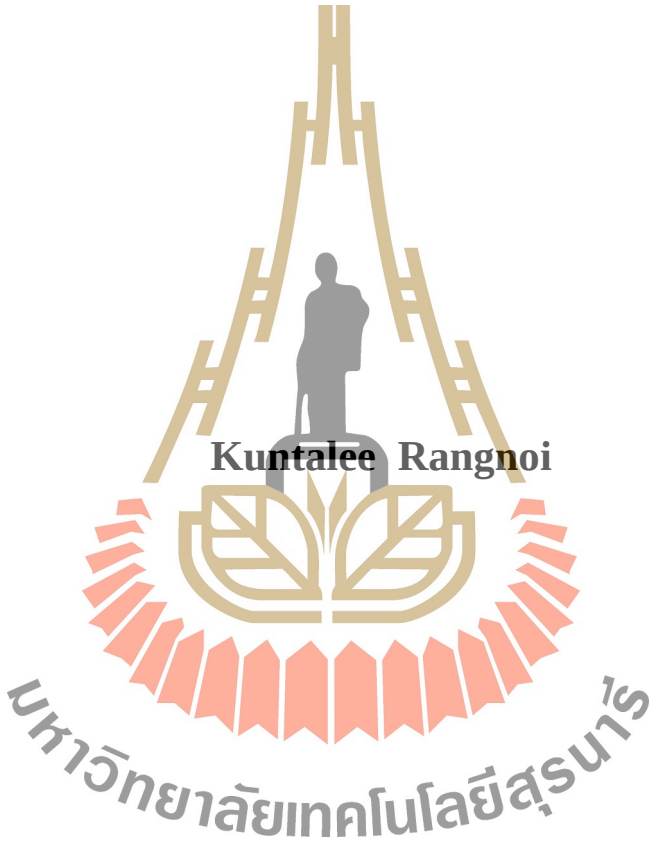
DEVELOPMENT OF RECOMBINANT ANTIBODY

FOR THE DETECTION OF AFLATOXIN



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นางสาวกุณฑลี ร่างน้อย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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DEVELOPMENT OF RECOMBINANT ANTIBODY FOR THE DETECTION OF AFLATOXIN

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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กุณฑลี ร่างน้อย : การพัฒนาแอนติบอดีดัดแปลงพันธุกรรมเพื่อใช้ในการตรวจสอบอะฟลาทอกซิน (DEVELOPMENT OF RECOMBINANT ANTIBOY FOR THE DETECTION OF AFLATOXIN) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร. มณฑารพ ยมาภัย, 128 หน้า.

อะฟลาทอกซินเป็นสารพิษที่ก่อให้เกิดมะเร็ง ซึ่งผลิตมาจากจากเชื้อรา *Aspergillus flavus* และ *A. parasiticus* พบปนเปื้อนในอาหารคนและสัตว์ รวมทั้งผลิตผลทางการเกษตรอื่นๆ ด้วยความเป็นพิษของอะฟลาทอกซินจึงทำให้ในหลายประเทศต้องมีการกำหนดระดับที่สามารถปนเปื้อนของอะฟลาทอกซินในผลผลิตทางการเกษตร รวมทั้ง อาหารมนุษย์และสัตว์เพื่อความปลอดภัย ดังนั้นจึงจำเป็นต้องมีวิธีการที่มีประสิทธิภาพ ในการตรวจหาอะฟลาทอกซินที่ปนเปื้อนในผลิตภัณฑ์ต่างๆ โดยวิธีทางอิมมูน เป็นวิธีการที่มีขั้นตอนที่สะดวก รวดเร็ว และมีความคุ้มทุนมากที่สุด วิธีการดังกล่าวนี้ อาทิเช่น การตรวจบนจานหลุมทดสอบ (อีไลซ่า) หรือแผ่นตรวจสอบ เป็นวิธีการที่ต้องใช้แอนติบอดีที่มีความจำเพาะ และความไว ในการจับกับอะฟลาทอกซินเพื่อใช้เป็นองค์ประกอบสำหรับตรวจสอบ จากงานวิจัยที่ผ่านมา ได้ทำการคัดเลือกแอนติบอดีเส้นเดี่ยวที่จำเพาะต่ออะฟลาทอกซิน ชื่อ YAF-C3 จากคลังเฟจที่แสดงแอนติบอดีมนุษย์แบบปฐมภูมิ ซึ่งเป็นคลังเฟจซึ่งแสดงแอนติบอดีมนุษย์แบบเส้นเดี่ยว (เอสซีเอฟวี) บนผิวเฟจ (คลังย่าโม) อย่างไรก็ตามเอสซีเอฟวีนี้ไม่สามารถตรวจจับอะฟลาทอกซินได้ในระดับที่นานาชาติยอมรับ ดังนั้น จึงได้มีการทำการสลับสับเปลี่ยนชิ้นส่วนแอนติบอดี เพื่อทำการปรับปรุงประสิทธิภาพของเอชซีเอฟวีนี้ ให้มีความจำเพาะ และมีความไวต่ออะฟลาทอกซินเพิ่มขึ้น โดยหลังจากทำการคัดเลือกจำนวน 3 รอบ พบว่า โคลนชื่อ sAFH-3E11, sAFH3F11 และ sAFH-3E3 มีความไวในการจับกับอะฟลาทอกซินได้ดีกว่าโคลนเริ่มต้นตั้งแต่ 3-7.5 เท่า โดยโคลน sAFH-3E3 มีความไวในการจับกับอะฟลาทอกซินมากที่สุด จึงได้นำโคลนนี้มาศึกษาโครงสร้างสามมิติ และศึกษากลไกการจับกันของโมเลกุล เพื่อเปรียบเทียบกับโคลนต้นแบบ ผลจากการวิเคราะห์ลำดับกรดอะมิโนของโคลนsAFH-3E3 พบว่ามีกรดอะมิโนที่แตกต่างจากโคลนเริ่มต้นที่บริเวณ แกนหลักชิ้นที่ 1 และ ซีดีอาร์ 1 ในส่วนของแอนติบอดีที่มีความแปรปรวนสูงสายหนัก รวมทั้งสิ้น 5 กรดอะมิโน หลังจากนั้น เอชซีเอฟวีแอนติบอดีนี้ได้นำมาดัดแปลงให้อยู่ในรูปแบบต่างๆ ได้แก่ เอชซีเอฟวีที่เชื่อมต่อกับเอนไซม์อาลคาไลฟอสฟาเตส (scFv-AP) เอชซีเอฟที่เชื่อมอยู่กับส่วนคงที่ของสายแอนติบอดี (scFv-Fc) และแอนติบอดีเต็มรูปแบบ (IgG) ซึ่งแอนติบอดีรูปแบบ scFv-AP สามารถผลิตได้ในอีโคไล ส่วน scFv-Fc และIgG สามารถถูกผลิตได้ปริมาณสูงในเซลล์มนุษย์ (HEK293-6E) จากนั้นได้นำแอนติบอดีรูปแบบเหล่านี้มาตรวจสอบความไวในการจับกับอะฟลาทอกซินด้วยวิธีอีไลซ่าแบบแข่งขัน ซึ่งพบว่ารูปแบบ scFv-AP มีความไวสูงที่สุด และมีความสะดวกที่สุด จึงมีความเหมาะสมที่จะพัฒนา

ใช้แอนติบอดีรูปแบบนี้ในการพัฒนาชุดตรวจสอบอะฟลาทอกซินด้วยวิธีอีไลซ่า นอกจากนั้นแล้วยังได้นำแอนติบอดีรูปแบบ IgG ที่มีความไวในการจับใกล้เคียงกับรูปแบบเอสซีเอฟวี มาพัฒนาด้วยการเชื่อมต่อกับอนุภาคทองและลาเท็กซ์ เพื่อใช้เป็นสารตรวจสอบบนแผ่นตรวจสอบ หลังจากทำการทดสอบโดยการเติมอะฟลาทอกซินที่ความเข้มข้นต่างๆลงในสารสกัดจากข้าวโพด แล้วนำมาตรวจสอบด้วยวิธีอีไลซ่า และวิธีอิมมูโนบนแผ่นตรวจสอบ พบว่าแอนติบอดีที่พัฒนามาได้นี้สามารถใช้ในการตรวจจับอะฟลาทอกซินบี 1 ได้ค่าต่ำสุดประมาณ 20 และ 5 นาโนกรัมต่อมิลลิลิตร ผลจากการศึกษาแสดงว่า แอนติบอดีที่พัฒนามาได้นี้ มีประสิทธิภาพเพียงพอที่จะนำมาพัฒนาให้เป็นชุดตรวจสอบสำหรับตรวจวิเคราะห์สารพิษอะฟลาทอกซินที่ปนเปื้อนในผลิตผลทางการเกษตรในรูปแบบต่างๆได้ต่อไปในอนาคต

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2559 ลายมือชื่ออาจารย์ที่ปรึกษา_____

KUNTALEE RANGNOI : DEVELOPMENT OF RECOMBINANT ANTIBODY FOR THE DETECTION OF AFLATOXIN. THESIS ADVISOR : PROF. MONTAROP YAMABHAI, Ph.D., 128 PP.

RECOMBINANT ANTIBODY/AFLATOXIN/CHAIN SHUFFLING/ELISA/ LATERALFLOW/IMMUNOASSAY

Aflatoxins are carcinogenic toxins that produced from *Aspergillus flavus* and *A. parasiticus*. These toxins are commonly found to contaminate in agricultural products, human food and animal feed. Because of their effects on human and animal health, many countries around the world have set their maximum level for aflatoxin contamination. Therefore, an effective method for aflatoxin detection is required. Immuno-based method, such as Enzyme linked immunosorbant assay (ELISA) and lateral flow immunoassay, is a rapid, sensitive and cost-effective method for the detection of aflatoxin. This method needs high sensitivity antibody as a detection reagent. Previously, an anti-aflatoxin scFv antibody, named YAF-C3 was isolated from a naïve phage display antibody library (YAMO library). However, this clone was not sensitive enough for detecting the aflatoxin at the limit concentration, provided by CODEX international standard legislation. Therefore, the chain shuffling and phage display techniques were used to improve the affinity and sensitivity of the scFv antibody (affinity maturation). After three round of affinity selection, three clones designated sAFH-3E11, sAFH3F11 and sAFH-3E3, showing 3-7.5-folds sensitivity improvement over the original scFv clone, were isolated. The three dimensional structure of clone sAFH-3E3 scFv which, showed the highest sensitivity was predicted

and the interaction with aflatoxin was studied by molecular docking. Amino acid sequence analysis indicated five amino acid mutations in framework 1 (FR1) and CDR1 regions of variable domain of heavy chain (V_H). In addition, the affinity matured scFv fragments were engineered to generate three formats of recombinant antibodies, i.e., scFv-AP, scFv-Fc and full length IgG. The scFv-AP was successfully expressed in *Eschericia coli*, while the scFv-Fc and IgG could be expressed at high yields in human cell (HEK293-6E). Competitive ELISA was performed to compare the sensitivity of these various formats. The scFv-AP showed the highest sensitivity in ELISA. Therefore, this format was further developed for the detection of aflatoxin in agricultural products. Moreover, the full-length antibody clone sAFH-3E3 IgG, which showed the similar sensitivity to scFv fragment, was conjugated with colloidal gold and latex particles, and used as detection reagent in a lateral flow immunoassay. Spike experiment using corn sample indicated that the detection limit of scFv-AP and IgG were 20 and 5 ng/ml for ELISA and lateral flow immunoassay, respectively. This improved clone has potential to be used as detecting reagent for aflatoxin contamination in agricultural products in various immune-detection kits in the future.

School of Biotechnology Student's Signature_____

Academic Year 2016 Advisor's Signature_____

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Kuntalee Rangnoi

CONTENTS

Page

CONTENTS (continued)

Page

CONTENTS (continued)

Page

CONTENTS (continued)

Page

CONTENTS (continued)

Page

CONTENTS (continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (continued)

Figure **Page**

LIST OF FIGURES (continued)

Figure **Page**

LIST OF FIGURES (continued)

Figure **Page**

LIST OF ABBREVIATIONS

Ab	Antibody
Abs	Absorbance
ABTS	2,2'-azino-bis (3-ethybenzthiazoline-6-sulphonic acid)
Ag	Antigen
Amp	Ampicillin
AP	Alkaline phosphatase
BIA	Biomolecular interaction analysis
bp	Base pairs
BSA	Bovine serum albumin
cfu	Colony forming units
CDR	Complementary determining region
CH_1	Constant heavy chain 1
CH_2	Constant heavy chain 2
CH_3	Constant heavy chain 3
CL	Constant light chain
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidyl triphosphates
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay

LIST OF ABBREVIATIONS (continued)

Fc	Constant region of an antibody molecule
Fv	Variable binding region of an antibody
HBS	HEPES buffered saline
HRP	Horse radish peroxidise
IgG	Immunoglobulin class
IMAC	Immobilised metal affinity chromatography
Kan	Kanamycin
Log	Logarithmicm
MW	Molecular weight
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PBST	Phosphate buffer saline/Tween
PEG	Polyethylene glycol
pH	Log of the hydrogen ion concentration
RT	Room temperature
scFv	Single chain Fv antibody derivative
SDS	Sodium dodecyl sulphate
SPR	Surface plasmon resonance
TMB	Tetramethylbenzidine dihydrochloride
UV	Ultraviolet

LIST OF ABBREVIATIONS (continued)

Units

µg	microgram
kDa	(kilo) Daltons
µl	microlitre
µM	micromoles
^{o}C	degrees Celcius
g	grams
hr	hours
kg	kilogram
L	litre
m	metre
M	molar
mg	milligram
min	minute
ml	millilitre
nM	nanomolar
pg	picograms
rpm	revolutions per minute
sec	seconds
v/v	volume per unit volume
w/v	weight per unit volume

CHAPTER I
INTRODUCTION

1.1 Significant of this study

Aflatoxins are highly toxic secondary metabolites produced by fungi strains *Aspergillus flavus* and *A. parasiticus*. The contamination of aflatoxin is found in various food products including peanuts, corn, oil seeds such as cottonseed, wheat, sorghum, almonds, walnuts, pecans, dried fruits, legumes, peppers, potatoes, rice, copra, filberts, milk, and milk products (Wild and Gong 2010). Aflatoxin B1 (AFB1) is the most toxic of all aflatoxins and is generally found in the highest concentration in food and animal feeds in many area of the world (Taylor et al. 1997). These toxins have been accused as the cause of high mortality in livestock and in some cases of death in human beings (Murjani 2003). They are listed as group I carcinogens to human by the International Agency for Research on Cancer (IARC). As a consequence, the routine screening of products destined for animal and human consumption are necessary. Several analytical methods for the determination of aflatoxins have been developed such as thin layer chromatography (TLC), gas liquid chromatography (LC), high-pressure liquid chromatography (HPLC), and mass spectrometry. However, these methods are laborious, time-consuming, require expensive instrumentations for analysis and clean-up of the samples (Kolosova et al. 2006). Immunological methods, which are simple, rapid, cost-effective and adaptable to the situation of developing countries, have been developed for quantitative

estimation of aflatoxin. These immunological methods need the polyclonal or monoclonal antibody as detection reagents. Productions of high affinity polyclonal and monoclonal antibodies need the immunization of animals, 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 antibody is attractive. In recombinant antibody technology, the recombinant antibody fragments are displayed on the surface of filamentous phage or other display format such as yeast (Boder and Wittrup 1997), bacterial surface (Fleetwood et al. 2013), or ribosome (Hanes and Plückthun 1997). Phage display antibody technology has been proposed to be beneficial for the development of convenient test kits for the detection of mycotoxin, which can be distributed to a wide range of customers and users at lower cost, resulting in a better quality of life of the population in the future (Yuan et al. 1997).

The most commonly used technology among the various *in vitro* strategies for the production of recombinant antibody 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, oligosaccharides, nucleic acid, toxins or low molecular weight compounds – haptens (Willats 2002, Yau et al. 2003).

Phage display antibody library can be produced from immune or non-immune source. The procedure for selecting phage is simple and inexpensive. This procedure is commonly called bio-panning method. Following bio-panning, the antibody can be inserted into vector and expressed in the bacteria, yeast or plants. Advantages of recombinant antibody technology are several (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* or cell lines, without the use of animals, (iv) the antibody gene can be further engineered to obtain increase affinity and modified specificity.

Human antibodies produced from phage display libraries typically tend to have low binding affinities (K_D) for hapten antigen, ranging from 10^{-7} to 10^{-9} M (Hudson and Souriau 2003). The antibodies selected from immune libraries may possess higher affinity than naïve, but it is not always the case, especially with hapten specific antibodies (Charlton et al. 2001). A higher-affinity antibody is important for efficient binding to the antigen for use as sensitive diagnostic reagents. Affinity maturation can be performed to improve the sensitivity, affinity and stability of recombinant antibody. The affinity maturation process involve introduction of diversity in antibody V gene, creation of a secondary mutant library from selected low affinity or, specificity clones, and selection of higher affinity from low affinity variants.

Several strategies can be used to affinity mature the antibodies derived from the original libraries. These include site-directed mutagenesis based on structural information, combinatorial mutagenesis of complementarity-determining regions (CDRs), random mutagenesis of the entire gene, and antibody shuffling (Clackson et al. 1991). Random mutagenesis consists of randomly mating the antibody, for example, gene mutations over V-domain genes, which can be derived from *Escherichia coli* mutator strain (Low et al. 1996), homologous gene rearrangements or error-prone PCR (Hawkins et al. 1992) whereas, site directed mutagenesis generally

directs or assigns mutations to chosen antibody gene sequence.

In this thesis, the scFv antibody specific to aflatoxin, previously selected from naïve phage display antibody library in our laboratory (Rangnoi et al. 2011) was used as a template for affinity maturation to enhance the affinity and sensitivity by chain shuffling technique. Two chain shuffling libraries were constructed and affinity selections or biopanning were performed. The scFv with greater binding property than the original scFv antibody was determined by competitive Enzyme-linked Immunosorbent Assay (ELISA). In addition, the scFv genes were engineered to generate different formats of antibody; namely, scFv-Fc, full-length IgG antibody, and scFv fuse to bacterial alkaline phosphatase (scFv-AP). These antibodies were used to detect aflatoxin in different assay formats. The scFv-AP was used as convenient one step detection reagents by competitive ELISA. ScFv-Fc and IgG were conjugated to colloidal gold and latex particles for using in Lateral Flow Immunochromatography Assay (LFIA). Finally, an efficient expression system for the production of recombinant antibody was developed for the detection of aflatoxins in agricultural and food products in the future.

1.2 Research objectives

The key objectives of this study are:

1. To improve the affinity and sensitivity of scFv antibody against aflatoxin.
2. To engineer anti-aflatoxin antibody so that it can be conveniently used as a convenient probe for the detection of aflatoxin.

1.3 Scope of the study

The affinity and sensitivity of anti-aflatoxin human scFv antibody was improved by chain shuffling technique. The source of antibody genes was from human origin. Experiments on the characterization of binders, binding affinity were conducted. The improved antibodies were engineered to generate different formats of antibody to be used as detection reagents in ELISA and lateral flow based assay.

1.4 References

Boder, E. T. and Wittrup, K. D. (1997). Yeast surface display for screening combinatorial polypeptide libraries. **Nature biotechnology** 15(6): 553-557.

Charlton, K., Harris, W. and Porter, A. (2001). The isolation of super-sensitive anti-hapten antibodies from combinatorial antibody libraries derived from sheep. **Biosensors and Bioelectronics** 16(9): 639-646.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D. and Winter, G. (1991). Making antibody fragments using phage display libraries. **Nature** 352(6336): 624-628.

Fleetwood, F., Devoogdt, N., Pellis, M., Wernery, U., Muyldermans, S., Ståhl, S. and Löfblom, J. (2013). Surface display of a single-domain antibody library on Gram-positive bacteria. **Cellular and Molecular Life Sciences** 70(6): 1081-1093.

Hanes, J. and Plückthun, A. (1997). In vitro selection and evolution of functional proteins by using ribosome display. **Proceedings of the National Academy of Sciences** 94(10): 4937-4942.

Hawkins, R. E., Russell, S. J. and Winter, G. (1992). Selection of phage antibodies by binding affinity: mimicking affinity maturation. **Journal of molecular**

biology 226(3): 889-896.

Hudson, P. J. and Souriau, C. (2003). Engineered antibodies. **Nature medicine** 9(1): 129-134.

Hust, M. and Dubel, S. (2004). Mating antibody phage display with proteomics. **Trends in Biotechnology** 22(1): 8-14.

Kolosova, A. Y., Shim, W.-B., Yang, Z.-Y., Eremin, S. A. and Chung, D.-H. (2006). Direct competitive ELISA based on a monoclonal antibody for detection of aflatoxin B1. Stabilization of ELISA kit components and application to grain samples. **Analytical and bioanalytical chemistry** 384(1): 286-294.

Low, N. M., Holliger, P. and Winter, G. (1996). Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain. **Journal of molecular biology** 260(3): 359-368.

Murjani, G. (2003). Chronic aflatoxicosis in fish and its relevance to human health. **Central Institute of Freshwater Aquaculture, India**.

Rangnoi, K., Jaruseranee, N., O'Kennedy, R., Pansri, P. and Yamabhai, M. (2011). One-Step Detection of Aflatoxin-B1 Using scFv-Alkaline Phosphatase-Fusion Selected from Human Phage Display Antibody Library. **Molecular biotechnology** 49(3): 240-249.

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(1): 28-43.

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(6): 698-700.

Wild, C. P. and Gong, Y. Y. (2010). Mycotoxins and human disease: a largely ignored global health issue. **Carcinogenesis** 31(1): 71-82.

Willats, W. G. T. (2002). Phage display: practicalities and prospects. **Plant molecular biology** 50(6): 837-854.

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(7): 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(1): 263.

CHAPTER II

LITERATURE REVIEWS

2.1 Aflatoxins

Aflatoxins 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 are produced during fungal infection of a susceptible crop in the field or after harvest. Aflatoxins can be produced 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, storage under optimal conditions for fungal growth and processing of the food (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).

Aflatoxins are crystalline structures that fluoresce under UV radiation. These toxins are hydrophobic in nature and can dissolve in various polar organic solvents including methanol, chloroform, dimethyl sulfoxide (DMSO), aqueous acetone and aqueous hexane-acetone-water azeotrope, which were developed for extraction procedures. They are extremely stable in the absence of light and UV radiation and heat stable, even at temperatures in excess of 100^{o}C. These toxins have closely similar

structures and form a unique group of highly oxygenated, naturally occurring heterocyclic compounds. Aflatoxins are broken down by oxidizing reagents such as sodium hyperchlorite, hydrogen peroxide and potassium permanganate resulting the loss of fluorescence.

There are four principle types of aflatoxin: B1, B2, G1 and G2, which are named for their respective innate fluorescent properties and mobility in chromatogram (Pitt 2000). The B designation of aflatoxins B1 and B2 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. The two additional aflatoxin M1 and M2 are major metabolites of aflatoxin B1 and B2 respectively. The aflatoxins M1 and M2 were first isolated from milk of lactating animals fed with aflatoxin. AFB1 has the highest toxicity, carcinogenicity, and mutagenicity, followed by G1, B2 and G2 with decreasing toxicity (Cortés et al. 2010).

2.1.1 Effect of aflatoxin contamination

Aflatoxins are often contaminated in agricultural products with fungi that can produce toxic metabolites. 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 AFM1 in milk, 10 ppb for AFB1 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) has established specific guidelines on acceptable level of aflatoxins present in human food as 20 ppb, in milk as 0.5 ppb and in corn for feeding animal as 20-300 ppb. The action level for most feedstuff is 20 ppb. The regulation

level for aflatoxin contamination in foods and animal feeds are varied depending on whether country setting limit for importer and exporter as shown in Table 2.1.

Table 2.1 Aflatoxin regulations in the world.

	Maximum level (ppb)			
	Foods		**Animal feed**	
	AFB1	**Total**	**AFB1**	**Total**
EU	2-8	4-15	20	-
USA (FDA)	-	20	-	20-200
Singapore	5	5	-	-
China	5-20	-	10-50	-
Malaysia	-	5-15	-	-
Japan	-	10		-
Indonesia	15	20	-	-
Korea	10	15	10-20	-

- Not report

Aflatoxins are both acutely and chronically toxic to animals and humans worldwide, causing acute liver damage, liver cirrhosis, induction of tumours and possibly mutagenesis and teratogenesls (Stoloff 1977). In humans, numerous studies have linked the incidence of primary hepatocellular carcinoma with the intake of aflatoxins, leading to the classification of AFB1 as Group 1 human carcinogen by WHO-IARC (World Health Organization and International Agency for Research on

Cancer) (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). In animals, aflatoxins have effect to the growth and are immunosuppressive. 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 poultry, they can cause an increase in liver and kidney weights, multifocal hepatic necrosis, biliary hyperplasia, diarrhoea, immunosuppression, decreased feed intake, and decreased weight gain (Rossi et al. 2012). 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).

Aflatoxins have been recently considered as an important sanitary problem because human can be exposed to aflatoxins from ingestion of plant derived foods that are contaminated with toxins and their metabolites (which are present in animal products such as milk, meat, visceral organs and eggs) (Versantvoort et al. 2005). The contaminated food will pose a potential health risk to human such as aflatoxicosis and cancer (Jeffrey and Williams 2005).

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, Lizárraga-Paulín et al. 2011).

2.1.2 Recent methods for detection of the aflatoxins

Rapid methods for the detection of aflatoxins are mostly used to determine the approximate level of contamination for the analyses sample. They are generally less sensitive and precise than laboratory methods like chromatography method (HPLC, TLC and GLC).

A widely used application based on specific antibodies is the enzyme-linked immunosorbent assay (ELISA). Generally, this technique involves the reaction of antigen and antibody in microplate wells. Competitive ELISA techniques are mainly used for determining aflatoxins because they are low molecular weight compound. After adding a substrate this test allows the quantification of the results by means of an optical ELISA reader. In indirect competitive ELISA, an antigen conjugated with a carrier protein such as bovine serum albumin (BSA), ovalbumin (OVA) is bound to microplate well. In the course of analysis, specific antibodies are added to the sample. The free antigen and the conjugated antigen incubated on well compete for binding to the specific antibodies in the solution. The antibodies labeled with an enzyme are added to determine the amount of bound antibodies. If there is no aflatoxin in the sample, the antibodies will bind to the aflatoxin conjugated with protein on the well, leading to a larger value of an OD. On the other hand, the competitive reaction between aflatoxin in the sample and the anchored aflatoxin will lead to a lower OD value. Moreover, the biosensor technology such as surface

plasmon resonance (SPR) biosensor is a promising attractive technology for rapid aflatoxin detection. The principle of surface plasmon resonance is based on the property that binding of materials to a surface can alter the refractive index near that surface. SPR devices measure the small changes in the angle, or intensity, of internally reflected light that result from the binding event. For this analytical method portable equipment may be used (Zheng et al. 2006).

Non-instrumental antibody based applications are lateral flow tests, dipstick tests or flow-through tests. This method is very simple, take few minutes for analyzing and easy for user. In contrast to ELISA, these applications use antigens or antibodies that are immobilized on carrier membranes instead of microtiter plates. The analyst content is indicated by appearance or absence.

2.2 Phage display antibody technology

Phage display antibody technology is an advance in generation and selection of recombinant antibodies from phage antibody library that produces from immune or non-immune source. Typically, the antibody fragments are displayed on surface of filamentous bacteriophage in difference formats, including scFv fragment, Fab fragment, Fv fragment and nanobodies (Fig 2.1). In conjunction with, the recombinant antibody fragment and coat protein (pIII) fusion product into phage coat exposes the antibody on the surface of phage while its coding material resides within the phage particle. Phage populations are exposed to the targets in order to selectively capture binding phage during the affinity selection (bio-panning). Throughout repetitive rounds of binding, washing, elution and amplification, the original diverse phage population is increasingly enriched by phages with a potential binding affinity to the target (Willats 2002).

2.2.1 Antibody fragments

There are different antibody formats that can be displayed on the surface of phage, including single chain Fv (scFv) fragment, Fab fragment, Fv fragment, and the variable fragment (V_{HH}) of single-domain heavy chain antibodies (HCAb) from camelids. The larger Fab fragment consists of V_H -C_H and V_L -C_L segments linked by disulfide bonds. The smaller Fv fragment is composed of the V_L and V_H domains. The recombinant version of the Fv is termed the single-chain variable fragment (scFv). A flexible $(Gly_4Ser)_3$ peptide linker, usually a 15 aa linker is used to join the two variable regions together, and expressed as a single polypeptide chain. The linker allows the association of the V_H and V_L to form the antigen-binding site. Recombinant monoclonal antibodies from human and various animals such as mouse, rabbit, chicken, sheep or camelids can be produced by amplified antibody gene using PCR. These antibody fragments are smaller in size and can easier to manipulate genetically and express in bacterial systems. Displayed antibody fragments remain functional for the purpose of affinity selection. As a result, specific antibody fragments and their coding sequences can be selected simultaneously from a diverse phage display antibody library. Schematic representations of natural immunoglobulin molecule and recombinant antibody fragments are presented in Fig 2.1.

