A phage display approach for rapid antibody humanization: Designed combinatorial V gene libraries. **Proceedings of the National Academy of Sciences of the United States of America** 95, 8910-8915.

Rapoza, M. P., and Webster, R. E. (1993): The filamentous bacteriophage assembly proteins require the bacterial SecA protein for correct localization to the membrane. **Journal of bacteriology** 175, 1856.

Rapoza, M. P., and Webster, R. E. (1995): The products of gene I and the overlapping in-frame gene XI are required for filamentous phage assembly. **Journal of Molecular Biology** 248, 627-638.

Robens, J. F., and Richard, J. L. (1992): Aflatoxins In Animal and Human Health, pp. 69-85. In W. W. George (Ed.): **Reviews of Environmental Contamination and Toxicology**, Springer-Verlag, New York.

Sánchez, L., Ayala, M., Freyre, F., Pedroso, I., Bell, H., Falcón, V., and Gavilondo, J. V. (1999): High cytoplasmic expression in E. coli, purification, and in vitro refolding of a single chain Fv antibody fragment against the hepatitis B surface antigen. **Journal of Biotechnology** 72, 13-20.

Sblattero, D., and Bradbury, A. (2000): Exploiting recombination in single bacteria to make large phage antibody libraries. **Nature biotechnology** 18, 75-80.

Schier, R., Bye, J., Apell, G., McCall, A., Adams, G. P., Malmqvist, M., Weiner, L. M., and Marks, J. D. (1996): Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. **Journal of Molecular Biology** 255, 28-43.

Sheedy, C., Roger MacKenzie, C., and Hall, J. C. (2007): Isolation and affinity maturation of hapten-specific antibodies. **Biotechnology Advances** 25, 333-352.

Sheets, M. D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J. C., and Marks, J. D. (1998): Efficient construction of a large nonimmune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. **Proceedings of the National Academy of Sciences** 95, 6157.

Simons, G. F., Konings, R. N., and Schoenmakers, J. G. (1981): Genes VI, VII, and IX of phage M13 code for minor capsid proteins of the virion. **Proceedings of the National Academy of Sciences** 78, 4194.

Skerra, A., and Plu¨ckthun, A. (1988): Assembly of a functional immunoglobulin Fv fragment in Escherichia coli. **Science** 240, 1038-1041.

Smela, M. E., Currier S. S., Bailey E. A., and M., E. J. (2001): The chemistry and biology of aflatoxin B1: From mutational spectrometry to carcinogenesis. **Carcinogenesis** 22, 535-545.

Smith, G. P. (1985): Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. **Science** 228, 1315.

Stoloff, L. (1977): Aflatoxins-an overview. **Mycotoxins in human and Animal Health**, 7–28.

Strachan, G., McElhiney, J., Drever, M., McIntosh, F., Lawton, L., and Porter, A. (2002): Rapid selection of anti-hapten antibodies isolated from synthetic and semi-synthetic antibody phage display libraries expressed in Escherichia coli. **FEMS Microbiology Letter** 210, 257–261.

Taylor, S. L., King, J. W., Greer, J. I., and Richard, J. L. (1997): Supercritical Fluid Extraction of Aflatoxin M1 from Beef Liver. **Journal of Food Protection** 60, 698-700.

Tomlinson, I. M., Williams, S. C., Corbett, S. J., Cox, J. P. L., and Winter, G. (1996): VBASE sequence directory. Cambridge, MRC Centre for Protein Engineering.

Van Egmond, H. P. (1989): Current situation on regulations for mycotoxins. Overview of tolerances and status of standard methods of sampling and analysis. **Food additives and contaminants** 6, 139.

Van Wezenbeek, P. M., Hulsebos, T. J., and Schoenmakers, J. G. (1980): Nucleotide sequence of the filamentous bacteriophage M13 DNA genome: comparison with phage fd. **Gene** 11, 129.

Vaughan, T. J., Williams, A. J., Pritchard, K., Osbourn, J. K., Pope, A. R., Earnshaw, J. C., McCafferty, J., Hodits, R. A., Wilton, J., and Johnson, K. S. (1996): Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. **Nature Biotechnology** 14, 309-314.

Waliyar, F., Reddy, S. V., and Lava-Kumar, P. (2009): Review of Immunological Methods for the Quantification of Aflatoxins in Peanut and Other Foods. **Peanut Science** 36, 54-59.

Ward, R. L., Clark, M. A., Lee, J., and Hawkins, N. J. (1996): Retrieval of human antibodies from phage-display libraries using enzymatic cleavage. **Journal of immunological methods** 189, 73-82.

Webster, R. E. (1996): Biology of the filamentous bacteriophage, pp. 1-20. In B. K. Kay (Ed.): **Phage display of peptides and proteins: A laboratory manual**, Academic Press, California.

Willats, W. G. T. (2002): Phage display: practicalities and prospects. **Plant molecular biology** 50, 837-854.

Winter, G., Griffiths, A. D., Hawkins, R. E., and Hoogenboom, H. R. (1994): Making Antibodies by Phage Display Technology. **Annual Review of Immunology** 12, 433-455.

Wogan, G. N. (1966): Chemical nature and biological effects of the aflatoxins. **Bacteriological Reviews** 30, 460.

Woychik, N. A., Hinsdill, R. D., and Chu, F. S. (1984): Production and characterization of monoclonal antibodies against aflatoxin M1. **Applied and Environmental Microbiology** 48, 1096.

Yang, L., Ding, H., Gu, Z., Zhao, J., Chen, H., Tian, F., Chen, Y. Q., Zhang, H., and Chen, W. (2009): Selection of Single Chain Fragment Variables with Direct Coating of Aflatoxin B1 to Enzyme-Linked Immunosorbent Assay Plates. **Journal of Agricultural and Food Chemistry** 57, 8927-8932.

Yau, K. Y. F., Lee, H., and Hall, J. C. (2003): Emerging trends in the synthesis and improvement of hapten-specific recombinant antibodies. **Biotechnology Advances** 21, 599-637.

Yuan, Q., Clarke, J. R., Zhou, H. R., Linz, J. E., Pestka, J. J., and Hart, L. P. (1997): Molecular cloning, expression, and characterization of a functional single-chain Fv

antibody to the mycotoxin zearalenone. **Applied and Environmental Microbiology** 63, 263.

BIOGRAPHY

Miss Kuntalee Rangnoi was born on November 14, 1983 in Phetchaburi, Thailand. She graduated with the Bachelor Degree of Animal Production Technology, Suranaree University of Technology, Thailand in 2005. She had opportunity to study Master Degree enrollment in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology (2005-2009). During her study, she has experience on her research for 6 months at Applied Biochemistry Group, school of Biotechnology and Biomedical Diagnostics Institute, Dublin City University, Dublin, Ireland. She had presented research work in Agricultural Biotechnology for Better Living and a Clean Environment (ABIG 2009), September 22-25, 2009, Queen Sirikit National Convention Center, Bangkok, Thailand (Poster presentation; in “Selection of scFv antibody fragment specific to Aflatoxin by using phage display technology”). She conducted to research in the topic of selection of monoclonal antibodies specific to aflatoxin from phage display antibody library.