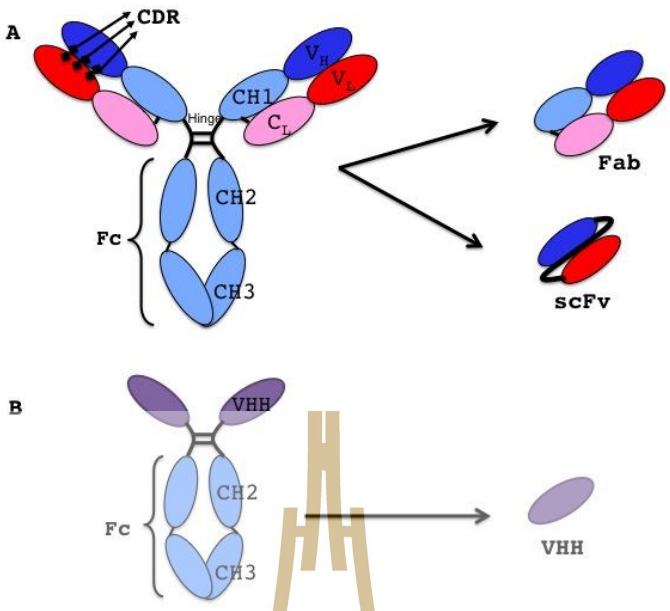


Figure 2.1 (A) Schematic diagram illustrating the structures of conventional human antibody molecule (IgG) and its different recombinant formats. CDR; Complementarity region (B) Naturally occurring single-domain heavy chain antibody (HCAb) from camelids and its variable domain V_H.

2.2.2 Phage display antibody library

Phage display antibody library can be prepared from immune and non-immune source. In general, two types of library can be used: naïve and immune. Immune libraries are created by cloning antibody genes from B cell of immunized human or animals such as mice (Tout et al. 2001), chickens (Andris-Widhopf et al. 2000), rabbits (Li et al. 2000, Kramer et al. 2002), camelids (Muyldermans and Lauwereys 1999) and sheep (Charlton et al. 2000). This library takes advantage of the fact that antibodies directed against antigen-specific antibody are enriched in such donors and they have been subjected to *in vivo* affinity maturation by the host immune

system (Clackson et al. 1991). However, the immune libraries is not always feasible, due to the lack of immune response to some antigen, the unpredictability of the immune response of the animal, ethical considerations in human antibodies and long time requirement.

Naïve libraries are derived from natural non-immunized human V genes, isolated from peripheral blood lymphocytes, bone marrow and spleen cells or from animal and human sources. These naïve libraries were used to isolated antibodies against multiple antigens because they are not biased towards any specific antigen. The affinities of isolated antibodies correlated with the library size. Therefore, it was necessary to construct very large libraries to obtain the high affinity antibodies (de Haard et al. 1999, Strachan et al. 2002). The next generations of non-immune libraries are synthetic or semisynthetic libraries. These libraries are built artificially by in vitro assembly of antibody gene segments (Desiderio et al. 2001). These can be constructed by generation light and heavy chain CDR3 diversity using synthetic oligonucleotides (Strachan et al. 2002).

2.2.3 Selection of phage antibody (bio-panning)

Bio-panning method was used to select the specifically binding clones from phage display antibody libraries. The phage antibodies libraries are incubated with immobilized target or interested antigen onto plastic tubes or Petri dishes (Marks et al. 1991), or specifically coated BIAcore sensorchip (Hoogenboom et al. 1998). After washing away a large portion of unbound phages, the phage clones bearing antibodies with specific binding properties to the target can be eluted in different ways with acidic solution such as glycine-HCl buffer, with basic solutions like triethylamine, by enzymatic cleavage of protease site engineered between the

antibody and gene III or by competition with excess antigen (Clackson et al. 1991). 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.

The optimal condition in selection specific phage antibody procedure can be adjusted. For example, high concentration of antigen, short washing times and elution were used in first round of selection that allows keeping the rare binders in the selectable pool. Stringent conditions are applied along with the growing number of selection cycle. The phage antibody with higher affinity may be enriched during rounds of selection by decreasing the concentration of antigen and washing times and also by using combinations of different elution steps.

2.2.4 Expression and purification of recombinant antibody

Recombinant antibody fragments (scFv, Fab) are expressed efficiently in many microorganisms, particularly in selected strains of *E. coli*. Especially, *E. coli* host strains such as BL21 or HB2151 and their derivatives become especially attractive for economical production of large amounts of sole scFv fragment (An et al. 2002). If the phagemid contain amber stop codon after the recombinant antibodies gene, the soluble recombinant antibodies can be produce by infecting *E. coli* strain HB2151 is nonsuppressor cells as they recognize an amber stop codon, engineered between the recombinant antibodies gene and gIII in the phagemid. Thus only express the recombinant antibodies will be expressed without the g3p protein. Soluble antibody fragments can be produced in culture supernatant, bacterial periplasm and

bacterial cytoplasm, depending on the signal sequence on N terminus of recombinant antibody.

E. coli. is the basic organism for antibody production. In contrast, the antibody fragment also have been expressed in mammalian cells (Uppala and Koivunen 2000), yeast like *Pichia pastoris* (Marty et al. 2001) and plant (Yuan et al. 2000). Mostly, the selectable of expression host must be consistent with final application of antibody molecule.

Immobilized metal ion affinity chromatography (IMAC) is commonly method for purification of bacterially produced antibodies because of its favorable cost /efficiency ratio (Blank et al. 2002) . This method is based on the binding affinity of metal ions such as Ni^{2+} or Cu^{2+} chelated to a chromatography matrix, which form complex with 6 histidine residues on the N- or C- terminus of an antibody fragment.

2.3 Affinity maturation

The low affinity of recombinant antibodies specific to hapten can be improved by mimicking *in vitro* somatic hypermutation called "Affinity maturation". Affinity maturation can generate extended and functional variability in recombinant antibodies (Borrebaeck and Ohlin 2002) and lead to selection of recombinant antibody fragments with increased affinity and specificity for target antigen. Several strategies have been described for affinity maturation of antibody fragment, namely random mutagenesis, site-directed mutagenesis and antibody shuffling.

2.3.1 Random mutagenesis

2.3.1.1 Error-prone PCR

Error-prone PCR uses low-fidelity polymerization PCR

conditions to introduce point mutations randomly over a gene sequence (Stemmer, 1994). Taq DNA polymerase is commonly used because it lacks proofreading activity. The error rate is further increased by employing unbalanced dNTP concentrations and reaction buffers containing Mn^{2+} (Cadwell and Joyce 1992). This technique has been successfully applied to the affinity maturation of an scFv directed against cardiac glycoside digoxigenin (Daugherty et al. 2000). More recently, the conjunction of error-prone PCR with an additional affinity maturation process has been described. Zahnd and collages have described a method involving ribosomal display, error-prone PCR and DNA shuffling for the affinity maturation of a peptide binding scFv, which lead to a 500-fold affinity improvement over its potential germ line precursor (Zahnd et al. 2004).

2.3.1.2 Bacterial mutator strain

E. coli mutator cells allows very rapid selection of antibody mutants with significant improvements in affinity or expression levels (Irving et al. 1996, Coia et al. 2001). This method consists of the selection of recombinant antibody from a library followed mutagenesis through amplification in a bacterial mutator strain of *E. coli* such as MutD5, which are a conditional mutant shown to produce single base substitutions (transversions or transitions) at high frequency compared to normal *E. coli* cells. Mutator strains can produce a large number of mutant recombinant antibody and can then be selected by phage-display or other methods (Irving et al. 1996). However, three to ten rounds of mutation or passage through mutator cells are required before high affinity mutant is selected (Coia et al. 2001). For example, three rounds of growth in mutator strain and subsequent display on phage surfaces for affinity selection, scFv specific to hapten 2-phenyl-5-oxazolone

with 100-fold increase in affinity were isolated (Low et al. 1996).

2.3.2 Site directed mutagenesis

Site-directed mutagenesis involves amino acid substitutions within one or more of the CDRs followed by the subsequent selection of clones with higher affinity for the target antigen. For example, Davies and Riechmann have investigated the effect of randomizing CDRs 1 and 2 residues in V_H domains specific for both haptens and protein antigens (Davies and Riechmann 1996). This research was successful in terms of generating randomized repertoires which were displayed on phage and affinity selected to improve antigen binding. Yang and associates reported increase the affinity of an anti-gp 120 Fab by 420 fold by mutating four CDRs and also described the largest affinity increase when optimizing CDR3 regions were observed (Yang et al. 1995). Similarly, the anti-ErbB2 antibody was mutated by sequentially targeting mutagenesis to the two CDR3 resulting a 1200-fold increase in affinity over the parental scFv was observed (Lou and Marks 2010). An alternative method of site-directed mutagenesis, termed “parsimonious mutagenesis” was described by Balint and Larrick (1993). In this method, all three CDRs of a variable region gene are simultaneously and thoroughly searched for improved variants in libraries of manageable size (Balint and Larrick 1993, Schier et al. 1996, Schier et al. 1996). In parsimonious mutagenesis, synthetic codons are used to mutate about 50 % of all targeted amino acids while keeping the other 50% of targeted residues intact (wild type) (Chames et al. 1998). Chames and associated (1998) used this technique to build the mutant library and selected the scFv specific to cytosol with up to eightfold better affinity and fivefold less cross-reactivity than the wild-type scFv.

2.3.3 Antibody Shuffling

2.3.3.1 DNA shuffling

DNA shuffling have been described by Stemmer (Stemmer 1994, Stemmer 1994). This shuffling technique involves digesting of a large antibody gene with DNase I and performing a PCR reaction in the absence of primers. Following several cycles, a full-length gene is produced by homologous or site-specific recombination. DNA shuffling was used to improve the production and function of a hapten-specific llama single-domain antibody fragment. The result show that one of the selected shuffled mutants had both increased 3.4 fold expression and 4 fold affinity while another clone had improved stability (van der Linden et al. 2000).

2.3.3.2 Chain shuffling

The heavy and light chains of antibody isolated from an immune library can be recombined, thereby generating a vast number of functional antibodies from an initially limited genetic repertoire (Kang et al. 1991). Chain shuffling has been used to obtain high affinity human scFv antibody that bound the hapten phenyloxazolone by increasing the affinity 300-fold from 3.0×10^{-7} M to 1.0×10^{-9} M (Marks et al. 1992). Typically, Shuffle the VL gene of the scFv is done first, because more of binding energy is usually contained within the VH. If the affinity of the affinity-matured light chain shuffled scFv is inadequate, the heavy-chain shuffling will process (Marks 2004). It has been reported that the scFv specific to halofuginone was improved 185 fold over the original scFv by using light chain shuffling (Fitzgerald et al. 2011).

2.4 Previous research on recombinant antibody against AFB_1

There have been some previous researches on the use recombinant antibodies for the detection aflatoxins. Some of them isolated anti-AFB_1 single chain fragment variables (scFvs) from a human lymphocyte antibody library and a semi-synthetic antibody library against AFB_1-BSA (Moghaddam et al. 2001, Pansri et al. 2009, Yang et al. 2009, Rangnoi et al. 2011). Daly et al. used AFB_1-dextran and AFB_1-BSA as antigens and panned murine anti-AFB_1 scFvs from an immunized phage displayed antibody library (Daly et al. 2002). The ELISA and SPR inhibition assays were used to determine analytic affinities and sensitivity of antibodies. The affinity and sensitivity of recombinant antibody against AFB1 from previous study were varies depend on selection method of recombinant antibodies from phage antibody libraries.

Because aflatoxins have a low molecular weight, they must be covalently linked to an immunogenic carrier protein such as BSA, which will elicit a strong immune response following immunization. The protein conjugate is also used at the screening and analytical stages of antibody production. So, several antibodies isolated bound to AFB1-BSA but not to AFB1 alone (Moghaddam et al. 2001). In 2009, Yang and associate develop new strategy on bio-panning by directly immobilized soluble AFB1 on to the microtiter plate instead of AFB1-BSA to get more chance to select an scFv antibody that bind to soluble AFB1 (Yang et al. 2009). Rangnoi and associate generated scFv fused to alkaline phosphatase (AP) specific to AFB1 resulted in 3 fold increase sensitivity. Another format of recombinant antibody against AFB1 have been reported. Fab antibody specific to AFB1 was used to compare the method to immobilized AFB1 on to microtiter plate. The sensitivity of Fab antibody was

improved when using the covalently immobilized AFB1 compare with passively absorbed AFB1-BSA (Edupuganti et al. 2013). In 2013-2014, the alpaca was immunized with AFB1-BSA or anti-AFB1 to produce VHH antibody. Wang and associate was successfully selected VHH, which is mimic to AFB1 binding site from VHH library that produced by injected high sensitivity of monoclonal antibody against AFB1 to alpaca (Wang et al. 2013). This VHH was immobilized as antigen instead of AFB1-BSA in ELISA assay. In the following year, the phage display VHH library was constructed from blood lymphocytes from alpaca immunized with AFB1-BSA. The high sensitivity VHH was obtained.

On acceptable level of aflatoxins contaminated in agricultural products by FDA, the high sensitivity of anti-AFB1 is needed. Several researches have been described low sensitivity of scFv-AFB1 (Moghaddam et al. 2001, Min et al. 2011, Rangnoi et al. 2011). The sensitivity and affinity of scFv can be improved by affinity maturation. So far, there are two publications, describing the improvement of scFv antibody specific to AFB1 by using affinity maturation by site directed mutagenesis (Li et al. 2012) and random mutagenesis (error prone PCR) (Min et al. 2015). The mutant scFv increased the sensitivity against AFB1 by 9-20 fold compare to original clone. The summary of previous reports on recombinant antibodies specific to aflatoxin is shown in Table 2.2. However, none of them has been used commercially for the detection of aflatoxin.

Table 2.2 Recombinant antibodies for aflatoxin selected from hybridoma cell lines or from phage libraries derived from naïve human and immunized animals.

Library	Antibody fragment	Assay platform	Affinity	Reference
Human naïve	scFv	SPR	K_D: 6 x 10^{-9} M	(Moghaddam et al. 2001)
Immunized mouse (splenocytes)	scFv	SPR	LOD: 3 ng/ml	(Daly et al. 2002)
Not published	scFv monomer/dimer	SPR	IC_{50}: 2 ng/ml, LOD: 0.3 ng/ml	(Dunne et al. 2005)
Human synthetic (Tomlinson I)	scFv	ELISA	IC_{50}: 0.4 µg/ml	(Wang et al. 2009)
Human synthetic (Tomlinson I+J)	scFv	ELISA	IC_{50}: 0.4 ng/ml	(Yang et al. 2009)
		SPR	K_D: 1.2 x 10^{-12} M	
Human naïve (Yamo-I)	scFv	ELISA	IC_{50}: 0.12 µg/ml	(Rangnoi et al. 2011)
	scFv-AP		IC_{50}: 0.034 µg/ml	
Human synthetic (Tomlinson I)	scFv-AP		IC_{50}: 0.14 µg/ml	

Table 2.2 (continued).

Library	Antibody fragment	Assay platform	Affinity	Reference
Immunized mouse (hybridoma)	scFv	SPR	K_D: 1.16 x 10^{-7} M	(Min et al. 2011)
Immunized mouse (hybridoma)	scFv	ELISA	IC_{50}: 0.02 ng/ml	(Li et al. 2012)
Immunized Alpaca	VHH	ELISA	IC_{50}: 0.16 ng/ml	(Wang et al. 2013)
Not published	Fab	ELISA	IC_{50}: 27 ng/ml	(Edupuganti et al. 2013)
Immunized mouse (hybridoma)	scFv	ELISA	IC_{50}: 0.01 ng/ml	(Li et al. 2013)
Immunized Alpaca	VHH	ELISA	IC_{50}: 0.75 ng/ml	(He et al. 2014)
Immunized mouse (hybridoma)	scFv	SPR	K_D: 9.2 x 10^{-8} M	(Min et al. 2015)

Abbreviations: K_D = equilibrium dissociation constant, IC_{50} = The half maximal inhibitory concentration

2.5 References

An, G., Dong, N., Shao, B., Zhu, M. and Ruan, C. (2002). Expression and characterization of the ScFv fragment of antiplatelet GPIIIa monoclonal antibody SZ-21. **Thrombosis research** 105(4): 331-337.

Andris-Widhopf, J., Rader, C., Steinberger, P., Fuller, R. and Barbas Iii, C. F. (2000). Methods for the generation of chicken monoclonal antibody fragments by phage display. **Journal of immunological methods** 242(1): 159-181.

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

Balint, R. F. and Larrick, J. W. (1993). Antibody engineering by parsimonious mutagenesis. **Gene** 137(1): 109-118.

Bedard, L. L. and Massey, T. E. (2006). Aflatoxin B1-induced DNA damage and its repair. **Cancer Letters** 241(2): 174-183.

Blank, K., Lindner, P., Diefenbach, B. and Plückthun, A. (2002). Self-immobilizing recombinant antibody fragments for immunoaffinity chromatography: generic, parallel, and scalable protein purification. **Protein expression and purification** 24(2): 313-322.

Borrebaeck, C. A. and Ohlin, M. (2002). Antibody evolution beyond Nature. **Nature biotechnology** 20(12): 1189-1190.

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

Cadwell, R. C. and Joyce, G. F. (1992). Randomization of genes by PCR mutagenesis. **Genome research** 2(1): 28-33.

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

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. **The Journal of Immunology** 161(10): 5421-5429.

Charlton, K. A., Moyle, S., Porter, A. J. R. and Harris, W. J. (2000). Analysis of the diversity of a sheep antibody repertoire as revealed from a bacteriophage display library. **The Journal of Immunology** 164(12): 6221-6229.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D. and Winter, G. (1991). Making antibody fragments using phage display libraries. **Nature** 352(6336): 624-628.

Coia, G., Hudson, P. J. and Irving, R. A. (2001). Protein affinity maturation in vivo using *E. coli* mutator cells. **Journal of immunological methods** 251(1): 187-193.

Cortés, G., Carvajal, M., Méndez-Ramírez, I., Ávila-González, E., Chilpa-Galván, N., Castillo-Urueta, P. and Flores, C. (2010). Identification and quantification of aflatoxins and aflatoxicol from poultry feed and their recovery in poultry litter. **Poultry science** 89(5): 993-1001.

Daly, S., Dillon, P., Manning, B., Dunne, L., Killard, A. and O'KENNEDY, R. (2002). Production and characterization of murine single chain Fv antibodies to aflatoxin B 1 derived from a pre-immunized antibody phage display library system. **Food and agricultural immunology** 14(4): 255-274.

Daugherty, P. S., Chen, G., Iverson, B. L. and Georgiou, G. (2000). Quantitative analysis of the effect of the mutation frequency on the affinity maturation of single chain Fv antibodies. **Proceedings of the National Academy of Sciences**

97(5): 2029-2034.

Davies, J. and Riechmann, L. (1996). Affinity improvement of single antibody VH domains: residues in all three hypervariable regions affect antigen binding. **Immunotechnology** 2(3): 169-179.

de Haard, H. J., van Neer, N., Reurs, A., Hufton, S. E., Roovers, R. C., Henderikx, P., de Bruïne, 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(26): 18218-18230.

Desiderio, A., Franconi, R., Lopez, M., Villani, M. E., Viti, F., Chiaraluce, R., Consalvi, V., Neri, D. and Benvenuto, E. (2001). A semi-synthetic repertoire of intrinsically stable antibody fragments derived from a single-framework scaffold. **Journal of molecular biology** 310(3): 603-615.

Dunne, L., Daly, S., Baxter, A., Haughey, S. and O'Kennedy, R. (2005). Surface Plasmon Resonance- Based Immunoassay for the Detection of Aflatoxin B1 Using Single- Chain Antibody Fragments. **Spectroscopy letters** 38(3): 229-245.

Edupuganti, S. R., Edupuganti, O. P., Hearty, S. and O'Kennedy, R. (2013). A highly stable, sensitive, regenerable and rapid immunoassay for detecting aflatoxin B 1 in corn incorporating covalent AFB 1 immobilization and a recombinant Fab antibody. **Talanta** 115: 329-335.

Fitzgerald, J., Leonard, P., Darcy, E., Danaher, M. and O'Kennedy, R. (2011). Light-chain shuffling from an antigen-biased phage pool allows 185-fold improvement of an anti-halofuginone single-chain variable fragment. **Analytical biochemistry** 410(1): 27-33.

Hartley, R., Nesbitt, B. F. and O'kelly, J. (1963). Toxic metabolites of Aspergillus flavus. **Nature** 198: 1056-1058.

He, T., Wang, Y., Li, P., Zhang, Q., Lei, J., Zhang, Z., Ding, X., Zhou, H. and Zhang, W. (2014). Nanobody-based enzyme immunoassay for aflatoxin in agro-products with high tolerance to cosolvent methanol. **Analytical chemistry** 86(17): 8873-8880.

Hoogenboom, H. R., de Bruïne, 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): 1-20.

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.

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

Jeffrey, A. and Williams, G. (2005). Risk assessment of DNA-reactive carcinogens in food. **Toxicology and applied pharmacology** 207(2): 628-635.

Kang, A. S., Jones, T. M. and Burton, D. R. (1991). Antibody redesign by chain shuffling from random combinatorial immunoglobulin libraries. **Proceedings of the National Academy of Sciences** 88(24): 11120-11123.

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

Kramer, K., Fiedler, M., Skerra, A. and Hock, B. (2002). A generic strategy for subcloning antibody variable regions from the scFv phage display vector

pCANTAB 5 E into pASK85 permits the economical production of Fab fragments and leads to improved recombinant immunoglobulin stability. **Biosensors and Bioelectronics** 17(4): 305-313.

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(10): 2746-2755.

Li, X., Li, P., Lei, J., Zhang, Q., Zhang, W. and Li, C. (2013). A simple strategy to obtain ultra-sensitive single-chain fragment variable antibodies for aflatoxin detection. **RSC Advances** 3(44): 22367-22372.

Li, X., Li, P., Zhang, Q., Li, Y., Zhang, W. and Ding, X. (2012). Molecular characterization of monoclonal antibodies against aflatoxins: a possible explanation for the highest sensitivity. **Analytical chemistry** 84(12): 5229-5235.

Li, Y., Cockburn, W., Kilpatrick, J. B. and Whitelam, G. C. (2000). High affinity ScFvs from a single rabbit immunized with multiple haptens. **Biochemical and Biophysical Research Communications** 268(2): 398-404.

Lizárraga-Paulín, E. G., Moreno-Martínez, E. and Miranda-Castro, S. P. (2011). Aflatoxins and Their Impact on Human and Animal Health: An Emerging Problem. **Afla- toxins-Biochemistry and Molecular Biology**: 255-282.

Lou, J. and Marks, J. D. (2010). Affinity maturation by chain shuffling and site directed mutagenesis **Antibody Engineering** (pp. 377-396): Springer.

Low, N. M., Holliger, P. and Winter, G. (1996). Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial

mutator strain. **Journal of molecular biology** 260(3): 359-368.

Marks, J. D. (2004). Antibody affinity maturation by chain shuffling. **Antibody Engineering: Methods and Protocols**: 327-343.

Marks, J. D., Griffiths, A. D., Malmqvist, M., Clackson, T. P., Bye, J. M. and Winter, G. (1992). By–Passing Immunization: Building High Affinity Human Antibodies by Chain Shuffling. **Nature Biotechnology** 10(7): 779-783.

Marks, J. D., Hoogenboom, H. R., Bonnert, T. P., McCafferty, J., Griffiths, A. D. and Winter, G. (1991). By-passing immunization: human antibodies from V-gene libraries displayed on phage. **Journal of molecular biology** 222(3): 581-597.

Marty, C., Scheidegger, P., Ballmer-Hofer, K., Klemenz, R. and Schwendener, R. A. (2001). Production of Functionalized Single-Chain Fv Antibody Fragments Binding to the ED-B Domain of the B-isoform of Fibronectin in *Pichia pastori*. **Protein expression and purification** 21(1): 156-164.

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

Min, W.-K., Kweon, D.-H., Park, K., Park, Y.-C. and Seo, J.-H. (2011). Characterisation of monoclonal antibody against aflatoxin B 1 produced in hybridoma 2C12 and its single-chain variable fragment expressed in recombinant Escherichia coli. **Food Chemistry** 126(3): 1316-1323.

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(1): 169-181.

Muyldermans, S. and Lauwereys, M. (1999). Unique single-domain antigen binding fragments derived from naturally occurring camel heavy-chain antibodies. **Journal of Molecular Recognition** 12(2): 131-140.

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(1): 6.

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(1): 184-192.

Rangnoi, K., Jaruseranee, N., O'Kennedy, R., Pansri, P. and Yamabhai, M. (2011). One-Step Detection of Aflatoxin-B1 Using scFv-Alkaline Phosphatase-Fusion Selected from Human Phage Display Antibody Library. **Molecular biotechnology** 49(3): 240-249.

Rossi, C. N., Takabayashi, C. R., Ono, M. A., Saito, G. H., Itano, E. N., Kawamura, O., Hirooka, E. Y. and Ono, E. Y. S. (2012). Immunoassay based on monoclonal antibody for aflatoxin detection in poultry feed. **Food chemistry** 132(4): 2211-2216.

Schier, R., Balint, R. F., McCall, A., Apell, G., Larrick, J. W. and Marks, J. D. (1996). Identification of functional and structural amino-acid residues by parsimonious mutagenesis. **Gene** 169(2): 147-155.

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- *erb* B-2 Single chain Fv Using Affinity-driven Selection. **Journal of molecular biology** 255(1): 28-43.

Stemmer, W. P. (1994). DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. **Proceedings of the National Academy of Sciences** 91(22): 10747-10751.

Stemmer, W. P. (1994). Rapid evolution of a protein in vitro by DNA shuffling. **Nature** 370(6488): 389-391.

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 letters** 210(2): 257-261.

Tout, N. L., Yau, K. Y. F., Trevors, J. T., Lee, H. and Hall, J. C. (2001). Synthesis of ligand-specific phage-display ScFv against the herbicide picloram by direct cloning from hyperimmunized mouse. **Journal of agricultural and food chemistry** 49(8): 3628-3637.

Uppala, A. and Koivunen, E. (2000). Targeting of phage display vectors to mammalian cells. **Combinatorial Chemistry & High Throughput Screening** 3(5): 373-392.

van der Linden, R., de Geus, B., Stok, W., Bos, W., van Wassenaar, D., Verrips, T. and Frenken, L. (2000). Induction of immune responses and molecular cloning of the heavy chain antibody repertoire of *Lama glama*. **Journal of immunological methods** 240(1): 185-195.

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(2): 139.

Versantvoort, C. H., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J. and Sips, A. J. (2005). Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. **Food and Chemical Toxicology** 43(1): 31-40.

Wang, T., Ding, H., Yang, L., Ji, Q., Chen, W. and Zhang, H. (2009). [Screening and identification of a single chain antibody fragment]. **Wei sheng wu xue bao= Acta microbiologica Sinica** 49(1): 135-140.

Wang, Y., Li, P., Majkova, Z., Bever, C. R., Kim, H. J., Zhang, Q., Dechant, J. E., Gee, S. J. and Hammock, B. D. (2013). Isolation of alpaca anti-idiotypic heavy-chain single-domain antibody for the aflatoxin immunoassay. **Analytical chemistry** 85(17): 8298-8303.

Willats, W. G. T. (2002). Phage display: practicalities and prospects. **Plant molecular biology** 50(6): 837-854.

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(19): 8927-8932.

Yang, W.-P., Green, K., Pinz-Sweeney, S., Briones, A. T., Burton, D. R. and Barbas III, C. F. (1995). CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. **Journal of molecular biology** 254(3): 392-403.

Yuan, Q., Hu, W., Pestka, J. J., He, S. Y. and Hart, L. P. (2000). Expression of a Functional Antizearalenone Single-Chain Fv Antibody in Transgenic ArabidopsisPlants. **Applied and environmental microbiology** 66(8): 3499-3505.

Zahnd, C., Spinelli, S., Luginbühl, B., Amstutz, P., Cambillau, C. and Plückthun, A. (2004). Directed in vitro evolution and crystallographic analysis of a peptide-binding single chain antibody fragment (scFv) with low picomolar affinity. **Journal of Biological Chemistry** 279(18): 18870-18877.

Zheng, M. Z., Richard, J. L. and Binder, J. (2006). A review of rapid methods for the analysis of mycotoxins. **Mycopathologia** 161(5): 261-273.

CHAPTER III

IMPROVEMENT OF THE SENSITIVITY OF HUMAN SCFV ANTIBODY AGAINST AFLATOXIN B1 BY CHAIN SHUFFLING AND MOLECULAR DOCKING ANALYSIS

3.1 Abstract

Recently an anti-aflatoxin scFv antibody, named YAF-C3 was isolated from a naïve human phage display library (Yamo library) in our laboratory (Rangnoi et al. 2011) This clone was expressed well in *Escherichia coli*. However, its binding affinity was not enough to detect the toxin at the limit concentration, provided by CODEX international standard legislation. The aim of this study was to improve the binding affinity of this clone by chain-shuffling technique and to study the molecular binding between antibody and AFB1. Two chain-shuffled scFv libraries were constructed from variable heavy (V_H) chains repertoire, amplified from the naïve library, recombined with variable light (V_L) chain of clone YAF-C3, and vice versa. Then, these two libraries were used to select for enhancing scFv antibody by standard bio-panning method. While heavy chain (V_H)-shuffled library yielded clones with improved binding affinity, none of the clones from light chain (V_L)-shuffled library showed improvement in the binding sensitivity. One clone, designated sAFH-3E3, showing 7.5-folds improvement in sensitivity over the original scFv clone, was

isolated. The affinity of this clone against AFB1-BSA conjugate was 2.29×10^{-9} M, which was 20-fold higher than that of the original scFv. Amino acid sequence analysis indicated that there are two amino acid mutations in framework 1 (FR1) and three mutation in CDR1 region. Homology modeling and molecular docking were used to compare the binding of this clone and the original clone. The results indicated that V_H is more important for AFB1 binding than V_L.

Key words: Aflatoxin, Single chain fragment variable (scFv), chain shuffling, Enzyme linked immunosorbant assay (ELISA), molecular docking

3.2 Introduction

Aflatoxins are carcinogenic toxins that can be produced as secondary metabolites from *Aspergillus flavus* and *Aspergillus parasiticus*. These toxins are regularly found to contaminate food and feed that contain peanut, chili, or maize. Because of their harmful toxicity, an effective method for the detection of aflatoxins below the legal limit is necessary. Enzyme linked immunosorbant assay (ELISA) is a technique for rapid and sensitive detection of aflatoxin. This method requires high sensitivity antibody as detection reagent.

Phage display technology is a powerful technique for producing monoclonal recombinant antibody fragment. The small fragment of antibody such as scFv fragement can be displayed on the surface of phage and easily selected for the desired binding property from phage display scFv antibody library. The human phage display antibody selected from human naïve library tend to produce low binding affinities (K_D) for hapten antigen (Irving et al. 1996). However, the antibody obtained from the original libraries can be further engineered by various techniques of affinity

maturation such as sites directed or random mutagenesis, and antibody chain shuffling. Successful improvements by affinity maturation have been reported., incluing as high as 1200-fold increase in affinity by mutation the two CDR3 (Lou and Marks 2010). Three to six-fold affinity improvement have been reported when using heavy chain shuffling technique to improve the scFv specific to preS1 of hepatitis B virus (Park et al. 2000).

In this study, the scFv antibody specific to aflatoxin B1, selected from human phage antibody library (Pansri et al., 2009), has been improved by affinity maturation. The original scFv antibody could detect aflatoxin at 0.04 µg/ml (40ppb) by competitive ELISA (Rangnoi et al, 2009). Since, the acceptable limits of aflatoxin contamination in agricultural product are 20 ppb (Shephard, 2008); therefore, this antibody was not sensitive enough to use as diagnostic reagent. To improve the sensitivity of this antibody, the chain shuffling technique was performed. A heavy and light chain shuffled library were constructed and used to select new specific scFv antibodies to AFB1. Three clones selected from heavy chain shuffling library that showed higher sensitivity than the original clones were obtained. One of the mutant scFv (sAFH-3E3) showed 7.5 fold and 20 fold higher than the original YAF-C3 clone, when analyzed by competitive ELISA and SPR analysis, respectively. Moreover, homology modeling and molecular docking were performed to elucidate the nature of antibody-AFB1 interactions.

3.3 Materials and Methods

3.3.1 Materials

All reagents were molecular grade or analytical grade. Ani-aflatoxin

scFv antibody YAF-C3 (YM1-C3) was produced in our laboratory. Aflatoxin B1 standards and AFB1 conjugated with BSA were obtained from Aokin (Germany). Bovine serum albumin (BSA), goat anti-mouse IgG-peroxidase antibody, hapten, polyethylene glycol 8000 (PEG 8000), Tween 20, and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Sigma (St. Louis, MO). 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) were obtained from Amresco (USA). *Escherichia coli* TG1 was used for cloning and amplification of phage; whereas *E. coli* HB2151 was used for the production soluble scFv fragments. Both *E. coli* were obtained from MRC, Cambridge, UK. Mouse anti-M13 –HRP, His probe-HRP and Protein L-HRP were purchased from Amersham-Pharmacia Biotech (Uppsala, Sweden), Thermo Fisher Scientific, USA and Sigma, Germany, respectively.

3.3.2 Construction of chain-shuffling libraries

The V_H and V_L repertoires were prepared from clone YAF-C3 and DNA extracted from YAMO library (Pansri et al. 2009). The variable heavy chain and light chain were amplified by PCR using *Taq* and *pfu* polymerase. Primer for amplification was shown in Table 2.1. PCR amplification was in a total volume of 50 µl, consisting of 1X *pfu* DNA polymerase buffer (Promega, USA), 0.2 mM dNTP (New England Biolabs (NEB, USA), 2.5 U of *Taq* DNA polymerase (NEB, USA), 1.5 U of *pfu* DNA polymerase (Promega, USA), 100 ng of DNA template, 1 µM of forward and reward primer (Table 1) under conditions: 95^{o}C for 2 min, 30 cycles of 95^{o}C for 1 min, 66^{o}C for 30 sec, 72^{o}C for 3 min and a final extension of 72^{o}C for 5 min.

The V_H gene of YAF-C3 were assembled to a pool of V_L genes from YAMO library, and vice versa to generate new variety of scFv fragment at linker sequence by PCR producing a library of scFv fragments of approximately 800 bp, as

decribeded by Pansri (Pansri et al. 2009). The assembled was performed in two steps. In the first step, assembly PCR with absence of primer in total volume of 40 µl consisted of 400 ng each of VH and VL, 1X *pfu* DNA polymerase buffer, 0.2 mM dNTP, and 1.2 U of *pfu* DNA polymerase. The mixture reaction was done at 95°C for 2 min, 25 cycles of 95°C for 1 min, 66°C for 30 sec, 72°C for 3 min and a final extension of 72°C for 5 min. Second step, the assembled scFv fragments were amplified by PCR with pull through primers (ShufFw5' and ShufRv3'). The PCR reaction in total volume 50 µl composed of 5 µl of assembled scFv, 1X of pfx50 buffer, 0.2 mM dNTP, 1µM of each forward and reward primer and 5 U of pfx50 DNA polymerase (Invitrogen, USA). PCR condition was performed at 94°C for 2 min, 30 cycles of 94°C for 15 sec, 66°C for 30 sec, 68°C for 1 min and a final extension of 68°C for 5 min. The scFv DNA fragment products were purified from gel using Wizard SV clean up kit (Promega, USA).

Table 3.1 Primers for amplification of heavy chain and light chain.

Primer	**Sequence**
VH amplification	
ShufFw5'	5' CCTTTCTTGCGGCCCAGCCGGCCATGGCC 3'
LinkBa3'	5' CCACCAGAGCCGCCGCCGCCGCTAC 3'
VL amplification	
LinkFw5'	5' GTAGCGGCGGCGGCGGCTCTGGTGG 3'
ShufRv3'	5' CCCGTGATGGTGATGATGATGTGCGGCCGCACC 3'

Sequences corresponding to *SfiI* and *NotI* restriction sites are underlined

The scFv DNA fragments and pMOD1 was sequentially digested with *NotI-HF* and *SfiI* (New England Biolabs, USA) at a ratio of 20 enzymes units per 1 µg DNA. The scFv DNA was ligated to 1 µg digested pMOD1 vector at vector:insert molar ratio of 1:2 ratio, with 2400 T4 DNA ligase Units (New England Biolabs, USA). Finally, ligated DNA was transformed into TG1 electrocompetent cells. Cells were grown on TYE agar plates containing 100 µg/ml ampicillin and 1 % glucose. Libraries were amplified and the phage display scFv antibody library was rescued with M13 helper phage. Then, phage particles were purified from bacterial supernatant by precipitation with 20% polyethylene glycol 8000 and 2.5 M NaCl, according to previously published method (Pansri et al. 2009).

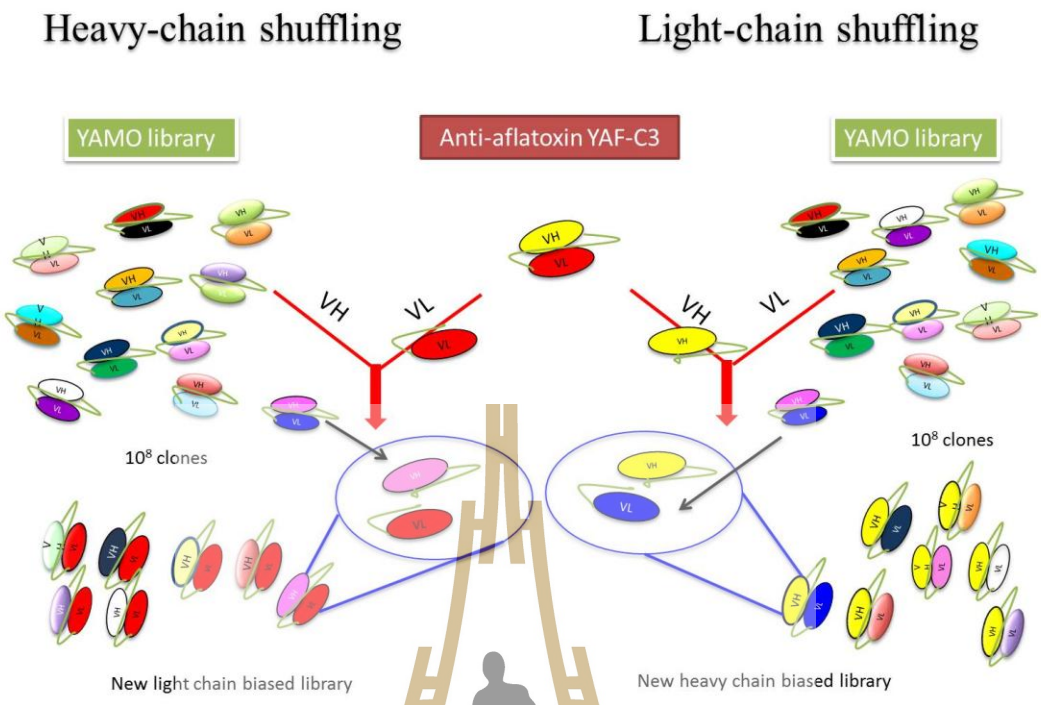


Figure 3.1 Illustration of variable chain shuffling library construction. For heavy chain shuffling library construction, variable heavy (V) chains repertoire, amplified from the naïve library (Yamo), recombined with variable light (VL) chain of clone YAF-C3 While, light chain shuffling library was constructed by amplification VH of clone YAF-C3 reassembled with VL from the naïve library.

3.3.3 Screening of phage and scFv antibody specific to aflatoxin

3.3.3.1 Bio-panning

Twenty µg of AFB1 conjugated BSA was immobilized on well of 96 well microtitre plates by dissolving in 100 µl of phosphate buffer saline (PBS) and incubated at 4^{o}C for overnight. The target was re-use for another round of bio-panning. The remaining sites on well were blocked with 2% skim powder milk, which

dissolved in PBS buffer (MPBS) for 1 hr at room temperature. Before adding to AFB1-BSA coated well, the chain-shuffling libraries were pre-incubated in 2% (w/v) skim milk powder and 1% (w/v) BSA in PBS at room temperature for 30 min. Then, the libraries were incubated with target for 1 hr at room temperature. Unbound phages were eliminated by washing wells with 10 times PBS containing 0.05% (v/v) Tween 20 (PBST), following by 10 times PBS. For 2nd and 3rd round of selection, the washing times were increased to 15 times with PBST, following 15 times with PBS, 20 times with PBST, following 20 times with PBS, respectively. Bound phages were eluted with 100 µl of 1 µg/ml trypsin buffer by incubating for 10 minutes at room temperature. Then, phage was incubated for another 10 minutes at room temperature with 50 µl of 100 mM glycine-HCl, pH 2 and neutralized with 50 µl of neutralize solution. Then, both elution solutions were mix together and then separately in half volume to infect *E coli* TG1 and HB2151. *E. coli* TG1 was used for phage amplification to use in the next round of bio-panning. Whereas *E. coli* HB2151 was use for induction scFv fragment. After first and third round of bio-panning, individual colony was randomly picked up from 2xYT agar plates supplemented with 100µg/ml ampicillin and 1% (w/v) glucose and tested the binding ability to AFB1-BSA by ELISA.

3.3.3.2 Confirmation phage and scFv positive clone by ELISA

After 1st and 3rd selection round, the individually phage clone was rescued as previous described (Rangnoi et al. 2011) before screening by indirect ELISA. Immuno 96 Wells Plate (Nunc, Denmark) was coated with 1 µg AFB1-BSA and 1% (w/v) BSA (negative control) in a volume of 100 µl of PBS. Then, plate was blocked as described in 3.3.3.1. After incubation for 1 hr at room temperature, the

wells were washed 3 times with PBS. After that, 100 µl of phage supernatant or scFv fragment and 50 µl PBS were added into each well of the plate. Plates were stand on the bench for 1 hr. After that, unspecific phages or soluble scFv fragment were washed away 3 times with PBST and 2 times with PBS. Subsequently, 100 µl HRP-anti-M13 (1:5000 dilutions in PBS) and HisProbe-HRP or ProteinL-HRP was added to each well to detect phage and soluble scFv fragment, respectively. After incubation for 1 hr at room temperature, the wells were washed again and 100 µl substrate solutions ABTS (AMRESCO, USA) or TMB (SIGMA, USA) was added to each well and incubated at room temperature for 15 mins-2 hrs. Absorbance was measured at 405 nm. When using TMB as substrate, the reaction was stopped with 10% (v/v) HCl after incubation for 10-30 min. The yellow color was developed after adding HCl. Absorbance was measured at 450 nm.

3.3.3.3 Competitive ELISA

Competitive or inhibition ELISA was performed as described in the normal ELISA methods, except that optimal amount of phage or scFv were pre-incubated with increasing amount of soluble AFB_1 before adding into previously coated, blocked, and washed wells of ELISA plates. For every assay, appropriate dilutions of Phage or scFv that showed a linear relationship by ELISA were used.

All antibody dilutions and standard AFB1 were performed in TBS buffer. Immuno 96 wells plate was coated with AFB1-BSA and block as described in 3.3.3.1 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 (AFB1) from 5 µg/ml to 0.019 µg/ml. After incubation at 37^{o}C 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 PBST and 2 times with PBS. Protein L-HRP or His Probe -HRP was added to wells and incubated for 1 hour at room temperature. Following this, the substrate solution was added as described in 3.3.3.2. The positive clone was showed decreasing signal when the concentration of AFB1 was increased.

3.3.4 Sequence analysis

Plasmid DNA 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: Yamo5' Fw 5'CAG GAA ACA GCT ATG ACC3' and 96 geneIII Rv 5'CCC TCA TAG TTA GCG TAA CG3'. Immunogenic analysis of variable regions was done using IGMT (Lefranc 1999) and V-Base database. The amino acid sequences were translated using Snapgene software.

3.3.5 Expression and purification of scFv fragment

A single colony of *E. coli* HB2151 containing pMOD phagemid bearing positive clones was inoculated into 5 ml 2XYT containing 100 μg /ml Ampicillin, 2% (w/v) glucose and was grown overnight at 37°C with shaking. In the next day, 2 ml of overnight culture was inoculated into 200 ml 2XYT broth, containing 100 μg/ml Ampicillin, 0.1% (w/v) glucose at 30°C with shaking for 3 hrs and 30 mins to reach OD600 is 0.9. After that, the culture was induced with 1mM IPTG and continued shaking for 6 hrs at 250 rpm. The scFv fragment can be found in periplasmic extraction.

For periplasmic extraction, the culture was centrifuged at 3,000 g for 20

mins at 4°C and the supernatant was discarded. The pellet was resuspended in 8 ml of cold periplasmic buffer (1xPBS, 1 M NaCl and 1 mM EDTA) and left on ice for 20 mins. The resuspended solution was spun at 3,000 g for 10 mins at 4°C. The supernatant which carrying the periplasmic fractions containing scFv fragments were collected and was added with $MgCl_2$ to be 1 mM as final concentration. Soluble scFv was purified by immobilized metal affinity chromatography (IMAC) using Ni-NTA by Akta purifier (AKTA pure, GE Healthcare). Before purification, cell debris was removed from periplamic supernatant by filtering through 0.2 µm. One ml His-Trap column (GE Healthcare) was equilibrated with 10 ml buffer (20 mM $NaPO_4$, 500 mM NaCl, 20 mM imidazole, pH 7.5). Then, the filtered periplasmic was loaded into the column. To remove loosely bound protein, the column was washed with the same buffer. The scFv was eluted from the column with buffer gradient buffer programme containing high concentration of imidazole (20 mM $NaPO_4$, 500 mM NaCl, 500 mM imidazole, pH 7.5). The antibody fraction was dialysed with PBS buffer using 10 kDa snakeskin dialysis tubing (ThermoScientific). Protein concentration was quantified by a Nanodrop ND2000 spectrophotome (Thermo Scientific).

3.3.6 Kinetic Binding of scFv antibodies by SPR

The kinetic binding of purified scFv antibody as performed by Biocore 3000 SPR instrument. The AFB-BSA was immobilized on the CM5 chip (GE health care, USA) using amide-coupling chemistry as described by instrument instruction (GE Healthcare). BSA was immobilized as a control in another flow cell on the same sensor chip. Unbound ligand was washed away with HEPES containing 0.01% Tween 20 and blocked the inactivated site by injection 30 µl of 1M ethanolamine, pH 8.5. Purified scFv at different concentrations were each injected over the captured AFB1-

BSA at constant flow of 30 µl/min for 3 min of contact time and 9 min of dissociation time. The captured surface was regenerated between each binding reaction with 15 mM NaOH at a flow rate of 30 µl/min for 1 min. BIAvaluation software was used to analyze the kinetic binding data and fit globally to a 1:1 binding model.

3.3.7 Antibody modeling and molecular docking

Homology modeling of the three-dimensional (3D) structures of scFv YAF-C3 and sAFH-3E3 as generated from the amino acid sequences using the SWISS-MODEL website (Biasini et al. 2014). The server chose the template by sequence identity analysis. After that, the sequences were processed by the server for modeling. Models were visualized with the program PyMOL (www.pymol.org). MolProbty website was used to analyse Ramachandran values of the scFv model. To understand the molecular interaction between AFB1 and the scFv antibody, GOLD software was used to analyze antigen-antibody docking. An AFB1 molecule was obtained from PubChem compound database (www.pubchem.ncbi.nlm.nih.gov). All scoring functions that were available for GOLD at the time of the study (ChemPLP, GoldScore, ChemScore, and Astex Statistical Potential (ASP)) were tested in separate runs. The active site for docking was defined follow as homology model of scFv at the position of CB, Ala33 incase of YAF-C3 antibody; CB, His33 atoms in the case of sAFH-3E3 within 6Å radius. The best scored solution (ChemPLP) was considered, and viewed in Discovery studio 2016 (BIOVIA, San Diego).

3.4 Results

3.4.1 Chain-shuffling libraries construction

The variable heavy chain and light chain were amplified from YAF-C3 and Yamo DNA by PCR using *Taq* and *pfu* polymerase. PCR products of the variable heavy chain (VH) and light chain (VL) showed at the size 350-400 bp. (Fig 3.2). The variable gene that amplified from Yamo DNA was shown smear band and have several non specific bands whereas the band of variable from YAF-C3 have a one clear band at the size 350 bp for VL and 400 bp for VH.

The variable genes were assembled with (G4S)3 peptide by overlap extension PCR, resulting in the scFv gene of approximately 800 bp. Therefore, the assembly of VH Yamo−Linker−VL YAF-C3 and VH YAF-C3−Linker−VL Yamo were amplified to create scFv fragments by pull-through PCR. There are 2 groups of scFv products for using to construct heavy chain and light chain shuffling libraries. The gel analysis after PCR reaction showed several non- specific products were amplified in addition to the 800 bp desired product (Fig 3.3). To get only correct band of scFv, all PCR products were loaded onto agarose gel and cut DNA at the size 800 bp. The gel purification was performed to get the 800 bp scFv gene.

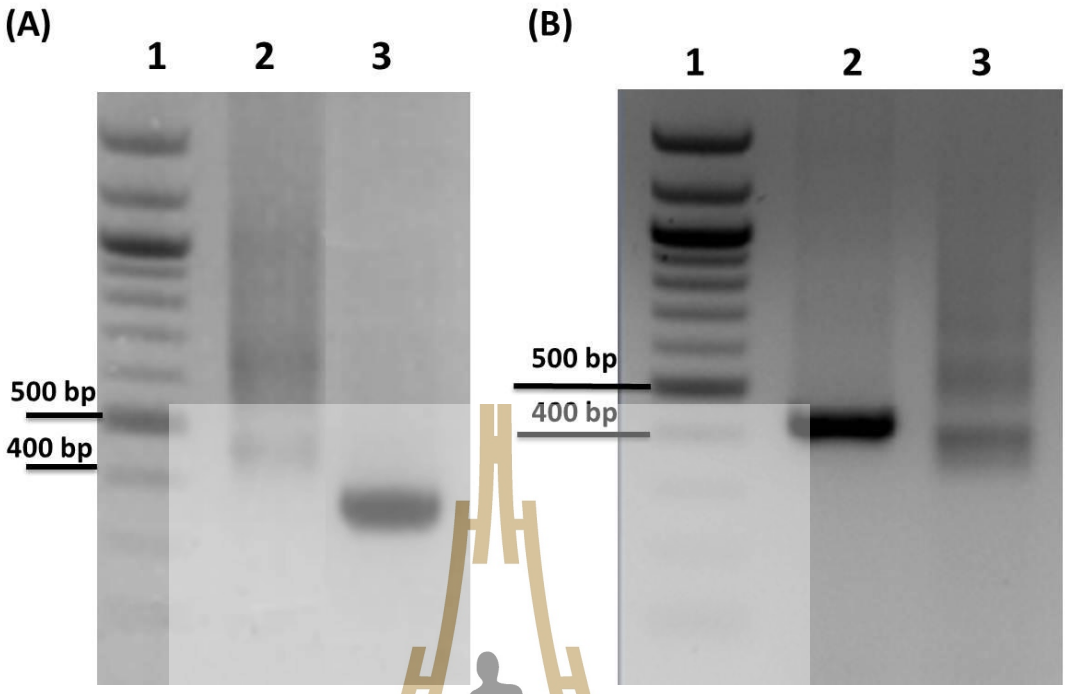


Figure 3.2 Agarose gel analysis of PCR of VH amplified from YamoI and VL amplified from YAF-C3 (A). Lane 1, DNA marker (100 bp ladder); lane 2, VH of YamoI; lane 3, VL of YAF-C3. PCR of VH amplified from YAF-C3 and VL amplified from YamoI (B). Lane 1, DNA marker (100 bp ladder); lane 2, VH of YAF-C3; lane 3, VL of Yamo.

The full length scFv fragments were ligated into pMOD1 vector at the *SfiI* and *NotI* sites. The agarose gel analysis of pMOD1 vector and two scFv fragments after *SfiI* and *NotI* digestion are shown in Fig 3.4. The ligate products were transformed into electrocompetent E. *coli* TG1 cells, rescued and the library size calculated. The heavy chain and light chain shuffling library size were 3.48×10^{5} and 2.26×10^{4} cfu/ml with 0.43% and 3.54% background, respectively.

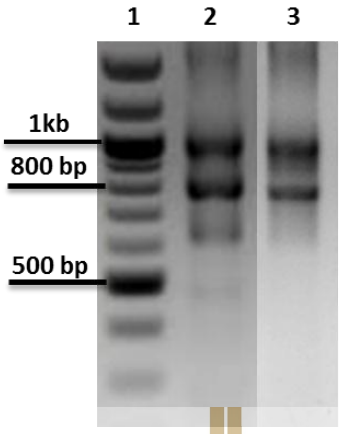


Figure 3.3 Agarose gel analysis of scFv products from pull-through PCR. Lane 1, DNA marker (100 bp ladder); lane 2, scFv of bias light chain for heavy chain shuffling construction; lane 3, scFv of bias heavy chain for light chain shuffling construction

Figure 3.4 Agarose gel analysis of *SfiI* and *NotI-HF* digestion products. pMOD, scFv bias heavy chain (A). Lane 1, DNA marker (1 kb ladder); lane 2 pMOD vector; lane 3, scFv fragment bias light chain. ScFv bias light chain (B) Lane 1, scFv fragment bias heavy chain; lane 2 DNA marker (100 bp ladder)

The qualities of libraries were checked by randomly picked 10-13 colonies. Plasmid of individually clone from heavy chain and light chain shuffling libraries were analyzed by restriction fragment analysis with *SfiI* and *NotI-HF* (Fig 3.5A) and PCR amplification, respectively (Fig 3.6A). Percent full length inserted scFv of H chain and L chain shuffling libraries were 100% and 69%, respectively.

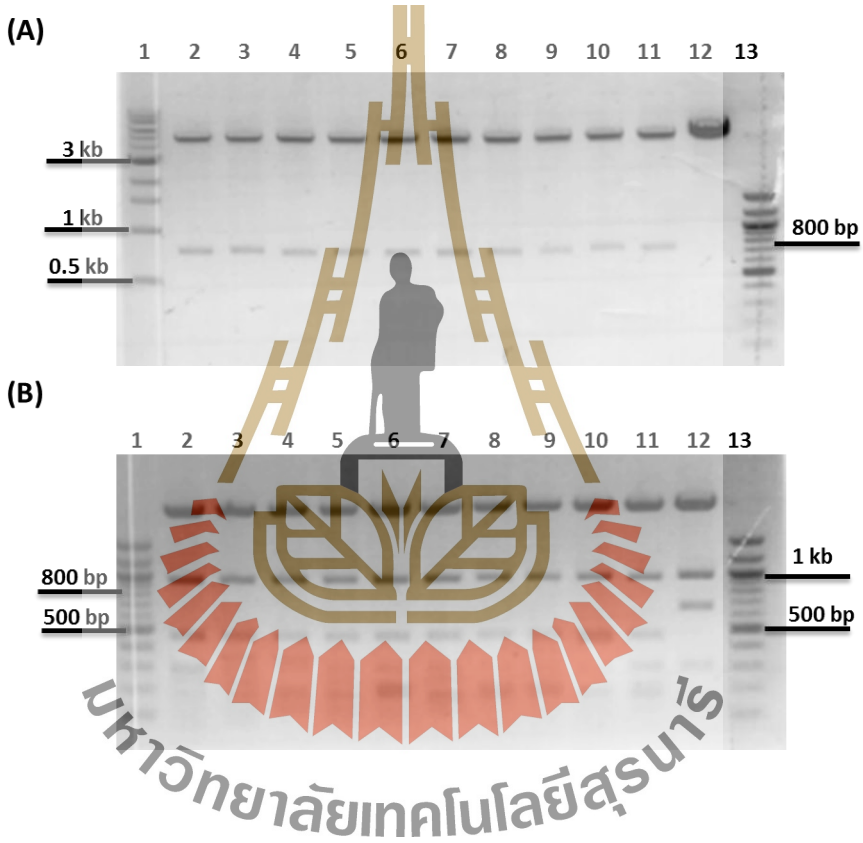


Figure 3.5 Agarose gel analysis of pre-panned plasmid of heavy chain shuffling library digested *SfiI* and *NotI-HF* (A). Lane 1, DNA marker (1 kb ladder); lane 2-12 randomly picked clones 1-11, respectively; lane 12, pMOD vector; lane 13, DNA marker (100 bp ladder). DNA fingerprint of heavy chain shuffling library (B). Plasmid of 10 clones were digested with *BstN1* at 60^{o}C for 3 hrs. Lane 1,13 DNA marker (100 bp ladder); lane 2-11, Clone 1-10, lane 12, pMOD vector.

The plasmid or PCR products of randomly picked clones from two libraries were digested with the *BstNI*. The fingerprint patterns of two libraries are shown in Fig 3.5B and 3.6B. To check the variety of scFv fragment in the libraries, plasmids of random clones were sequenced. After sequencing, the IGMT and V -Base database were used to indicate the family and CDR of variable region. For heavy chain shuffling library, ten random clones are different which are revealed 4 different VH gene families (VH1, VH3, VH4 and VH5) (Table 3.2) and VL are identical to VL from YAF-C3, which is belonged to VK1 (IGKV1-39*01). In case of light chain shuffling library, seven random clones were analyzed. The result showed that VH are identical to VH from YAF-C3, which is belonged to VH3 (IGHV1-3*01) and five VL gene families (Vκ1, Vκ3, and Vλ2, Vλ3, Vλ6) (Table 3.3). These two libraries were further amplified and used for screening the improved sensitivity clones.

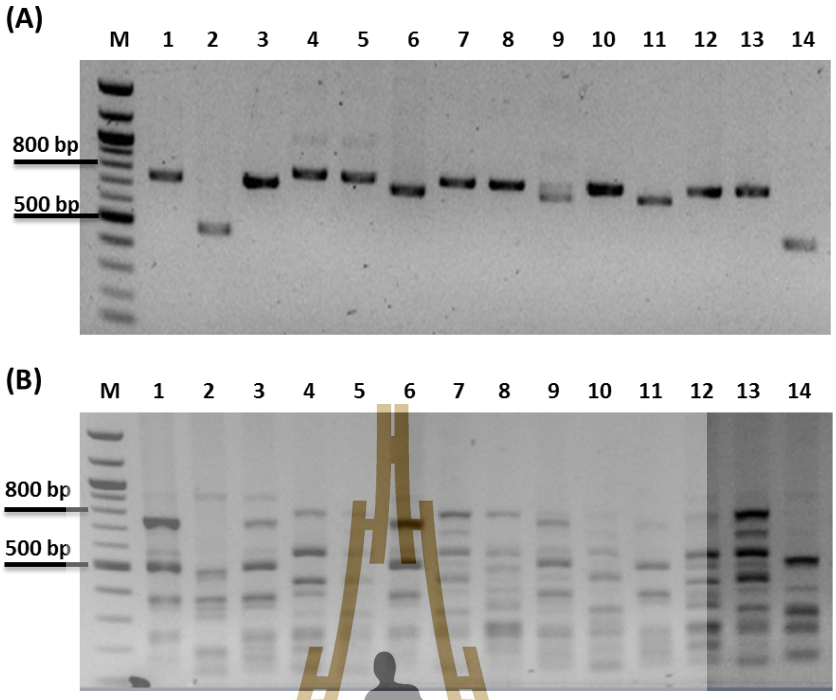


Figure 3.6 Agarose gel analysis of PCR products amplified from pre-panned plasmid of light chain shuffling (A). Lane M, DNA marker (100 bp ladder); lane 1-14 randomly picked clones 1-14, respectively. DNA fingerprint of light chain shuffling library. PCR products of 14 clones were digested with *BstN1* at 60°C for 3 hrs (B). Lane M, DNA marker (100 bp ladder); lane 1-14, Clone 1-14.

Table 3.2 Amino acids sequences of pre-panned heavy chain shuffling library clones.

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
sAFH-1	EVQLVQSGAEVKKPGS SVKVSCKAS	GGTFSSYA	ISWVRQAPG QGLEWMGI	IKPSGGTT	TYAQKFQGRLTMTRDTSTR TVYVELSSLASEDTAVYYC	ARDPRGYSYPR DYGMDV	IGHV1-46*01	14	VH1
sAFH -2	EVQLVESGAEVKTPGA SVKVSCKAS	GYTFTGYY	MHWVRQAPG QGLEWMGW	INPNSGGT	NYAQKFQGRVTMTRDTSIS TAYMKLSILRSEDTAVYY	ARDRGTPATSM VRGVPFGMDV	IGHV1-2*02	6	VH1
sAFH-3	QVQLVESGAEVKKPGE SLKISCKGS	GYSFTSYW	IGWVRQMPG KGLEWMGI	IYPGDSDT	RYSPSFQGQVTISADRSIS TAYLQWSSLKASDTAMYYC	ARRMVRGAPDA FDI	IGHV5-51*01	3	VH5
sAFH-4	EVQLVQSGAEVKKPGS SVKVSCKAS	GGTFSSYA	ISWVRQAPG QGLEWMGW	INPNSDGT	NYAQKFQGRVTMTRDTSIS TAYMELSRLRSDDTAVYYC	ARMDWTEMATI PGDY	IGHV1-2*02	9	VH1
sAFH-5	QVQLQESGPGLVKPSE TLSLTCTVS	GGSIYPYY	WSWIRQSPG KGLEWIGY	IYYNGAT	NDNPSLRSRVTMSVDTSKN QLSLRLSSVTAADTAIYYC	ARVAKDDYNSG ALD	IGHV4-59*01	11	VH4
sAFH-6	*VQLVQSGAEVKQPGA SVKVSCKAS	GYTFTSYG	INWVRQATG QGLEWMGW	MNPNSGNA	GYAQKFQGRVTMTRDTSIS TAYMELSSLRSDDTAVYYC	ASVYSSGGY	IGHV1-8*01	7	VH1
sAFH-7	QVQLVQSGGGVVQPGR SLRLSCVAS	GFSFSTYA	MHWVRQAPG KGLQWVAL	ISYDGTNQ	YYGDSVKGRFTISRDNAKN SLYLQMNSLRAEDTAVYYC	ARDPVYSSSWY GY	IGHV3-30-3*01	11	VH3
sAFH-8	QVQLVQSGGGLVKPGE SLRLSCTTS	GFTFSSYA	MHWVRQAPG KGLEWVAV	ISYDGSNK	YYADSVKGRFTISRDNSKN TLYLQMNSLRAEDTAVYYC	AKPTGIQLWLL S	IGHV3-30*04	7	VH3
sAFH-9	QVQLMQSGGGLIQPGG SLRLSCVAS	GFTFSNYA	MTWVRQAPG KGLEWVSG	VSGVGSHT	YYADSVKGRFTISRDNSKN TLYLQMNSLRAEDTAVYYC	AKEDRVYGSGS YYKDAFDI	IGHV3-23*01	12	VH3
sAFH-10	EVQLVESGGGLVQPGG SLRLSCAAS	GFTFRNHA	ITWVRQAPG KGLEWVSG	ISASGTST	YYADSVEGRFTISRDNSKN TLYLQMSSLRVDDTATYYC	AKGFSGSYLDY FDP	IGHV3-23*04	13	VH3

Table 3.3 Amino acids sequences of pre-panned light chain shuffling library clones.

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
sAFL1-2	*SALTQPASVSGCPGQ SITISCTGT	SSDVGSYNL	VSWYQQRPG KAPKLMIY	EVS	KRPSGASNRFSGSKSGNTAS LTISGLQAEDEADYYC	QSYDGSSWV	IGLV2-23*02	8	VL2
sAFL1-3	SSELTQDPAVSVALGQ TVRITCQGD	SLRSYY	ASWYQQKPG QAPVLVIY	QDS	KRPSGIPERFSGSNSGNTAT LTISGTQAMDEADYYC	QAWDSSTAV	IGLV3-19*01	16	VL3
sAF1-10	QSALTQPASVSGSPGQ SITISCTGS	NSDIGAYYF	VSWYQQHPD KPPKLMIY	NVS	HRPSGVSDRFSGSKSGNTAS LTISGLQAEDEADYYC	CSYAGSSTPL V	IGLV2-23*02	12	VL2
sAFL2-12	DIQMTQSPSSFSASTG DRVTITCRAS	QGISSY	LNWYQQKPG KAPRLLIY	AAS	TLQSGVPSRFSGSGSGTDFT LTISSLQPEDFATYYC	QQSYSTPPYS	IGKV1-39*01	5	VK1
sAFL1-8	NFMLTQPHPVSESPGK TVSISCTGS	SGSIASNY	VQWYQQRPG SSPTTVIY	EDN	QRPSGVPDRFSGSIDSSSSS ASLTISGLKTEDEADYYC	QSYDNSNRAV	IGLV6-57*01	5	VL6
sAFL2-4	NFMLTQPHPVSESPGK TVTISCTRS	SGSIASNY	VQWYQQRPG SSPTTVIY	EDN	QRPSGVPGRFSGSIDSSSNS ASLTISGLKTGDEADYYC	QSYDGANQWL	IGLV6-57*01	5	VL6
sAFL2-8	EIVLTQSPGTLSSSPG ERATLSCRAS	QSVSSSY	LAWY*QKPG QAPRLLIY	GAS	TRATGIPARFSGSGSGTDFT LTISSLEPEDFAVYYC	QQYGSSLWT	IGKV3-20*01	6	VK3

3.4.2 Screening of AFB1 binder

The shuffling libraries were grown to use for selecting scFv specific to Aflatoxin. The results in Table 3.4 showed the overview of selection antibody specific to AFB1 from two libraries. Three rounds of selection were performed. After first and third round of selection, the positive phages and soluble scFv clones that bind to AFB1-BSA were monitored by Indirect ELISA. To obtain antibody that bind to soluble aflatoxin B1, the competitive ELISA was performed.

Table 3.4 Overview of screening of antibody against AFB1.

scFv library source	**Heavy chain shuffling**	**Light chain shuffling**
Number of clone after 1st panning	2.1×10^4	3.2×10^4
Number of clone after 2nd panning	2.1×10^6	2.0×10^7
Number of clone after 3rd panning	1.0×10^5	3.0×10^7
Number of positive phage clones after 1st round	93/95	N/A
Number of positive phage clones after 3rd round	95/95	51/96
Number of soluble scFv clones expressed after 1st round	90/95	72/96
Number of soluble scFv clones expressed after 3rd round	89/95	91/96
Number of scFv fragments that bound soluble AFB1	32/32	17/17
Number of scFv producing different clones	9/18	3/17
Number of original scFv	8/18	13/17
Number of scFv with improved binding properties	3/9	0/3

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

3.4.2.1 Indirect ELISA

Three rounds round of bio-panning of both libraries were performed and out put of each round of selection determined (Table 3.4). During bio-panning process the AFB1-BSA was reused and the number of washing was increased. After 1st and 3rd round of bio-panning, 95-96 clones were randomly picked and screened the specific binding to AFB1-BSA by phage and scFv ELISA using mouse anti-M13 antibodies conjugated to HRP and His Probe-HRP for detection. The phage and scFv clones were scored as positive by ELISA when the absorbance value on AFB1-BSA coated wells was at least two times higher than binding of phage to 1% (w/v) BSA coated-wells. From 1st and 3rd round ELISA result, it was determined that more than 50% of the clones were AFB1-BSA specific. The positive phage and soluble scFv clones were enriched from 1st round of selection. The numbers of positive clones in each round of selection are shown in Table 3.4.

3.4.2.2 Competitive ELISA

The AFB1 binder was screened by competitive ELISA. The scFv fragments were produced by infecting phage to *E. coli* HB2151 as described by Rangnoi et al, 2011. Phage and soluble scFv after 1st and 3rd round in total 32 clones selected from heavy chain shuffling library, which showed positive binding to AFB1-BSA on indirect ELISA assay were analyzed the binding to soluble AFB1 compare to original clone (YAF-C3). Competitive ELISA results showed that 4 clones, namely, sAFH-3E11, sAFH-3E3, sAFH-3F11 and sAFH-3D4 performed higher sensitivity to AFB1 than that of original clone (Fig 3.8). All of them are the clone derived from 3rd round of selection whereas no clone from 1st round selection showed higher sensitivity than YAF-C3 (Fig 3.7).

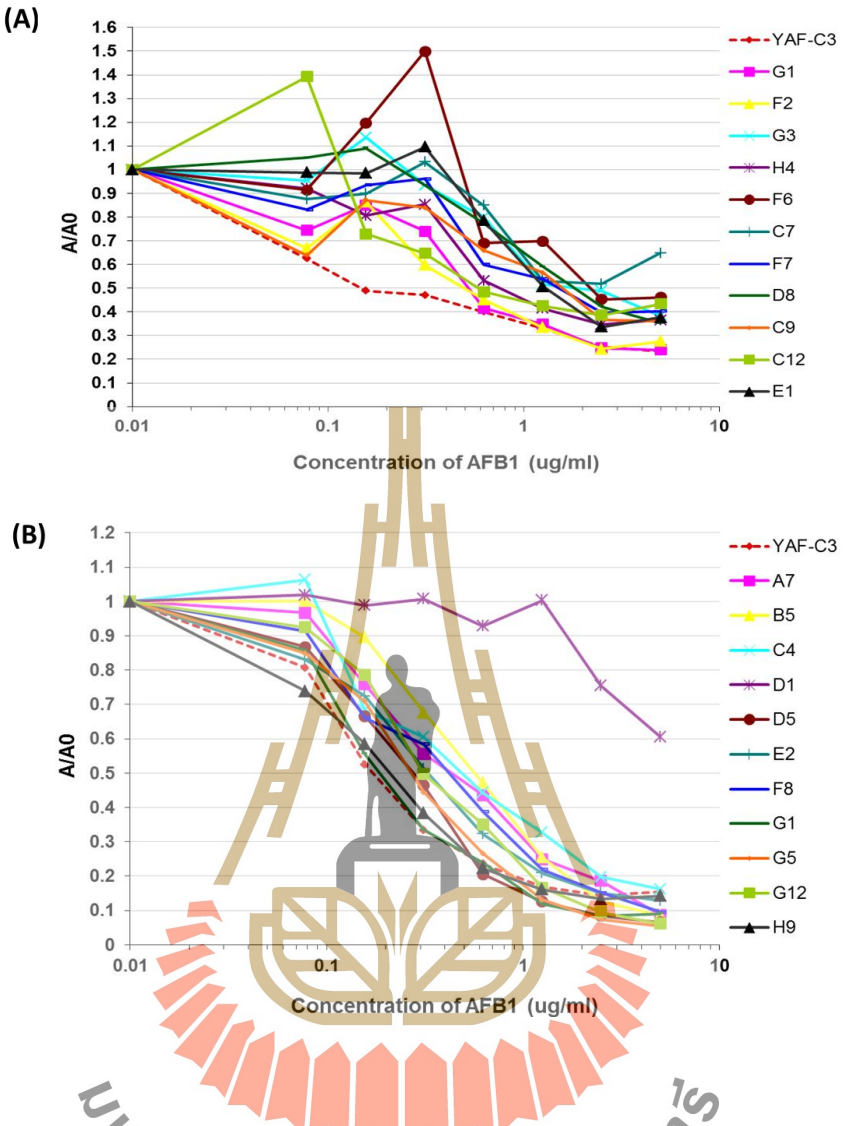


Figure 3.7 Competitive ELISA analysis of phages (A) and soluble scFv (B) antibody AFB1-specific clones, which selected from 1st round bio-panning from heavy chain shuffling library. An optimal dilution of phage and soluble scFv antibodies was incubated with decreasing concentrations of AFB1 from 5,000 to 2.28 ng/ml. Any bound phage and scFv antibody was detected by anti-M13 HRP-labelled and His Probe-HRP, respectively. The results are shown as A/A0, where the absorbance values of the evaluated samples (A), are normalised by expressing them as a function of the blank standard (A_0).

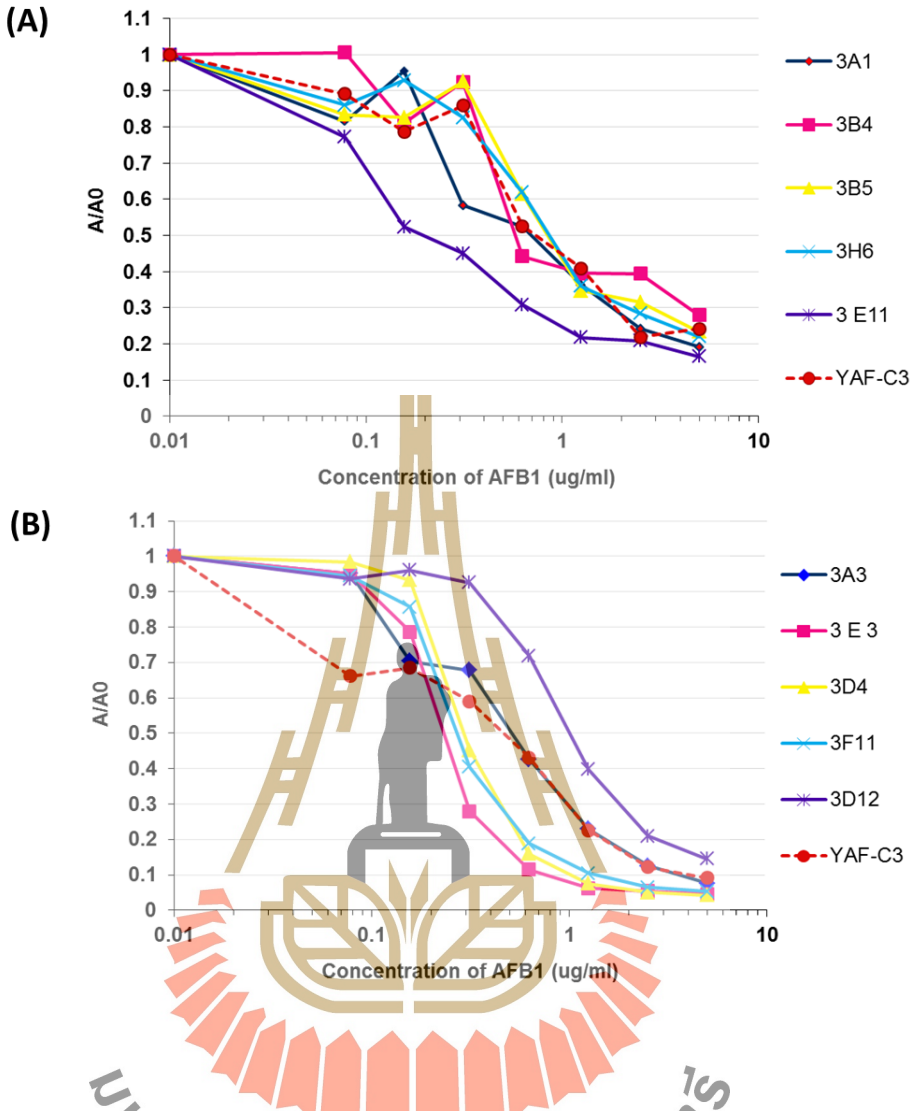


Figure 3.8 Competitive ELISA analysis of phages (A) and soluble scFv (B) antibody AFB1-specific clones, which selected from 3rd round bio-panning from heavy chain shuffling library. An optimal dilution of phage and soluble scFv antibodies was incubated with decreasing concentrations of AFB1 from 5,000 to 2.28 ng/mL. Any bound phage and scFv antibody was detected by anti-M13 HRP-labelled and His Probe-HRP, respectively. The results are shown as A/A0, where the absorbance values of the evaluated samples (A), are normalised by expressing them as a function of the blank standard (A_0).

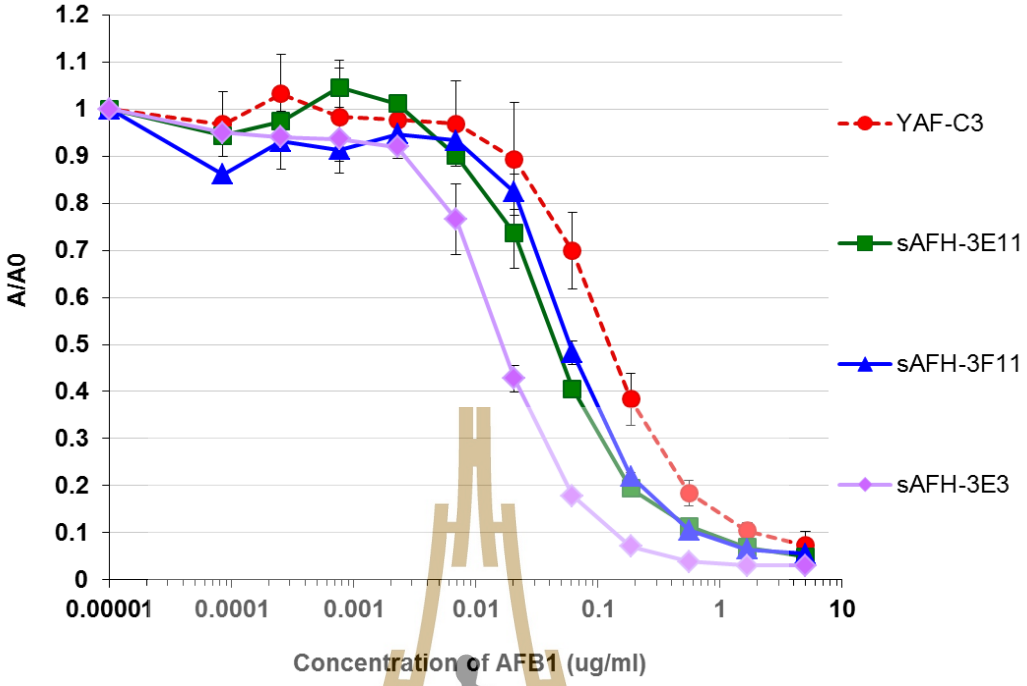


Figure 3.9 Competitive ELISA analysis of soluble scFv antibody heavy chain shuffled clone sAFH-E3, sAFH-3E11, sAFH-3F11 and YAF-C3 (parent) AFB1-specific clones. An optimal dilution of soluble scFv antibodies was incubated with decreasing concentrations of AFB1 from 5,000 to 0.028 ng/ml. Any bound phage and scFv antibody was detected by Protein L-HRP, respectively. The results are shown as A/A0, where the absorbance values of the evaluated samples (A), are normalised by expressing them as a function of the blank standard (A_0). The IC_{50} values for the scFv YM1-C3, sAFH-3E11, sAFH-3F11and sAFH-3E3 were found to be 0.15, 0.05, 0.06 and 0.017 µg/ml, respectively.

After that, plasmids of these 4 clones were sequenced. After sequencing, there are 3 clones (sAFH-3E11, sAFH-3E3 and sAFH-3F11), showed different amino acid. Three clones were confirmed the sensitivity compare to the original clone by competitive ELISA (Fig 3.10). The Ic_{50} of improved heavy chain shuffled cloned are vary between 0.02-0.06 μg/ml which is 2.5- 7.5 fold greater than parental clone (YAF-C3).

Phage and soluble scFv after 1st and 3rd round in total 17 clones selected from light chain shuffling library, which showed positive binding to AFB1-BSA on indirect ELISA assay were also analyzed the binding to soluble AFB1 compare to the original clone (YAF-C3). Competitive ELISA results indicated that 5 clones, namely sAFL-B2, sAFL-3B2, sAFL-3B12, sAFL-3H4 and sAFL-H7 showed higher sensitivity to AFB1 than that of original clone (Fig 3.10).

The amino acid from all of 17 clones were sequenced. There are only 3 clones, namely sAFL-3B2, sAFL-3B12, sAFL-3H1 showed different amino acid from YAF-C3. These 3 clones were confirmed the sensitivity compare to the original clone by competitive ELISA (Fig 3.11). No clone was shown the improved sensitivity to soluble AFB1.

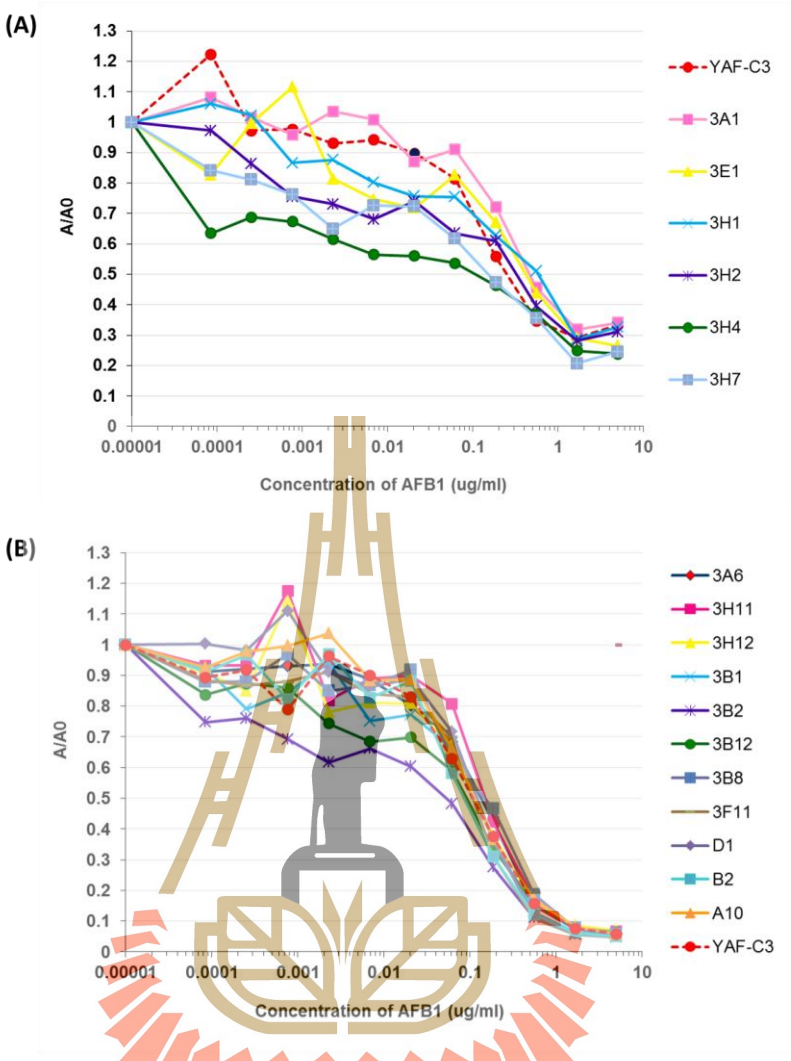


Figure 3.10 Competitive ELISA analysis of phages (A) and soluble scFv (B) antibody AFB1-specific clones, which selected from 1st and 3rd round bio-panning from light chain shuffling library. An optimal dilution of phage and soluble scFv antibodies was incubated with decreasing concentrations of AFB1 from 5,000 to 0.028 ng/ml. Any bound phage and scFv antibody was detected by anti-M13 HRP-labelled and His Probe-HRP, respectively. The results are shown as A/A0, where the absorbance values of the evaluated samples (A), are normalised by expressing them as a function of the blank standard (A_0).

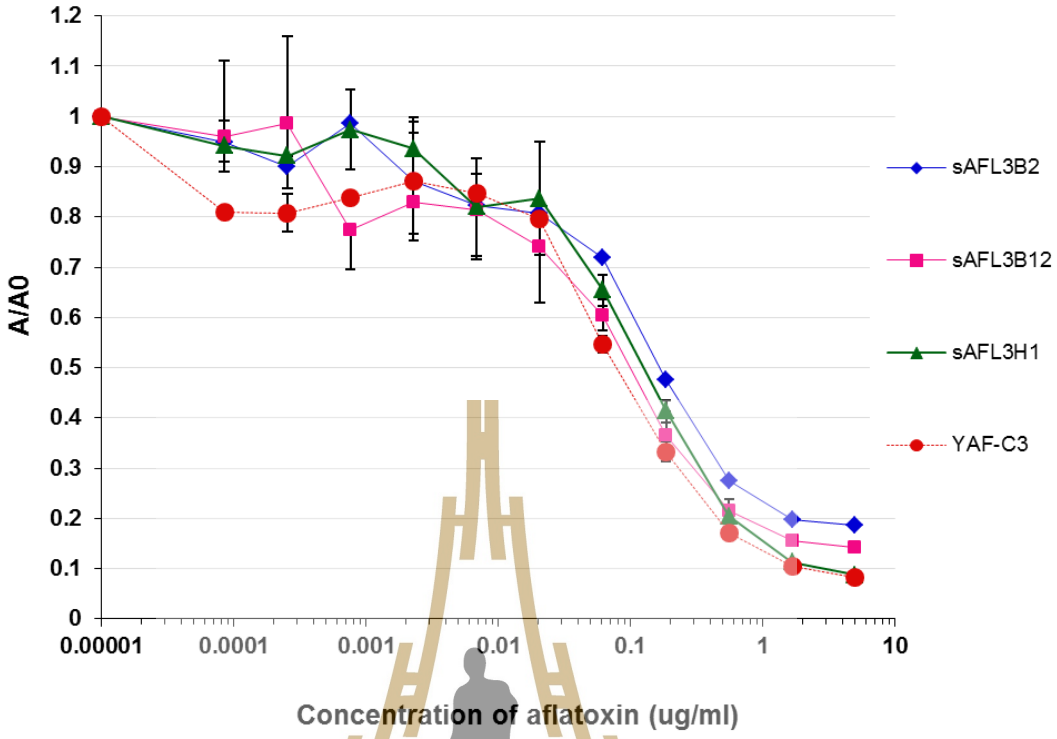


Figure 3.11 Competitive ELISA analysis of scFv antibody AFB1-specific clones, which selected from 1st and 3rd round bio-panning from light chain shuffling library. An optimal dilution of soluble scFv antibodies was incubated with decreasing concentrations of AFB1 from 5,000 to 0.028 ng/ml. Any bound and scFv antibody was detected by His Probe-HRP, respectively. The results are shown as A/A0, where the absorbance values of the evaluated samples (A), are normalised by expressing them as a function of the blank standard (A_0). The IC_{50} values of light chain shuffled clones (sAFL-3B2, sAFL-3B12 and sAFL-3H1) and parent clone were found to be 0.18, 0.11, 0.12 and 0.08 μg/ml, respectively.

3.4.3 Sequence analysis of shuffled clones

Eighteen heavy chain shuffled and seventeen light chain shuffled clones that can be inhibited by soluble aflatoxin B1 in competitive ELISA were sent to Macrogen company (Macrogen, Korea) for sequencing. The DNA sequences were translated to amino acid using Snapgene software and were aligned using Clustal Omaga software. The origin of the V gene and complementarity determining region (CDR) were determined using V BASE immunoglobulin V gene database (Tomlinson et al. 1996) and IGMT database. The closest germline sequence for anti-aflatoxin scFv antibody could be identified by comparison to database. There are 50% and 88.24% shuffled clones selected from heavy chain shuffled and light chains are identical to amino acids of YMAF-C3 (Table 3.5 and 3.6). The VH and VL of shuffled clones belonged to VH3 (IGHV1-3*01) and VK1 (IGKV1-39*01), which are the same family as original clone (YAF-C3).

For heavy chain shuffled clones, the VH gene of all of 18 clones were classified as human heavy chain family VH1 with derived germline V1-3*01 and V1-69*01. From four improved heavy chain shuffled clones (sAFH-3E11, sAFH-3E3, sAFH-3F11 and sAFH-3D4), DNA sequencing results indicated that sAFH-3E3 and sAFH-3D4 are identical. The deduced amino acid sequences of VH of improved clones (sAFH-3E11, sAFH-3E3 and sAFH-3F) are illustrated in Figure 3.12. There are 3-5 amino acids different from original clone at position in framework 1 and CDR1.

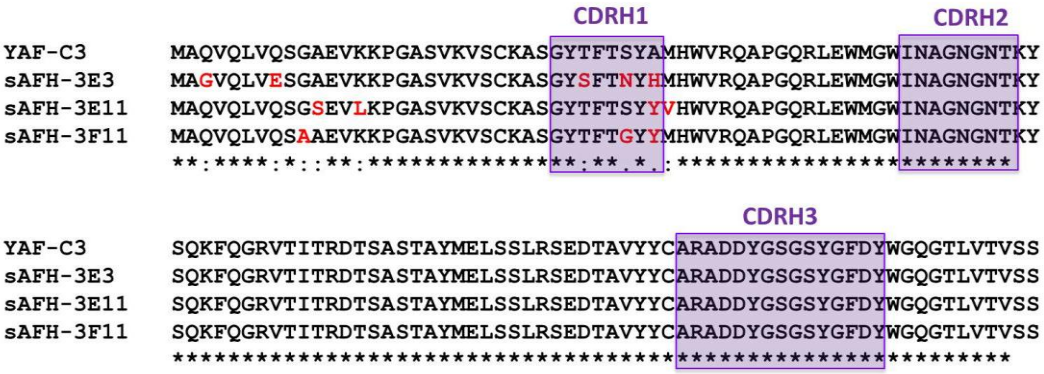


Figure 3.12 Multiple alignments of amino acid sequences of the VH of heavy chain shuffled clones. The alignment was performed using the Clustal Omega database. The highlighted sequences in purple box show the CDRs of VH of the recombinant antibody fragments. The amino acids sequence, which showed the different from original clone (YAF-C3) were indicated in red letter. (*) Symbol indicated that amino sequence are identical in the row; (:) symbol, one amino acid different; (.) symbol, more than one amino acid different in the row.

For light chain shuffled clones, VL fell into human kappa light chain family Vκ1 derived germline V1-39*01. The analysis showed that 3 clones were different from YAF-C3. The deduced amino acid sequences of VL of improved clones (sAFL-3B2, sAFL-3B12 and sAFL-H1) are illustrated in Figure 3.12. There are 1-2 amino acids different from original clone at position in framework 1 and 4 not in CDR region.

The CDR is critical importance in the interaction of an antibody with its respective antigen. Three improved heavy chain shuffled clones have 1-3 amino acids different from original clone in CDR1 region, which shown different sensitivity to AFB1. sAFH-3E3 is shown the highest sensitivity than others in competitive ELISA also represented the highest amino acids different from YAF-C3. The interaction between recombinant scFv fragment antibody to aflatoxin were described in 3.4.5.

Figure 3.13 Multiple alignments of amino acid sequences of the VH of light chain shuffled clones. The alignment was performed using the Clustal Omega database. The highlighted sequences in green box show the CDRs of VH of the recombinant antibody fragments. The amino acids sequence, which showed the different from original clone (YAF-C3) were indicated in red letter. (*) Symbol indicated that amino sequence are identical in the row; (:) symbol, one amino acid different; (.) symbol, more than one amino acid different in the row.

Table 3.5 Amino acids sequences of selected heavy chain shuffling library clones.

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
sAFH-D1	QVQLVQSGAEVKKPGASVKVSCKAS	GYTFTSYG	ISWVRQAPGQGLEWMGG	IIPIFGTA	NYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVYYC	ARADDYGSGSYGFDY	IGHV1-69*01	4	VH1
YAF-C3, sAFH-E2. sAFH-C4, sAFH -A7, sAFH -G12, sAFH -H9, sAFH -3H6, sAFH -3D12, sAFH -3A3	QVQLVQSGAEVKKPGASVKVSCKAS	GYTFTSYA	MHWVRQAPGQRLEWMGW	INAGNGNT	KYSQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYC	ARADDYGSGSYGFDY	IGHV1-3*01	0	VH1
sAFH -G1	QVQLVQSGAEVKKPGASVKVSCKAS	GYTFTSYA	MHWVRQAPGQRLEWMGW	INAGNGNT	KYSQKFKGRVTITRDTSASTAYMELSSLRSEDTAVYYC	ARADDYGSGSYGFDY	IGHV1-3*01	1	VH1
sAFH -B5, sAFH -D5	EVQLLQSAAEVKKPGASVKVSCKAS	GYTFTSYA	MHWVRQAPGQRLEWMGW	INAGNGNT	KYSQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYC	ARADDYGSGSYGFDY	IGHV1-3*01	3	VH1
sAFH -G5	EVQLVESGAEVKKPGSSVKVSCKAS	GYTFTSYA	MHWVRQAPGQRLEWMGW	INAGNGNT	KYSQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYC	ARADDYGSGSYGFDY	IGHV1-3*01	3	VH1
sAFH -F8	QVQLVQSGSELKKPGASVKVSCKAS	GYTFTSYA	MHWVRQAPGQRLEWMGW	INAGNGNT	KYSQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYC	ARADDYGSGSYGFDY	IGHV1-3*01	2	VH1
sAFH -3E11	QVQLVQSGSELKKPGASVKVSCKAS	GYTFTSYY	VHWVRQAPGQRLEWMGW	INAGNGNT	KYSQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYC	ARADDYGSGSYGFDY	IGHV1-3*01	4	VH1

Table 3.5 (continued).

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
sAFH -3F11	QVQLVQSAAEVKKPG ASVKVSCKAS	GYTFTGYY	MHWVRQAP GQRLEWMG W	INAGNGNT	KYSQKFQGRVTITRDTSA STAYMELSSLRSEDTAVY YC	ARADDYGSG SYGFDY	IGHV1-3*01	3	VH1
sAFH -3E3, sAFH -3D4	GVQLVESGAEVKKPG ASVKVSCKAS	GYSFTNYH	MHWVRQAP GQRLEWMG W	INAGNGNT	KYSQKFQGRVTITRDTSA STAYMELSSLRSEDTAVY YC	ARADDYGSG SYGFDY	IGHV1-3*01	5	VH1

Table 3.6 Amino acids sequences of selected light chain shuffling library clones

Clone	FR1	CDR1	FR2	CDR2	FR3	CDR3	Germline	Amino acid different from germline	Family
sAFL-3H1	DIQMTQSPSSLSASVGD RVTITCRAS	QSISSY	LNWYQQKPG KAPRLLIY	AAS	SLQSGVPSRFSGNGSG TDFTLTISSLQPEDFAT YYC	QQSYSTPYA	IGKV1-39*01	2	VK1
YAF-C3, sAFL-B2, sAFL-A10, sAFL-D1, sAFL-3H11, sAFL-3H12, sAFL-3H2, sAFL-3F11, sAFL-3B8, sAFL-3B1, sAFL-3A6, sAFL-3A1, sAFL-3H4, sAFL-3H7	DTVMTQSPSSLSASVG DRVTITCRAS	QSISSY	LNWYQQKPG KAPRLLIY	AAS	SLQSGVPSRFSGNGSG TDFTLTISSLQPEDFAT YYC	QQSYSTPYA	IGKV1-39*01	4	VK1
sAFL-3B2	DTVMTQSPSSLSASVG DRVTITCRAS	QSISSY	LNWYQQKPG KAPRLLIY	AAS	SLQSGVPSRFSGNGSG TDFTLTISSLQPEDFAT YYC	QQSYSTPYA	IGKV1-39*01	4	VK1
sAFL-3E1, sAFL-3B12	DTVMTQSPSSLSASVG DRVTITCRAS	QSISSY	LNWYQQKPG KAPRLLIY	AAS	SLQSGVPSRFSGNGSG TDFTLTISSLQPEDFAT YYC	QQSYSTPYA	IGKV1-39*01	4	VK1

3.4.4 Production and characterization of scFv fragments

Clone sAF-3E3, sAF-3E11, sAF-3F11 and the original were expressed and purified as described in 3.4.5. The antibody fragment was purified from periplasmic fraction using HIS Trap column, and analyzed by SDS-PAGE (Figure 3.14). The SDS-PAGE result showed that the scFv- hexahistidine fusion protein of about 32 kDa was expressed, but the contaminated bands were visible. Each clone showed different level of expression. Clone sAF-3E3, sAF-3E11, sAF-3F11 and YAF-C3 were expressed at concentration of 3.85, 3.08, 6.36 and 4.96 mg/l, respectively of the culture medium.

After purification, the binding kinetics of all 4 clones was determined using BIAcoreTM 300 system. Original scFv (YAF-C3) showed K_D value at 5.22×10^{-8} M. Binding affinity of heavy chain shuffled clones showed 3-20 folds improvement relative to YAF-C3, following to the K_D values of improved scFv cloned were 3- 20 folds lower than YAF-C3. The scFv sAFH-3E3 is the best affinity-matured scFv, indicating by its had the lowest K_D (2.29×10^{-9} M) values (Table 3.7). The affinity enhancement was depended on a combination of an increase in K_{on} and a decrease in K_{off}.

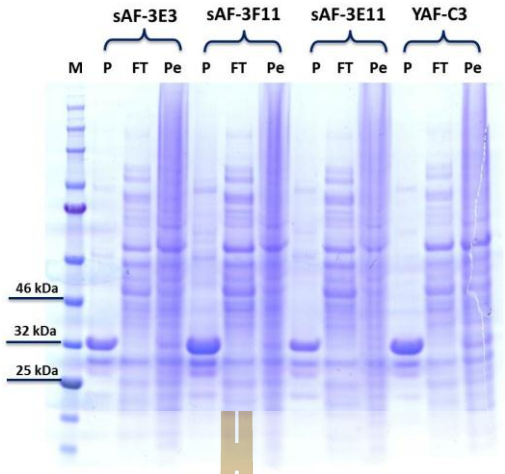


Figure 3.14 His affinity chromatography purified fractions from periplasmic expression of scFv of YAF-C3, sAFH-3E11, sAFH-3F11 and sAFH-3E3 in *E.coli* HB2151. M, Precision Plus Protein™ Dual Color Standards; lane Pe, periplasmic fraction lane FT, flow-through fraction; lane P, purified scFv antibody. Twenty microliters per lane of purified proteins were analyzed on 12% polyacrylamide gels and stained with coomassie blue. The size of scFv is shown approximately 32 kDa.

Table 3.7 Binding kinetics of scFv antibodies by SPR.

scFv	Kon (M-1 S-1)	Koff (S-1)	K_D (M)
YAF-C3	2.97×10^{4}	1.55×10^{-3}	5.22×10^{-8}
sAFH-3E11	3.56×10^{4}	6.69×10^{-4}	1.88×10^{-8}
sAFH-3F11	1.4×10^{4}	1.62×10^{-4}	1.16×10^{-8}
sAFH-3E3	1.29×10^{5}	2.95×10^{-4}	2.29×10^{-9}

Kon association constant, Koff dissociation constant and KD equilibrium dissociation constant (KD = Koff/Kon)

3.4.5 Molecular docking

The homology models were generated based on template (PDB code: 3uzq1A) with the solution of 1.6 Å. The percentage of a sequence identity with template of YAF-C3 and sAFH-3E3 were 64.41 and 63.98, respectively. The model was submitted for the Ramachandran plot analysis of MolProbity website. The plot showed that the YAF-C3 and sAFH-3E3 had 93.33% in flavored regions, 6.2% in allowed regions and 0.8% in the outlier regions. The three dimensional structure of scFv YAF-C3 and sAFH-3E3 were depicted in Fig 3.15. The mutant amino acids of sAFH-3E3 and the corresponding position in original scFv YAF-C3 were located in FR1 and CDR1 of heavy chain region. Base on the amino acid sequence and 3D structure, His33 of sAFH-3E3 was considered to be binding site for docking analysis compared with Ala33. Figure 3.16 revealed that the VH domain of both antibodies was the most contacts to AFB1.

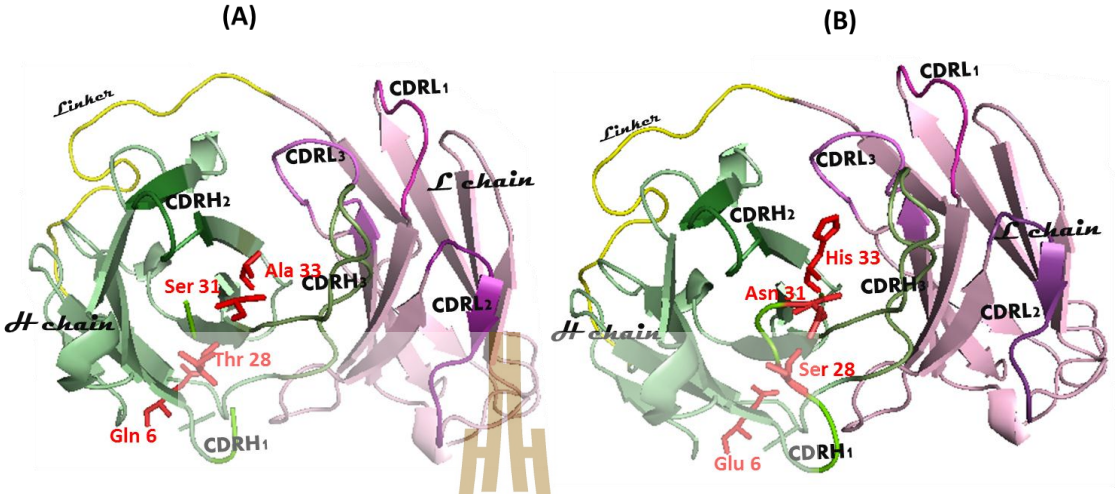


Figure 3.15 Three-dimensional structure prediction of scFv YAF-C3 (A) and scFv sAFH-3E3 (B). The V_H and V_L are shown in green and pink, respectively. The complementarity-determining region (CDR) of V_H and V_L were labeled. Mutated Amino acid residues and its number in sAFH-3E3 and the corresponding position in original scFv YAF-C3 are indicated in red color.

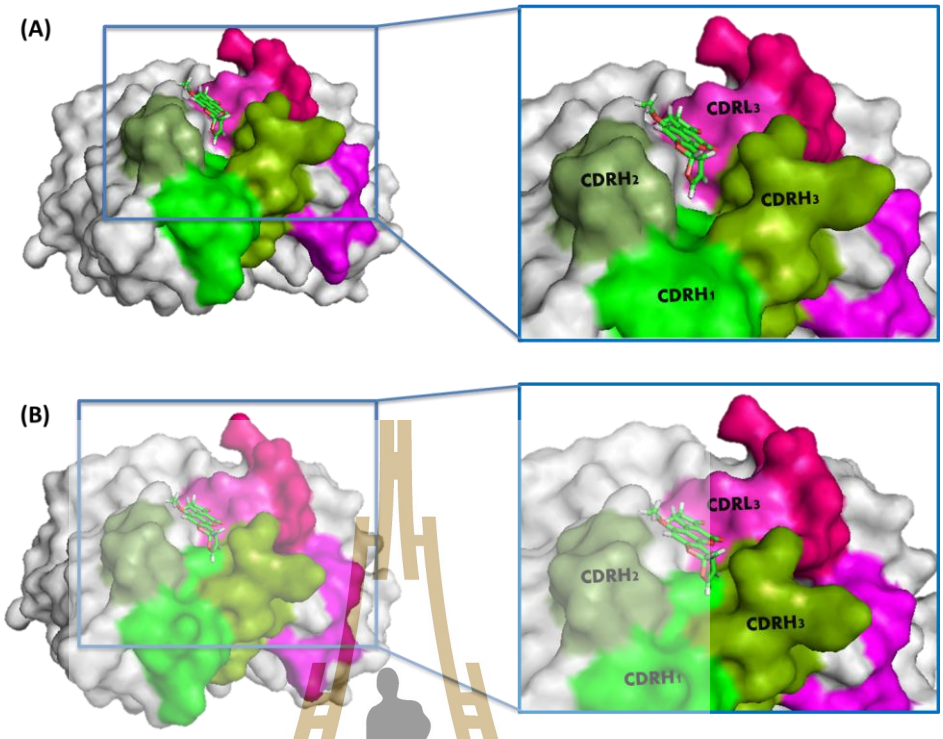


Figure 3.16 The binding pockets of molecular docking with AFB1: scFv YAF-C3 (A) and sAFH-3 (B). The complementarity-determining region (CDR) of V_H and V_L were labeled. The close up views of AFB1 interacted with antibody showed that the AFB1 was surrounded by amino acids from the VH domain. CDR of VH and VL showed as green and pink color, respectively. This figure was generated in PyMoL.

The common interaction with AFB1 for these two scFvs showed that four hydrogen bond are formed (one in Thr-L231 of CDR L3, two in Tyr-L233 of CDR L3 and one in ASN-57 CDR H2) and T- shape π stacking interaction of aromatic side chain of Trp-H50 of FR H2 formed with benzofuranyl ring of AFB1 (Fig 3.17 and 3.18).

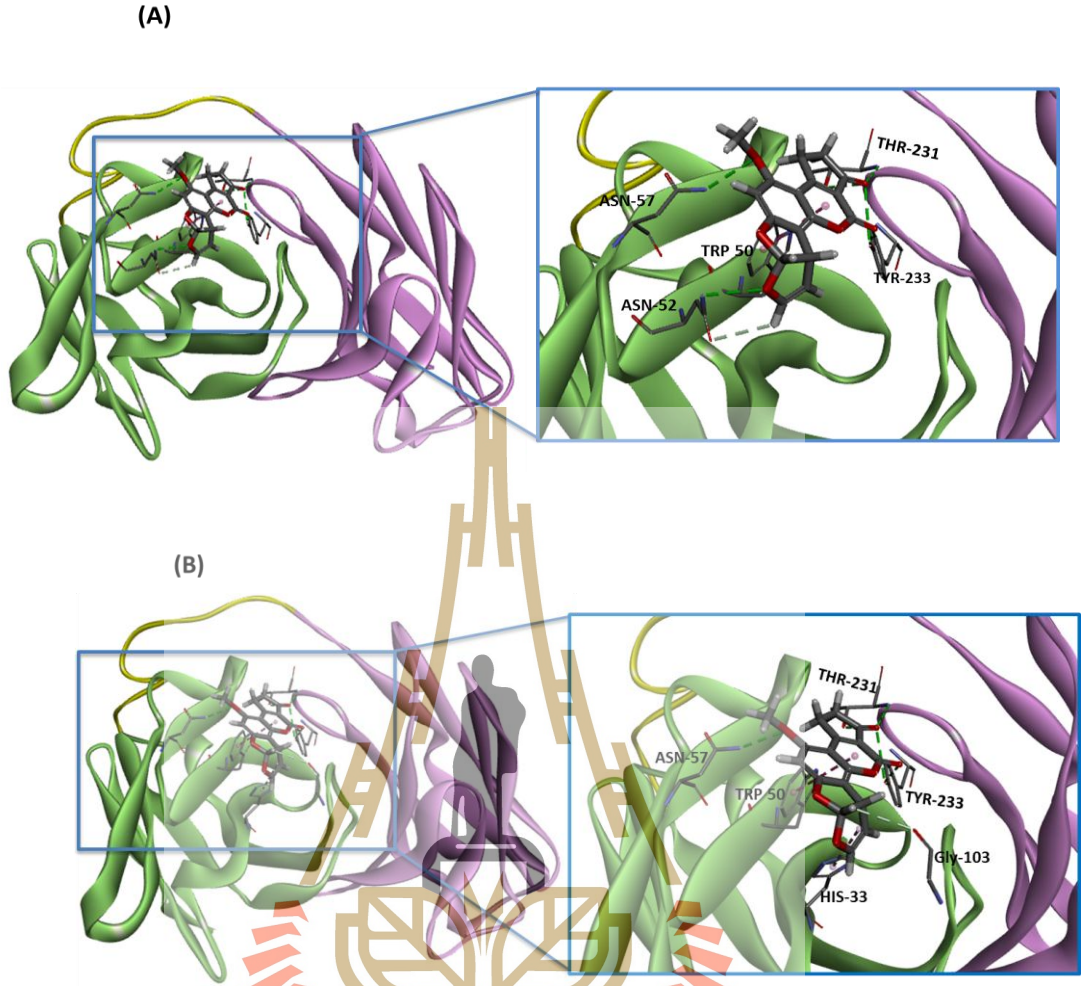


Figure 3.17 Comparison of the molecular docking of the scFv YAF-C3 (A) and sAFH- 3E3 (B) antibodies with aflatoxin B1 generated using Gold software. The heavy chain, light chain and linker are shown in green, pink and yellow, respectively. The amino acid residues involved in the interactions with the AFB1 are labeled. Hydrogen bonds are shown as green dash lines. His-H33 and Gly-H103 of sAFH-3E3 forms π interaction and carbon hydrogen bond whereas Asn-H52 forms hydrogen bond with AFB1 around the same area. The figure was generated from Discovery studio 2016.

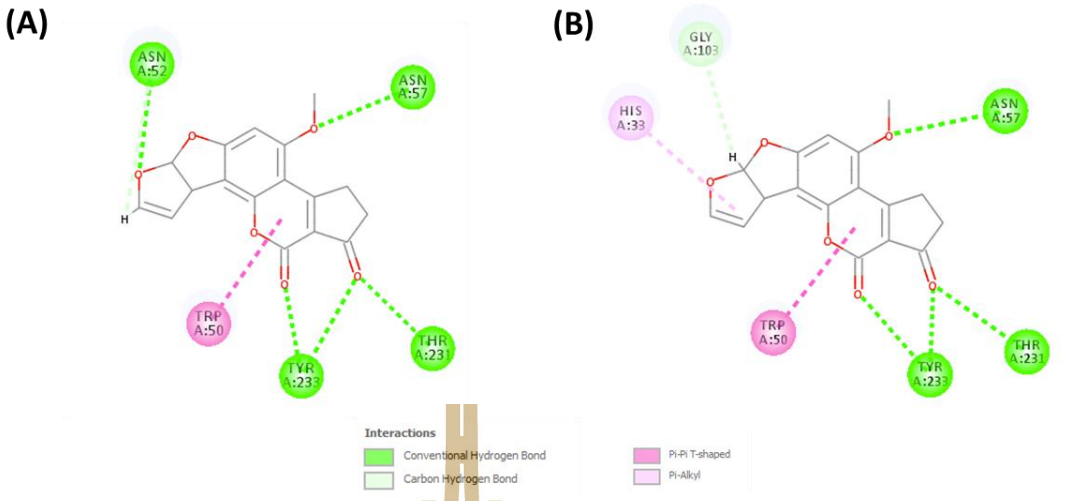


Figure 3.18 Two dimensional diagram of ligand (AFB1) interaction to scFv YAF-C3 (A) and sAFH-3E3 (B). The figure was generated from Discovery studio 2016.

The different interaction between scFv YAF-C3 and sAFH-3E3 included Gly-103 in CDR H3 of YAF-C3 form carbon-hydrogen bond with AFB1 and π alkyl interaction at His 33 in CDR H3 while, ASN-52 in FR2 of YAF-C3 form H-bond with AFB1 (Fig 3.19).

Because of the large hydrophobic fragments of aflatoxin, the hydrophobic interaction might be another important factor to increase the binding affinity between scFv and AFB1. The result indicated that the mutant amino acids from Ala-33 to His-33 may be the key amino acid influencing for the binding. As shown in Fig. 3.20, His-33 showed the hydrophobic interaction with AFB1.

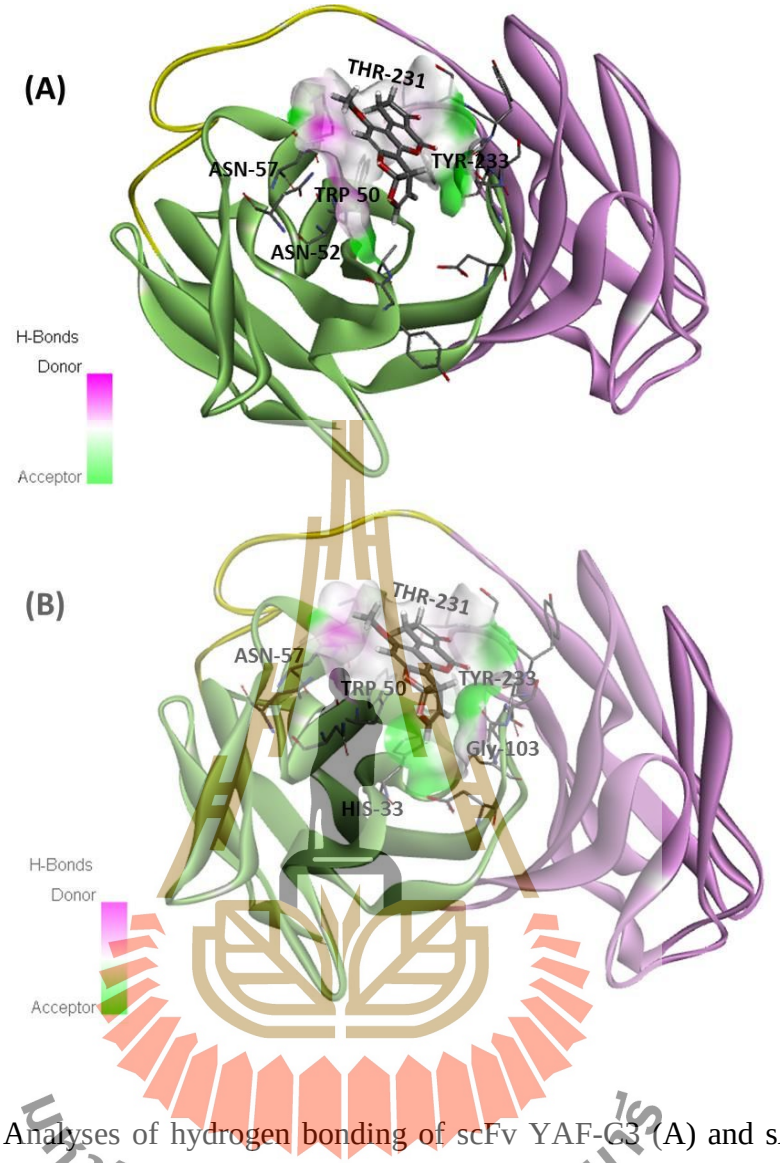


Figure 3.19 Analyses of hydrogen bonding of scFv YAF-C3 (A) and sAFH-3E (B) with aflatoxin B1. The heavy chain, light chain and linker are shown in green, pink and yellow, respectively. The amino acid residues involved in the interactions with the AFB1 are labeled. The figure was generated from Discovery studio 2016.

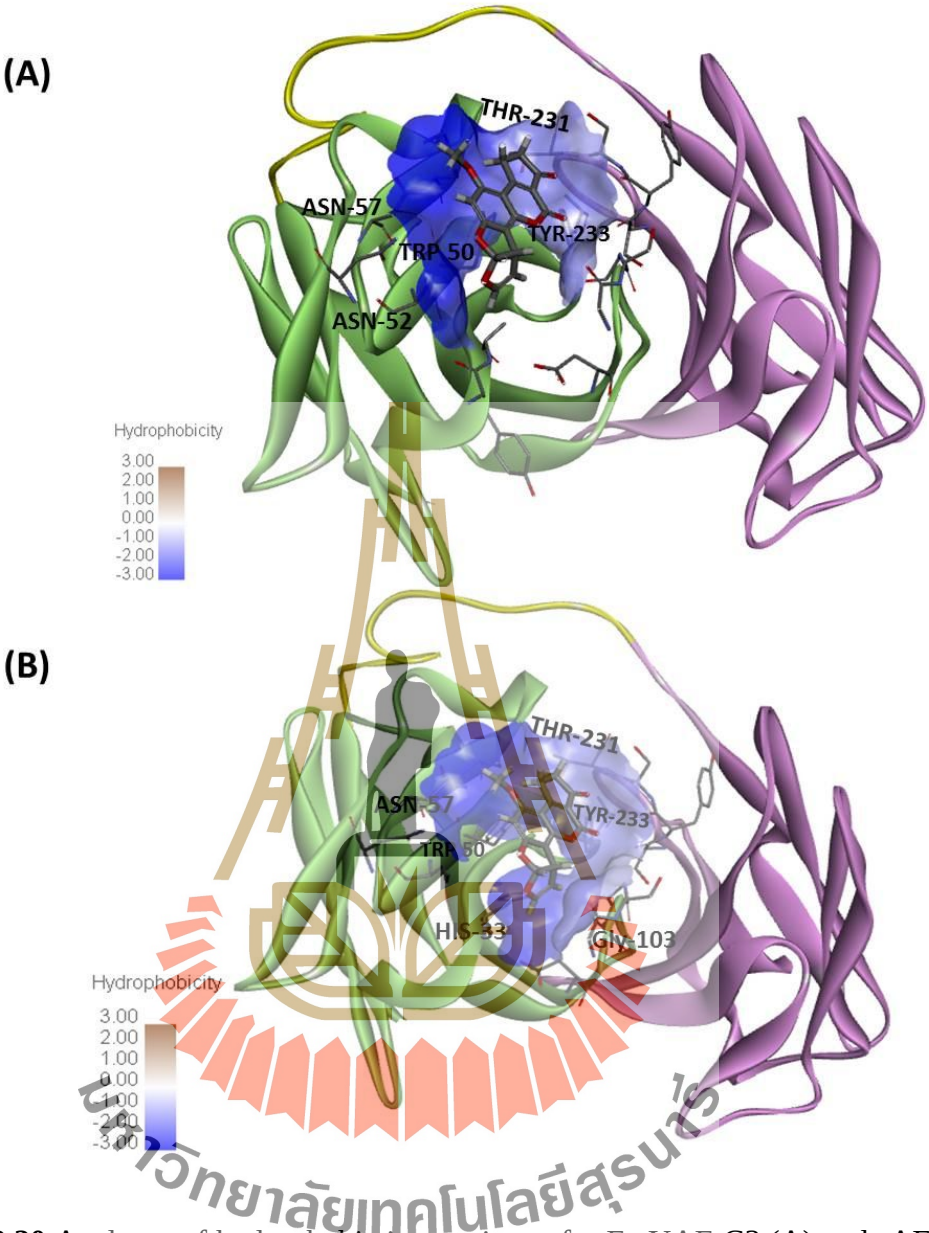


Figure 3.20 Analyses of hydrophobic interactions of scFv YAF-C3 (A) and sAFH-3E (B) with aflatoxin B1. The heavy chain, light chain and linker are shown in green, pink and yellow, respectively. The amino acid residues involved in the interactions with the AFB1 are labeled. The figure was generated from Discovery studio 2016.

3.5 Discussions

The chain-shuffling technique was used to improve the recombinant antibody scFv human anti-aflatoxin for both the affinity and sensitivity. The two chain shuffling libraries (heavy and light chain shuffling) were constructed by re-assembling VH of parent clone with VL of pre-selected Yamo I library and vice versa to get the new varieties of scFvs. The H chain and L chain shuffled library containing 3.48×10^5 and 2.26×10^4 independent clones, respectively was subjected to 3 rounds of panning against AFB1-BSA immobilized on the microtiter plate. Then, the libraries were processed to select AFB1 binder antibody. After selection and DNA sequencing analysis, at least fifty percentages of selected clones showed the same DNA sequence with YAF-C3, which are belonged to VH3 (IGHV1-3*01) and VK1 (IGKV1-39*01). The reason why all the clones that were selected were the same as original contruct could be because the libraries are small. Three clones selected from heavy chain shuffling library were found to have higher sensitivity and affinity than its parent (YAF-C3); whereas no clone from light chain shuffling library was isolated. Among these three clones, sAFH-3E3 show the highest sensitivity and affinity than others compare to parental clone. This is the same result with Park et al described that they identify clone from H-chain shuffling library with improve affinity 2.8-6.5 fold parental clone (Park et al. 2000). The increase of affinity is not to high as another group reported which they did the light chain shuffling with the group of selected binding clone to enhance sensitivity 185 fold over the original scFv (Fitzgerald et al. 2011). In addition, the CDR site directed mutagenesis can be increased 420 fold affinity binding (Yang et al. 1995). Thus, it is suggested that I should use the pool of V gene repertoires not only one clone and merging with another mutagenesis such as

error prone PCR and CDR mutagenesis to improve in the affinity achieved by chain shuffling. The sequencing result of these three improved clones showed that there are 3- 5 amino acids mutation in the framework 1 and CDR 1 of heavy chain. sAFH-3E3 showed the highest number of amino acids mutation. The heavy chain may affect to the binding of AFB1 which is correlated with previous report (Li et al. 2012). They have been revealed from crystal structures of several antibody antigen complexes that the VH have an important role for the most of molecular interactions between antigens (Morea et al. 1998, Xu and Davis 2000).

The three-dimensional models of scFv mutant and parental were predicted and studied the molecular interaction with AFB1. After analyzing binding interaction between scFv and AFB1, the result showed that the hydrogen bonding and hydrophobic interactions are important roles for the affinity binding which is in agreement with previous work (Li et al. 2012). From the 3D model, the pocket binding site to AFB1 was surrounded with heavy chain. This is in agreement with the result observed in ELISA. The amino acid were mutated in heavy chain and affected to the binding to AFB1. This was consistent with previous study by Xu and Davis and López-Requena et al demonstrate that all three-hypervariable regions in the VH domain affect the antigen binding (Xu and Davis 2000, López-Requena et al. 2007). SAFH-3E3 showed 5 amino acids mutation in the framework 1 and CDR 1 of VH. The amino acid His33 located in the pocket binding appears to be important amino acid involved in the hydrophobic interaction with AFB1. The sensitivity was improved may be because of this amino acid. This hypothesis can approve by site directed this amino acid to be another amino acid such as Phe (Phenylalanine) and tested the sensitivity by competitive ELISA in the future. The improvement clone was further engineered to different formats of antibody for aflatoxin detection in different

assay format in the next chapter.

3.6 Conclusions

In conclusion, the chain shuffling technique was successfully employed to enhanced affinity and sensitivity of scFv YAF-C3. The clone from heavy chain shuffling library namely, sAFH-3E3 showed 20 fold improvement in affinity. Three dimensional model of this clone and parent clone were predicted and the molecular docking with AFB1 was conducted. The result is in good agreement with the results observed in competitive ELISA experiment that VH has higher influence for the binding to AFB1 than the VL.

3.7 References

Biasini, M., Bienert, S., Waterhouse, A., Arnold, K., Studer, G., Schmidt, T., Kiefer, F., Cassarino, T. G., Bertoni, M. and Bordoli, L. (2014). SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. **Nucleic acids research**: gku340.

Fitzgerald, J., Leonard, P., Darcy, E., Danaher, M. and O'Kennedy, R. (2011). Light-chain shuffling from an antigen-biased phage pool allows 185-fold improvement of an anti-halofuginone single-chain variable fragment. **Analytical biochemistry** 410(1): 27-33.

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

Lefranc, M. (1999). The IMGT unique numbering for immunoglobulins, T-cell receptors, and Ig-like domains. **Immunologist** 7(4): 132-136.

Li, X., Li, P., Zhang, Q., Li, Y., Zhang, W. and Ding, X. (2012). Molecular characterization of monoclonal antibodies against aflatoxins: a possible explanation for the highest sensitivity. **Analytical chemistry** 84(12): 5229-5235.

López-Requena, A., Rodríguez, M., de Acosta, C. M., Moreno, E., Puchades, Y., González, M., Talavera, A., Valle, A., Hernández, T. and Vázquez, A. M. (2007). Gangliosides, Ab1 and Ab2 antibodies: II. Light versus heavy chain: An idiotype-anti-idiotype case study. **Molecular immunology** 44(5): 1015-1028.

Lou, J. and Marks, J. D. (2010). Affinity maturation by chain shuffling and site directed mutagenesis **Antibody Engineering** (pp. 377-396): Springer.

Morea, V., Tramontano, A., Rustici, M., Chothia, C. and Lesk, A. M. (1998). Conformations of the third hypervariable region in the VH domain of immunoglobulins. **Journal of molecular biology** 275(2): 269-294.

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(1): 1.

Park, S.-G., Lee, J.-S., Je, E.-Y., Kim, I.-J., Chung, J.-H. and Choi, I.-H. (2000). Affinity maturation of natural antibody using a chain shuffling technique and the expression of recombinant antibodies in Escherichia coli. **Biochemical and biophysical research communications** 275(2): 553-557.

Rangnoi, K., Jaruseranee, N., O'Kennedy, R., Pansri, P. and Yamabhai, M. (2011). One-Step Detection of Aflatoxin-B1 Using scFv-Alkaline Phosphatase-Fusion Selected from Human Phage Display Antibody Library. **Molecular biotechnology** 49(3): 240-249.

Tomlinson, I. M., Walter, G., Jones, P. T., Dear, P. H., Sonnhammer, E. L. and Winter, G. (1996). The imprint of somatic hypermutation on the repertoire of human germline V genes. **Journal of molecular biology** 256(5): 813-817.

Xu, J. L. and Davis, M. M. (2000). Diversity in the CDR3 region of V H is sufficient for most antibody specificities. **Immunity** 13(1): 37-45.

Yang, W.-P., Green, K., Pinz-Sweeney, S., Briones, A. T., Burton, D. R. and Barbas III, C. F. (1995). CDR walking mutagenesis for the affinity maturation of a potent human anti-HIV-1 antibody into the picomolar range. **Journal of molecular biology** 254(3): 392-403.

CHAPTER IV

ENGINEERING OF DIFFERENT FORMAT OF ANTIBODY FOR AFLATOXIN DETECTION

4.1 Abstract

Three clones (sAFH-3E11, sAFH-3F11 and sAFH-3E3) and one parental clone (YAF-C3) of human scFv antibodies against aflatoxin B1 (AFB1), previously isolated and engineered by chain shuffling technique in our lab as described in chapter III were converted into various formats, i.e., scFv-AP fusions, scFv-Fc, and whole IgG molecules. These antibodies were expressed in their appropriate hosts, purified to apparent homogeneity with the yield of scFv-Fc and IgG approximately 270 and 490 mg/l, respectively and approximately 0.7 and 2.3 mg/l for scFv-AP and scFv, respectively. After that the binding properties of various formats of these antibodies were characterized by competitive ELISA. The results indicated that scFv-AP fusion formats showed the highest sensitivity. SAFH-3E3 AP clone showed the best performance by competitive ELISA at detection limit of 20 ng/ml. In addition, the sAFH-3E3 clone in IgG format was selected to test for the binding property by Lateral Flow Immunochromatography assay (LFIA). Different conditions for toxin extraction and antibody conjugations were tested. The results showed that PBS is the best extraction solution and conjugation with latex lead to better performance than conjugation with colloidal gold. The limit of detection when using lateral flow strip test with the recombinant antibody was 5 ng/ml. In conclusion these results indicated

that the sAFH-3E3 recombinant antibody has a potential to be applied for use in commercial test kit. It could eventually replace the use of conventional monoclonal antibody and reduce the cost for the production of lateral flow test strip in the future.

Key words: Aflatoxin, recombinant antibody, Enzyme linked immunosorbant assay (ELISA), Lateral flow immunochromatography assay (LFIA)

4.2 Introduction

Aflatoxins are highly toxicity produced from *Aspergillus Flavus* and *Aspergillus parasiticus*, which can be found in agricultural communities and animal feed. Aflatoxin B1 is the most harmful to human and animal health by causing to liver cancer. AFB2, AFG1 and AFG2 are also frequently found and have toxicity. Therefore, the maximum level for AFB1 and for total aflatoxins (AFB2+AFG1 and AFG2) have been set by European union and all over the worlds (Shim et al. 2007).

Enzyme linked immunoassay and lateral flow base assay are fast detection method, sensitive and easy to use for analytical aflatoxin. These methods used the antibody as the important chemical for detection. The antibody should be sensitive enough to use in the assay.

There are several recombinant antibodies were produced and successfully to used for aflatoxin detection in ELISA assay. Various format of antibody was used, for example, scFv fragment (Wang et al. 2009, Yang et al. 2009, Rangnoi et al. 2011, Li et al. 2012, Li et al. 2013, Min et al. 2015), Fab (Edupuganti et al. 2013) and VHH (Wang et al. 2013, He et al. 2014).

In this study, the recombinant scFv antibody was engineer to different format of antibody and used in different assay. After engineering all the formats were tested by competitive ELISA. SAFH-3E3 showed the highest sensitivity. This clone showed the

same sensitivity in every format. Therefore, scFv was fused to bacterial alkaline phosphatase to develop one-step detection ELISA by decreasing step for adding of secondary antibody. In addition, it was converted to full length IgG and used in lateral flow assay.

4.3 Materials and Methods

4.3.1 Materials

All reagents were molecular grade or analytical grade. Ani-aflatoxin scFv antibody YAF-C3, sAFH-3E11, sAFH-3F11 and sAFH-3E3 were produced in our laboratory. Aflatoxin B_1, B_2, G_1, G_2, M_1 standards and hapten AFB_1 conjugated with BSA were obtained from Aokin, Germany. TMB substrate (3, 3′, 5, 5′-tetramethylbenzidine), PNPP substrate (*p*-Nitrophenyl Phosphate) and Protein L peroxidase HRP were obtained from Sigma (St. Louis, MO). *Escherichia coli* HB2151 was obtained from the MRC, Cambridge, UK, and used for production soluble scFv fragments. Goat anti human IgG (H+L) was purchased from Jackson ImmunoResearch Inc. (PA, USA).

4.3.2 Construction mammalian expression vector

The pTT28 (NRC, USA) was used as a basic frame for the construction of mammalian expression (Fig 4.1). This vector contains the CMV promoter, the human signal peptide and ampicillin resistance. The constant heavy chain and light chain was generated from pRom108-3D6 H chain (gift from David's lab, BOKU, Austria) and MIS104 (pTT5-3D6 L chain, gift from Florain's lab, BOKU, Austria), respectively. All of PCR reaction was set up containing 0.2 μM each of primers, 0.2 mM dNTP,

0.25 µl (1.25 U) of one Taq polymerase (NEB, USA), 0.5 µl of vector template and MilliQ water to 50 µl. Primers for mammalian vector construction are listed in table 4.1.

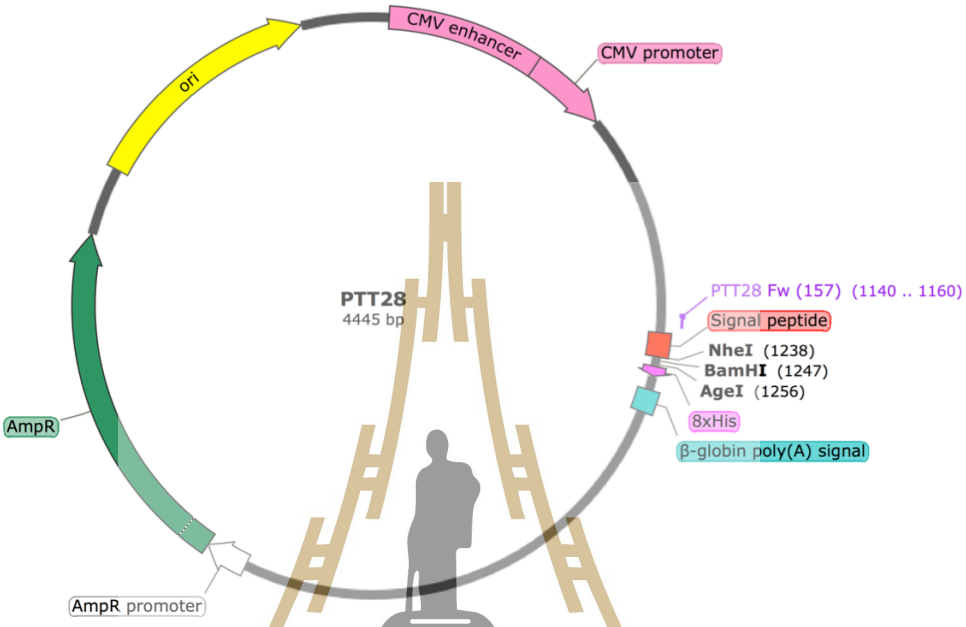


Figure 4.1 Map of pTT28. The arrow shows the position of CMV promoter and ampicillin resistance genes.

4.3.2.1 scFv-Fc vector

For the generation of scFv-Fc vector, the Fc region (Hinge-CH2-CH3) was amplified from pRom108 by PCR with primer introducing *BspEI* site at 5' end (Fc-BspEIFw) and *AgeI* site at 3' end (CH-AgeIRv) of Fc gene and cloned into pTT28 via *BspEI* and *AgeI* (NEB, USA) *sites*. Moreover, the scFv gene of each three mutant clones (sAFH-3E11, sAFH-3F11 and sAFH-3E3) and parent clone (YAF-C3) were amplified with primer introducing *NheI* site at 5' end and *BspEI* site at 3' end of scFv gene by PCR and then cloned into PTT28 vector at *NheI* and *BspEI* sites. This scFv-Fc vector was designed as KR-scFv-Fc vector.

at 3’ end of scFv gene by PCR and then cloned into PTT28 vector at *NheI* and *BspEI* sites. This scFv-Fc vector was designed as KR-scFv-Fc vector.

4.3.2.2 IgG vector

For the generation of IgG expression vector, the heavy chain and light chain expression vector were separately generated. Constant heavy chain was amplified from pRom108 3D6 with primer introducing *NheI* site at 5’ end (CH-NheIFw) and *AgeI* site at 3’ end (CH-AgeIRv) of constant heavy chain gene and cloned into pTT28 at *NheI* and *AgeI* (NEB, USA) sites. In addition, VH each three mutant clones (sAFH-3E11, sAFH-3F11 and sAFH-3E3) and parent clone (YAF-C3) were amplified and then also clone in to PTT28 vector at *NheI/NheI*. This heavy chain expression vector was called KR-HC.

For light chain vector, the Kappa (κ) light chain was amplified from MIS104 with primer introducing *BsiWI* site at 5’ end and *AgeI* site at 3’ end of constant heavy chain gene and cloned into pTT28 at *BsiWI* and *AgeI* (NEB, USA) sites. All of four clones have the same VL sequence which are YAF-C3 VL. This YAF-C3 VL was amplified and clone into PTT28 at *NheI/BsiWI* sites. This light chain (κ) expression vector was called KR-LC.

After digestion the insert and vector with restriction enzyme, the vector and insert was ligated using T4 DNA ligase (400U/µl, NEB, USA) at 25°C for 1 hour and then was multiplied by transformation of *E. coli* Top 10. The individually colonies were picked and cultured overnight for plasmid preparation using Nucleo spin plasmid (MACHEREY-NAGEL, Germany) according to the manufacturer’s protocol. The plasmids were confirmed by DNA sequencing. After checking sequence, the plasmid was amplified in *E. coli* Top 10 in 100 ml culture

volume to prepare the large concentration of plasmid by using Nucleo Bond extra Midi (MACHEREY-NAGEL, Germany) for using in large-scale expression.

Table 4.1 Oligonucleotides for construction scFv-Fc and IgG vector.

Primer	**Sequence**
Fc-BspEIFw	5'AGT CTC CGG AGA GCC CAA GAG CTG CGA C3'
CH-NheIFw	5'ATG CGC TAG CAC CAA GGG CCC CAG CGT GTT CC3'
CH-AgeIRv	5'GCC ACC GGT TCA CTT GCC GGG GGA CAG GCT3'
C3 VLNheIFw	5'AGT GCC GCT AGC GAC ACC GTG ATG ACC CAG TCT3'
LC conAgeIRv	5'AGT GCC ACC GGT CTA ACA CTC TCC CCT GTT3'
C3 VHNheIFw	5'GCC GCT AGC CAG GTG CAG CTG GTG CAG TC3'
3E3 VHNheIFw	5'AGT GCC GCT AGC GGG GTG CAG CTG GTG GAG TC3'
VL BspEIRv	5'GCA CAG TCC GGA ACG TTT GAT CTC CAC CTT GGT3'
C3 VHNheIRv	5'TTG GTG CTA GCT GAG GAG ACG GTG ACC AGG G3'
C3 VLNheIFw	5'AGT GCC GCT AGC GAC ACC GTG ATG ACC CAG TCT3'

Sequences corresponding to *NheI, BspEI*, *BsiWI* and *AgeI* restriction sites are underlined

4.3.3 Construction of scFv fused with AP

The three improved scFv gene from the phagemid vector were inserted into an alkaline phosphatase vector (pKP300ΔIII) (Pershad et al. 2011) between the *NcoI* and *NotI* sites. The DNA of scFv fragments and pKP300ΔIII vector was double digested with *NcoI* (10U/µl, NEB, USA) and *NotI* (10U/µl, NEB, USA) enzymes, to generate compatible sticky ends. The reactions were incubated at 37°C for 3 hours. The reactions were heat inactivated at 65°C for 20 minutes. After heat inactivate enzyme, the *NcoI/NotI* digested vector was dephosphorylated by adding 0.5 µl of CIP enzyme (10U/µl, NEB, USA) and incubated at 37°C for 1 hour. The inserts and vectors were separated from stuffer fragments by gel electrophoresis followed by illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare, USA). The scFv DNA was ligated into pKP300ΔIII vectors at a 3:1 ratio. The ligation reaction was incubated at 25°C for 2 hours and heat inactivate enzyme at 65°C for 10 minutes. Then, the ligation reaction was transformed into 100 µl of *E. coli TG1* cells. After that the transformed cells were spread on LB plates containing 100 µg/ml ampicillin and incubated for overnight at 37°C. Two individual colonies of each clone were picked and analyzed by double digestion with *NcoI/NotI*. Moreover, the construct was confirmed by automated DNA sequencing.

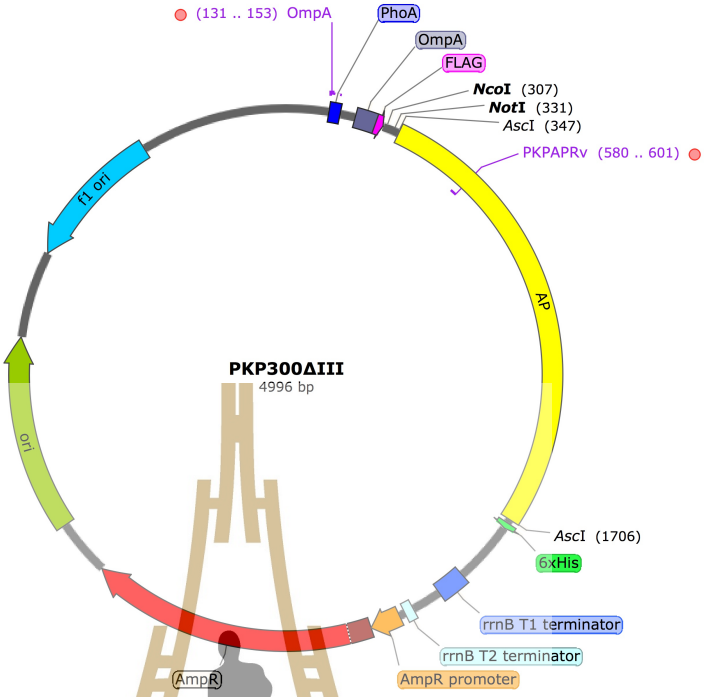


Figure 4.2 Map of pKP300ΔIII. The arrow shows the position of PhoA promoter, alkaline phosphatase and ampicillin resistance genes.

4.3.4 Expression and purification antibody

4.3.4.1 scFv-AP expression in *E.coli*

E. coli TG1 harbored pKP300ΔIII-scFv expression vector was grown onto LB Agar supplemented with 100 µg/ml ampicillin. Individual colony was picked with sterilized toothpicks into 5 ml of LB media containing 100 µg/ml ampicillin at 37°C, 250 rpm. After incubation for overnight, one hundreds microliters of overnight culture was grown into 100 ml of low phosphate medium supplemented with 100 µg/ml ampicillin (1:1000 dilution). ScFv-AP was expressed at 30°C for 18-20 hrs at 250 rpm. After 18-20 hrs, the cultures were centrifuged at 8,000 rpm for 10

minutes at 4°C. The pellet was resuspended in 8 ml of cold periplasmic buffer (1xPBS, 1 M NaCl and 1 mM EDTA) and left on ice for 20 mins. The resuspended solution was spun at 3,000 g for 10 mins at 4°C. The supernatant which carrying the periplasmic fractions containing scFv fragments were collected and was added with $MgCl_2$ to be 1 mM as final concentration. The periplasmic fraction was used for protein purification and analyzed on SDS-PAGE.

ScFv-AP was purified by immobilized metal affinity chromatography (IMAC) using Ni-NTA by Akta purifier (GE Healthcare, USA). Before purification, cell debris was removed from cell lysate by filtering through 0.45 µm. One ml His-Trap column (GE Healthcare, USA) was equilibrated with 10 ml buffer (20 mM Tris-HCl, 500 mM NaCl, 20 mM imidazole, pH 7.9). Then, the filtered periplasmic fraction was loaded into the column. To remove loosely bound protein, the column was washed with the same buffer. The scFv was eluted from the column with buffer gradient buffer programme containing high concentration of imidazole (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.9). The antibody fraction was dialysed with TBS buffer containing 1 mM $MgCl_2$ by using 10 kDa snakeskin dialysis tubing (Thermo Scientific, USA). Protein concentration was quantified by a Nanodrop ND2000 spectrophotome (Thermo Scientific, USA). 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

4.3.4.2 IgG and scFv-Fc expression in HEK293-6E cells

The HEK293-6E (Human Embryonic Kidney) cells were used for scFv-Fc and IgG expression. This cell was maintained in a shaker flask with

orbital shaker at 37°C. Before transfection, the cells was passaged and cultured in F17 media (Invitrogen, USA) supplemented with 4 mM L-Glutamine, 0.1% Pluronic F68 and 25 µg/ml G418 until the cell density around 1.7-2.0 × 10^6 cells/ml.

The cell was transfected with plasmid in culture volume 25 ml F17 media using 125 ml flask. The HEK-293-6E cells was transfected with 1 µg/ml plasmid DNA by adding a complex solution of plasmid DNA and PEI (polyethylenimine) (Polysciences, Germany) at a ratio 1:2 ratio. In case of IgG expression, the HEK-293-6E cells was transfected with equimolar amounts of heavy chain and light chain vectors. For example, 0.5 µg/ml of each heavy chain and light chain plasmid DNA was used. The cells were feed with TN1 medium (Tekniscience, Canada) in final concentration 0.5% after culture for 24 hrs at 37°C, 5% CO_2 with shaking at 130 rpm. After 48 hrs, the culture was spun at 1,300 rpm for 5 mins. The scFv-Fc or IgG was found in supernatant. The supernatant was filtered with 0.45 µm filter.

Human IgG and scFv-Fc were purified on a 1 ml HiTrap Protein-A HP column (Amersham, Uppsala Sweden). The column was equilibrated with 20 ml PBS buffer. Then, the filtered supernatant was loaded to column at a flow rate of 1 ml/min. To remove loosely bound protein, the column was washed with the same buffer. The scFv was eluted from the column with buffer gradient buffer programme containing high concentration of imidazole (Glycine-HCl, pH 3.5). The elution fractions were neutralized with 2 M Tris–HCl pH 8.0. The antibody fraction was dialysed with PBS buffer by using 10 kDa snakeskin dialysis tubing (Thermo Scientific, USA). Protein concentration was quantified by a Nanodrop ND2000 spectrophotome (Thermo Scientific, USA). 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.

4.3.5 Competitive ELISA for different formats of antibody

4.3.5.1 scFv-AP

Competitive ELISA was performed as described in Rangnoi et al., 2011. The ELISA plate was immobilized with 10 µg/ml AFB1-BSA and blocked with 2% skimmed milk. After 1 hr incubation, plate was washed three times with TBS. The optimal amount of scFv-AP were pre-incubated with increasing amount of soluble AFB1 ranging from 0.028 to 5000 ng/ml at 37°C for 30 mins before adding into previously coated, blocked, and washed wells of ELISA plates. For every assay, appropriate dilutions of scFv-AP that showed a linear relationship by direct ELISA was used. After that, plate was washed 3 times with TBST and 2 times with TBS. The PNPP substrate was added to plate. The OD was measured at 405 nm.

Standard curves were plotted as absorbance (A) vs. logarithm of analyte concentration. A half-maximum inhibition (IC_{50}) was estimated at 50% A/A_0. Detection limit was determined as the concentration corresponding to 70% A/A_0.

Cross-reactivity with another types of aflatoxin and other mycotoxin; ochratoxin A and Zearalenone were tested. ScFv-AP antibodies were assayed for binding against a range of soluble aflatoxins; B1, B2, G1, G2, M1, ochratoxin A (OTA) and Zearalenone (ZON). Stock solutions of aflatoxins and other mycotoxinswere were prepared in 100% (v/v) acetonitrile and methanol, respectively and diluted using TBST. The assays were performed following the competitive

ELISA protocol, as described above, for each of the aflatoxins tested.

4.3.5.2 IgG and scFv-Fc

The competitive ELISA of IgG scFv-Fc was done followed as 4.3.5.1 except on the detection step. After washing, the secondary antibody Goat anti human IgG (H+L) was added into plate and incubated for 1 hr. After that, the plate was washed again and the TMB substrate was added. The reaction was stopped with 10% (v/v) HCl after incubation for 10-30 min. The yellow color was developed after adding HCl. Absorbance was measured at 450 nm.

4.3.6 Kinetic binding of antibodies by SPR

The kinetic bindings of purified scFv-Fc and IgG antibodies were performed by Biocore 3000 SPR instrument as described in 3.3.6.

4.3.7 Passive adsorption of antibodies to colloidal gold and latex microparticles

4.3.7.1 Colloidal gold

Colloidal gold was synthesized as signal reagent for the test system in a particle size 40 nm (BBI solutions, UK). Anti aflatoxin antibody (IgG) clone sAFH-3E3 and YAF-C3 were labeled with colloidal gold. The proper pH of buffer and concentration of antibodies must be determined to complex with the gold. The colloidal gold in different buffer; borate buffer pH 8.55 and 0.2 M MES buffer pH 6.5 were used to couple with different concentrations of antibodies. After ligand absorption, the solution was blocked with 1% BSA. Excess ligand and aggregation proteins were removed by centrifugation at 9000 g, 20^{o}C for 20 min. After that, 6%

BSA and 10% NaN_3 were added for stabilizing the conjugated. The antibody- gold complex was diluted to 5%- 10 % before used.

4.3.7.2 Latex particles

One hundred microliters of green color microsphere (70 nm and 169 nm, MERKS, Germany) were diluted in 10 mM borate buffer pH 8.7. The mixture was centrifuged at 10,000 rpm for 5 min. The supernatant was carefully discarded. The pellet was resuspended in 500 µl of 10 nM borate buffer pH 8.7. For the passively absorb antibodies solutions IgG and scFv-Fc clone sAFH-3E3 were added to the particles pellet that resuspended with borate buffer and incubated for 90 min at room temperature by rocking the solutions. After incubation, 10% BSA was added to cover the binding site of particles, further incubation for 45 minute. Then, the particles was washed with buffer and centrifuged. The pellet was dissolved with 100 mM borate buffer pH 8.7. The antibody- latex particles complex was diluted in 1:3 dilution before used.

4.3.8 Lateral Flow Immunochromatography assays (LFIA) test antibodies coupling with colloidal gold and latex particles

The AgraStrip AFLA was provided by Romer Labs Division Holding GmbH (Tulln, Austria), which was used to test the antibodies conjugating with colloidal gold and latex particles. LFIA base on competitive ELISA, the test strip were contained mouse monoclonal antibody and aflatoxin conjugated BSA on to control line and test line, respectively (Fig 4.3). Gold and latex particles complex were diluted with running buffer before use. Ten gram of Corn sample (Romer labs, Austria) was weight into bag and 20 ml of 70% methanol or PBS buffer were added. The bags were shaked for 1 and 2 minute for 70% methanol and PBS buffer

extraction, respectively. After that, the samples were settled by standing the bag for 2 minutes. Before testing with the lateral flow assay, the extraction solution was diluted in 1:10 with PBS buffer and 1:20 with 70% Methanol. Fifty microliters of antibody-latex particles complex were mixed with 50 µl of extraction solution in microwell by pipetting up and down. One test strip was put into one microwell and allow test strip to develop color for 3 minutes. The spike AFB1 analysis, different concentrations of AFB1 (100, 50, 25 and 6.25 ppb) were added to the sample extract solution and was used to mixed with antibody-latex particles complex or antibody conjugated colloidal gold into the well. Then, the AgraStrip was dipped to the well containing antibody-latex particles complex.

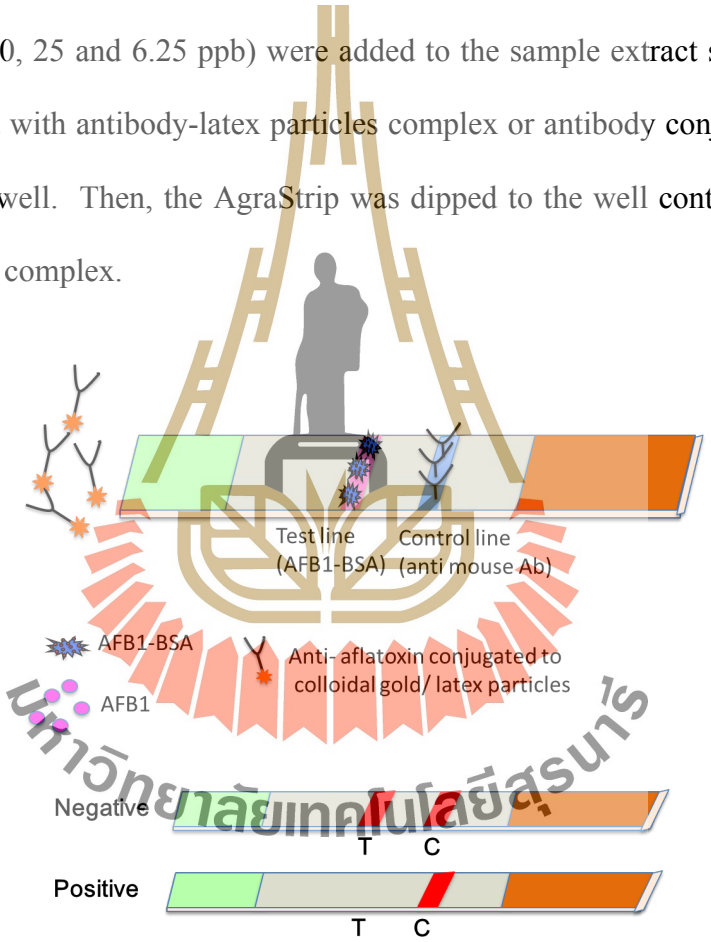


Figure 4.3 Schematic illustration of a lateral flow assay for the detection of aflatoxin. The visualization of bound antibody occurs at the test line (AFB1-BSA) and control line (anti mouse IgG) by using anti-aflatoxin-colloidol gold or latex particles. After testing, the color on control line alway visible whereas on the test line appears depend on concentraiotn of aflatoxin.

4.3.9 Spike analysis of scFv-AP in ELISA format

The ten-gram of corn sample with 0 ppb AFB1 (Trilogy, USA) was added with 100 ml 70% methanol. The glass bottle was vortex for 3 mins and allowed to stand to let the matrix go down for 10- 20 mins. The solutions were filtered with Whatman filter paper No.1. The AFB1 standard was spiked in filtered solution to be final concentration 2-200 ng/ml. The spiked solutions were diluted 1 in 3 with TBST before analysis. After that, scFv-AP competitive ELISA was performed as described in 4.3.5.1 by using this spiked AFB1 instead of AFB1 standard. This spiked AFB1 was also used to test with commercial ELISA test kit, ScreenEZ (Thailand) and Romerlabs (Austria). A competitive curve between A/A0 and concentration of spiked AFB1 was plotted. The IC_{50} and detection limit was estimated from the graph.

4.3.10 scFv-AP stability

To test the storage condition of scFv-AP antibody, it was separately kept at 4°C and -20°C. After storage time (1, 2 weeks and 1, 3 months), the indirect ELISA was performed. Plate was immobilized with 10 µg/ml AFB1-BSA, blocked and washed as described in 3.3.3.2. Ten and twenty microliters of scFv-AP (sAFH-3E3) that kept at 4°C and -20°C, respectively were added into the plate. After that, plate was incubated at room temperature for 1 hr. The plate was washed with TBST and TBS and then PNPP was added. The yellow color was developed. The OD was measured at 405 nm.

4.4 Results

4.4.1 Mammalian expression vectors for expression antibodies

All vectors used the same backbone vector (pII28) consisted of a bacterial origin of replication (ori) and the ampicillin resistance gene (AmpR) for plasmid amplification and selection in *E. coli*. This vector contained with gene fragments encoding CMV promoter which is suitable for expression in HEK 293-6E cells and the secretory human signal peptide for protein secretion from the cell.

The mammalian expression vector pKR-scFv-Fc (Fig 4.4) was used to convert scFv to scFv-Fc. The scFv gene of 4 clones was located on this vector at *NheI* and *BspEI* sites. This vector can be applied to used as backbone vector for producing scFv-Fc by inserting another scFv at *NheI* and *BspEI* sites.

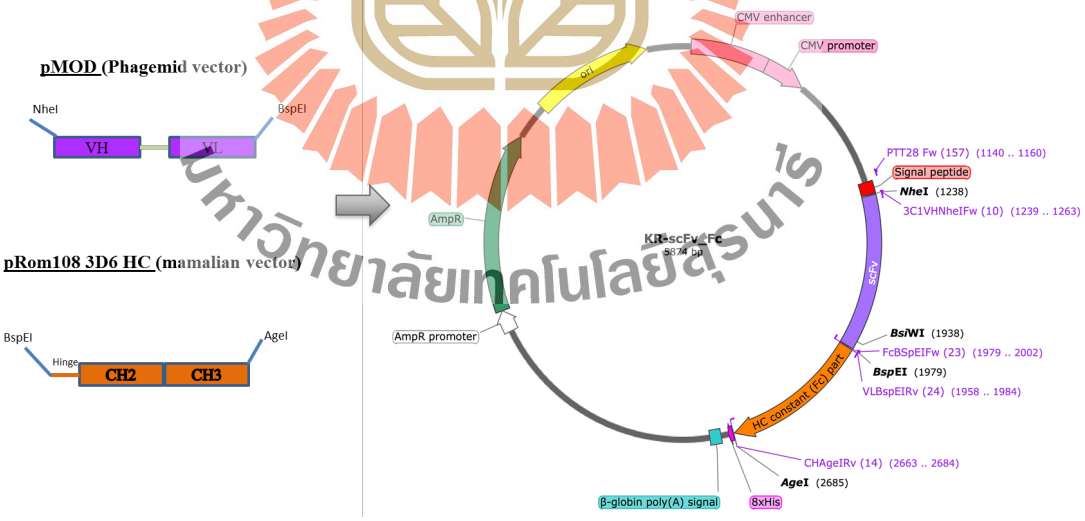


Figure 4.4 Diagram of construction of Plasmid map of pKR-scFv-Fc. The restriction sites for cloning scFv genes and Fc region are *NheI*/*BspEI* and *BspEI* /*AgeI*, respectively.

Human IgG kappa was produced from monocistronic vectors pKR-HC (Fig 4.5A) and pKR-LC (Fig 4.5B). This mammalian IgG vector can be used to express the IgG kappa by cloning the VH genes with *NheI*/*NheI* sites to express the human IgG heavy chain and VL kappa gene via *NheI*/*BsiWI* restriction sites to express kappa light.

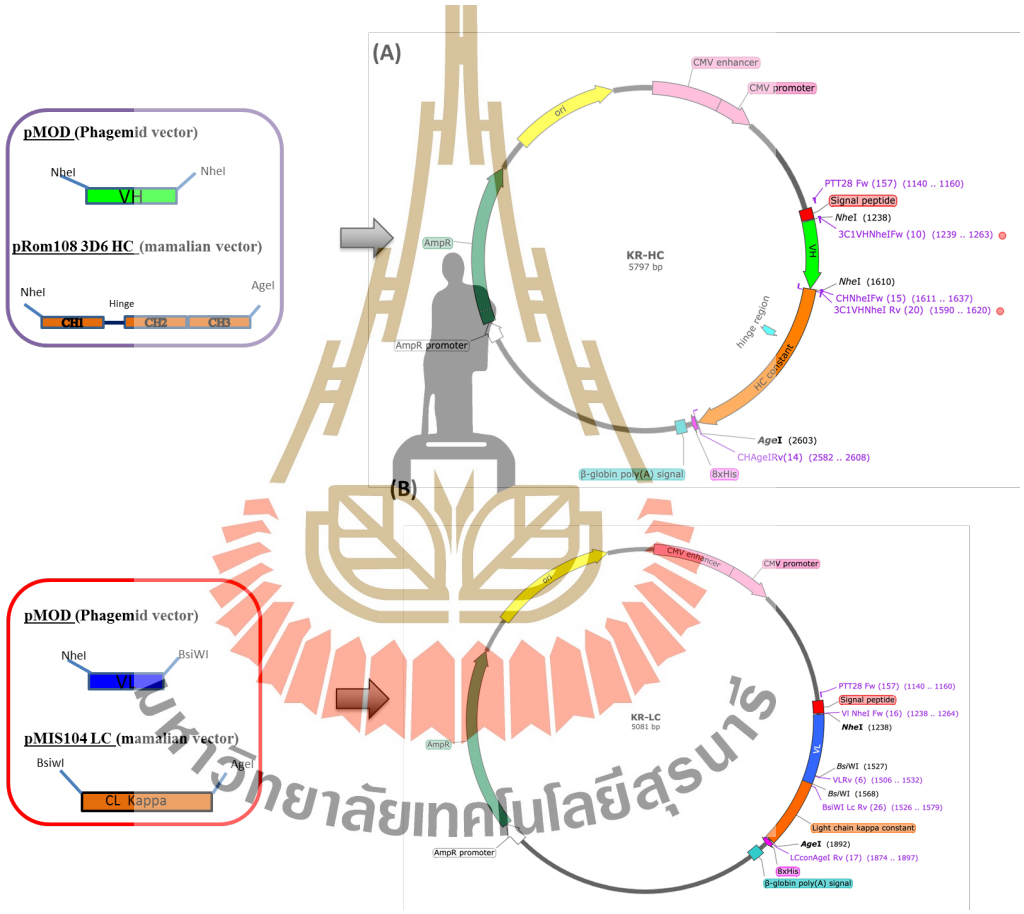


Figure 4.5 Diagram of construction of Plasmid map of IgG expression vector. Heavy chain vector (KR-HC) (A) The restriction sites for cloning VH genes and constant heavy chain are *NheI*/*NheI* and *NheI*/*AgeI*, respectively. Kappa light chain vector (KR-LC) (B) The VL genes and constant kappa light chain can be cloned via *NheI*/*BsiWI* and *BsiWI*/*AgeI*, respectively.

4.4.2 Expression and purification of antibodies in different format

The scFv antibody was engineered to be scFv-AP, which expressed in the same system (*E. coli*) as scFv. Moreover, it was engineered to be scFv-Fc and IgG formats, which expressed in mammalian cells. scFv expression and purification was done as described in Chapter 3. ScFv-AP was expressed in *E. coli* TG1 in low phosphate media under the control of PhoA promotor. After that, scFv-AP was purified from periplasmic by IMAC using His affinity chromatography. The purified fractions were run SDS-PAGE, followed by staining with coomassie blue. The scFv-AP bands appeared and were expected the size approximately 80 kDa (Fig. 4.6). The yields of four different scFv-AP ranged from 0.7 – 2.7 mg/l.

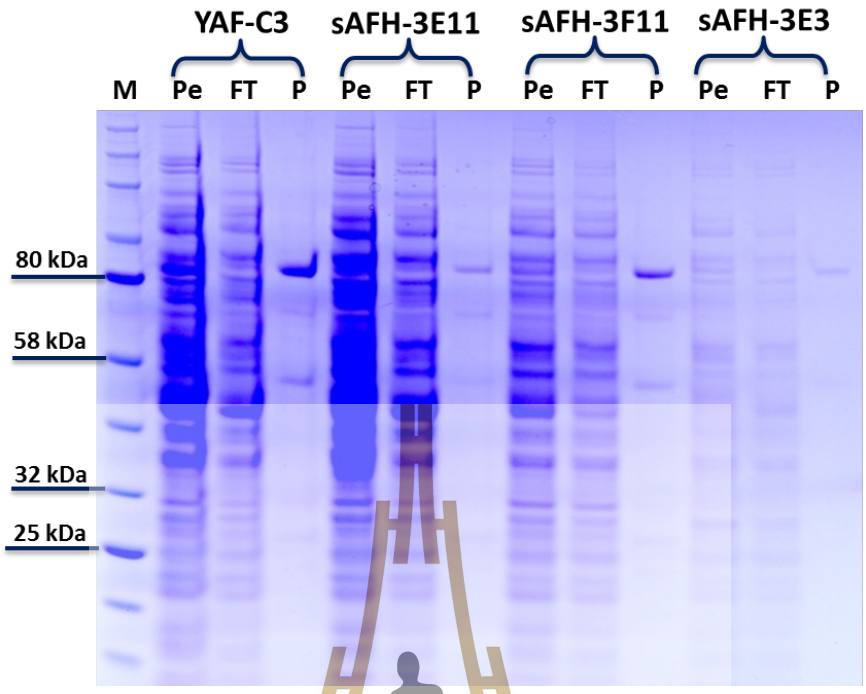


Figure 4.6 His affinity chromatography purified fractions from periplasmic expression of scFv-AP of YAF-C3, sAFH-3E11, sAFH-F11 and sAFH-3E3 in *E.coli* HB2151. M, Precision Plus Protein™ Dual Color Standards; lane Pe, periplasmic fraction; lane FT, flow-through fraction; lane P, purified scFv antibody. Fifteen microliters per lane of purified proteins were analyzed on 12% polyacrylamide gels and stained with coomassie blue. The size of scFv-AP is shown approximately 80 kDa.

ScFv was converted into IgG and scFv-Fc format by cloning into mammalian expression vector. IgG and scFv-Fc were produced in HEK293-6E cells in 25 ml scale. These antibody formats were purified by protein A affinity chromatography. Purity and apparent molecular weight of purified antibodies was assessed by SDS-PAGE analysis. Under non-reducing conditions, apparent molecular

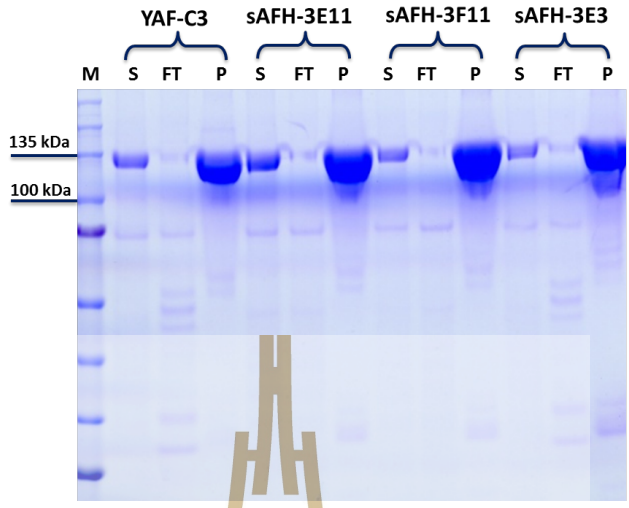
sizes were found approximately 120 and 90 kDa for scFv-Fc and IgG, respectively (Fig. 4.7). The molecular sizes of IgG corresponding to the heavy and light chains suggest the secreted antibodies are properly folded and glycosylated. Yields of four different scFv-FC and IgG were between 270 to 389 mg/l and 277 to 480 mg/l, respectively. The antibody yields of each format are shown in Table 4.2.

The expression cassette in different format of antibody and its resulting proteins were shown in Fig 4.8.

Table 4.2 Comparison of antibody yields purified by IMAC.

	Yields of purified antibody (mg/L culture volume)			
	scFv	**scFv-AP**	**scFv-Fc**	**IgG**
YAF-C3	4.96	2.72	276.48	289.2
sAFH-3E11	3.08	1.11	489.6	388.8
sAFH-3F11	6.36	1.69	448	313.2
sAFH-3E3	3.85	0.69	372	269.2

(A)



(B)

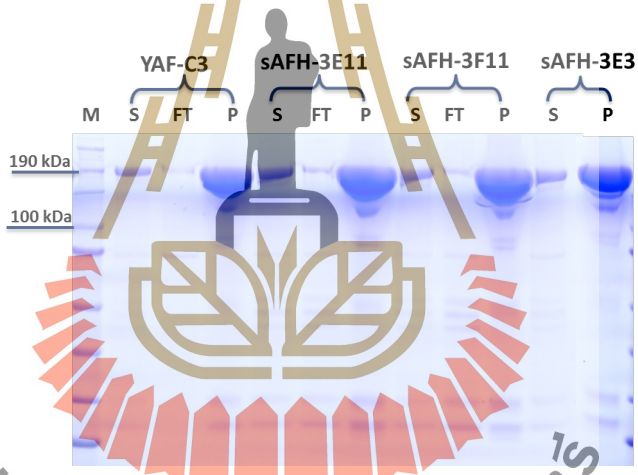


Figure 4.7 Protein-A affinity chromatography purified fractions from supernatants of transient expression of scFv-Fc (A) and IgG (B) of YAF-C3, sAFH-3E1, 1sh3F11 and sh3E3 in HEK293-6E. M, Precision Plus Protein™ Dual Color Standards, lane S, supernatant; lane FT, flow-through fraction; lane P, purified scFv antibody. Fifteen microliters per lane of purified proteins were analyzed on 12% polyacrylamide gels and stained with coomassie blue. The size of IgG is shown approximately 150 kDa and scFv-Fc approximately 120 kDa.

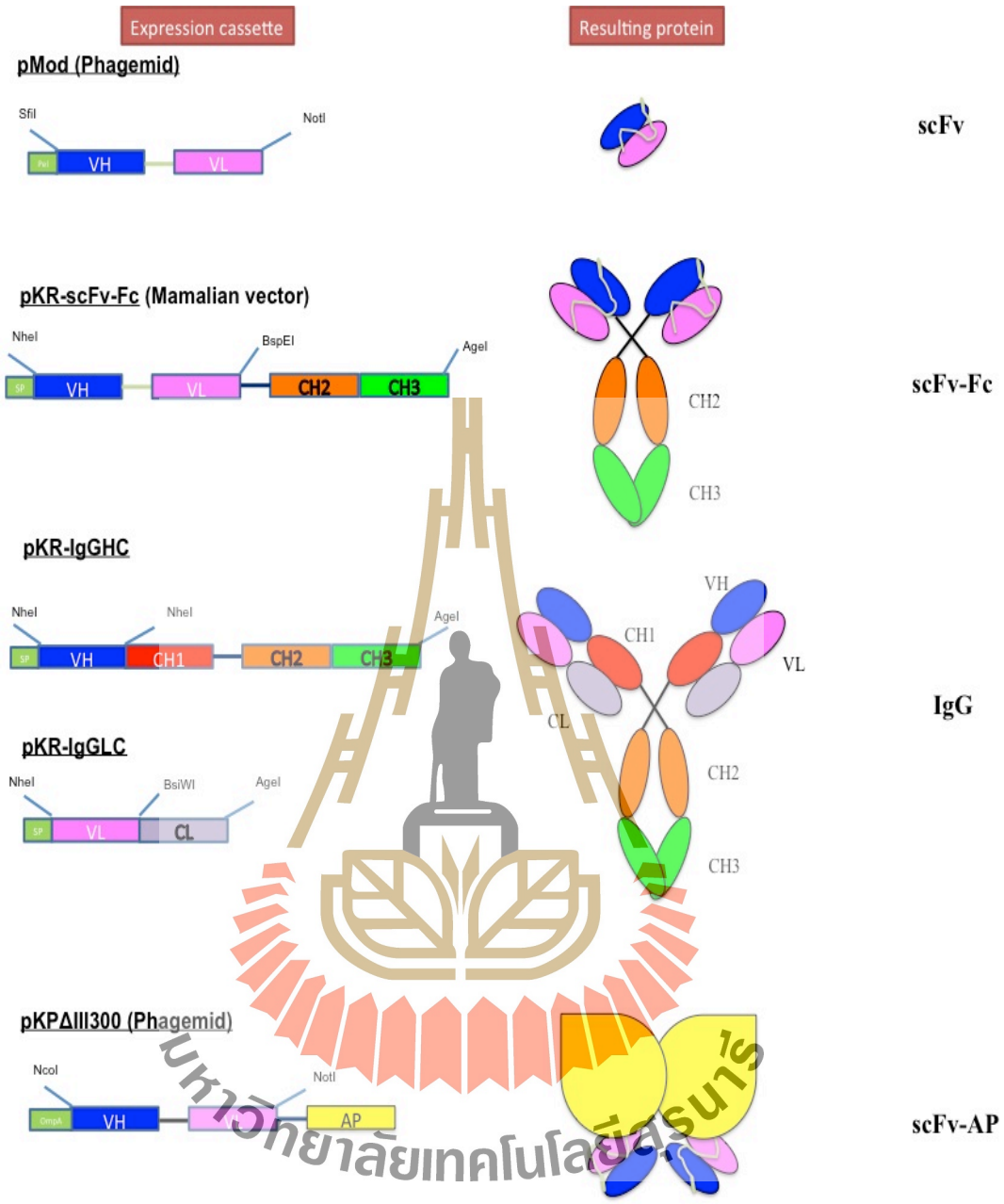


Figure 4.8 Illustration of phage display and mammalian vectors used for expression scFv, scFv-AP, scFv-Fc and IgG.

4.4.3 Characterization of antibodies in different format

4.4.3.1 Competitive ELISA

To determine the sensitivity of various formats of four clones (YAF-C3, sAFH3E11, sAFH3F11 and sAFH3E3) antibodies, indirect competitive ELISA was performed. The graphs were plotted between absorbance value (expressed as A/A_0) and concentration of AFB1 (Fig. 4.9). The IC_{50} values for the scFv, scfv-AP, scFv-Fc and IgG were concluded in Table 4.2. From the result showed that the sensitivity of antibody can be changed when it was transformed to another format. All clones showed that the scFv-AP format has the highest sensitivity to aflatoxin varied between 0.007-0.06 µg/ml. ScFv-Fc format of Clone sAFH3E11 and sAFH3F11 has IC_{50} value close to scFv formats whereas IgG format gave lower sensitivity than scFv. In contrast, the parent YAF-C3 IgG format performed higher sensitivity than scFv and scFv-Fc formats. SAFH-3E3 has the highest sensitivity than others. The sensitivity of scFv-Fc and IgG of this clone are similarly to scFv format. Therefore, in this study showed that the engineering of scFv to be IgG and scFv-Fc didn't help to improve the sensitivity of antibody.

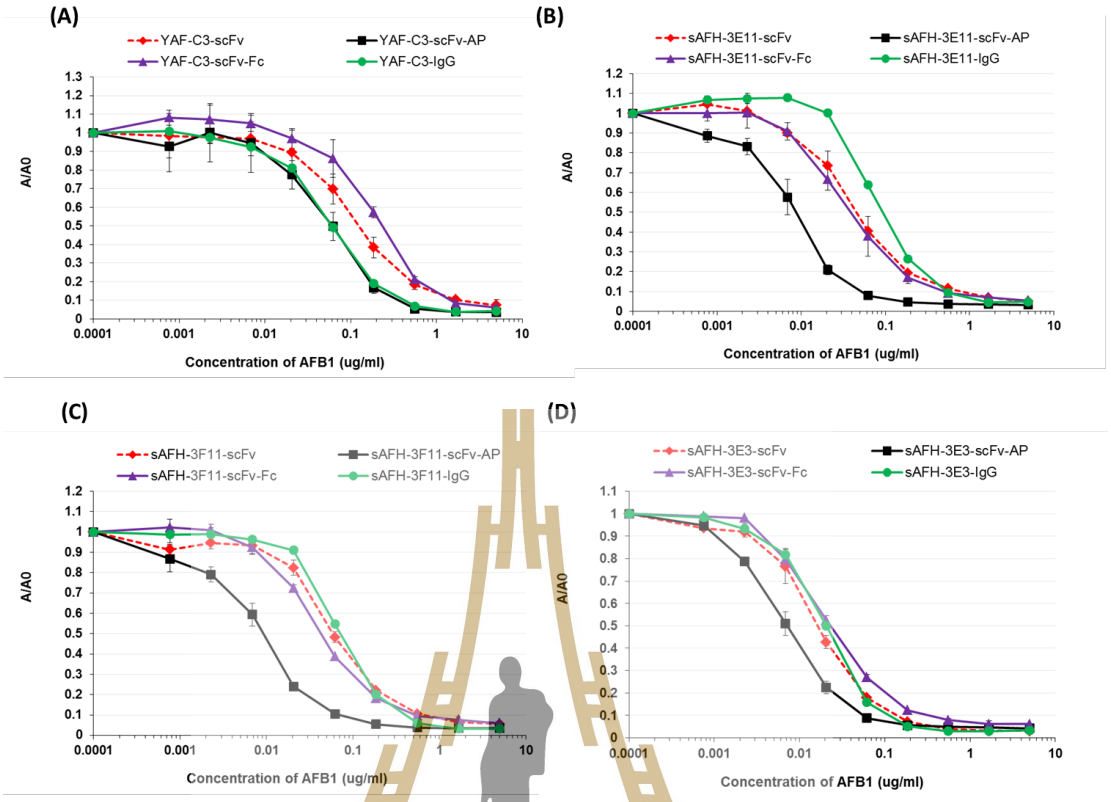


Figure 4.9 Comparison of binding properties of various formats (scFv, scFv-AP, scFv-Fc and IgG) of antibody of clone YAF-C3 (A), sAFH3E11 (B), sAFH3F11 (C), sAFH3E3 (D) by competitive ELISA. Various concentration of soluble AFB1 from 5.0 µg/ml to 0.085 ng/ml were incubated with different formats (scFv, scFv-AP, scFv-AP and IgG) of antibodies at 37°C for 30 min before addition to wells of Immuno 96 MicroWell plates, coated with 10 µg/ml AFB1-BSA. The plates were washed with TBST and TBS after 1 h incubation. Bound antibodies were demonstrated by colorimetric detection using the AP substrate, pNPP for scFv-AP and TMB substrate for another formats of antibody. Absorbance values (expressed as A/A0) were plotted against the logarithm of AFB1 concentration. The Ic_{50} values were concluded on Table 4.1.

Table 4.3 IC_{50} of different formats of antibody.

	IC_{50} (µg/ml)			
	scFv	**scFv-AP**	**scFv-Fc**	**IgG**
YAF-C3	0.120	0.060	0.230	0.060
sAFH-3E11	0.042	0.009	0.038	0.090
sAFH-3F11	0.055	0.009	0.040	0.070
sAFH-3E3	0.018	0.008	0.022	0.022

Cross-reactivity of four scFv-AP clones were determined against structurally related aflatoxins (aflatoxin B2, G1, G2 and M1) and other mycotoxins. The results showed that the scFv-AP showed no cross reactivity with OTA and ZON (data not shown). All scFv-AP performed high degrees of cross-reactivity with related aflatoxin especially AFG1 varies between 70-155% (Table 4.3) and low degrees with AFB1 (<4%). The parent clone has the degree of cross reactivity in decreasing order as AFG1>AFB2>AFG2 whereas the three mutant clones showed the order of cross reactivity as AFG1>AFG2>AFB2. The highest sensitivity clone to AFB1 (sAFH-3E3) showed high percentage of cross reactivity with AFG1, AFG2 and AFB2 more than 70%.

Table 4.4 Results of cross-reactivity of four scFv-AP against aflatoxin.

	Cross-reactivity (%)				
	AFB1	**AFB2**	**AFG1**	**AFG2**	**AFM1**
YAF-C3	100	30	70	17.14	0
sAFH-3E11	100	33.33	90	45.45	0.5
sAFH-3F11	100	22.5	81.25	52.94	0.31
sAFH-3E3	100	75	155	78.13	3.87

4.4.3.2 SPR analysis

Purified scFv, scFv-Fc and IgG were determined the affinity by using Biacore instrument. The aflatoxinB1–BSA was coated on chip. The various concentrations of antibody were injected and analyzed the kinetic binding. The result showed that the scFv-Fc and IgG format had a higher affinity than scFv format (Table 4.3). When compare this two formats of antibody between mutant clones and parental clone, it was indicated that the overall KD values of three mutant clones were 10-1,000 lower than YAF-C3, indicating an approximately 10-1000 folds improvement in affinity to parent clone.

Table 4.5 Binding kinetics of different formats of antibody by SPR.

	K_D (M)		
	scFv	**scFv-Fc**	**IgG**
YAF-C3	5.22×10^{-8}	5.98×10^{-9}	4.15×10^{-9}
sAFH-3E11	1.88×10^{-8}	1.55×10^{-12}	2.96×10^{-10}
sAFH-3F11	1.16×10^{-8}	1.19×10^{-11}	1.85×10^{-11}
sAFH-3E3	2.29×10^{-8}	1.54×10^{-10}	2.28×10^{-10}

KD equilibrium dissociation constant

4.4.4 Determining optimal buffer and antibody concentration for antibody conjugating with colloidal gold and latex particle

4.4.4.1 Colloidal gold

The colloidal gold in different buffer; borate buffer pH 8.55 and 0.2 M MES buffer pH 6.5 were used to couple with 0.5 µg/ml antibody to find the proper pH and buffer for forming gold-antibody complex. After coupled, the red color mean had the succeeded gold-antibody conjugate, where the antibodies protect the colloidal gold particles from the salt. Whereas, blue-grey solution mean they have aggregated gold particles in them and those mixtures do not have enough antibodies to protect the colloidal gold from the aggregating properties of salt. The result showed that 0.5 µg/ml sAFH-3E3 IgG and 0.5 µg/ml BSA in 0.2 M borate buffer was considered to be suitable to coupling with antibody-colloidal gold (Fig. 4.10).

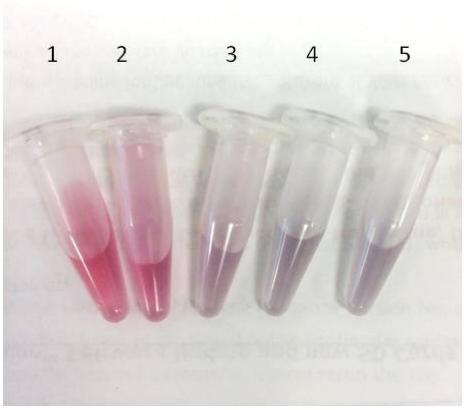


Figure 4.10 Colloidal gold coupling with antibody. Borate buffer pH 8.55 and MES buffer pH 6.5 were used for coupling the colloidal gold and antibody. 1, 0.2 M borate buffer; 2, 0.5 µg/ml sAFH-3E3 IgG and 0.5 µg/ml BSA in 0.2 M borate buffer; 3, 0.5 µg/ml sAFH-3E3 IgG in 0.2 M borate buffer; 4, 0.5 µg/ml YAF-C3 IgG in MES buffer; 5, 0.5 µg/ml sAFH-3E3 IgG in MES buffer.

4.4.4.2 Latex particles

Firstly, The optimal concentration of antibody conjugating with latex particles had to be determined. Different concentrations (0.05, 0.1, 0.25 and 0.5 mg/ml) of sAFH-3E3 IgG and scFv-Fc were coupled with latex particle followed as Bruning et al. (1999) method. After coupling, the antibody- latex particles complex was diluted in 1:3 dilution before using in LFIAs (AgraStrip). After three minutes, AgraStrip were read with the naked eyes with green coloration. The control line and test line was not appeared on the AgraStrip when testing with ScFv-Fc-latex complex. But when testing with IgG-latex complex, the test line became visible (Fig. 4.11). It is shown that at 0.05 mg/ml of antibody, the test line was appeared. Therefore, we chose

this concentration to couple with latex particles. Secondly, The concentration at 0.05 mg/ml of both antibodies forms was used to conjugate with different sizes of latex particles (0.07 and 0.169 µm). The result showed that 0.07 µm latex particles were suitable to couple with 0.05 mg/ml IgG and used in LFIAs as shown in Fig. 4.12.

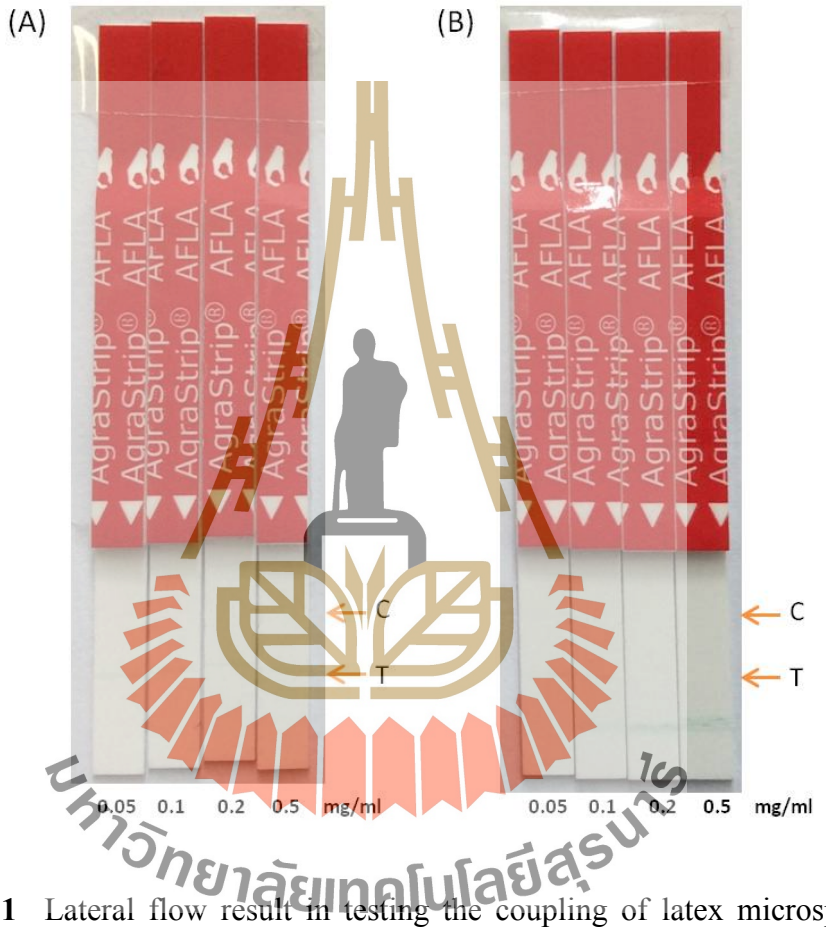


Figure 4.11 Lateral flow result in testing the coupling of latex microsphere with different concentration of antibody sAFH-3E3 (A) IgG and (B) scFv-Fc. The strip showed the green colour line of control line (C) and test line (T). The colour in Test line was showed when no AFB1 contamination.

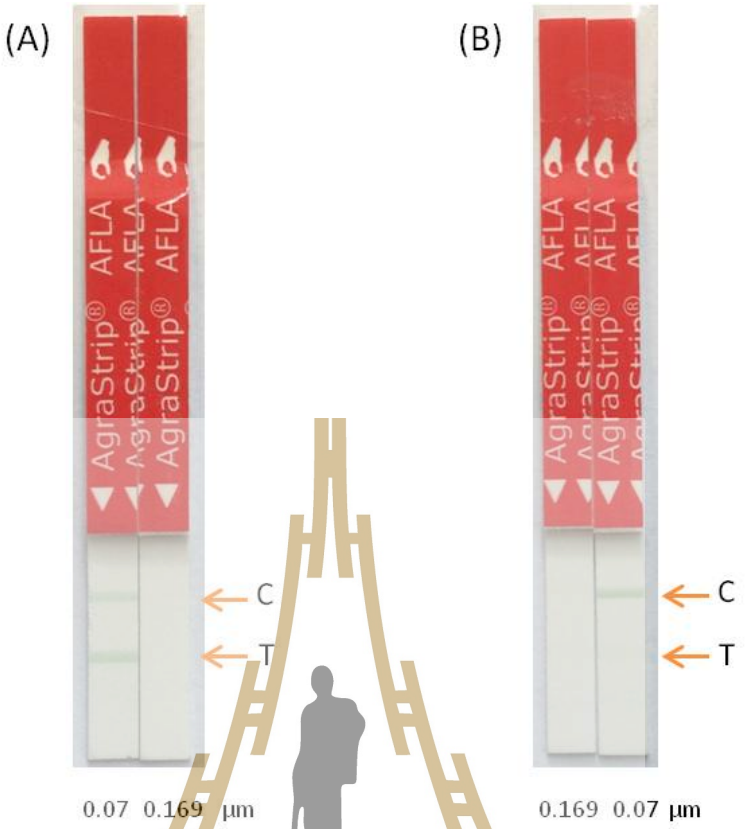


Figure 4.12 Lateral flow result in testing the coupling in different size of latex microsphere with antibody sAFH-3E3 (A) IgG and (B) scFv-Fc. The strip showed the green colour line of control line (C) and test line (T). The colour in Test line was showed when no AFB1 contamination.

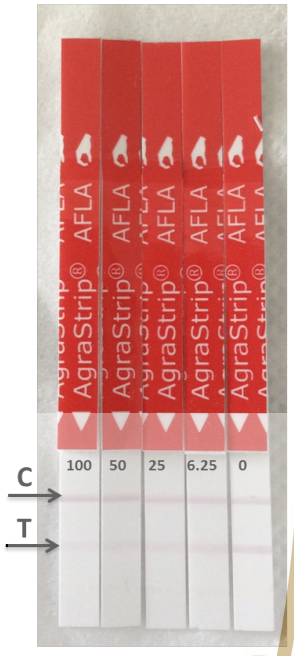
4.4.5 Analysis of lateral-flow based assay for the detection of aflatoxin

Quantitative detection of AFB1 was studied using a AgraStrip. Colloidal gold or latex particle conjugated to sAFH-3E3 IgG acted as the probe, anti-mouse mAb served as secondary antibody, which can bind to sAFH-3E3 IgG and AFB1-BSA acted as the competitive antigen.

4.4.5.1 Colloidal gold

SAFH-3E3 IgG-colloidal gold conjugates were performed efficiency as detection probe in LFIA for aflatoxin detection. The corn samples were extracted with PBS buffer. The different concentrations of AFB1 were spiked into extraction solutions. After that, spiked solutions were mixed with IgG labeled with colloidal gold and put into the microwell. Then, strip was dipped into the mixture solution. The IgG-colloidal gold flowed along the membrane where encountered the coated AFB_1-BSA and were captured. The red color was appeared on test line (T). When some AFB_1 was present in the sample, the binding of the IgG-colloidal gold to the AFB1-BSA was inhibited and the colour of the test line faded. The control line should always be visible because of the reaction between colloidal gold- sAFH-3E3 IgG and anti mouse IgG, which was considered to be an indicator of the good functionality of the test. The strip was evaluated value using AgraStrip reader (Romer Labs, Austria). The limit of detection was 6 ppb (Fig. 4.13C).

(A)



(B)

Concentration of AFB1 [ppb in solution]	Peak area [Units]
0	3550519
6,25	2485904
25	1841290
50	1292314
100	1252168

(C)

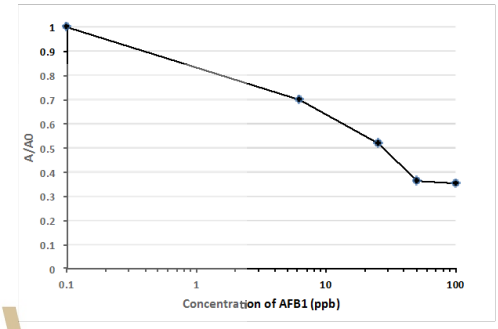


Figure 4.13 Colloidal gold coupling testing with corn sample spiked with AFB1 standard. (A) Lateral flow result in testing in different concentration of AFB1. The strip showed the red colour line of control line (C) and test line (T). The colour in Test line was decreasing when the concentration of AFB1 was increased. (B) The value when measure the strip test with machine showed as peak areas units. (C) Graph was plotted from A/A_0 against log of concentration of aflatoxin. A= peak area value of each concentration AFB1 standard, A_0 = peak area value of at 0 ppb AFB1 standard.

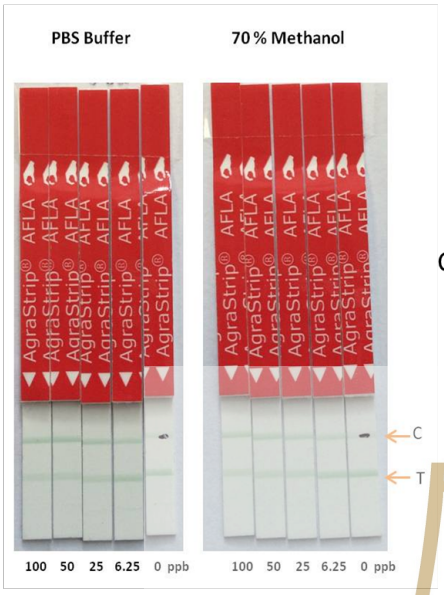
4.4.5.2 Latex particles

SAFH-3E3 IgG –latex particle was used in LFIA to test for aflatoxin detection instead of colloidal gold-IgG as described above. In this assay, corn samples were extracted with different buffer; PBS and 70% methanol. Reference

corn sample contaminated with AFB1 98.7 ppb and 0 ppb were extracted and tested with LFIA. The antibody-latex particle conjugates combined with AFB1-BSA and anti-mouse monoclonal antibody can be detected with the naked eye with green coloration as test line and control line. Absence of color at test line is an indication for the presence of analyte while appearance of color both at test and control lines indicates a negative result. The strip can be used to detect AFB1 contamination at 98.7 ppb when PBS buffer was used as extraction buffer as shown in Fig. 4.14.

Figure 4.14 Lateral flow result in testing sAFH-3E3 IgG – latex microsphere complex with extraction corn samples contaminated with AFB1 0 and 98.7 ppb by different extraction buffer; (A) PBS buffer and (B) 70% methanol. The strip showed the colour line of control line (C) and test line (T).

(A)



(B)

Concentration AFB1 [ppb in solution]	water extract Peak area [Units]	70% methanol Peak area [Units]
0	2357222	2489174
6.25	1583236	2146974
25	1064466	1376369
50	845380	1322567
100	766005	1234216

C)

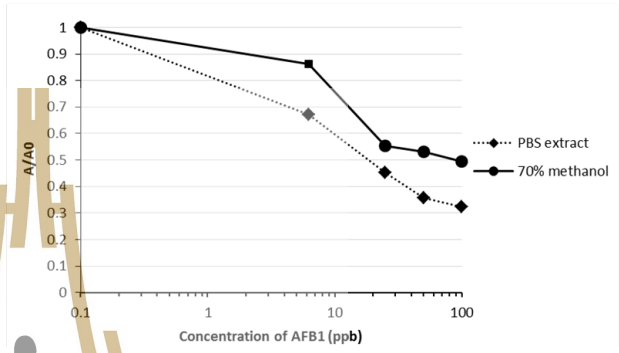


Figure 4.15 Latex microsphere coupling testing with corn sample spiked with AFB1 standard by using different extraction buffer. (A) Lateral flow result in testing in different concentration of AFB1. The strip showed the red colour line of control line (C) and test line (T). The colour in Test line was decreasing when the concentration of AFB1 was increased. (B) The value when measure the strip test with machine showed as peak areas units. (C) Graph was plotted from A/A_0 against log of concentration of aflatoxin; A= peak area value of each concentration AFB1 standard, A_0 = peak area value of at 0 ppb AFB1 standard.

For quantitative result, strip reader was used to measure the test strip after testing with different concentration of AFB1 standard which spiking in a blank sample. The intensity of the test lines decreased with increasing AFB1 concentrations (Fig. 4.15A). Curve was plotted from the value. The curve was slope down when the concentration of AFB1 was increased (Fig. 4.15C). The result showed

that the IC_{50} values were 18 ng/ml and 70 ng/ml when extracting with PBS buffer and 70% methanol, respectively. From this result can conclude that, PBS buffer is suitable for use as extraction buffer in this assay. The strip test can be used for aflatoxin detection at 5 ppb as limit detection.

4.4.6 Determining aflatoxin detection in ELISA format by using scFv-AP (spike experiment)

To compare the sensitivity for aflatoxin detection between our scFv-AP and commercial test kit, the competitive ELISA was performed by using spiked sample instead of standard AFB1. The IC_{50} and limit of detection was determined from graph plotted with A/A0 versus log of concentration of spiked AFB1 (Fig 4.16). The result showed that the IC_{50} of ScreenEZ, Romer labs test kits and sAFH-3E3 AP, were 5, 8 and 80 ng/ml, respectively. The detection limits of them were 0.4, 4 and 20 ng/ml.

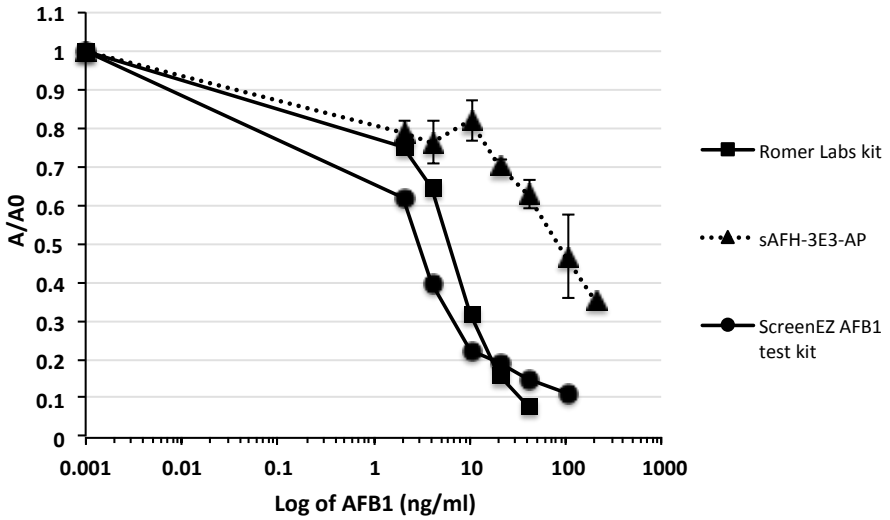


Figure 4.16 ELISA from spike experiment sAFH-3E3 compare with test kit from two company.

4.4.7 scFv-AP stability

After purification, the scFv-AP sAFH-3E3 was separately kept at 4°C and freeze in -20°C. The indirect ELISA was performed to test the activity of scFv-AP. The result showed that scFv-AP activity was lost after 2 weeks. In contrast, the activity still remains when kept it at -20°C (Fig 4.17). To keep the antibody in long term, it should be added with glycerol and keep in-20°C.

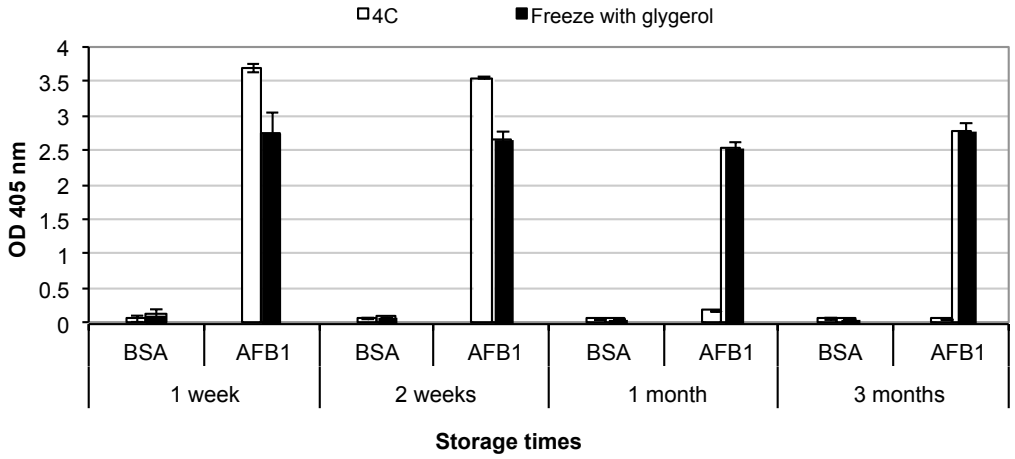


Figure 4.17 ELISA of scFv-AP with different storage temperature and different times (1 week, 2 weeks, 1 month, 2 months).

4.5 Discussion

In this study, One original clone and three mutant clones with improved binding affinity using chain-shuffling technique were successfully converted into different formats i.e., IgG, scFv-Fc and scFv-AP, expressed in human cell and *E. coli* cell and purified by affinity chromatography. The expression level of antibody showed the improvement when expression in mammalian cell whereas expression in *E. coli* still low.

Binding sensitivity of different formats of antibodies to free AFB1 was evaluated by competitive ELISA. The result showed that scFv-AP showed the highest sensitivity in ELISA. The scFv fused with AP, which it known as form dimer. Pohl and colleaque reported that the increase avidity can increase hundred fold of antigen binding (Pohl et al. 2012). This may be the reason of the improved sensitivity in this format. The cross-reactivity of scFv-AP of each clone showed that all of the mutated clone has the order to bind to other aflatoxins as G1>G2>B2>M1 whereas the parent clone can bind to aflatoxin G1>B2>G2>M1. The mutant clones were able to bind AFG1 as AFB1 or even better. This is in agreement with Moghaddam (2001) (Moghaddam et al. 2001). All of them showed low percentages binding to AFM1 and not bind to another mycotoxins (ochratoxin and zealarenone).

When conversions the scFv fragment to the full length IgG and scFv-Fc, the sensitivity of each clone was different. ScFv-Fc format of clone sAFH3E11 and sAFH3F11 has retained sensitivity as scFv formats whereas IgG format showed lower sensitivity than scFv. In contrast, the parent YAF-C3 IgG format showed the higher sensitivity than scFv and scFv-Fc formats (Fig 4.9). The result is correlated to several previous reports say that conversion of antibody fragments back to full length IgG or liked IgG format (scFv-Fc) can result in similar or improved antigen binding(Ames et al. 1995, Huls et al. 1999, Liu et al. 2007). Moreover, previous study has reported the scFv was loss of affinity after conversion scFv fragment to IgG (Menzel et al. 2008).

The sAFH-3E3 clone has the highest sensitivity as shown by an IC_{50} value between 0.008-0.02 μg/ml, depending on the formats of antibody. Among this, the scFv-AP showed the highest sensitivity. Therefore, this format could be developed to use as convenient one-step detection probe in ELISA format kit. The sensitivity of

scFv fragment and IgG and scFv-Fc were similar. So, This scFv-Fc and full length IgG antibody was conjugated to colloidal gold and latex particle and test with lateral flow strip. The result showed that the IgG format is better form for using in this experiment. The scFv-AP and IgG format were successfully employed in ELISA assay and lateral flow immonoassay format for the detection of aflatoxin from corn samples in spike experiment.

Although, the sensitivity of our antibody was not good as the commercial test kit, but it is enough to use as the detection reagent in both ELISA and lateral flow strip test under the maximal level provide by CODEX. As the antibody in the test kit are monoclonal antibody and polyclonal antibody, which they need the animal for production and they are still have limitation point such as polyclonal can be showed different sensitivity when it was produced in different lots. In contrast, the production of recombinant antibody is much more easier than the convention method because no need to used animal and easy reproducible in bacteria or animal cells. The scFv-AP and IgG will be used to test with the contaminated aflatoxin sample (corn and peanut) in the future.

4.6 Conclusion

In conclusion we have engineered recombinant antibody in different formats; i.e., scFv-AP, scFv-Fc and IgG to be used as reagents for the detection of AFB1. The sAFH-3E3 showed the highest sensitivity in every formats of antibody by competitive ELISA. ScFv-AP had the highest sensitivity so this format is the most suitable format for aflatoxin detection in ELISA assay. In addition, cojutation of sAFH-3E3 IgG with latex particles was shown to be the most suitable formats for lateral flow-based assay.

The detection limit of antibody in scFv and IgG format in ELISA format and strip test was approximately 20 and 5 ng/ml, respectively.

4.7 References

Ames, R. S., Tornetta, M. A., Deen, K., Jones, C. S., Swift, A. M. and Ganguly, S. (1995). Conversion of murine Fabs isolated from a combinatorial phage display library to full length immunoglobulins. **Journal of immunological methods** 184(2): 177-186.

Edupuganti, S. R., Edupuganti, O. P., Hearty, S. and O'Kennedy, R. (2013). A highly stable, sensitive, regenerable and rapid immunoassay for detecting aflatoxin B 1 in corn incorporating covalent AFB 1 immobilization and a recombinant Fab antibody. **Talanta** 115: 329-335.

He, T., Wang, Y., Li, P., Zhang, Q., Lei, J., Zhang, Z., Ding, X., Zhou, H. and Zhang, W. (2014). Nanobody-based enzyme immunoassay for aflatoxin in agro-products with high tolerance to cosolvent methanol. **Analytical chemistry** 86(17): 8873-8880.

Huls, G. A., Heijnen, I. A., Cuomo, M. E., Koningsberger, J. C., Wiegman, L., Boel, E., Loyson, S. A., Helfrich, W., van Berge Henegouwen, G. P. and van Meijer, M. (1999). A recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. **Nature biotechnology** 17(3): 276-281.

Li, X., Li, P., Lei, J., Zhang, Q., Zhang, W. and Li, C. (2013). A simple strategy to obtain ultra-sensitive single-chain fragment variable antibodies for aflatoxin detection. **RSC Advances** 3(44): 22367-22372.

Li, X., Li, P., Zhang, Q., Li, Y., Zhang, W. and Ding, X. (2012). Molecular characterization of monoclonal antibodies against aflatoxins: a possible explanation for the highest sensitivity. **Analytical chemistry** 84(12): 5229-5235.

Liu, B., Conrad, F., Roth, A., Drummond, D. C., Simko, J. P. and Marks, J. D. (2007). Recombinant full-length human IgG1s targeting hormone-refractory prostate cancer. **Journal of Molecular Medicine** 85(10): 1113-1123.

Menzel, C., Schirrmann, T., Konthur, Z., Jostock, T. and Dübel, S. (2008). Human antibody RNase fusion protein targeting CD30+ lymphomas. **Blood** 111(7): 3830-3837.

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

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(1): 169-181.

Pershad, K., Sullivan, M. A. and Kay, B. K. (2011). Drop-out phagemid vector for switching from phage displayed affinity reagents to expression formats. **Analytical biochemistry** 412(2): 210-216.

Pohl, S. C., Schwarz, S., Frenzel, A. and Schirrmann, T. (2012). A cassette vector system for the rapid cloning and production of bispecific tetravalent antibodies. **Antibodies** 1(1): 19-38.

Rangnoi, K., Jaruseranee, N., O'Kennedy, R., Pansri, P. and Yamabhai, M. (2011). One-Step Detection of Aflatoxin-B1 Using scFv-Alkaline Phosphatase-Fusion Selected from Human Phage Display Antibody Library. **Molecular biotechnology** 49(3): 240-249.

Shim, W., Yang, Z., Kim, J., Kim, J., Kang, S., Woo, G., Chung, Y., Eremin, S. A. and Chung, D. (2007). Development of immunochromatography strip-test using nanocolloidal gold-antibody probe for the rapid detection of aflatoxin B1 in grain and feed samples. **Journal of microbiology and biotechnology** 17(10): 1629.

Wang, T., Ding, H., Yang, L., Ji, Q., Chen, W. and Zhang, H. (2009). [Screening and identification of a single chain antibody fragment]. **Wei sheng wu xue bao= Acta microbiologica Sinica** 49(1): 135-140.

Wang, Y., Li, P., Majkova, Z., Bever, C. R., Kim, H. J., Zhang, Q., Dechant, J. E., Gee, S. J. and Hammock, B. D. (2013). Isolation of alpaca anti-idiotypic heavy-chain single-domain antibody for the aflatoxin immunoassay. **Analytical chemistry** 85(17): 8298-8303.

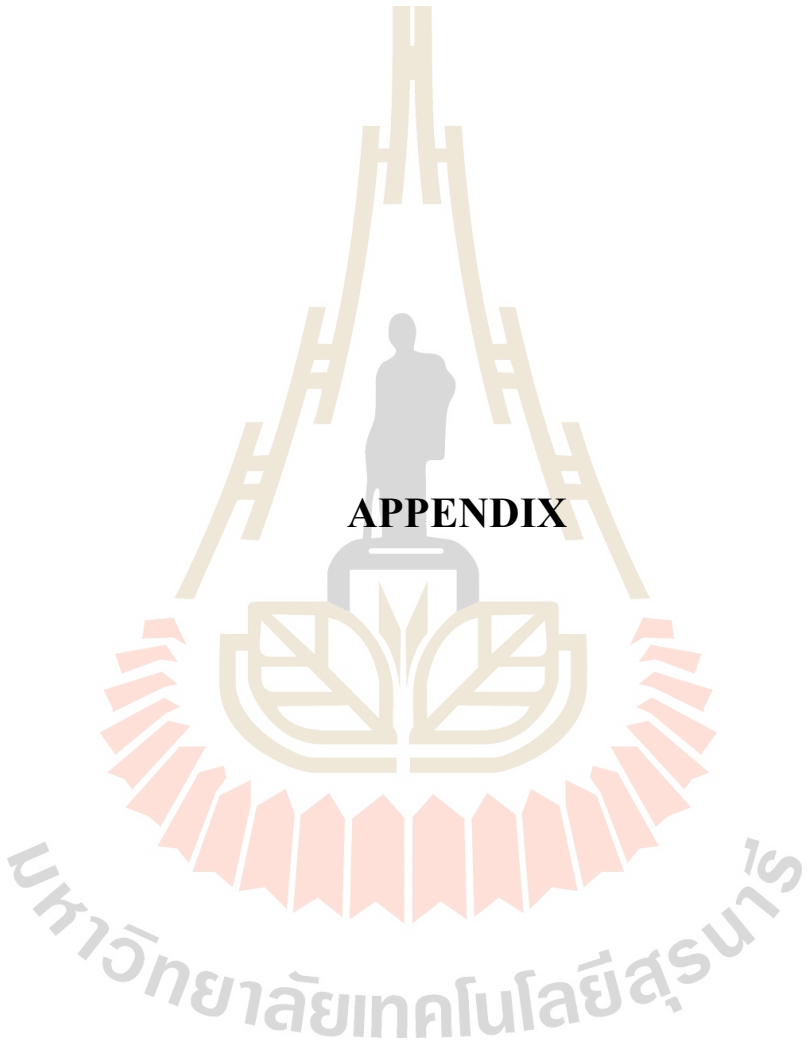
Yang, L., Ding, H., Gu, Z., Zhao, J., Chen, H., Tian, F., Chen, Y. Q., Zhang, H. and Chen, W. (2009). Selection of single chain fragment variables with direct coating of aflatoxin B1 to enzyme-linked immunosorbent assay plates. **Journal of agricultural and food chemistry** 57(19): 8927-8932.

CHAPTER V

CONCLUSION

1. The property of anti-Aflatoxin B1 (AFB1) scFv antibody could be improved by chain shuffling technique (heavy chain shuffling).
2. Up to 7.5-fold improvement in affinity and sensitivity was achieved.
3. Antibody expression level could also be improved when converted into full-length IgG and expressed in human cells (HEK 293-6E).
4. Various formats of antibody (free scFv, scFv-Fc, IgG, and scFv-AP) showed different binding sensitivity by competitive ELISA.
5. Antibody in the form of scFv-AP fusion is the most suitable format for the detection of AFB1 by ELISA-based method.
6. Antibody in the form of IgG is suitable for assembly into a lateral flow test kit (strip test).
7. A bio-innovation for the detection of aflatoxin contamination based on recombinant antibody could be developed for monitoring aflatoxin contamination in agricultural products in the future.

APPENDIX



APPENDIX

LISTS OF PATENTS

1. **Thai Patent Application No. 1601005973**

   Yamabhai M., Rangnoi, K.

   Title: Recombinant human single chain anti-aflatoxin scFv antibody

2. **Thai Patent Application No. 1601005974**

   Yamabhai M., Rangnoi, K.

   Title: Human single chain anti-aflatoxin scFv antibody – alkaline phosphatase fusion for use as one step detection probe

BIOGRAPHY

Miss Kuntalee Rangnoi was born on November 14, 1983 in Phetchaburi, Thailand. She graduated with the Bachelor Degree of Animal Production Technology, in 2005. She received her Master's Degree in School of Biotechnology from Suranaree University of Technology in 2009. After graduation, she has been employed under the position of research assistance by School of food technology and School of biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Thailand. In 2012-2016, she had opportunity to study Doctural Degree enrollment in School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology. During her study, she received scholarship names "Technology Grants" (Sandwich program) from Austria to experience on her research for 1 year at Christian Doppler Laboratory for Antibody Engineering, University of Natural Resources and Life Sciences, Vienna (BOKU). She also had another 2 months experience on her research at Applied Biochemistry Group, school of Biotechnology and Biomedical Diagnostics Institute, Dublin City University, Dublin, Ireland. She had presented research work in The 2nd Asean Microbial Biotechnology Conference (AMBC 2016), August 3-4, 2016, Sanur Paradise Plaza, Bali, Indonesia (Oral Presentation; Improvement of single chain fragment variable (scFv) antibody for aflatoxin detection by chain-shuffling). The 5th School of Biotech International colloquium September 22, 2016, Suranaree University of Technology, Thailand (Oral presentation; in "Antibody engineering for AFB1 detection").